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Fd Cosmet. Toxicol. Vol. 11, p. i. Pergamon Press 1973. Printed in Great Britain

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ARTICLES OF GENERAL INTEREST

TOXICOLOGY: ABSTRACTS AND COMMENTS

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Research Section

Evolution, au Cours du Temps, de Quelques Effets du Bordeaux S et du Jaune de Beurre sur l'Organisme du Rat

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(Reçu le 26 juin 1972)

Résumé—Le Bordeaux S et le Jaune de Beurre sont, tous deux, des colorants azoïques. Le premier est hydrosoluble et il est utilisé comme additif alimentaire. Le second est liposoluble; c'est un cancérogène du foie de rat. On a administré des quantités croissantes de chacun de ces composés à des rats mâles dès le sevrage et comparé, après 1–8 mois du traitement, leurs effets sur quelques enzymes hépatiques. Les animaux traités par le colorant alimentaire paraissent peu différents des rats témoins. En revanche, le Jaune de Beurre abaisse l'activité de la phosphatase du 6-phosphorylglucose et stimule parfois la biosynthèse des déshydrogénases du 6-phosphoryl-glucose et du 6-phosphorylgluconate. L'action du Jaune de Beurre varie au cours du temps d'administration et l'on peut confirmer biochimiquement les études histologiques: la cancérisation du foie consiste en une succession de processus de destruction et de régénération cellulaire. D'autre part, l'activité azoréductasique n'est pas influencée de la même façon par le cancérogène selon le type de substrat. Il est bien connu que l'activité réductrice est diminuée avec un substrat liposoluble, mais les résultats présentés dans cet article montrent que l'azoréductase est, en revanche, augmentée avec un substrat hydrosoluble. Ce fait pose la question de savoir s'il y a plusieurs azoréductases dans le foie.

INTRODUCTION

Notre propos est de comparer, dans les mêmes expériences, les effets de deux colorants azoïques sur l'organisme du rat. Le Bordeaux S (Amaranthe; C.I. (1956) no. 16185) est un composé hydrosoluble utilisé comme additif alimentaire. Le Jaune de Beurre (*p*-diméthylaminoazobenzène; DAB; C.I. (1956) no. 11020) est un aminoazoïque liposoluble; il a fait et fait encore l'objet de nombreuses recherches puisqu'il constitue un matériau commode pour l'étude de la cancérisation chimique expérimentale. Le Jaune de Beurre est aujourd'hui interdit pour les produits alimentaires mais il était autrefois ajouté à la margarine.

Feuer, Golberg et Le Pelley (1965) ont montré que des méthodes biochimiques permettent de déceler une action toxique avant que des modifications histopathologiques soient observables. Notre travail relève de cette conception puisqu'il porte sur les modifications de l'activité de quelques enzymes hépatiques. A notre connaissance, pour le Bordeaux S, aucune étude de cette sorte n'a été entreprise.

Nous observons les effets du colorant alimentaire et du cancérogène sur l'activité potentielle de la phosphatase et de la déshydrogénase du 6-phosphorylglucose ainsi que sur la déshydrogénase du 6-phosphorylgluconate. Selon la thèse de Feuer *et al.* (1965) les hépatotoxiques réduisent toujours l'activité de la phosphatase.

En outre, dans une étude antérieure (Manchon et Lowy, 1968), nous avons indiqué que l'activité de la déshydrogénase du 6-phosphorylglucose semble stimulée par le Méthyl-orange. S'il y a effectivement une induction enzymatique avec ce colorant, en est-il de même avec d'autres azoïques hydrosolubles et particulièrement avec un additif alimentaire autorisé, le Bordeaux S? Un autre intérêt de l'étude des déshydrogénases du cycle des pentoses est que ces enzymes fournissent du NADPH, lequel est nécessaire pour les réactions de transport des électrons dans les microsomes (cf. Gillette, 1971).

Les azoïques sont métabolisés dans le foie, *in vitro*, par coupure de la double liaison réunissant les atomes d'azote; il se forme deux amines (Mueller et Miller, 1948). Le système enzymatique intervenant dans cette réaction est l'azoréductase dont on sait qu'elle met en jeu la chaîne microsomale de transport des électrons, en particulier, la réductase NADPH-cytochrome *c* et le cytochrome P_{450} (Hernandez, Mazel et Gillette, 1967b). Dans ce travail, nous mesurons l'activité de l'azoréductase ainsi que celle de la réductase NADPH-cytochrome *c* chez des animaux traités par le Bordeaux S ou par le Jaune de Beurre.

Il est bien connu que l'effet d'un composé sur l'organisme dépend largement de la quantité reçue et qu'il n'est pas indifférent que celle-ci soit ingérée dans un laps de temps court ou long. Nous sacrifions donc des animaux d'âge différent (2, 3, 4 et 9 mois), ce qui nous permet d'étudier l'évolution des effets des colorants au cours du temps; nous n'avons pas étudié l'influence du moment de la mise au régime: tous les animaux traités reçoivent les azoïques dès le sevrage.

METHODES EXPERIMENTALES

Animaux et traitement. Nous travaillons avec des rats mâles de souche Wistar CF. Nous recevons les animaux au sevrage à 30 jours. Après 2 jours d'acclimatation à notre animalerie, nous commençons de leur donner un régime semi-synthétique que nous préparons au laboratoire. La composition en poids de ce régime a été antérieurement précisée dans cette revue (Albrecht, Manchon et Lowy, 1972). Les rats témoins reçoivent ce régime sans autre addition. Les animaux traités ont à leur disposition le régime de base auquel nous incorporons soit 0,3 ou 1,0% de Bordeaux S (BS) soit 0,12% de Jaune de Beurre (JB). La durée du traitement par les colorants étant variable, nous avons calculé la quantité moyenne d'azoïque ingérée par rat pour chaque groupe expérimental: rats âgés de 2 mois au moment du sacrifice, 1,4 g de BS (0,3%) ou 0,6 g de JB; 3 mois, 3 g de BS (0,3%) ou 1,2 g de JB; 4 mois, 16 g de BS (1%) ou 1,9 g de JB; 9 mois, 43 g de BS (1%) ou 5,1 g de JB.

Après 16–18 h. de jeûne, les animaux sont pesés et sacrifiés par décapitation. Le foie est prélevé, lavé et pesé. Nous préparons un homogénéisat de cet organe à 25% (poids/volume), par broyage (20.000 tours/min) à l'"Ultra-Turrax" pendant 30 sec dans un milieu refroidi dont la composition est 0,154 M-ClK et 1 mM-EDTA à pH 7,6. Nous centrifugeons cette préparation à 12.500 g pendant 30 min à 2°C et nous recueillons le liquide surnageant qui est exempt de mitochondries (absence d'activité succino-oxydasique), Nous déterminons la teneur en protéines de ce surnageant en appliquant la méthode de Lowry, Rosebrough, Farr et Randall (1951).

Phosphatase de 6-phosphorylglucose (*E.C. 3-1-3-9*). Nous utilisons la méthode décrite par Langdon et Weakley (1955). La quantité de phosphate minéral formée est estimée selon la technique de Briggs (1924). La concentration du surnageant dans le milieu d'incubation est de 4,6 mg foie/ml, le volume total étant de 1,1 ml.

Déshydrogénases du 6-phosphorylglucose (*E.C. 1-1-1-49*) *et du 6-phosphorylgluconate* (*E.C. 1-1-1-43*). L'activité potentielle de ces enzymes est déterminée selon la méthode de

Glock et McLean (1953), modifiée par Fitch, Hill et Chaikoff (1959). La concentration du surnageant est de 178,5 μg de foie/ml, le volume total étant de 2,0 ml.

Azoréductase (*E.C. 1-1-6-7*). Nous incubons pendant 15 min en anaérobiose stricte, un milieu comportant du surnageant, en présence de Bordeaux S. Après défécation par l'acide trichloracétique, nous déterminons le pourcentage de disparition du colorant par la mesure, à 520 nm, de la différence des densités optiques au début et à la fin de l'incubation (Manchon, Gradnauer et Lowy, 1964). La concentration finale des composants du milieu d'incubation est 50 mM-phosphate monosodique, 20 mM-nicotinamide, 1 mM-EDTA, 0,1 mM-NADP, 1,125 mM-6-phosphorylglucose, 0,2 mM-Bordeaux S et surnageant, 50 mg de foie/ml. Le milieu est tamponné à pH 7,5. La température est de 37°C. Le volume total est de 5,0 ml.

Réductase NADPH-cytochrome c (*E.C. 1-6-2-3*). Pour déterminer l'activité potentielle de cette enzyme, nous nous inspirons du travail de Hernandez, Gillette et Mazel (1967a). Nous incubons, dans des cuves de verre de 1 cm de chemin optique, un milieu comportant du surnageant, en présence de cytochrome c^{3+}. Nous mesurons, à 550 nm pendant 3 min, l'amplitude de la variation de la densité optique, correspondant à la formation de cytochrome réduit. La concentration finale des composants du milieu d'incubation est 50 mM-phosphate monosodique, 1 mM-EDTA, 1 mM-CNK, 0,042 mM-NADPH, 0,050 mM-cytochrome c^{3+} et surnageant, 0,333 g de foie/ml. Le milieu est tamponné à pH 7,6. La température est de 30°C. Le volume total est de 3,0 ml. La lecture est faite contre une cuve qui comporte le milieu complet à l'exception du NADPH. Avec Hernandez *et al.* (1967a) nous définissons l'unité de réductase NADPH-cytochrome *c* comme la quantité d'enzyme nécessaire pour produire un changement de densité optique de 1,0 à 550 nm dans le système considéré.

RESULTATS

Caractéristiques des animaux

Rats traités au Bordeaux S. L'administration de colorant alimentaire ne paraît pas modifier sensiblement le poids vif des animaux, quelles que soient les quantités de Bordeaux S ingérées ou la durée du traitement (Tableau 1). Le poids des foies n'est pas non plus généralement différent de celui des témoins. Cependant, chez les rats de 3 mois, nous remarquons que la quantité de protéines par organe est significativement réduite (17%) alors que leur concentration n'est pas modifiée (Tableau 2). Il est donc possible qu'il y ait, chez ces animaux, une légère atrophie hépatique.

Rats traités au Jaune de Beurre. L'administration du cancérogène produit un amaigrissement sensible des animaux, notamment dès le début du traitement (Tableau 1). Il donne aussi une hypertrophie du foie, laquelle s'accentue au cours du temps. Les rats de 9 mois présentent tous d'ailleurs des tumeurs très volumineuses. L'hépatomégalie se déclare bien dès le début de l'administration du colorant. Cependant, les animaux de 3 mois (quantité ingérée, 1,2 g) ont un poids du foie qui ne semble pas différent de celui des témoins. D'autre part, le Jaune de Beurre abaisse la concentration des protéines du surnageant de foie chez les animaux de 2 et 9 mois (Tableau 2); il est, en revanche, sans effet apparent chez ceux de 3 et 4 mois.

Activités enzymatiques

Rats traités au Bordeaux S. Le colorant alimentaire ne semble pas modifier sensiblement l'activité de la phosphatase du 6-phosphorylglucose (Tableau 3). Néanmoins, il est possible

Tableau 1. *Poids corporel et poids des foies des rats mâles traités au Bordeaux S ou Jaune de Beurre*

Age des animaux (mois)	Poids (g†) des rats nourris avec un régime contenant du			Poids des foies (g†) des rats nourris avec un régime contenant du		
	0 (rats témoins)	Bordeaux S	Jaune de Beurre	0 (rats témoins)	Bordeaux S	Jaune de Beurre
2	197,6 ± 3,89 (20)	199,7 ± 3,89 (20)	153,3 ± 4,49** (15)	5,14 ± 0,137 (20)	5,15 ± 0,137 (20)	7,13 ± 0,158** (15)
3	235,9 ± 8,98 (8)	210,0 ± 10,4 (6)	184,0 ± 10,4** (6)	6,53 ± 0,388 (8)	5,50 ± 0,336 (6)	7,44 ± 0,336 (6)
4	323,6 ± 7,93 (12)	317,2 ± 7,93 (12)	247,4 ± 8,68** (10)	7,39 ± 0,322 (12)	7,33 ± 0,322 (12)	9,70 ± 0,352** (10)
9	393,0 ± 14,3 (12)	420,0 ± 15,0 (11)	359,0 ± 22,2 (5)	9,79 ± 0,278 (12)	9,58 ± 0,291 (11)	15,73 ± 0,431** (5)

†Moyenne, écart-type sur la moyenne (nombre d'animaux).
Les valeurs marquées avec des astérisques diffèrent significativement de celles des témoins: $^{**}P < 0{,}01$.

Tableau 2. *Concentration des protéines du surnageant et quantité de protéines du surnageant par foie des rats mâles traités au Bordeaux S ou Jaune de Beurre*

Age des animaux (mois)	Concentration des protéines (g/100 g de foie†) chez les rats traités au			Quantité de protéines par foie (g†) chez les rats traités au		
	0 (rats témoins)	Bordeaux S	Jaune de Beurre	0 (rats témoins)	Bordeaux S	Jaune de Beurre
2	17,83 ± 0,350 (20)	17,66 ± 0,350 (20)	16,27 ± 0,404** (15)	1,024 ± 0,0348 (20)	1,025 ± 0,0348 (20)	0,872 ± 0,0401** (15)
3	17,15 ± 0,378 (8)	18,19 ± 0,437 (6)	17,01 ± 0,437 (6)	1,195 ± 0,0644 (8)	0,988 ± 0,0744* (6)	1,171 ± 0,0744 (6)
4	17,29 ± 0,310 (12)	17,36 ± 0,310 (12)	16,60 ± 0,340 (10)	1,415 ± 0,0759 (12)	1,378 ± 0,0759 (12)	1,614 ± 0,0831 (10)
9	18,09 ± 0,335 (12)	17,59 ± 0,349 (11)	16,44 ± 0,518* (5)	1,744 ± 0,0627 (12)	1,758 ± 0,0655 (11)	2,445 ± 0,0972** (5)

†Moyenne, écart-type sur la moyenne (nombre d'animaux).
Les valeurs marquées avec des astérisques diffèrent significativement de celles des témoins: $^{*}P < 0{,}05$; $^{**}P < 0{,}01$.

Tableau 3. *Activités de la phosphatase et de a déshydrogénase du 6-phosphorylg ucose et de' a déshydrogénase du 6-phosphorylg.uconate du foie des rats mâles traités au Bordeaux S ou Jaune de Beurre*

Age des animaux (mois)	Phosphatase du G6P (μmoles de P/min/100 mg protéines†) chez les rats traités au			Déshydrogénase du G6P (μmoles de NADPH/min/100 mg protéines†) chez les rats traités au			Déshydrogénase du 6PG (μmoles de NADPH/min/100 mg protéines†) chez les rats traités au		
	0 (rats témoins)	Bordeaux S	Jaune de Beurre	0 (rats témoins)	Bordeaux S	Jaune de Beurre	0 (rats témoins)	Bordeaux S	Jaune de Beurre
2	10,45 ± 0,344 (20)	10,16 ± 0,344 (20)	8,11 ± 0,397** (15)	3,49 ± 0,396 (20)	3,11 ± 0,396 (20)	5,42 ± 0,457** (15)	5,82 ± 0,292 (20)	5,72 ± 0,292 (20)	6,98 ± 0,337* (15)
3	8,66 ± 0,466 (8)	7,80 ± 0,538 (6)	7,17 ± 0,538 (6)	4,11 ± 0,565 (8)	3,61 ± 0,715 (5)	5,76 ± 0,653 (6)	4,98 ± 0,241 (8)	4,87 ± 0,305 (5)	5,38 ± 0,278 (6)
4	8,68 ± 0,269 (10)	8,25 ± 0,269 (10)	5,48 ± 0,322** (7)	3,26 ± 0,380 (12)	3,32 ± 0,380 (12)	3,53 ± 0,416 (10)	7,22 ± 0,378 (12)	6,69 ± 0,378 (12)	8,17 ± 0,414 (10)
9	6,59 ± 0,306 (12)	6,57 ± 0,319 (11)	3,98 ± 0,474** (5)	2,27 ± 0,278 (11)	2,01 ± 0,278 (11)	4,68 ± 0,412** (5)	5,95 ± 0,297 (11)	4,91 ± 0,297* (11)	9,76 ± 0,441** (5)

G6P = 6-Phosphorylglucose 6PG = 6-Phosphorylgluconate

†Moyenne, écart-type sur la moyenne (nombre d'animaux).
Les valeurs marquées avec des astérisques diffèrent significativement de celles des témoins: $*P < 0{\cdot}05$; $**P < 0{,}01$.

Tableau 4. *Activités de l'azoréductase et de la réductase NADPH-cytochrome* c *du foie des rats mâles traités au Bordeaux S ou Jaune de Beurre*

Age des animaux (mois)	Azoréductase (nmoles de Bordeaux S mét./min/100 mg protéines†) chez les rats traités au			Réductase NADPH-cytochrome *c* (unités/min/100 mg protéines†) chez les rats traités au		
	0 (rats témoins)	Bordeaux S	Jaune de Beurre	0 (rats témoins)	Bordeaux S	Jaune de Beurre
2	8,23 ± 3,56 (19)	88,0 ± 3,56 (19)	102,2 ± 4,15** (14)	16,44 ± 0,947 (12)	17,03 ± 0,947 (12)	16,0 ± 1,24 (7)
3	90,7 ± 2,67 (8)	89,3 ± 3,38 (5)	100,6 ± 3,09 (6)	—	—	—
4	88,4 ± 4,91 (12)	85,7 ± 4,91 (12)	112,6 ± 5,38* (10)	10,39 ± 0,525 (12)	10,26 ± 0,525 (12)	9,31 ± 0,525 (10)
9	82,5 ± 4,73 (12)	76,4 ± 5,18 (10)	112,8 ± 7,33** (5)	9,80 ± 0,593 (7)	10,92 ± 0,702 (5)	9,93 ± 0,702 (5)

†Moyenne, écart-type sur la moyenne (nombre d'animaux).
Les valeurs marquées avec des astérisques diffèrent significativement de celles des témoins: $*P < 0{,}05$; $**P < 0{,}01$.

que l'aminoazoïque abaisse légèrement l'activité de cette enzyme chez les rats de 3 mois. Si l'on exprime l'activité de la phosphatase non plus pour 100 mg de protéines, mais par foie entier, on trouve 102,2 $\pm$ 5,05 μmoles de phosphate minéral/min (huit animaux) pour les témoins et 76,0 $\pm$ 5,83 μmoles (six animaux) pour les rats traités par le Bordeaux S; la différence est très significative. Le Bordeaux S ne paraît jamais modifier les activités de la déshydrogénase du 6-phosphorylglucose, de l'azoréductase et de la réductase NADPH-cytochrome *c* (Tableaux 3 et 4). Par contre, il est possible que le colorant alimentaire inhibe la biosynthèse de la déshydrogénase du 6-phosphorylgluconate (Tableau 3) chez les animaux qui en reçoivent de fortes quantités (43 g; rats de 9 mois).

Rats traités au Jaune de Beurre. L'activité de la phosphatase du 6-phosphorylglucose est très nettement réduite par le colorant cancérogène, à l'exception des rats de 3 mois pour lesquels elle n'est pas modifiée (Tableau 3). Le Jaune de Beurre induit la biosynthèse de la déshydrogènase du 6-phosphorylglucose chez les animaux de 2 et 9 mois mais il n'a pas d'effet apparent chez ceux de 3 et 4 mois. L'activité potentielle de la déshydrogénase du 6-phosphorylgluconate est accrue pour les rats de 2 et 9 mois et peut-être pour ceux de 4 mois, mais elle n'est pas différente de l'activité des témoins chez les animaux de 3 mois. Le Jaune de Beurre paraît augmenter toujours l'activité de l'azoréductase (avec le Bordeaux S comme substrat) mais, là aussi, il semble que l'effet de l'aminoazoïque soit moins sensible pour les rats âgés de 3 mois (Tableau 4). Enfin, la réductase NADPH-cytochrome *c* n'est pas influencée par le traitement cancérogène.

DISCUSSION

Effets physiopathologiques du Bordeaux S

Le colorant alimentaire ne paraît pas produire de variations importantes et certaines. Tout au plus, nous pouvons dire que, chez les rats traités de 3 mois (quantité de colorant ingérée, 3 g), il y a une tendance à l'atrophie hépatique et à un ralentissement de la synthèse protéique; nous constatons un abaissement de la quantité de protéines par foie. L'organisme animal n'est donc pas tout à fait indifférent à ce composé et des modifications caractéristiques de l'intoxication peuvent apparaître, tel que l'abaissement d'activité de la phosphatase du 6-phosphorylglucose. Cependant, il faut souligner que ce phénomène est passager et réversible, puisqu'il ne se manifeste plus si l'on prolonge le traitement. Un autre effet possible du Bordeaux S est une réduction de l'activité potentielle de la déshydrogénase du 6-phosphorylgluconate chez les rats qui en ont reçu de fortes quantités (quantité ingérée, 43 g; durée du traitement, 9 mois). S'il y a effectivement une réduction, faut-il la considérer comme un indice de la toxicité du Bordeaux S? Nous pensons que non; d'autre part, parce que cet abaissement d'activité est faible, d'autre part, chez ces animaux, aucun des autres caractères étudiés n'est modifié et, en particulier, la phosphatase du 6-phosphorylglucose. Administré oralement, le colorant alimentaire, qui est un composé polaire, ne doit pénétrer que très difficilement à l'intérieur des tissus (Brodie et Hogben, 1957; Radomski et Mellinger, 1962); ce qui expliquerait en partie son innocuité.

Dans des travaux antérieurs (Manchon et Lowy, 1965 et 1968), nous avions expliqué l'action du Méthylorange sur le cycle des pentoses et sur le système d'oxydoréduction du NADP par un accroissement du fonctionnement de la déshydrogénase du 6-phosphorylglucose. Or, nous constatons, par la présente expérience, que le Bordeaux S n'induit pas la biosynthèse de cette enzyme. Ces résultats sont-ils contradictoires ou signifient-ils qu'il y a

effectivement une différence d'action entre les deux colorants? Rappelons que le Méthyl-orange a une structure chimique qui le rapproche du Jaune de Beurre.

Des études ultérieures seront nécessaires pour préciser ce point.

Effets physiopathologiques du Jaune de Beurre

Il suffit de quelques semaines d'administration de Jaune de Beurre pour que les rats soient amaigris et pour que leur foie soit hypertrophié; ceci est en accord avec les observations de nombreux chercheurs (Allard, de Lamirande et Cantero, 1957; Trams, Inscoe et Resnik, 1961). Cependant, après 2 mois d'administration du colorant (quantité totale ingérée, 1,2 g), nous remarquons à la fois un ralentissement de la croissance du tissu hépatique et de la perte du poids vif. Dès 1955, Weber et Cantero (1955) remarquaient que le Jaune de Beurre abaisse l'activité de la phosphatase du 6-phosphorylglucose. Les études histochimiques de Hadjiolov (1968 et 1969) montrent que l'hépatome n'est cependant pas dénué d'activité. Notre travail confirme que l'aminoazoïque diminue l'activité de la phosphatase dans un surnageant de foie. Le colorant est donc bien toxique selon les critères définis par Feuer *et al.* (1965). Néanmoins, nos résultats indiquent qu'il y a une discontinuité dans l'action du Jaune de Beurre. Les rats traités pendant 2 mois (quantité ingérée, 1,2 g) ont, en effet, une activité phosphatasique qui est identique à celle des animaux témoins du même âge. D'autre part, comme nous savons qu'il y a des analogies certaines entre l'effet *in vivo* des hépatotoxiques sur cette enzyme et l'effet *in vitro* des phospholipases (Feuer et Golberg, 1967), on peut penser que le Jaune de Beurre altère la structure des phospholipoprotéines du réticulum endoplasmique.

Nous constatons que le cancérogène produit parfois une augmentation de l'activité potentielle de la déshydrogénase du 6-phosphorylglucose et de la déshydrogénase du 6-phosphorylgluconate. Ces observations sont en accord avec les données de la littérature (Adamson et Fouts, 1961; Poirier et Pitot, 1970). Néanmoins, nos résultats font apparaître (comme pour la phosphatase) une évolution au cours du temps des effets du Jaune de Beurre sur ces enzymes.

Il semble donc que l'on puisse décomposer l'action de l'aminoazoïque, chez les rats mâles, en trois stades suivant la durée de son administration. Au début du traitement (rats âgés de 2 mois), tous les caractères que nous étudions sont différents de ceux des témoins; les animaux traités paraissent donc rapidement intoxiqués. Ensuite, chez les rats de 3 et 4 mois, les effets du colorant sont beaucoup moins sensibles; particulièrement, chez ceux de 3 mois pour lesquels un seul caractère (poids vif) est modifié sur les neuf que nous observons. Enfin, le dernier stade (animaux de 9 mois) concerne des rats qui présentent tous des hépatomes: nous constatons évidemment des perturbations biologiques importantes. Ainsi, nous confirmons biochimiquement les observations des morphologistes cancérologues (Lacassagne, 1966): la cancérisation du foie consiste en une succession de processus de destruction et de régénération cellulaire.

Mueller et Miller (1950) ont montré que l'administration de Jaune de Beurre à des rats diminue l'activité de l'azoréductase. Ce fait a été maintes fois confirmé (Rosenberg et Emanoil-Ravicovitch, 1965; Yanai et Kuretani, 1968). Cependant, ces chercheurs mesurent toujours l'activité de l'enzyme avec le Jaune de Beurre lui-même comme substrat ou avec un autre aminoazoïque liposoluble de structure très voisine. Dans notre travail, nous mesurons l'activité azoréductasique avec un substrat hydrosoluble, le Bordeaux S, et nous constatons que le traitement cancérogène augmente au contraire l'activité réductrice. Nous avons par ailleurs confirmé l'abaissement d'activité de l'azoréductase mesurée avec le

Jaune de Beurre (Albrecht *et al.* 1972). Nos expériences ne permettent évidemment pas d'affirmer qu'il y ait deux azoréductases hépatiques. Mais, s'il en est ainsi, on peut supposer, suivant la théorie de Monod, Changeux et Jacob (1963), que l'aminoazoïque inactive un répresseur endogène de l'azoréductase du Bordeaux S et active un répresseur de l'azoréductase du Jaune de Beurre. Cette hypothèse n'impliquerait d'ailleurs pas obligatoirement que ces deux répresseurs spécifiques appartiennent à deux espèces chimiques parfaitement distinctes.

Y aurait-il plusieurs azoréductases? Les composés qui sont métabolisés par les enzymes microsomales peuvent être classés en deux types (I et II) selon les modifications spectrales qu'ils exercent sur le cytochrome P_{450} (Remmer, Schenkman, Estabrook, Sasame, Gillette, Narasimhulu, Cooper et Rosenthal, 1966). Gigon, Gram et Gillette (1969) ont révélé que les substrats du second type ralentissent la réduction du P_{450} par le NADPH dans les microsomes hépatiques alors que les composés du type I accélèrent cette réduction. Parli et Mannering (1971) ont montré que les aminoazoïques produisent des liaisons du type II avec les microsomes. On peut alors penser que l'action du Jaune de Beurre est un peu comparable à celle des hydrocarbures polycycliques (cf. Mannering, 1971). Dans ce cas, l'administration de l'aminoazoïque modifierait la structure d'une partie du P_{450} "natif" et induirait la synthèse d'une nouvelle hémoprotéine. Si le site de liaison du cytochrome avec le Bordeaux S demeure intact, le colorant alimentaire sera réduit plus facilement parce qu'il sera en présence d'une quantité plus importante d'hémoprotéine. En revanche, pour expliquer le ralentissement de la réduction du Jaune de Beurre, on admettra que l'hémoprotéine "anormale" (P_{450} transformé, plus cytochrome induit) a perdu le site de liaison de l'aminoazoïque (type II). Ce serait alors une liaison du type I qui serait caractéristique de la nouvelle hémoprotéine, ce qui affaiblit notre hypothèse puisque précisément l'on sait que cette liaison est plus fragile que celle de type II (Mannering, 1971). On voit que l'hypothèse de l'existence d'une seule azoréductase soulève beaucoup de difficultés.

D'autre part, nous remarquons que si le traitement cancérogène modifie l'activité azoréductasique, il n'a pas d'influence en revanche sur l'activité de la réductase NADPH-cytochrome *c*. Ainsi, nous renforçons la thèse de Hernandez *et al.* (1967b) selon laquelle la réductase NADPH-cytochrome *c* intervient dans l'activité azoréductasique mais ne la recouvre pas entièrement. Il est d'ailleurs vraisemblable que, dans la chaîne microsomale de transport des électrons, un ou plusieurs intermédiaires se placent entre la réductase NADPH-cytochrome *c* et le P_{450} (Davies, Gigon et Gillette, 1969).

CONCLUSIONS

Chez les rats traités par le colorant alimentaire (Bordeaux S), nous observons parfois quelques rares modifications physiopathologiques; mais, comme celles-ci sont à la fois peu importantes et passagères, nous en concluons que le Bordeaux S ne paraît pas toxique selon les critères explorés par nos méthodes.

Les animaux traités par le colorant cancérogène (Jaune de Beurre) constituent en quelque sorte des témoins positifs. Ce colorant produit des modifications du métabolisme cellulaire qui sont un des aspects de sa toxicité et de son pouvoir cancérogène. L'action de l'aminoazoïque varie au cours de l'administration; il est possible de caractériser biochimiquement les périodes de régénération et de dégénérescence cellulaire. Chez les rats traités par le Jaune de Beurre, l'activité azoréductasique, mesurée avec un substrat hydrosoluble, est augmentée. L'activité, mesurée avec un substrat liposoluble, est diminuée. Y-a-t-il plusieurs azoréductases hépatiques?

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Time-related changes in some effects of amaranth and butter yellow in rats

Abstract—Amaranth and butter yellow are both azo dyes. The former is water-soluble and is used as a food additive. The latter is fat-soluble; it is a hepatocarcinogen in the rat. Each of these compounds was administered to male rats in the diet from weaning and their effects on several hepatic enzymes were compared after 1–8 months of treatment. The animals treated with the food colouring differed little from the controls. On the other hand, butter yellow reduced the activity of glucose 6-phosphatase and at some stages induced the biosynthesis of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. The action of butter yellow varied with the duration of administration and confirmed biochemically histological studies showing hepatocarcinogenesis to consist of succeeding processes of cellular destruction and regeneration. Azoreductase activity was affected differently by the carcinogen according to the type of substrate. It is well known that reducing activity is decreased with a fat-soluble substrate, but the results presented in this paper show, in contrast, that azoreductase activity is increased with a water-soluble substrate. This raises the question as to whether there are several azoreductases in the liver.

Zeitabhängige Änderungen einiger Wirkungen von Amaranth und Buttergelb bei Ratten

Zusammenfassung—Amaranth und Buttergelb sind Azofarbstoffe. Der erstere ist wasserlöslich und wird als Lebensmittelfarbstoff verwendet. Der letztere ist fettlöslich und wirkt bei der Ratte als Hepatocarcinogen. Jede dieser Verbindungen wurde an männliche Ratten im Futter von der Zeit des Absetzens an verabreicht und die Wirkungen aud verschiedene Leber-Enzyme wurden nach 1 bis 8 Monate währender Verabreichung verglichen. Die Tiere, die den Lebensmittelfarbstoff erhalten hatten, unterschieden sich wenig von den Kontrolltieren. Andererseits verminderte Buttergelb die Aktivität von Glucose-6-phosphatase und induzierte in einigen Stadien die Biosynthese von Glucose-6-phosphatdehydrogenase und 6-Phosphogluconatdehydrogenase. Die Wirkung von Buttergelb war während der Verabreichung unterschiedlich und bestätigte biochemisch die histologischen Untersuchungen, welche ergaben, dass die Hepatocarcinogenese aus aufeinanderfolgenden Prozessen der Zellenzerstörung und -regeneration bestand. Die Azoreduktaseaktivität wurde je nach der Substrattype von dem Carcinogen unterschiedlich beeinflusst. Es ist bekannt, dass die reduzierende Aktivität bei einem fettlöslichen Substrat abnimmt, aber die in dieser Abhandlung dargestellten Ergebnisse zeigen im Gegensatz dazu, dass die Azoreduktaseaktivität bei einem wasserlöslichen Substrat zunimmt. Daraus ergibt sich die Frage, ob verschiedene Azoreduktasen in der Leber vorhanden sind.

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Metabolism and Elimination of Sulphite by Rats, Mice and Monkeys

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Abstract—The fate of ingested sulphite was investigated in rats, mice and monkeys using dose levels of 10 and 50 mg SO_2/kg administered as $NaHSO_3$ mixed with $Na_2{}^{35}SO_3$. Most (70–95%) of the ingested [^{35}S]sulphite was absorbed from the intestine and voided in the urine of all three species within 24 hr. Most of the remaining ^{35}S was eliminated in the faeces, the rate of this elimination being species-dependent. Residual ^{35}S in the animal carcasses after 1 wk accounted for 2% or less of the administered dose in all cases. No free sulphite was detected in rat urine even after administration of a single oral dose as high as 400 mg SO_2/kg. Induction of liver sulphite oxidase could not be demonstrated after a single dose of 200 mg SO_2/kg nor after 50 or 200 mg SO_2/kg/day given for 30 days. In addition, electron-microscopic examination of both the liver and kidneys of these same animals showed no abnormality.

INTRODUCTION

The subject of food additives and their safety has generated much interest and concern recently. Part of this concern is due to the lack of basic knowledge about how these various chemicals behave in the body and the fear that we may be doing irreparable damage to ourselves without realizing it. There is no uncertainty about the presence of one food additive, sulphur dioxide and sulphur dioxide-containing materials, in our daily diet. Compounds such as sodium or potassium sulphite, bisulphite and metabisulphite, in addition to sulphur dioxide itself, are widely used as bacteriostatic agents, bleaching or anti-browning agents and antioxidants. When these compounds are dissolved in water, they dissociate into bisulphite (HSO_3^-) and sulphite (SO_3^{2-}) ions, the relative proportions of each depending upon the pH of the solution. In the presence of water and the organic constituents of foods or beverages, the possibilities for their chemical reaction are numerous, the three most common being oxidation to sulphate (Postgate, 1963; Schroeter, 1963), addition across carbonyl groups (Braverman, 1953; Ingles, 1959) and addition to disulphide linkages in proteins (Milligan & Swan, 1962; Parker & Kharasch, 1959). The human body is consequently presented with many compounds in greater concentration than would normally be found in food, and the actual amount of ingested unbound sulphite is unknown. The fate of any free sulphite in the body is of interest in view of recent findings that it is capable of producing genetic mutation, as indicated by *in vitro* tests showing bisulphite alteration of uracil, cytosine and yeast RNA, as well as mutation in phage λ and *Escherichia coli* (Hayatsu & Miura, 1970; Hayatsu, Wataya & Kai, 1970; Hayatsu, Wataya, Kai & Iida, 1970; Mukai, Hawryluk & Shapiro, 1970; Shapiro, Cohen & Servis, 1970; Shapiro, Servis & Welcher, 1970).

In this paper, we report a study of the ability of three species of animal to eliminate ingested sulphite and the capacity of the rat in particular to cope with large doses of this chemical.

EXPERIMENTAL

Methods of sulphite analysis

Sulphite in aqueous solution was assayed either by the colorimetric method based on pararosaniline, as described by the Public Health Service (1963), or by iodometric titration (Pierce, Haenisch & Sawyer, 1962). Pararosaniline hydrochloride was purchased from Fischer Scientific Co., Pittsburgh, Pa, and was purified according to Scaringelli, Saltzman & Frey (1967). These methods were used to test the stability of aqueous solutions of Na_2SO_3, $NaHSO_3$ and mixtures of the two compounds. The solutions, prepared with doubly glass-distilled water to give a final sulphite concentration of 0·44 mM, were allowed to stand in open vessels at room temperature and aliquots were removed for analysis at intervals between 0 and 24 hr.

A third method of sulphite analysis, using radioactive *N*-[1-^{14}C]ethylmaleimide (^{14}C-NEM) was used for urine samples, as described by Mudd (1968). Radioactive ^{14}C-NEM, specific activity 9·8 mC/m-mole, was purchased from New England Nuclear, Boston, Mass. Unless otherwise specified, urine analysis was carried out under nitrogen in a 50 μl reaction mixture, which contained the urine sample and 40 nmoles ^{14}C-NEM in 50 mM-phosphate, pH 7·4. The phosphate buffer had been previously flushed with nitrogen for 15 min and maintained under a nitrogen atmosphere until its addition to the reaction mixture. Since only small volumes of the ^{14}C-NEM were needed for each reaction mixture, the purchased reagent was diluted with cold 95% ethanol just prior to its addition to the reaction mixture to facilitate volume measurement. These dilutions were in the 3–7-fold range for the experiments described. If there was any delay before the urine sample was added, reaction mixtures were stored in ice until used. For any given experiment, this time period did not exceed 3 hr.

After addition of the sample, the nitrogen-flushed tubes were incubated for at least 30 min at room temperature before being spotted on paper for chromatography. Descending chromatography was carried out on Whatman no. 1 chromatography paper for 16 hr using *tert*-butanol–88% formic acid–water (70:15:15, by vol.) as solvent. D,L-Methionine was used as a control marker adjacent to sample spots and was located by the use of a 0·1% aqueous potassium permanganate spray. The sample strips were scanned for radioactivity on a Vanguard Model 880 Low-Background Autoscanner. Quantitation was achieved by triple elution of the ^{14}C-NEM-SO_3^- area of the chromatogram with water by capillary action followed by counting of the eluate in Bray's solution. Control experiments indicated that virtually 100% of the radioactivity was recovered in the eluate. A linear relationship existed between the number of nmoles sulphite added to the reaction mixture and the eluted radioactivity tested, between 0 and 14 nmoles sulphite.

In control experiments related to the ^{14}C-NEM-SO_3^- assay, difficulty was encountered in obtaining a quantitative recovery of the unreacted ^{14}C-NEM after paper chromatography. A preliminary investigation of this problem indicated that the unreacted reagent was unstable in both acidic and basic chromatographic solvent systems, those tested being *tert*-butanol–88% formic acid–water (70:15:15, by vol.), 95% ethanol–pyridine (95:5, v/v), and 95% ethanol–28·8% ammonium hydroxide (95:5, v/v). In contrast, a known amount

of the ^{14}C-NEM-SO_3^- addition compound chromatographed in the *tert*-butanol–formic acid–water system was subsequently eluted from the paper with complete recovery of radioactivity.

The difference in stability between NEM and its sulphite addition compound was further demonstrated when a small aliquot of each was spotted on chromatography paper, followed by an aliquot of 1 M-hydrochloric acid. The acid caused complete loss of radioactivity from the free unreacted reagent upon evaporation, but no loss from the addition compound. Apparently, the free reagent was degraded to yield volatile components containing the radioactivity. To verify that intact reactive ^{14}C-NEM was present in excess of all the reactive components in urine samples, routine runs were carried out with a control reaction mixture to which excess unlabelled sulphite was added. The amount of radioactivity found in the sulphite-NEM peak from this control reaction mixture after chromatography was several times greater than the summation of all radioactive components found in urine samples. Thus sufficient reagent was present to react with any sulphite present in the treated urine samples.

Sulphite-balance experiments

Rodents. Albino rats (90–100 g body weight) were obtained from the Holtzman Co., Madison, Wisc., and albino mice (15 g body weight) from C. L. Rolfsmeyer Co., Madison, Wisc. The animals were housed in stainless-steel metabolism cages, with a screen attached to the cage bottom beneath the existing wire floor to facilitate separation of food, urine and faeces, and were fed *ad lib.* with Wayne Lab-Blox, purchased from Allied Mills Inc., Chicago, Ill.

Using all-glass feeding tubes of approximately 15 ml capacity, twelve rats were given $NaHSO_3$ mixed with tracer amounts of $Na_2{}^{35}SO_3$ (from New England Nuclear) in a dose of 50 mg SO_2/kg (giving $3{\cdot}37 \times 10^7$ counts/min) in 5·0 ml 0·5% glucose. Generally the animals were dosed at 16.00 hr and after the dose had been consumed, usually by 22.00 hr, water was provided *ad lib.* The longest time taken for dose consumption was less than 14 hr. Groups of three rats were killed at the end of 24 and 48 hr from the time of administration, during which periods urine and faeces were collected, and the remaining two groups of three rats were killed 1 and 2 wk after dosing. All animals were killed by a blow on the head and were frozen whole prior to the determination of the radioactivity remaining in the carcass. For determination of levels of activity in the urines and faeces, food was separated from faeces manually and from urine by filtration through Whatman no. 1 filter paper on a Büchner funnel. The cage floor, screen and funnel were washed with distilled water and the washings were added to the urine. Any food that might have been contaminated with urine was soaked in water and filtered and the filtrate was also added to the urine. The experiment was repeated in another group of rats, using a dose of 10 mg SO_2/kg.

Mice were also given a dose of 50 mg SO_2/kg and the experiments were conducted in essentially the same way as the rat experiments, except that the dose was administered in 0·5 ml of drinking-water and groups of six to eight mice were used for urine and faeces collections and killed at each time interval.

Monkeys. Rhesus monkeys (*Macaca mulatta*) were provided by the Primate Research Center, Madison, Wisc. The five females and one male, each weighing approximately 6 kg, were housed in stainless-steel metabolism cages, which permitted separation of urine and faeces. Food and water were provided *ad lib.* Each received by stomach tube a single dose

of 50 mg SO_2/kg in the form of 15 ml of a $Na_2{}^{35}SO_3$–$NaHSO_3$ solution ($3{\cdot}3 \times 10^8$ counts/min) in 0·5% glucose. Urine and faeces were collected at 24-hr intervals for 5 days, and analysed for radioactivity. The collecting funnel was rinsed with water after each daily collection and the rinsings were added to the urine sample. At the end of the experiment, each cage was washed thoroughly, the washings were separated into a solid and liquid fraction by filtration through Whatman no. 1 filter paper, and the solid and liquid fractions were tabulated respectively as part of the faeces and urine collected on day 5. In the case of the solids, the filter paper was dissolved as described below along with the solids, prior to sampling for measurement of radioactivity.

Study of sulphite excretion in urine

Three types of experiment were carried out in an attempt to define the level at which sulphite ingestion would lead to excretion of unaltered sulphite in rat urine.

The first consisted of feeding 100-g rats daily for 5 days with 5 ml of either 0·5% glucose, 5 mg SO_2 as $NaHSO_3$ in 0·5% glucose, or 20 mg SO_2 as $NaHSO_3$ in 0·5% glucose. These doses (0, 50 and 200 mg/kg) were given each morning at 09.00 hr and when they had been consumed, fresh unsulphited water was provided. A 10 μl sample of freshly voided urine was taken when each rat had consumed 90–100% of the dose. These samples, obtained on days 1, 2, 3 and 5, were treated with ^{14}C-NEM for sulphite analysis as described on p. 186.

In another experiment, groups of six rats were fed doses of $NaHSO_3$ daily for 30 days at a level of 50 or 200 mg SO_2/kg. On day 30, the rats were given their usual dose and from each dose group, as well as from a control group, two rats, one male and one female that had finished at least 50% of their daily dose in 4 hr, were sampled (13·5 μl) for urinary sulphite content.

Finally, a short-term study was carried out in which two rats (100 g body weight) were each given by stomach tube a 400 mg SO_2/kg dose of $NaHSO_3$ in 0·5% glucose. Urine samples of 25 μl were obtained at 30 min and 1, 2 and 3 hr after dose administration and were assayed for sulphite. The 85 μl reaction mixture under nitrogen contained 52 nmoles ^{14}C-NEM and the urine sample in 57 mM P_i, pH 7·4.

Freshly voided urine samples

Freshly voided urine samples were easily obtained from rats by placing the animal on a clean glass plate. This usually led to urination. If not, the animal was drawn slowly backward by the tail or was elevated slightly to raise his back legs off the glass. Urine samples were drawn up immediately into a micropipette and discharged into the reaction mixture within 15 sec of voidance.

Preparation of samples for radioactivity determination

Urine solutions were sampled directly for oxidation. Each frozen carcass was ground in a meat grinder and placed in a large beaker. The faeces and carcass were liquefied by solution in modified Pirie's reagent (Pirie, 1932) consisting of 3 vols conc. nitric acid and 1 vol. 60% perchloric acid and 13·3 g magnesium nitrate/100 ml of the acid mixture. Generally, the reagent was added cold to the carcass in a 1:1 (v/w) ratio and to the faeces in a 15:1 (v/w) ratio. The solutions were then heated slowly in a sand bath to 150°C with vigorous stirring when necessary to dissipate any foam produced. Faeces solutions were then sampled directly for further oxidation. Carcass solutions were transferred while still warm into a separating

funnel and were permitted to cool to room temperature. A residual foam, which collected at the surface, was found to contain negligible radioactivity. The liquid phase was separated from the foam and sampled for further oxidation.

The final oxidation of all samples was carried out in triplicate according to the method of Jeffay, Olubajo & Jewell (1960). Radioactivity was determined in a Packard Tri-Carb Liquid Scintillation Spectrophotometer, series 314-E at 3°C. The counting efficiency was monitored by addition of an internal standard.

Sulphite-oxidase induction

One-day study. Twenty-four male rats (200 g body weight) were dosed by stomach tube with 200 mg SO_2/kg as 3 ml of a 0·5% glucose solution of $NaHSO_3$. Groups of three rats were killed by a blow on the head at 3-hr intervals. The livers were weighed and prepared for assay of sulphite oxidase and protein determination (see below).

Thirty-day study. Eighteen rats were divided into three groups each of three males and three females. Each day at 15.30 hr, the animals were weighed and given a dose of either 0·5% glucose (5 ml/100 g body weight), 50 mg SO_2/kg as $NaHSO_3$ in 0·5% glucose at a concentration of 1 mg SO_2/ml, or 200 mg SO_2/kg as $NaHSO_3$ in 0·5% glucose at a concentration of 4 mg SO_2/ml. When these doses had been consumed, usually by 22.00–24.00 hr, fresh unsulphited drinking-water was provided until the next morning. The drinking-water was removed between 07.00 and 09.00 hr and the animals were deprived of liquid until the afternoon dosing time. All rats were killed on the morning of day 31, the livers were weighed and samples of both liver and kidney were removed for microscopic examination. The remaining parts of the livers were prepared for sulphite-oxidase assay and protein determination.

Sulphite-oxidase assays and protein determinations. The rat livers were minced with scissors and then homogenized with 15 vols cold (−20°C) acetone in a Waring blender for 2 min at top speed. The homogenate was suction-filtered and the filter-cake was washed with another 5 vols acetone followed by a small volume of ethyl ether. The filter cake was then crumbled and sifted through screen. After most of the ether had been allowed to evaporate, the dry powder was stored at −20°C in an evacuated desiccator over solid sodium hydroxide.

Approximately 25 mg of the dry powder was stirred continuously for 30 min at room temperature with 1·0 ml 0·01 M-K_2HPO_4 and 1×10^{-4} M-EDTA, pH 7·2. This extract was then centrifuged for 10 min in an International Clinical Centrifuge at full speed and the supernatant was assayed for sulphite oxidase by the method of Cohen & Fridovich (1971). Cytochrome *c* was used as the electron acceptor, as this system is unaffected by non-specific sulphite oxidation (MacLeod, Fridovich & Handler, 1961). One unit of enzyme activity is defined as that amount which causes an absorbance change of 0·10/min at 550 nm. Supernatant protein contents were determined by the method of Lowry, Rosebrough, Farr & Randall (1951) using crystalline bovine serum albumin from Sigma Chemical Co. (St. Louis, Mo.) as a standard.

Microscopy

Rat livers and kidneys were fixed, sectioned and examined by light and electron microscopy as described by Allen, Carstens, Norback & Loh (1970).

RESULTS

Levels of intake

As a starting point for these experiments, information was needed about the quantity of sulphite normally ingested by man in his everyday diet*. Our estimates are based on the sulphite content of various foods, together with the quantity of each consumed. These estimates are shown in Table 1, along with the acceptable daily intake and the conditionally acceptable intake recommended by the Joint FAO/WHO Expert Committee on Food Additives (1965).

Table 1. *Estimated daily human intake of dietary sulphite in the United States*

Levels of intake	mg/kg*	mg/70-kg man*
US average	0·10	7·2
US maximum	1·7	120
FAO/WHO "acceptable"	0·35	25
FAO/WHO "conditionally acceptable"	1·5	105

*Expressed as SO_2.

The estimated *per capita* consumption of SO_2-containing foods was based on a total population of 200 million people. When wine and beer were being considered, the drinking population was assumed to be 75% of the total. Solid foods and non-alcoholic beverages contributed approximately 2 mg SO_2/day to the total, while alcoholic beverages accounted for the remainder (beer 1·2 and wine 4·0 mg SO_2/day). The "US maximum" designation in Table 1 indicates the upper end of the normal range of intake.

Stability of sulphite in solution

Most of the SO_2 consumed in the adult diet is derived from beverages. The method of choice for its administration in animal experiments would therefore be to include it in the drinking-water. A prerequisite for experiments of this nature is the determination of the stability of sulphite in aqueous solutions under the conditions of its intended use. The results of such an investigation are shown in Figs 1 and 2. Figure 1 is based on the colorimetric pararosaniline method and Fig. 2 on the iodometric titration method. The results obtained by both methods of analysis were similar. A negligible amount of sulphite remained in solutions of Na_2SO_3 after a 24-hr period, while $NaHSO_3$ solutions were much more stable. As has been indicated by other investigators, the stability of $NaHSO_3$ solutions with respect to oxidation could be further enhanced by addition of various compounds (Bigelow, 1898; Young, 1902). In Fig. 1, 0·5% glucose is shown to enhance the stability of a $NaHSO_3$ solution to such a degree that negligible sulphite loss occurred in a 24-hr period; myo-inositol had a similar effect, as shown in Fig. 2. The similarity of the effect produced by these two compounds indicates that stabilization by glucose is not due *per se* to the formation of a carbonyl-bisulphite addition compound, since such addition is not possible with myo-inositol. Tested in this way, concentrations of bisulphite up to 20 mM in 0·5% glucose showed at most a 6% loss in 24 hr.

*Dietary intakes and experimental dosages are given in terms of the equivalent amounts of SO_2.

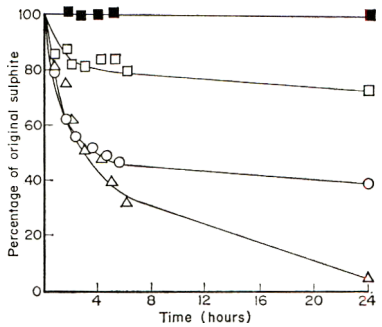

FIG. 1. Stability of sulphite in aqueous solutions. Na_2SO_3 (△), $NaHSO_3$ (□) and a mixture of the two in doubly glass-distilled water (○) or in 0·5% aqueous glucose (■) were prepared to give a final concentration of 0·44 mM-sulphite. The solutions were allowed to stand in open vessels at room temperature and, at intervals, aliquots were removed for sulphite analysis by the pararosaniline method.

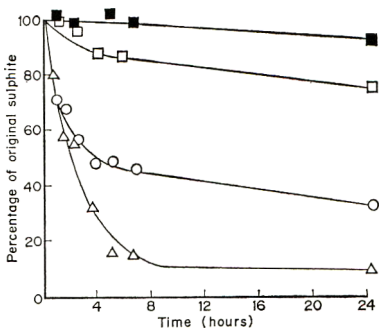

FIG. 2. Stability of sulphite in aqueous solutions. Na_2SO_3 (△), $NaHSO_3$ (□) and a mixture of the two in doubly glass-distilled water (○) or in 0·5% aqueous myo-inositol (■) were prepared to give a final concentration of 0·44 mM-sulphite. The solutions were allowed to stand in open vessels at room temperature and, at intervals, aliquots were removed for sulphite analysis by the iodine titration method.

Sulphite-balance experiments with rats, mice and monkeys

Rats. The results of the study of the fate of ^{35}S given to rats in a single dose of 50 mg SO_2/kg are shown in Table 2. The highest and lowest values for the percentage of the dose

Table 2. *Disposition of ^{35}S given to rats in a single dose of bisulphite*

Group no.	Time after dosing (days)	Total radioactivity (% of administered dose) in			Recovery (%)
		Urine	Faeces	Carcass	
1	1	74–79	4–17	9–21	100–105
2	2	75–84	13–18	4–7	97–104
3	7	—	—	2	—
4	14	—	—	1	—

Values given are the highest and lowest found in groups of three rats (body weight 91–97 g). All were given a dose of 50 mg SO_2/kg in 5 ml 0·5% glucose, as $NaHSO_3$ containing $3·37 \times 10^7$ counts/min as $Na_2{}^{35}SO_3$.

contained in the urine, faeces and carcass are presented. Three experiments of this type have given similar results, in that 70–80% of the dose appeared in the urine in 24 hr and the carcass contained on average about 15% after 24 hr, 6% after 48 hr, 2% after 1 wk and 1% after 2 wk. The overall recovery for each of the rats for which this was determined was 100 ± 8%. The experiments were carried out at a dose level of 10 as well as 50 mg SO_2/kg. A comparison of the results (Fig. 3) shows a similar rate of elimination at both dose levels. The height of the bars represents mean values and the maximum and minimum variations are indicated.

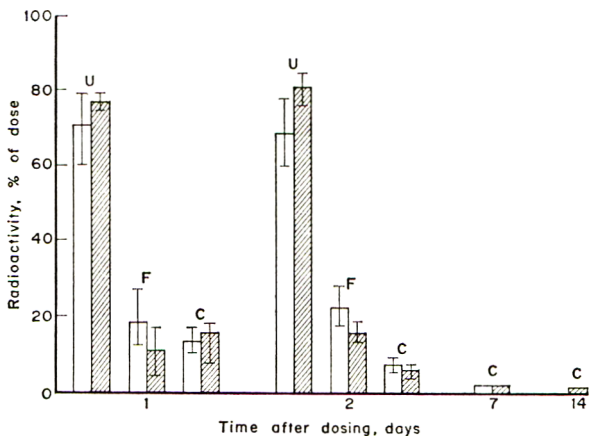

FIG. 3. Disposition of ^{35}S administered to rats. A single dose of $NaHSO_3$ in 0·5% aqueous glucose containing tracer $Na_2{}^{35}SO_3$ was administered to rats in their drinking water. The content of sulphite was equivalent to either 10 (□) or 50 (▨) mg SO_2/kg body weight. The urine (U), faeces (F) and/or carcass (C) content of ^{35}S was determined as indicated and is presented as a percentage of the dose.

Mice. The results of the balance experiments in mice are shown in Table 3. Mean values are given for the percentage of the administered dose found in urine, faeces and carcass, with the range displayed by individual animals indicated in parentheses. As might be expected from their faster metabolic rate, mice eliminated sulphite more quickly than did rats. Both sexes appeared to behave identically in these experiments.

Table 3. *Disposition of ^{35}S given to mice in a single dose of bisulphite*

Group no.	No. of mice/group	Time after dosing (days)	Total radioactivity (% of administered dose) in			Recovery (%)
			Urine	Faeces	Carcass	
1	7	1	78·7 (72–86)	15·6 (12–37)	3·1 (2–5)	100·1 (92–104)
2	6	2	80·8 (77–84)	14·8 (12–18)	1·8 (1·5–2)	97·6 (94–100)
3	8	7	—	—	0·83 (0·7–1·1)	—
4	8	14	—	—	0·36 (0·3–0·5)	—

Values are expressed as the means (with ranges in parentheses) for the numbers of animals stated. All mice were given a dose of 50 mg SO_2/kg in 0·5 ml drinking water, as $NaHSO_3$ containing 1·24 or 1·80 × 10^7 counts/min as $Na_2{}^{35}SO_3$.

Monkeys. The results of radioactivity determinations on the urine and faeces of monkeys given a single dose of 50 mg SO_2/kg labelled with ^{35}S are presented in Table 4. The physical relationship and structure of the cages did not eliminate all possible urine cross-contamination between monkeys housed in adjacent cages; this fact very probably accounts for the high individual variation and high and low total recoveries in the case of individual animals (Table 4). It is clear, however, that virtually all of the ^{35}S was eliminated from the body in 3 days. An efficient voidance of the dose via the urinary tract was demonstrated in the first 24 hr. In contrast to rats and mice, which consistently eliminated 15–20% of the administered dose in their faeces, the greatest cumulative amount found in the faeces of these six monkeys was 6%, the remainder being recovered in the urine. This may be indicative of a species difference or, alternatively, could be attributable to the different method of dosage.

Table 4. *Disposition of ^{35}S administered to monkeys as a single dose of sodium bisulphite*

Time after dosing (days)	Recovery (% of administered radioactivity) in	
	Urine	Faeces
1	94·9 (80·0–105·9)	1·8 (0·3–3·0)
2	3·2 (2·6–4·0)	2·2 (0·7–3·0)
3	1·1 (0·7–1·4)	0·4 (0·2–1·0)
4	0·6 (0·3–0·8)	0·2 (0·0–0·3)
5	0·7 (0·2–1·4)	0·1 (0·0–0·1)
Total recovery ...105·2% (91–115)		

Values are expressed as the means (with ranges in parentheses) for groups of six monkeys given by stomach tube a dose of 50 mg SO_2/kg in 15 ml 0·5% glucose, as $NaHSO_3$ containing $3·3 \times 10^8$ counts/min as $Na_2{}^{35}SO_3$.

Sulphite in urine

It was of interest to try to define what level of ingested sulphite could be tolerated by rats before they would start to excrete unaltered sulphite in their urine. No indication of any sulphite excretion was found in rats given up to 200 mg SO_2/kg/day either for 5 or for 30 days. In the short-term study carried out to investigate the possibility that in the previous experiments sulphite present in the urine could have been voided before sampling time, chromatography of urine samples taken 30 min–3 hr after dosing revealed no detectable increases in sulphite excretion compared with samples taken before SO_2 administration. Control reaction mixtures, to which excess sulphite was added at the end of the 3-hr study as described on p. 187, indicated that if sulphite were indeed present, it would form the ^{14}C-NEM-SO_3^- product under these conditions. We conclude, therefore, that at no time during the course of these experiments did we saturate the animals' ability to oxidize the administered sulphite. In view of this finding, it was of interest to see whether the animals were capable of metabolizing sulphite at these levels with the amount of sulphite oxidase normally found in their bodies or whether they had available to them a readily inducible enzyme system on which they could draw.

Sulphite-oxidase induction

Possible sulphite-oxidase induction was first studied over a 1-day period using a dose of 200 mg SO_2/kg. The results of this short-term study are shown in Table 5. Since the specific activity in all the livers studied was nearly identical, it was obvious that no enzyme induction occurred in the short time allowed. A 30-day experiment was then undertaken using dose

Table 5. *Sulphite-oxidase induction experiment carried out in rats over 24 hr*

Group no.	Time after dosing (hr)	Mean liver weight (% of body weight)	Mean enzyme activity (units/mg protein)
1	0	4·87	4·58
2	3	4·49	4·40
3	6	4·53	4·51
4	9	4·19	4·42
5	12	4·25	4·21
6	15	5·08	4·72
7	18	5·08	4·43
8	21	4·94	4·60

Values are means for groups of three rats.

levels of 50 and 200 mg SO_2/kg/day. All animals appeared healthy throughout the 30-day course of the experiment and the growth rates of all the rats in the three groups were identical, with one exception. The results are shown in Table 6, except for this one animal which showed a difference in growth rate.

This rat was a male in the group given the high dose level. He gained very little weight for 6 days and then began losing weight until his death on day 13. His liver, unfortunately not removed until several hours after death and frozen at −20°C for 2 wk prior to study, was found to be somewhat large, being 6·1% of his total body weight of 81 g. Assay of the liver for sulphite oxidase showed only half the specific activity (2·63 units/mg protein) of that found in the other experimental animals. Whether this enzyme level represented the amount that would have been found in this animal when the liver was fresh or a decreased level due to freezing and deterioration of the liver after death is not known.

Table 6. *Sulphite-oxidase induction experiment carried out in rats over 30 days*

Group	Dose (mg SO_2/kg)	Sex	Mean liver weight (% of body weight)	Mean enzyme activity (units/mg protein)
1	0	M	4·23	5·12
		F	4·07	5·92
2	50	M	4·43	4·83
		F	4·08	5·89
3	200	M	4·70	4·67
		F	4·03	5·66

Values are means for three males or three females in each group.

It is clear from the data in Table 6 that the levels of sulphite administered did not induce the production of increased amounts of sulphite oxidase. Apparently the existing enzyme levels are fully capable of metabolizing the amounts of sulphite administered, in that no enzyme induction was observed and no free unaltered sulphite was excreted in the urine, as described earlier.

Microscopy

Examination of both the livers and kidneys of these animals by light microscopy showed no lesion in either tissue that was attributable to consumption of bisulphite by the rats. A general survey was conducted with the electron microscope as well, in which approximately nine different sections from each tissue were evaluated; all were found to be normal.

DISCUSSION

Our estimates of SO_2 consumption in the United States represent the *per capita* consumption of adults only, since children are not included in the figures used for wine and beer consumption. These values are of necessity very approximate and probably low, since a complete list of SO_2-containing foods together with their annual consumption levels was not available. However, we did attempt to account for all major SO_2 sources. A detailed list of the figures used in formulating this estimate is not presented, for a more complete study is in progress and is expected to be published shortly (in the FDA review of GRAS list substances). Our estimate is of the same order of magnitude as that reported by Bigwood (1968) for the daily SO_2 consumption in Belgium. The major difference between the two is found in his value for SO_2 consumed in alcoholic beverages, viz. 14 mg/day yielding a *per capita* total of 16·3 mg/day, as compared to our estimate of 5·2 mg/day with a *per capita* total of 7·2 mg/day.

Glucose was chosen to protect sulphite solutions from air oxidation because it occurs in the body and in foods and would not be expected to cause any adverse effect. The dissociation constant for the glucose–bisulphite addition compound is of the order of $6{\cdot}4 \times 10^{-1}$, so that the reaction is readily reversible (Burroughs & Whiting, 1960). This was apparent in sulphite analysis with the pararosaniline colorimetric assay and in assays of sulphite oxidase, where the presence of glucose did not alter the extent or rates of reaction. In balance experiments, sulphite was administered to rats and mice in the drinking-water, rather than by stomach tube, to approximate more closely the mode of intake in man. An unusually large or concentrated dose would have produced an unnatural situation for the animal to cope with and might have altered the results or produced injury. This would have been the preferred method of administration with monkeys as well, but a reliable all-glass feeding system was not available and administration by stomach tube was the only feasible method for supplying a measured dose.

The number of animals used in these studies was relatively small. Greater numbers would undoubtedly have narrowed the range of individual values observed. Our objective, however, was primarily to establish the general overall trend with respect to the disposition of orally administered inorganic sulphite. From these studies, several points seem clear.

Sulphite is readily absorbed from the digestive tract of mice and rats, since about 70% of the administered radioactivity appeared in the urine in 24 hr. The similar voidance rate at both the 10 and 50 mg/kg dose levels indicates that neither appears to overload the body's capacity to metabolize sulphite, even though these doses are 100–500 times greater than our

estimates for the average daily intake by man. The relatively good recovery in balance studies in both the rat and mouse shows that very little, if any, ingested SO_2 is eliminated by exhalation. The residual isotope that remains in the carcass could be bound to membrane proteins or other body components with a slow turnover rate or it could be concentrated in a specific tissue. These points are at present under investigation. In monkeys, the absorption of sulphite from the intestinal tract appears to be somewhat more efficient than in rats and mice. However, as already discussed, this may be due to the use of different methods for dose administration.

All attempts to detect free sulphite in the urine of treated rats failed. Sulphite in the urine should be a valid indication of saturating the capacity for sulphite metabolism, since the work of Irreverre, Mudd, Heizer & Laster (1967) showed that when sulphite oxidase was deficient in an infant, excess sulphite was eliminated by this route. A minor urinary component, which reacts with ^{14}C-NEM and chromatographs in the vicinity of the sulphite–NEM product, made exact quantitation difficult, but control experiments indicated that sulphite at a concentration of 0·1 mM could be detected above background on a chromatogram scan with this assay system. In the experiment in which a single dose of 400 mg/kg was given, an aliquot of urine was assayed which was large enough to permit the detection of 40 nmoles sulphite/ml. Assuming that the daily urinary volume for a 100-g rat is about 8·0 ml, we could have detected 320 nmoles total sulphite or 0·05% of the total dose administered. In the remaining experiments, if the same assumptions are made, the detection limit is 0·25% for a 200 mg/kg dose and 1% for a 50 mg/kg dose.

The capacity of mammals to metabolize sulphite appears quite large. Wilkins, Greene & Weller (1968) showed that in dogs, the liver alone could metabolize at least 50 mg SO_2/kg/hr, and studies of MacLeod, Farkas, Fridovich & Handler (1961) on rats have shown that the liver is not the only tissue readily able to metabolize appreciable quantities of sulphite. Thus, the doses of sulphite used in our study may not have been large enough to produce sulphite in the urine. This considerable metabolic capacity already in existence may also account for our inability to produce demonstrable sulphite-oxidase induction. Higher doses were not investigated in this study, as the levels used greatly exceeded our estimated daily SO_2 intake by the public in this country. The normal microscopic appearance of the liver and kidneys of rats maintained on high doses of sulphite over a period of time further support the implication of the biochemical data, that the animal is apparently under no stress at these levels of sulphite.

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Métabolisme et élimination du sulfite par le rat, la souris et le singe

Résumé—On a étudié chez des rats, des souris et des singes la destinée du sulfite ingéré. On a employé à cette fin des doses de 10 et de 50 mg SO_2/kg administré sous forme de $NaHSO_3$ mélangé de $Na_2{}^{35}SO_3$. La plus grande partie (70–95%) du [^{35}S]-sulfite ingéré a été absorbée à partir de l'intestin et déversée dans les 24 h dans l'urine des trois espèces animales en cause. La plus grande partie du ^{35}S restant a été éliminée avec les fèces, le taux de cette élimination dépendant de l'espèce animale. Le ^{35}S résiduel présent après 1 semaine dans les carcasses des animaux a représenté dans tous les cas 2% ou moins de la dose administrée. On n'a pas décelé de sulfite libre dans l'urine des rats, même après l'administration d'une dose orale unique, même aussi élevée que 400 mg SO_2/kg. L'induction de l'oxydase du sulfite dans le foie n'a pas pu être mise en évidence après une dose unique de 200 mg SO_2/kg ni après 30 jours d'administration de 50 ou de 200 mg SO_2/kg/jour. De plus, l'examen au microscope électronique du foie et des reins de ces mêmes animaux n'a révélé aucune anomalie.

Stoffwechsel und Ausscheidung von Sulfit durch Ratten, Mäuse und Affen

Zusammenfassung—Das Schicksal aufgenommenen Sulfits wurde bei Ratten, Mäusen und Affen mittels Verabreichung in Dosierungen von 10 und 50 mg SO_2/kg, verabreicht als $NaHSO_3$ gemischt mit $Na_2{}^{35}SO_3$, untersucht. Das meiste (70–95%) des aufgenommenen [^{35}S]Sulfits wurde aus dem Darm aufgenommen und von allen drei Arten innerhalb von 24 Stunden mit dem Urin ausgeschieden. Der grösste Teil des restlichen ^{35}S wurde mit den Faeces ausgeschieden, wobei die Geschwindigkeit dieser Ausscheidung abhängig von der Tierart war. Das in den Tierleichen nach 1 Woche gefundene ^{35}S betrug 2% oder weniger der verabreichten Dosis in allen Fällen. Im Rattenurin wurde kein freies Sulfit gefunden, selbst wenn eine orale Einzeldosis bis zu 400 mg SO_2/kg verabreicht worden war. Die Induktion von Lebersulfitoxydase konnte weder nach einer Einzeldosis von 200 mg SO_2/kg noch nach 50 oder 200 mg SO_2/kg/Tag, für die Dauer von 30 Tagen verabreicht, nachgewiesen werden. Ausserdem zeigte die elektronenmikroskopische Untersuchung der Leber und der Nieren dieser gleichen Tiere keine Anomalie.

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Antioxidants and Carcinogenesis: Butylated Hydroxytoluene, but not Diphenyl-*p*-phenylenediamine, Inhibits Cancer Induction by *N*-2-Fluorenylacetamide and by *N*-Hydroxy-*N*-2-fluorenylacetamide in Rats*

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Abstract—The effect of butylated hydroxytoluene (BHT) and diphenyl-*p*-phenylenediamine (DPPD) on cancer induction by *N*-2-fluorenylacetamide (FAA) and its *N*-hydroxy derivative (N-OH-FAA) has been studied in two strains of rat. Groups of Charles River rats were fed carcinogen and antioxidant in a molar ratio of 1:30 (223 ppm FAA or 239 ppm N-OH-FAA (1 mM) and 7850 ppm DPPD or 6600 ppm BHT) in Wayne Chow for 24 wk (males) or 32 wk (females). Male and female rats then continued on control diet for another 12 wk.

With FAA alone 70% of male rats had hepatoma and 20% of females had mammary adenocarcinoma; with N-OH-FAA, 60% of males had hepatoma and 70% of females had mammary adenocarcinoma. The simultaneous administration of DPPD failed to alter cancer induction by these agents in male or female rats. However, BHT reduced the incidence of hepatoma in males to 20% when the carcinogen was FAA. With N-OH-FAA, BHT lowered the incidence of liver tumours to 15% and of mammary cancer in females to 35%.

Similar data on inhibition by BHT were obtained using different levels of carcinogens in rats of the Fischer strain. Equimolar levels of sulphate failed to increase liver-tumour incidence in animals given N-OH-FAA and BHT. Also, BHT failed to depress the excretion of free urinary inorganic sulphate.

Liver- and oesophageal-tumour induction with 51 ppm diethylnitrosamine in drinking water for 24 wk was not affected by BHT or DPPD, nor was tumour induction by propane sultone.

INTRODUCTION

Several antioxidants are widely used as preservatives for both industrial products and foodstuffs. Vitamin E or α-tocopherol is a naturally occurring antioxidant. There have been numerous studies on the physiological and chronic effects of such materials by themselves, including tests of their safety in the human environment (Dacre, 1970; Deichmann, Clemmer, Rakoczy & Bianchine, 1955; Gaunt, Feuer, Fairweather & Gilbert, 1965; Joint FAO/WHO Expert Committee on Food Additives, 1962; Pascal, 1969; Pascal, Durand & Penot, 1970; Pascal & Terroine, 1969; Tappel, 1970). However, there have been relatively few experiments on the interaction of these materials with other potentially harmful chemicals.

The present paper describes results obtained in studies on the effect of two antioxidants,

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diphenyl-*p*-phenylenediamine (DPPD), employed chiefly as an antioxidant in industrial products, and butylated hydroxytoluene (BHT), so used in foodstuffs, especially in oils and fats. Their interactions with two chemical carcinogens inducing tumours in a variety of target organs in rats, namely *N*-2-fluorenylacetamide (FAA) and its metabolically derived proximate carcinogen, *N*-hydroxy-*N*-2-fluorenylacetamide (N-OH-FAA), were examined. The effect of DPPD and BHT on carcinogenesis induced by propane sultone and diethylnitrosamine (DEN) was also studied.

EXPERIMENTAL

Male and female weanling Charles River CD SPF rats were maintained on a diet of Wayne laboratory meal until they were 6 wk old, when they were assigned to the various experimental and control groups. (Details of all dose schedules are tabulated in the Results section.) The rats were housed in large plastics cages, initially in groups of three and subsequently in groups of two, on corn-cob bedding with free access to water and diet, under conditions of controlled lighting and temperature. The animals were inspected daily, and were weighed weekly in the first 3 months of the experiments and then every other week.

Male rats in this test series were fed the experimental diets for 24 wk and females for 32 wk, and were then given the control diet of Wayne Laboratory Blox. Males were killed at wk 37 and females at wk 44.

In another series of experiments, male rats of the Fischer strain were procured at weaning and were treated as the Charles River rats, described above. However, they were maintained on their respective diets for only 16 wk and continued on control diet for another 10 wk, at which time they were killed. Urine analyses were performed on rats of this strain to determine whether dietary administration of 5000 ppm BHT affected the excretion of free or bound sulphate.

All animals were carefully autopsied at the end of the test. Selected organs and liver, kidneys and spleen were weighed. All tissues, including liver, kidneys, spleen, heart, urinary bladder, mammary-gland tissue and all grossly abnormal tissues were fixed in 10% buffered formalin. The tissues were routinely processed for histological examination by conventional techniques. Sections were stained with haematoxylin and eosin and studied microscopically.

Diets were prepared in two steps. The finely powdered chemical was mixed with ground Wayne laboratory meal to prepare a concentrate, and this was then incorporated in the remainder of the meal in a V-blender. Diets were freshly prepared at least once a week and were stored in a cold room until they were offered to the animals.

Other groups of rats fed DPPD or BHT or control diet were given the carcinogen, DEN, in the drinking water at a level of 51 ppm for 24 wk. Additional animals on these diets were given 40 mg propane sultone/kg by gavage once a week for 33 wk. These animals were killed after a further 11 wk under control conditions.

RESULTS

Effect of BHT on liver cancer induction by FAA and N-OH-FAA in Charles River rats

When FAA was fed alone, seven of ten rats bore hepatomas and two had hyperplastic nodules with atypia, giving a total of nine animals with liver lesions (Table 1). With N-OH-

Table 1. *Effect of BHT and DPPD on induction of neoplasms and other proliferative lesions by FAA and N-OH-FAA in male and female Charles River rats*

Carcinogen* and dietary level (ppm)	Antioxidant* and dietary level (ppm)	No. of rats/group	Nos of animals affected with: Lesions of liver parenchyma†		Mammary lesions†		Other lesions†
			Hyperplasia	Hepatoma	Adenoma	Adeno-carcinoma	
			Males				
0	0	20	0	0	0	0	0
0	DPPD, 7850	10	0	0	0	0	0
0	BHT, 6600	10	0	0	0	0	0
FAA, 223	0	10	2	7	0	0	Cystic biliary hyperplasia 1
FAA, 223	DPPD, 7850	20	4	13	0	0	Cystic biliary hyperplasia 2; biliary adenoma 2; biliary carcinoma 1; glioma 2
FAA, 223	BHT, 6600	20	7	4	0	0	Cystic biliary hyperplasia 5; biliary adenoma 1; alveolar adenoma, lung 1; granulocytic leukaemia 1; squamous cell carcinoma, ear duct 1; papilloma, urinary bladder 1
N-OH-FAA, 239	0	10	3	6	0	0	Cystic biliary hyperplasia 6; biliary adenoma 1
N-OH-FAA, 239	DPPD, 7850	20	3	11	0	0	Cystic biliary hyperplasia 10; biliary adenoma 4; granulocytic leukaemia 1; malignant lymphoma 1
N-OH-FAA, 239	BHT, 6600	20	3	3	0	0	Cystic biliary hyperplasia 4; biliary carcinoma 2; adenocarcinoma, colon 1; biliary adenoma 4
			Females				
0	0	20	0	0	0	0	0
0	DPPD, 7850	10	0	0	0	0	0
0	BHT, 6600	10	0	0	0	(1)‡	0
FAA, 223	0	10	3	0	0	2 (1)‡	Cystic biliary hyperplasia 3
FAA, 223	DPPD, 7850	20	4	1	5	7	Cystic biliary hyperplasia 2; biliary adenoma 3; theca cell tumour, ovary 1
FAA, 223	BHT, 6600	20	4	0	1	6	Cystic biliary hyperplasia 10; biliary adenoma 3; squamous cell carcinoma, ear 1
N-OH-FAA, 239	0	10	2	0	1	7 (3)‡	Cystic biliary hyperplasia 2; biliary adenoma 1
N-OH-FAA, 239	DPPD, 7850	20	1	0	1	11	Cystic biliary hyperplasia 6; biliary adenoma 1; squamous cell carcinoma, ear duct 2; transitional cell papilloma, kidney 1, urinary bladder 1
N-OH-FAA, 239	BHT, 6600	20	0	0	1	7	Cystic biliary hyperplasia 3; squamous cell carcinoma, ear duct 1; follicular adenoma, thyroid 1; focal mucosal hyperplasia, urinary bladder 2

*Diets with the agents were fed to male rats for 24 wk and to females for 32 wk. Surviving males were killed at 37 wk and females at 44 wk.
†Only the most abnormal lesion in any tissue is listed.
‡No. of rats with metastases.

FAA, six of the ten rats had hepatoma or hepatocellular carcinoma, one with metastases. In addition, three animals had hyperplastic nodules and seven had cystic biliary hyperplasia or adenoma.

In a group of 20 male rats fed FAA and BHT, only four had hepatoma. There were seven rats with hyperplastic nodules and six with cystic biliary hyperplasia or adenoma. In a group of 20 rats fed BHT and N-OH-FAA, only two had hepatoma and one had hyperplastic nodules with atypia. Two additional rats had carcinoma of the bile ducts and four more had adenoma of the bile ducts. Three had hyperplastic nodules and four had cystic biliary hyperplasia.

In ten female rats fed FAA there were no liver cancers, but three animals had hyperplastic nodules in the liver and three more had cystic biliary hyperplasia. With N-OH-FAA, there

were again no liver cancers. Two animals had hyperplastic nodules and three had cystic biliary hyperplasia or adenoma. In a group of 20 females given BHT and FAA, there were three biliary adenomas. The non-cancerous lesions included four rats with hyperplastic nodules and ten with cystic biliary hyperplasia. In the group of 20 females fed BHT plus N-OH-FAA there were no liver cancers, but three rats had cystic biliary hyperplasia.

Effect of DPPD on liver-tumour formation

This antioxidant failed to affect the production of liver tumours by FAA or N-OH-FAA. Rats given DPPD in addition to the carcinogen exhibited an incidence of liver tumours (hepatomas or hyperplastic nodules with atypia) similar to that seen in those fed carcinogen alone. Precancerous lesions such as hyperplastic nodules were also present to a similar extent. In these groups, females showed a single liver tumour and presented a picture in respect of non-malignant liver lesions similar to that in female rats administered the carcinogens alone.

Effect of BHT on mammary-tumour formation with FAA and N-OH-FAA

Under the conditions of this experiment, two of ten rats fed FAA exhibited adenocarcinoma of the mammary gland, including one case with metastases to the lung. Seven of ten females given N-OH-FAA had adenocarcinomas, including three with metastases. One had a mammary adenoma and one a fibroadenoma.

One female given BHT alone had mammary adenocarcinoma which metastasized to the lung. Six of the 20 rats fed the mixture of BHT and FAA had mammary adenocarcinoma, and seven of the 20 given BHT plus N-OH-FAA had adenocarcinoma and one had an adenoma.

Miscellaneous tumours

In addition, these various groups of rats had single tumours at sites such as the ear duct, colon, urinary bladder and renal pelvis (transitional cell papilloma), granulocytic leukaemia, theca cell ovarian tumour, alveolar pulmonary adenoma and lymphoma, but none of these could be related to an effect of the antioxidant in groups of rats fed the carcinogens.

Effect of BHT on liver tumour formation in male rats of the Fischer strain

Administration of 150 ppm FAA or 160 ppm N-OH-FAA induced liver lesions in all or almost all of the animals. Administration of 5000 ppm BHT decreased the carcinogenic effect of either compound on the liver to a moderate extent. Simultaneous administration of additional sodium sulphate in the diet failed to affect this inhibition with either carcinogen (Table 2). Also, in contrast to *p*-hydroxyacetanilide (Büch, Rummel, Pfleger, Eschrich & Texter, 1968; Yamamoto, Williams, Richardson, Weisburger & Weisburger, 1973), BHT failed to affect excretion of free or bound sulphates by rats of this strain (Table 3).

Effect of DPPD on carcinogenicity of propane sultone

In this series, in which rats were given a lower level (40 mg/kg) of this carcinogen for a shorter period of time (44 wk) than in an earlier study (Ulland, Finkelstein, Weisburger, Rice & Weisburger, 1971), there was a low incidence of gliomas, mammary tumours and intestinal tumours. DPPD, however, failed to affect the relative yield of tumours at these various sites.

Table 2. *Effect of BHT and sodium sulphate on liver-tumour induction by FAA and N-OH-FAA in male rats of the Fischer strain*

Carcinogen and dietary level (ppm)	Dietary level of sodium sulphate (ppm)	Dietary level of BHT (ppm)	No. of rats/group	No. of animals with liver lesions	
				Hyperplasia	Hepatoma
0	0	0	4	0	0
0	0	5000	5	0	0
FAA, 150	0	0	10	1	9
FAA, 150	0	5000	15	13	2
FAA, 150	8400	5000	12	11	1
N-OH-FAA, 160	0	0	11	4	7
N-OH-FAA, 160	0	5000	22	11	11
N-OH-FAA, 160	8400	5000	17	13	4

The chemicals were fed for 16 wk followed by the control diet of Wayne Laboratory Chow for a further 10 wk.

Table 3. *Failure of BHT to affect urinary excretion of free inorganic sulphate in a 24-hr period by male Fischer rats*

Chemical and dietary level (ppm)	No. of rats	Volume of urine† (ml)	Urinary sulphate				
			Total† (mg)	Free		Bound*	
				(mg)†	(% of total)	(mg)†	(% of total)
0	4	9·8 ± 0·5	65 ± 5	52 ± 7	79	13 ± 2	21
BHT, 5000	4	11·8 ± 0·3	54 ± 1	46 ± 2	82	10 ± 2	18
p-OHAA‡, 8800	2	13·1 ± 0·1	47 ± 2	24 ± 3	52	23 ± 1	48

*Released by hydrolysis at 100°C for 20 min at pH 1.
†Means ± SEM, for nos of rats shown.
‡*p*-Hydroxyacetanilide, which combines with sulphate (Büch *et al.* 1968; Yamamoto *et al.* 1973) and was therefore used as a positive control.

Effect of DPPD or BHT on tumour induction by DEN

DEN by itself induced a high yield of hepatocellular carcinoma with metastases, as well as some cancers in the oesophagus. Neither BHT nor DPPD altered this carcinogenic effect (Table 4).

DISCUSSION

The salient results obtained in an examination of the effect of two commercial antioxidants on the activity of a number of carcinogens indicated that some of these mixtures did and others did not interact as regards the carcinogenicity of the agents tested. Interpretation of the results gave some clues on the underlying mechanisms. Liver-tumour induction by the carcinogens FAA or N-OH-FAA was reduced with the antioxidant BHT. Mammary-tumour formation was decreased with BHT only when the carcinogen was N-OH-FAA. At first, it seemed as though BHT, which has a phenolic hydroxy group, could modify the

Table 4. *Effect of BHT and DPPD on the induction of tumours and other proliferative lesions in Charles River rats given 51 ppm DEN in the drinking water*

Antioxidant and dietary level (ppm)	No. of rats/group	No. of animals with					
		Liver lesions		Oesophageal lesions*			
		Hyperplasia	Hepatoma†	Atypical hyperplasia	Papilloma	Carcinoma	Other lesions
				Males			
0	10	—	10 (9)	—	1	4	Cystic biliary hyperplasia 1
DPPD, 7850	20	—	20 (9)	—	3	—	Focal mucosal hyperplasia, urinary bladder 1
BHT‡, 6600	20	1	17 (7)	2	1	10	Tubular adenoma, kidney 1; biliary adenoma 1; gastric papilloma 1
				Females			
0	10	—	10 (4)	4	—	1	Cystic biliary hyperplasia 1; squamous cell carcinoma, forestomach 1
DPPD, 7850	20	—	19 (11)	1	—	—	Tubular adenoma, kidney 2; biliary adenoma 1; cystic biliary hyperplasia 3
BHT‡, 6600	20	—	19 (9)	—	—	4	Tubular adenoma, kidney 2; gastric papilloma 1; cystic biliary hyperplasia 2

*Only the most abnormal lesion in any single tissue is listed for each animal.
†Nos in parentheses are the nos of rats with metastases, usually to the lungs.
‡Simultaneous controls given 6600 ppm BHT without DEN had no lesions.
All groups were given 51 ppm DEN in the drinking water for 24 wk and survivors were killed 11 wk later.

carcinogenicity of FAA and N-OH-FAA by the binding of sulphate ion, which is known to affect cancer induction to some extent in the liver (De Baun, Smith, Miller & Miller, 1970; Weisburger, Yamamoto, Williams, Grantham, Matsushima & Weisburger, 1972) though not in the mammary gland (Irving, Janss & Russell, 1971). However, tests of this concept indicated that BHT probably did not operate thus, for the excretion of free sulphate in the urine was unchanged by BHT intake.

It is equally improbable that the antioxidant properties of BHT played a role. If they were involved, the equally effective antioxidant, DPPD, should have acted likewise, but it had no effect on cancer incidence.

Wattenberg (1972a) has recently reported on the effect of certain antioxidants on chemical carcinogenesis by polycyclic hydrocarbons in specific target organs. BHA at a level of 10,000 ppm in the diet inhibited forestomach-tumour formation in mice due to benzo[*a*]-pyrene or 7,12-dimethylbenz[*a*]anthracene (DMBA). However, BHT at a similar level had only a borderline effect in the Ha/ICR mouse strain. Nonetheless, in A/HEJ mice a level of only 5000 ppm BHT led to a significant reduction in tumours. In another system, a single dose of 200 mg BHT/ml in oil given prior to an effective tumour-inducing dose of DMBA significantly lowered the incidence of mammary tumours in female Sprague–Dawley rats and also inhibited the adrenal necrosis typically seen. In skin-tumour formation in mice, on the other hand, antioxidants had no effect.

It has been reported in a number of relevant experiments that rats pretreated with DPPD, BHT or other antioxidants were partially protected against a hepatotoxic dose of carbon tetrachloride (Cawthorne, Bunyan, Sennitt, Green & Grasso, 1970; Di Luzio, 1967; Di Luzio & Costales, 1965; Hartman, Di Luzio & Trumbull, 1968). Likewise, antioxidants prevented ethanol-induced fatty liver and abnormal triglyceride metabolism. Gallagher (1962) reported previously that antioxidants maintained normal levels of purine nucleotides in the liver under these conditions. Also, BHT and other antioxidants increased the average survival time of mice and rats, especially as a function of the composition and amount of dietary fat fed to these animals (Harman, 1968 & 1969). Further, Frankfurt, Lipchina,

Bunto & Emanuel (1967) demonstrated that administration of 3000 ppm BHT inhibited the induction of liver tumours by a carcinogenic azo dye in two strains of rats, which showed a 65–90% hepatoma incidence when fed the dye alone. Concurrently, the amount of carcinogen bound to tissue protein was decreased.

While some investigators have interpreted their results as a function of the antioxidant properties of the agents fed or administered, it would seem that our own results, in the main, cannot be explained on this basis.

Others have described the powerful induction of enzymes in rat liver by BHT (Botham, Conning, Hayes, Litchfield & McElligott, 1970; Gilbert, Martin, Gangolli, Abraham & Golberg, 1969). That this feature of BHT is responsible for decreasing the carcinogenicity of FAA and N-OH-FAA is shown in the following paper (Grantham, Weisburger & Weisburger, 1973). Briefly, it seems that BHT induces enzymes like glucuronyl transferase, which increase the formation of glucosiduronic acid leading to excretion of the carcinogen metabolites. Certain other enzyme inducers like phenobarbitone given at the same time as the carcinogen have acted likewise (Matsushima, Grantham, Weisburger & Weisburger, 1972; Peraino, Fry & Staffeldt, 1971).

While DPPD has not been tested for enzyme-inducing potential under these conditions, our results suggest that it would be inactive.

Considering that carcinogens such as DEN also require biochemical oxidation, of course of a different type from FAA and its derivatives, it is somewhat curious that BHT failed to affect the carcinogenicity of this agent. For a different tumour system, Wattenberg (1972b) did note that antioxidants inhibited DEN carcinogenesis. On the other hand, propane sultone, being a direct-acting alkylating agent which does not require biochemical modification, should not have been affected by enzyme inducers. However, if these agents could also act as nucleophilic trapping agents there should have been an effect, but none was found.

The antioxidants administered at high levels for a long time appeared to delay certain ageing processes and reduce so-called spontaneous tumour incidence under certain conditions. This was ascribed to the possible trapping of free radicals (Harman, 1968 & 1969). While this is possible, our current results show that cancers induced by certain carcinogens can be inhibited not by a free-radical trapping process, but mainly by decreased biochemical activation and increased detoxification of these agents.

BHT and similar antioxidants have not yet found universal approval for stabilizing certain components of diets ingested by man. For example, the Joint FAO/WHO Expert Committee on Food Additives (1962) gave only conditional acceptance to BHT and felt that additional studies on the effect of BHT on lipid metabolism were indicated. The combined data now available from a sizable body of studies indicate that antioxidants administered to animal model systems more often than not decrease a toxic, and indeed as we show here, a carcinogenic effect (Wattenberg, 1972a,b). Lower levels may have a more discrete but certainly not an adverse effect. Also, in chemical structure and physiological properties such synthetic agents are similar to the essential and useful Vitamin E. It would seem, in the light of the potentially beneficial effect, that the current attitude to the use of all such agents needs to be re-examined.

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Les antioxydants et la carcinogenèse: L'hydroxytoluène butylé, mais non la diphényl-*p*-phénylènediamine, inhibe chez le rat l'induction de cancers par le *N*-2-fluorénylacétamide et par le *N*-hydroxy-*N*-2-fluorénylacétamide

Résumé—On a étudié sur deux souches de rats l'effet de l'hydroxytoluène butylé (BHT) et de la diphényl-*p*-phénylènediamine (DPPD) sur l'induction de cancers par le *N*-2-fluorénylacétamide (FAA) et son dérivé *N*-hydroxy (N-OH-FAA). On a incorporé le carcinogène et l'antioxydant dans le rapport molaire 1:30 (223 ppm FAA ou 238 ppm N-OH-FAA (1 mM) et 7850 ppm DPPD ou 6600 ppm BHT) à l'aliment (Wayne Chow) et servi celui-ci pendant 24 semaines à des mâles et pendant 32 semaines à des femelles Charles Rivers. Mâles et femelles ont ensuite reçu le régime alimentaire témoin pendant 12 semaines.

70% des mâles qui avaient consommé l'aliment additionné uniquement de FAA ont contracté des hépatomes et 20% des femelles soumises au même régime ont fait des adénocarcinomes mammaires. Des hépatomes ont été constatés chez 60% des mâles et des adénocarcinomes mammaires chez 70% des femelles qui avaient consommé l'aliment additionné uniquement de N-OH-FAA. L'administration simultanée de DPPD n'a pas pu modifier l'induction de cancers par ces deux agents ni chez les mâles ni chez les femelles. Le BHT, par contre, a réduit à 20% la fréquence des hépatomes chez les mâles qui avaient consommé le FAA. Dans le cas du N-OH-FAA, le BHT a réduit la fréquence des tumeurs du foie à 15% et celle des cancers mammaires chez les femelles à 35%.

On a obtenu des données similaires quant à l'inhibition par le BHT lorsqu'on administrait différentes doses de carcinogènes à des rats de souche Fischer. Des taux équimolaires de sulfate n'ont pas pu faire augmenter la fréquence des tumeurs du foie chez les animaux qui recevaient le N-OH-FAA et le BHT. Le BHT n'a pas plus fait diminuer l'excrétion urinaire de sulfate inorganique libre.

L'induction de tumeurs du foie et de l'oesophage par 51 ppm de diéthylnitrosamine ajoutées pendant 24 semaines à l'eau de boisson n'a pas été modifiée par le BHT ni par le DPPD. Le propane-sulfone aussi est resté sans effet sur l'induction de tumeurs.

Antioxydantien und Carcinogenese: Butyliertes Hydroxytoluol, aber nicht Diphenyl-*p*-phenylendiamin hemmt die Induktion von Krebs durch *N*-2-Fluorenylacetamid und durch *N*-Hydroxy-*N*-2-fluorenylacetamid bei Ratten

Zusammenfassung—Der Einfluss von butyliertem Hydroxytoluol (BHT) und Diphenyl-*p*-phenylendiamin (DPPD) auf die Krebsinduktion durch *N*-2-Fluorenylacetamid (FAA) und sein *N*-Hydroxyderivat (N-OH-FAA) wurde an zwei Rattenstämmen untersucht. Gruppen von Charles-River-Ratten erhielten Carcinogen und Antioxydans bzw. Alterungsschutzmittel in einem Molverhältnis von 1:30 (223 ppm FAA oder 239 ppm N-OH-FAA (1 mM) und 7850 ppm DPPD oder 6600 ppm BHT) in Wayne Chow auf die Dauer von 24 Wochen (männliche) oder 32 Wochen (weibliche Tiere). Die männlichen und die weiblichen Ratten erhielten dann noch 12 Wochen lang Kontrollfutter.

Mit FAA allein wurde bei 70% der männlichen Ratten Hepatom und bei 20% der weiblichen Ratten mammäres Adenocarcinom induziert, mit N-OH-FAA bei 60% der [männlichen Tiere Hepatom und bei 70% der weiblichen mammäres Adenocarcinom. Die gleichzeitige Anwendung von DPPD änderte die Krebsinduktion durch diese Mittel bei den männlichen und den weiblichen Ratten nicht. BHT verminderte jedoch die Häufigkeit von Hepatom bei männlichen Tieren auf 20%, wenn das Carcinogen FAA war. War das Carcinogen N-OH-FAA, dann senkte BHT die Häufigkeit von Lebertumoren auf 15% und die von mammärem Carcinom bei weiblichen Tieren auf 35%.

Ähnliche Daten der Inhibition durch BHT wurden bei Verwendung verschiedener Carcinogenkonzentrationen bei Ratten des Fischer-Stammes erzielt. Äquimolare Konzentrationen von Sulfat erhöhten nicht die Lebertumorhäufigkeit bei Tieren, die N-OH-FAA und BHT erhielten. BHT verursachte auch keine verminderte Ausscheidung freie anorganischen Sulfats im Urin.

Die Induktion von Leber- und Speiseröhrentumoren mit 51 ppm Diäthylnitrosamin im Trinkwasser auf die Dauer von 24 Wochen wurde von BHT oder DPPD nicht beeinflusst, ebenso wenig die Induktion durch Propansulton.

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Effect of the Antioxidant Butylated Hydroxytoluene (BHT) on the Metabolism of the Carcinogens *N*-2-Fluorenylacetamide and *N*-Hydroxy-*N*-2-Fluorenylacetamide

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Abstract—The effect of the antioxidant butylated hydroxytoluene (BHT) on the metabolism of *N*-2-fluorenylacetamide (FAA) and *N*-hydroxy-*N*-2-fluorenylacetamide (N-OH-FAA) was studied in male and female rats. The administration of 6600 ppm BHT in the diet for 4 wk increased the liver-to-body weight ratio and led to the excretion in the urine of a larger percentage of a single ip dose of either carcinogen. This higher level of excretion was accounted for chiefly by glucuronic acid conjugates. Sulphuric acid esters were decreased. Also lower were the levels of radioactivity in blood, in liver and bound to liver DNA 48 hr after injection of the labelled carcinogen. It is concluded that BHT increases the detoxification metabolites of FAA and N-OH-FAA, and thus lowers the amount of a given dose available for activation reactions.

INTRODUCTION

The carcinogenicity of agents of the aromatic amine type, typified by *N*-2-fluorenylacetamide (FAA), is strongly dependent on a number of endogenous and exogenous factors (Miller, 1970; Weisburger, 1973; Weisburger & Weisburger, 1958). Thus, the incidence and types of tumours, the latent period and other relevant factors are dependent on the species, strain and sex of the test animal as well as on the presence of dietary and other chemical factors. Among these, certain antioxidants were of interest, for such substances are now widely used to stabilize industrial products as well as foodstuffs (Lundberg, 1962; Stuckey, 1968). In studying the effect of two typical antioxidants, diphenyl-*p*-phenylenediamine and butylated hydroxytoluene (BHT), it was discovered that the former had no effect on the carcinogenicity of FAA or its *N*-hydroxy metabolite (N-OH-FAA) in rats. On the other hand, BHT given in the diet with the carcinogen in a molar ratio of 30:1 considerably decreased the carcinogenicity of FAA and of N-OH-FAA in the liver and the mammary gland, two key target organs of these agents in male and female rats (Ulland, Weisburger, Yamamoto & Weisburger, 1973).

The question arose by which mechanism one but not the other antioxidant exerted this effect. It is improbable that the difference was due specifically to the antioxidant properties of these agents, since only one elicited a depressing effect. Also, a lead was the high dose levels required. Antioxidants would typically operate at lower doses. Thus, it seemed necessary to consider the possibility that BHT modified the carcinogenic response by its ability to induce liver enzymes, an ability already demonstrated in connexion with studies of the metabolism of other drugs and agents (Gaunt, Feuer, Fairweather & Gilbert, 1965;

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Gilbert & Golberg, 1965; Sharratt, Gangolli & Grasso, 1970). We now report that this is the principal, although perhaps not the exclusive, scheme by which BHT reduces the carcinogenicity of FAA, namely by increasing detoxification metabolites. Consequently, less of the carcinogen is available for the required activation reactions.

EXPERIMENTAL

Materials. BHT was obtained through the courtesy of Dr. J. P. Frawley of Hercules, Inc., Wilmington, Delaware. FAA was purchased from Aldrich Chemical Co. and N-OH-FAA was procured through the kind cooperation of Dr. H. B. Wood, Jr., Drug Development Branch, National Cancer Institute. Isotopically labelled FAA and N-OH-FAA were purchased from New England Nuclear Co., Boston, Mass., and β-glucuronidase (Type II) was from Sigma Chemical Co., St. Louis, Mo. All other reagents, and standard metabolites for the evaluation of products by thin-layer and paper chromatography, were available from earlier studies (Matsushima, Grantham, Weisburger & Weisburger, 1972).

Treatment of animals. Twelve male and 12 female 6-wk-old Charles River CD rats were fed 6600 ppm BHT, mixed in the diet, for 4 wk. Twelve male and 12 female rats were maintained as controls on Wayne laboratory meal (Ulland *et al.* 1973). After the 4-wk period, six male and six female rats pre-fed BHT, as well as six of each sex from the controls, were given a single ip injection of labelled FAA in a dose of 22·3 mg/kg (specific activity 0·48 mC/m-mole). Another group of six male and six female rats pre-fed BHT, and also six controls of each sex were given a single ip dose (23·9 mg/kg) of labelled N-OH-FAA (specific activity 0·50 mC/m-mole).

The animals were transferred to metabolism cages (Acme Metal Co., Cincinnati, Ohio) permitting separate collection of urine and faeces. Urines were collected in ice-cold receivers for 2 time periods, 0–24 and 24–48 hr. At 48 hr the rats were killed under light ether anaesthesia by withdrawal of blood from the abdominal aorta. The livers were removed and weighed, and an aliquot was homogenized and assayed for radioactivity. DNA was isolated from the liver homogenate of the male rats, and the specific radioactivity was determined (Matsushima *et al.* 1972).

Analysis of metabolites. Samples of 0–24 hr urines were analysed for metabolites of FAA and N-OH-FAA by methods previously described (Matsushima *et al.* 1972). Briefly, after determination of the total radioactivity in a sample, urines were extracted with ether to remove the free metabolites. The aqueous phases were subjected to hydrolysis with β-glucuronidase and the metabolites liberated, formerly present as glucosiduronic acids, were extracted with ether. Lastly, mild acid hydrolysis, followed by neutralization and ether extraction gave information on sulphuric acid conjugates. Determination of radioactivity was carried out in a liquid scintillation spectrophotometer with external standardization. Determination of protein and nucleic acid were carried out as already described.

RESULTS

Distribution of radioactivity

Compared with control animals, both male and female rats pre-fed BHT exhibited considerable liver enlargement (Table 1), as already found in previous studies such as that of Gilbert & Golberg (1965). More total radioactivity was present in the livers of male than

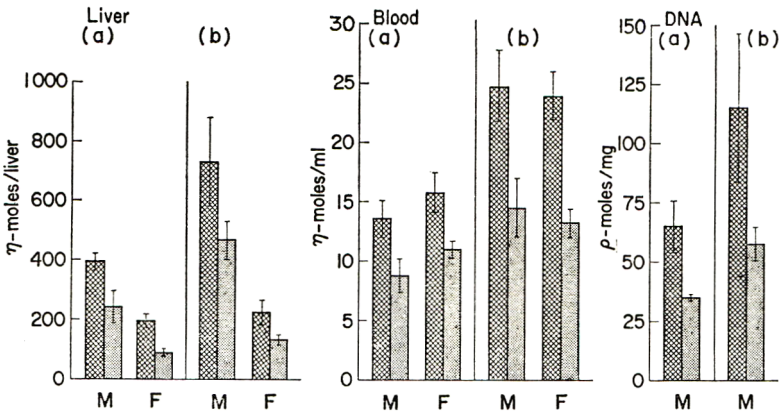

FIG. 1. Levels of radioactivity representing metabolites of FAA (a) and of N-OH-FAA (b) in the liver and blood of males and females and bound to liver DNA in males only, 48 hr after an ip injection of the labelled carcinogen in rats fed a diet containing 0 (control; ⊠) or 6600 ppm BHT (▦) for the previous 4 wk. Data are means ±SEM for groups of six rats.

of female rats (Fig. 1). It was also higher in rats given N-OH-FAA than in those given FAA. In all these cases, however, pre-feeding with BHT reduced the total amount of radioactivity found in the livers. Likewise, radioactivity in the blood and blood plasma collected from the rats at 48-hr exhibited the same relative effects, except that the levels were almost identical in males and in females. Significantly, BHT pre-feeding decreased circulating carcinogens.

Table 1. *Body and liver weights, and urinary radioactivity in male and female rats pre-fed 6600 ppm BHT in the diet for 4 wk and injected with a single dose of labelled FAA or N-OH-FAA*

Treatment		Body weight (g)	Liver weight (% of body weight)	^{14}C in urine (% of dose) at	
Diet	Injection			24 hr	48 hr
		Males			
—	FAA	396 ± 20	3·6 ± 0·3	40·9 ± 3·3	3·9 ± 0·7
BHT	FAA	363 ± 19	5·6 ± 0·4	51·8 ± 9·6	3·4 ± 1·5
—	N-OH-FAA	370 ± 18	3·3 ± 0·2	40·8 ± 0·7	7·2 ± 1·4
BHT	N-OH-FAA	335 ± 23	5·2 ± 0·5	63·8 ± 2·8	1·6 ± 0·4
		Females			
—	FAA	242 ± 9·2	3·7 ± 0·2	42·8 ± 2·1	3·6 ± 0·5
BHT	FAA	225 ± 11	5·5 ± 0·1	67·7 ± 1·4	2·1 ± 0·3
—	N-OH-FAA	228 ± 18	2·9 ± 0·2	41·7 ± 3·7	3·0 ± 0·1
BHT	N-OH-FAA	235 ± 6·0	5·2 ± 0·5	68·7 ± 4·2	2·3 ± 0·1

Values are the means for groups of six rats ±SEM.

DNA from male rats only was isolated and purified. In this instance, in parallel with the situation in whole liver, DNA had a higher level of radioactivity in animals injected with N-OH-FAA than in those injected with FAA (Fig. 1). In both cases, and indeed more so with N-OH-FAA, BHT reduced the amount of carcinogen bound firmly to DNA. Since

the amount of activity bound to DNA paralleled the total liver radioactivity in males, the same was probably true for females. Therefore, the DNA isolation step was omitted.

Urinary radioactivity

Both male and female rats excreted similar amounts of radioactivity, representing metabolites of the injected FAA or N-OH-FAA (Fig. 2). Most of the isotope was found in the first 24 hr (Table 1). In rats of both sexes, BHT increased very considerably the amount of radioactivity excreted from both carcinogens. In females, about 70% of the carbon-14 was eliminated in the 48-hr period.

Irrespective of whether BHT was fed or not, there were relatively few changes as regards the urinary excretion of the free metabolites, which amounted to only a small proportion

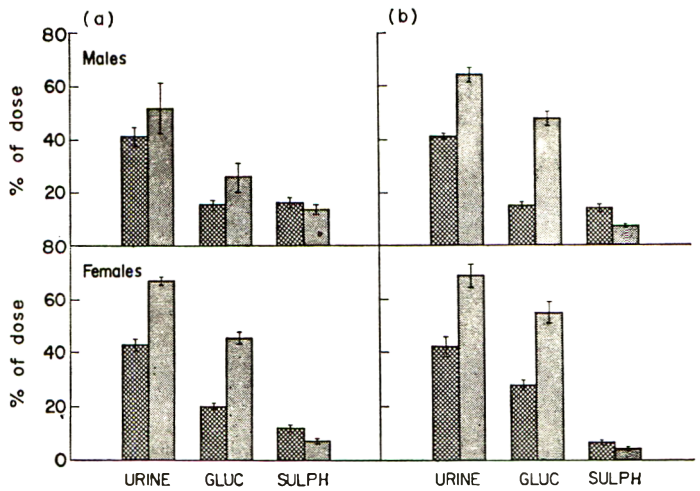

FIG. 2. Levels of radioactivity representing metabolites of FAA (a) and of N-OH-FAA (b) excreted in the urine in a 24-hr period after a single ip dose of the labelled carcinogen administered to male and female rats fed a diet containing 0 (control; ⊠) or 6600 ppm BHT (▦) for the previous 4 wk. Data are means ±SEM for groups of six rats and are also presented separately for glucuronic acid (GLUC) and sulphuric acid (SULPH) conjugates.

(1–4%) of the dose. It was in the fractions corresponding to the glucosiduronic acids and the sulphuric acid conjugates that major differences were found. Thus, male rats had similar levels of glucuronides and sulphates whether injected with FAA or with N-OH-FAA (Fig. 2). However, pretreatment with BHT almost doubled the percentage of a dose of FAA excreted as a glucuronide, and almost tripled the proportion with N-OH-FAA. In the latter cases, there was a corresponding decrease in sulphate ester excretion, which we have shown to be due chiefly to the ester of 7-OH-FAA (Grantham, 1967; Weisburger *et al.* 1961).

The balance of the metabolites were the so-called water-soluble metabolites, currently the object of intensive study to determine their nature. It seems most likely that they were present in the form of mercapturic acids. They showed relatively little variation as a function of agent or pretreatment.

As was observed before, female rats excreted in the urine a higher proportion of the dose when N-OH-FAA was injected than after the FAA injection and with both carcinogens

the proportion excreted was higher than in male rats (Weisburger, Yamamoto, Glass, Grantham & Weisburger, 1968). Correspondingly, in most instances there were small quantities of the sulphate esters, a finding accounted for by a lower level of liver sulphotransferase (DeBaun, Miller & Miller, 1970; Jackson & Irving, 1972). In female rats, BHT pretreatment led to considerable stimulation of the formation and excretion of glucosiduronic acids, more so with N-OH-FAA than with FAA, and again this was paralleled by a reduction in sulphate esters (Fig. 2).

Major metabolites in the glucuronic acid fraction excreted in male rats

The major metabolites of FAA in the glucuronic acid fraction, as resolved by thin-layer and paper chromatography, were metabolites hydroxylated in the 7-, 5- and N-positions (Fig. 3). Minor metabolites were those hydroxylated in positions 3 and 1. Pretreatment of

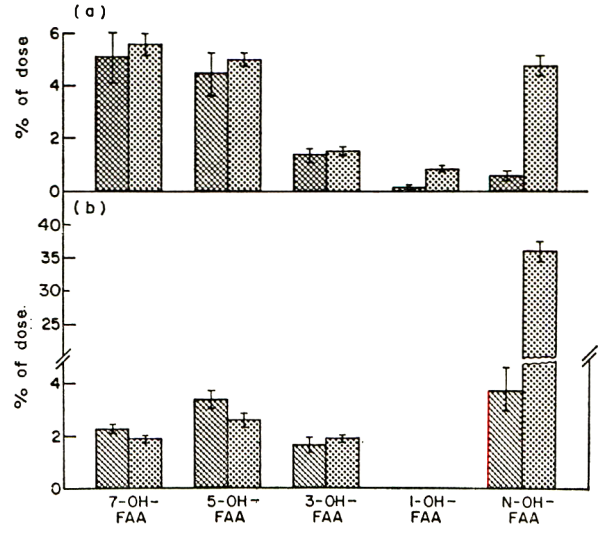

FIG. 3. Major metabolites of FAA (a) and N-OH-FAA (b) in the glucosiduronic acid fraction of the urine of male rats given a diet containing 0 (control; ▨) or 6600 ppm BHT (▩) for 4 wk before ip injection of the labelled carcinogen. The amounts of ring-hydroxylated derivatives of FAA, such as *N*-2-(7-hydroxy-2-fluorenyl)acetamide (7-OH-FAA) and *N*-hydroxy-*N*-2-fluorenylacetamide (N-OH-FAA) are expressed as a percentage of the radioactivity administered.

male rats with BHT failed to have any significant effect on the metabolites hydroxylated at the 3-, 5- and 7-positions and found in the urine as glucuronic acids, but increased very significantly the metabolite hydroxylated on the nitrogen.

Compared with the picture seen with FAA, there were lower amounts of metabolites hydroxylated at the 7-, 5-, 3- and 1-positions when N-OH-FAA was injected, but, as was to be expected, there were larger amounts of the *N*-hydroxy derivative. BHT pretreatment again failed to affect the production of ring-hydroxylated metabolites, but increased appreciably the amount of the *N*-hydroxy derivative excreted as the glucuronide. This finding shows that glucuronic acid conjugation rapidly ties up the N-OH-FAA reaching the liver and withdraws it from further metabolic reaction.

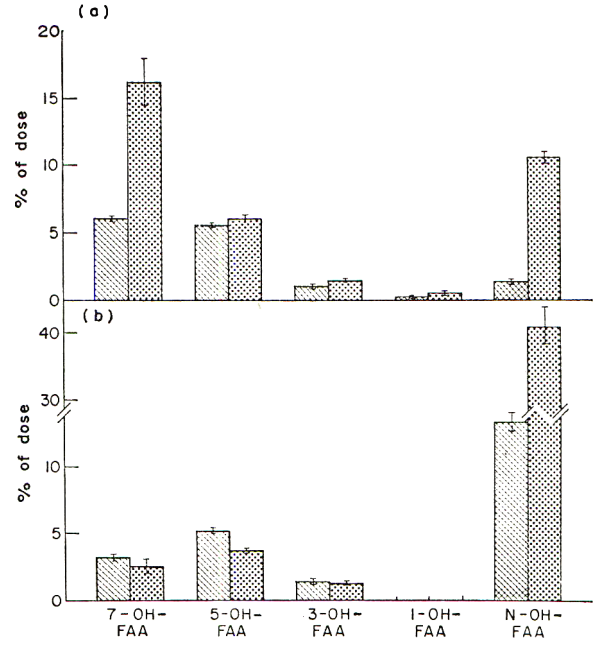

FIG. 4. Major metabolites of FAA (a) and N-OH-FAA (b) in the glucosiduronic acid fraction of the urine of female rats given a diet containing 0 (control; ▨) or 6600 ppm BHT (▦) for 4 wk before ip injection of the labelled carcinogen. The amounts of ring-hydroxylated derivatives of FAA are expressed as a percentage of the radioactivity administered.

Major metabolites in the glucuronic acid fraction excreted in female rats

In females injected with FAA, the picture was similar to that seen in male rats, except that the relative amounts of ring- and *N*-hydroxylated metabolites were different (Fig. 4). In addition, BHT increased considerably the excretion not only of the *N*-hydroxy but also of the 7-hydroxy derivatives as glucuronides. However, when N-OH-FAA was injected, pretreatment with BHT increased only the excretion of the *N*-hydroxy derivative as glucuronide.

Sulphuric acid esters

As was found previously (Grantham, 1967; Weisburger *et al.* 1961) the main metabolite excreted as sulphate was the 7-hydroxy derivative (Fig. 5). With injection of FAA, pretreatment with BHT did not have much effect on the amount of sulphate ester of 7-OH-FAA excreted by male rats, but caused a decrease in female rats. BHT pretreatment also lowered the amount of 5-OH-FAA sulphate in both sexes.

After injection of N-OH-FAA, less of the various ring-hydroxylated metabolites appeared in the urine than was the case when FAA was injected, a finding reported earlier (Grantham, 1967; Weisburger *et al.* 1961). In this instance, BHT pretreatment lowered the 7- and 5-OH-FAA metabolites in the sulphate ester fraction in males and females.

DISCUSSION

Antioxidants are interesting substances from a number of points of view. Their wide use in the environment suggests that there is a need for fundamental information on the

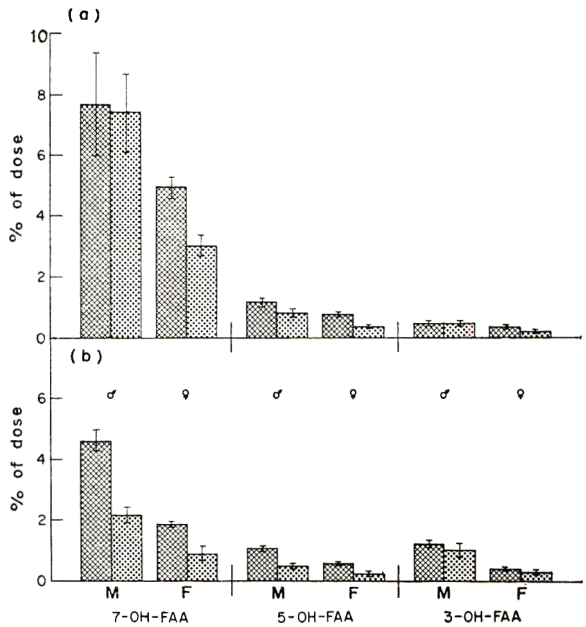

FIG. 5. Major metabolites of FAA (a) and N-OH-FAA (b) in the sulphate ester fraction of the urine of male and female rats given a diet containing 0 (control; ⊠) or 6600 ppm BHT (▦) for 4 wk before ip injection of the labelled carcinogen. The amounts of ring-hydroxylated derivatives of FAA are expressed as a percentage of the radioactivity administered.

mechanisms of their actions in biological systems. We have now shown that the administration of fairly high dose levels of BHT to rats leads to enzyme induction in the liver, as measured by changes in the urinary levels of key carcinogen metabolites. These findings complement earlier studies (Cawthorne, Bunyon, Sennitt, Green & Grasso, 1970; Gaunt *et al.* 1965*; Gilbert & Golberg, 1965; Sharratt *et al.* 1970; Ulland *et al.* 1973). This action had already been established with other substances. In the present case, it affects the metabolism of typical carcinogenic aromatic amines like FAA and its active *N*-hydroxy metabolite, by increasing the excretion of detoxification metabolites as glucuronic acid conjugates, and hence by making less of the administered materials available for activation reactions. In a pioneering paper, Deichmann, Clemmer, Rakoczy & Bianchine (1955) recorded an increased excretion of glucuronic acid in the urine of rabbits given BHT. There is also a parallel increase in urinary ascorbic acid (Gaunt *et al.* 1965).

Similar results were obtained recently with another type of enzyme inducer, phenobarbitone, which likewise increased glucuronic acid conjugation and led to lower levels of active sulphate esters (Matsushima *et al.* 1972). Thus, as we now report, radioactivity from carcinogens was lower in the liver and also as forms bound to liver DNA.

It is not known whether BHT also acted by virtue of its antioxidant potential, which would centre chiefly around the trapping of one-electron intermediates (Harman, 1969). While such agents have been proposed as activated forms of certain chemical carcinogens (Wilk & Girke, 1968), it would appear that the carcinogens used in our study are activated mainly by conversion to highly reactive electrophilic reagents such as the sulphuric acid

*And other papers in *Food and Cosmetics Toxicology*, 1965, **3**, no. 3.

ester or an *O*-acetyl ester of the *N*-hydroxy derivative (Bartsch, Miller & Miller, 1972; DeBaun *et al.* 1970; King & Phillips, 1972; Weisburger, Yamamoto, Williams, Grantham, Matsushima & Weisburger, 1972).

The current report dealing with the biochemical modification of the metabolism of FAA and N-OH-FAA by BHT demonstrates that the antioxidant augments detoxification processes. The companion paper (Ulland *et al.* 1973) dealing with the inhibiting effect of BHT on the carcinogenicity of these agents suggests that BHT certainly does not exert a harmful effect, and may in fact be beneficial. This, taken together with information establishing that trace amounts of antioxidants such as BHT are not only safe themselves but may stabilize perishable foods and essential micronutrients, indicates that their status in the human environment deserves favourable consideration.

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Effet d'un oxydant, l'hydroxytoluène butylé (BHT) sur le métabolisme de deux carcinogènes, le *N*-2-fluorénylacétamide et le *N*-hydroxy-*N*-2-fluorénylacétamide

Résumé—On a étudié chez des rats, mâles et femelles, l'effet d'un oxydant, l'hydroxytoluène butylé (BHT), sur le métabolisme du *N*-2-fluorénylacétamide (FAA) et du *N*-hydroxy-*N*-2-fluorénylacétamide (N-OH-FAA). Le BHT administré pendant 4 semaines à raison de 6600 ppm du régime a fait augmenter le poids du foie par rapport à celui du corps et entraîné l'excrétion urinaire d'une proportion plus élevée d'une dose ip unique de chacun de ces carcinogènes. Les principaux responsables de cette augmentation de l'excrétion étaient des conjugués de l'acide glucuronique. Les esters de l'acide sulfurique avaient diminué. Les taux de radioactivité du sang et du foie et de l'ADN du foie étaient aussi moins élevés 48 h après l'injection du carcinogène marqué. On conclut de ceci que le BHT fait augmenter les métabolites de détoxication du FAA et du N-OH-FAA et fait ainsi diminuer la quantité disponible d'une dose donnée pour des réactions d'activation.

Einfluss des Oxydationsschutzmittels butyliertes Hydroxytoluol (BHT) auf den Stoffwechsel der Carcinogene *N*-2-Fluorenylacetamid und *N*-Hydroxy-*N*-2-fluorenylacetamid

Zusammenfassung—Der Einfluss des Oxydationsschutzmittels butyliertes Hydroxytoluol (BHT) auf den Stoffwechsel von *N*-2-Fluorenylacetamid (FAA) und *N*-Hydroxy-*N*-2-fluorenylacetamid (N-OH-FAA) wurde an männlichen und weiblichen Ratten untersucht. Die Verabreichung von 6600 ppm BHT im Futter auf die Dauer von 4 Wochen erhöhte das Gewichtsverhältnis der Leber zum Körper und führte zur Ausscheidung eines höheren Prozentsatzes einer einzelnen ip-Dosis von jedem der beiden Carcinogene im Urin. Diese höhere Ausscheidungskonzentration erklärte sich hauptsächlich durch Glucuronsäurekonjugate. Schwefelsäureester wurden vermindert. Niedriger waren auch die Konzentrationen der Radioaktivität im Blut, in der Leber und in der Leber gebundener DNA 48 Stunden nach der Injektion von markiertem Carcinogen. Daraus wird der Schluss gezogen, dass BHT die Entgiftungsmetaboliten von FAA und N-OH-FAA vermehrt und damit die Menge, die von einer verabreichten Dosis für Aktivierungsreaktionen zur Verfügung steht, vermindert.

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Short-term Peroral Toxicity of Undegraded Carrageenan in Pigs

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Abstract—Undegraded carrageenan was administered orally to pigs at levels of 0 (control), 50, 200 and 500 mg/kg body weight/day for 12 wk. No effects attributable to carrageenan treatment were seen with respect to behaviour, body-weight gain, feed utilization, haematology, blood chemistry, urine analysis or organ weights. The treatment resulted, however, in changes in the intestinal flora: the total counts of aerobic bacteria were decreased in the colon and rectum and the number of Lactobacilli was reduced in the rectum. No ulcerative colitis or erosions of the mucous membrane of caecum and colon were observed. A few focal areas with an irregular surface were observed in the colonic mucosa of some of the pigs given 200 or 500 mg carrageenan/kg. A shift was seen in the cellular infiltration pattern of the mucosa of the large intestine of pigs from all the groups given carrageenan. The slight changes in the intestinal mucosa are not considered to be a significant toxic effect.

INTRODUCTION

Carrageenan is derived from seaweed sources, mainly *Chondrus crispus* and *Gigartina stellata*. Chemically, it is a sulphated polysaccharide consisting mainly of galactose and anhydrogalactose units. Carrageenan is available as a degraded and an undegraded product. Undegraded carrageenan is widely used as a texture-modifying food additive. An ADI has been established by the Joint FAO/WHO Expert Committee on Food Additives (1970), mainly on the basis of life-span studies in rats and mice (Nilson & Wagner, 1959). Carrageenan may be degraded (Morgan & O'Neill, 1959) to give a product with a molecular weight of less than 30,000. Preparations of degraded carrageenan have been used extensively for the treatment of gastric ulcer in man.

Induction of intestinal lesions was reported in 1969, in guinea-pigs, rabbits, rats and mice given degraded carrageenan in the drinking-water and in guinea-pigs given undegraded carrageenan (Marcus & Watt, 1969; Watt & Marcus, 1969). The question of a possible health hazard to man was therefore raised, especially in view of the widespread use of undegraded carrageenan as a food additive. For this reason we undertook a short-term feeding study in pigs with undegraded carrageenan.

Recently several authors have demonstrated erosive or ulcerogenic activity in the large intestine of several mammalian species, and the scope and findings of these studies are summarized in Table 1. Elsewhere, the biological properties of carrageenan, the effects in different animal species and the importance of these data to man have recently been reviewed (Di Rosa, 1972; *Food and Cosmetics Toxicology*, 1971).

EXPERIMENTAL

Carrageenan. Undegraded carrageenan kindly supplied by Copenhagen Pectin Factory Ltd., Denmark, had the following specification: Carrageenan Genulacta type K 100°,

Table 1. *Summary of studies on the influence of orally administered degraded and undegraded carrageenan on the large intestine in mammalian species*

Reference	Summary of findings in							
	Guinea-pig	Rabbit	Rat	Mouse	Rhesus monkey	Squirrel monkey	Ferret	Pig
Nilson & Wagner (1959)			o*	o*				
Marcus & Watt (1969 & 1971)	±D ?U†	±D ?U†	±D ?U†	(±)D ?U†				
Watt & Marcus (1969, 1970a,b & 1971)	±D ±U	±D						
Maillet, Bonfils & Lister (1970)	±D		oD oU	oD oU				
Sharratt, Grasso, Carpanini & Gangolli (1970 & 1971)	±D ±U	±*	oD oU	oD oU		o*	o*	
Tournut (1970)								o*
Benitz, Abraham, Golberg & Coulston (1972)	±D oU		±D oU		±D oU			

± = Ulcerations or erosions (±) = Other lesions o = No change D = Degraded carrageenan U = Undegraded carrageenan

*Nature of the carrageenan used is not clearly indicated.

†The effect of undegraded carrageenan is not clearly indicated.

batch no. 039240, produced from *Chondrus crispus* of Canadian origin; molecular weight (number average) about 200,000.

Animals. Danish Landrace Pigs, 39–53 days old at arrival from an SPF breeding colony, were kept individually, without bedding, in pens at 19 ± 1°C and a relative humidity of 55–65% with an air-change 8 times/hr and artificial light from 08.00–16.30 hr. Half of the floor surface was kept at 28°C.

Diets. The pigs were fed a balanced, pelleted (or powdered for one group) diet consisting of 74·4% barley, 16% soya meal, 5% skim-milk powder, 2% meat- and bone-meal, 2·4% mineral mixture and 0·2% vitamin supplement and having a protein content of 15%. Bacteriological examination confirmed the absence of potential pathogens and gave a total count of less than 33 × 10^3/g. The diet was offered *ad lib.* twice daily at 08.30 and 15.30 hr. Water also was provided *ad lib.*

Experimental design. After an accommodation period of 22 days, groups of three males and three females were given undegraded carrageenan for 83 days according to body weight. Three groups were given carrageenan as a jelly (10 g/kg/day) mixed with the diet pellets at levels of 50, 200 or 500 mg/kg/day. The jellies were prepared weekly: carrageenan mixed with hot 10% sucrose solution was kept at 100°C for 1 hr and then stored in a refrigerator. Bacteriological examination showed less than 60 bacteria/g during storage. A fourth group received carrageenan (500 mg/kg/day) and sucrose (1 g/kg/day) mixed with the powdered diet. A control group of three males and three females was given the corresponding amount of sucrose. In the data for clinical chemistry, control values were included from an extra four pigs of similar age and maintained under identical conditions.

Observations. Clinical symptoms were recorded twice daily. Body weight was recorded weekly, after which the carrageenan intake for each animal was adjusted. Food intake was recorded twice daily.

Clinical chemistry. Blood samples were collected from the jugular vein 16 and 9 days prior to and 5, 19, 47 and 75 days after the commencement of carrageenan administration.

The parameters examined in whole blood from all animals were glucose (Hultman, 1959; Hyvärinen & Nikkilä, 1962), haemoglobin (van Kampen & Zijlstra, 1961), packed cell volume, total red cell and total white cell counts (Coulter Counter, F_N) and a differential count. Serum from all animals was analysed for total protein (Gornall, Bardawill & David, 1949), creatinine (Bonsnes & Taussky, 1945), urea (Chaney & Marbach, 1962), sodium and potassium (by flame photometry) and calcium (by atomic absorption spectrophotometry). The serum activities of glutamic–oxalacetic and glutamic–pyruvic transaminases (Karmen, Wróblewski & LaDue, 1955) and of alkaline phosphatase (Kind & King, 1954) were determined.

Regression analyses were performed on all control data. A positive correlation with time ($P < 0{\cdot}01$) was observed for all parameters measured, except for glucose, urea, alkaline phosphatase, packed cell volume, total red and white cell and differential counts.

Urine samples were collected during the last 14 days of the treatment and examined for glucose, protein, ketones, heme-pigments and bilirubin (Ames sticks). The pH and the specific gravity were also measured.

Pathology. The pigs were sacrificed by exsanguination under CO_2 anaesthesia. At autopsy, gross abnormalities were noted, and the kidneys, adrenals, spleen, liver, heart, lungs, thymus, thyroid, pancreas, ovaries or testes, brain and pituitary were weighed. Samples of these organs (from the brain, samples of cerebral cortex, hippocampus, cerebellum, pons and medulla oblongata) and of the aorta, mandibular gland, stomach, duodenum, jejunum, ileum, ileo-caecal valve, caecum, colon, rectum, mesenteric lymph node, uterus, fallopian tube, epididymis, seminal vesicle, bulbourethral gland, urinary bladder, skin, sciatic nerve, triceps and longissimus dorsi muscles, femoral bone, mammary gland, spinal cord, cervical and lumbar enlargement, eye, and renal, hepatic, bronchial, cervical superficial and submandibular lymph nodes were fixed in 4% buffered formalin. Paraffin-wax sections of these tissues from all the animals were stained with haematoxylin and eosin. Sections from the intestinal tract were stained with iron haematoxylin–van Gieson, and sections of the colon with toluidine blue. Frozen sections of liver were stained with Oil Red O.

Table 2. *Body weight and feed utilization in pigs fed 0–500 mg undegraded carrageenan/kg/day for 83 days*

Dose level (mg/kg/day)	Mean body weight (kg) at wk							Feed utilization (g feed consumed/kg weight gained)
	0	2	4	6	8	10	12*	
0	15	22	29	38	51	64	76	2040
50	17	24	33	41	54	65	77	2210
200	17	24	31	40	53	65	78	2050
500	14	21	28	37	49	61	72	2130
500†	15	23	31‡	42‡	54§	67§	78§	2030§

*Terminal body weight.
†Dry carrageenan in powdered diet.
‡Five animals.
§Four animals.
Values are means for groups of six (three male and three female) pigs unless otherwise indicated.

Table 3. *Haematological findings before treatment and on day 75 in pigs fed 0–500 mg undegraded carrageenan kg/day for 83 days*

	Mean values* in pigs fed undegraded carrageenan at levels (mg/kg/day) of					
	0†	0	50	200	500	500‡
Parameter — No. of pigs tested. . . .	30	6	6	6	6	4
Haemoglobin (mmol/litre)	6·90 (5·60–9·47)	8·67 (7·77–9·28)	8·42 (7·68–9·03)	8·53 (8·12–8·91)	8·44 (7·73–9·39)	8·21 (7·41–8·60)
Erythrocytes (10^6/mm^3)	6·94 (5·02–8·39)	6·79 (6·57–7·00)	6·57 (5·79–7·11)	6·61 (6·18–6·80)	6·53 (6·09–7·09)	6·84 (6·66–7·10)
Packed cell volume (%)	39 (32–45)	45 (41–49)	44 (39–48)	45 (42–48)	44 (41–48)	46 (44–48)
Mean corpuscular volume (μm^3)	56 (39–68)	67 (60–70)	67 (63–71)	67 (63–72)	67 (67–74)	67 (62–70)
Total leucocytes (10^3/mm^3)	18·5 (11·4–26·9)	16·9 (13·0–19·7)	18·4 (15·9–21·0)	17·8 (15·5–20·1)	16·2 (12·4–18·7)	17·5 (14·9–19·4)
Lymphocytes (%)	57 (31–96)	74 (64–82)	71 (62–82)	75 (66–80)	76 (68–80)	80 (64–97)
Monocytes (%)	9 (2–18)	4 (3–6)	5 (2–8)	3 (2–6)	6 (2–9)	3 (1–5)
Neutrophils (%)	34 (1–59)	20 (14–29)	22 (13–31)	21 (15–31)	17 (11–25)	18 (3–30)
Eosinophils (%)	1 (0–4)	2 (0–3)	1 (0–3)	1 (0–5)	1 (0–3)	0 (0–2)
Basophils (%)	1 (0–3)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)

*Values are the means for the numbers of animals (males and females) shown, with the range in parentheses.
†Mean pretreatment values for the 30 animals from all groups. All other values are for samples taken on day 75.
‡Dry carrageenan in powdered diet.

Table 4. *Clinical chemistry data determined before treatment and on day 75 in pigs fed 0–500 mg undegraded carrageenan/kg/day for 83 days*

	Mean values* in pigs fed undegraded carrageenan at doses (mg/kg/day) of					
	0†	0	50	200	500	500‡
Parameter§ No. of pigs tested. . . .	34	9‖	6	6	6	4
GOT (IU)	29 (12–87)	18 (7–36)	23 (17–32)	24 (12–38)	21 (13–33)	28 (13–56)
GPT (IU)	22 (9–38)	17 (13–26)	18 (13–25)	19 (13–23)	18 (15–24)	18 (15–22)
AP (KA U/litre)	167 (89–250)	145 (98–227)	123 (104–140)	153 (125–195)	149 (118–174)	132 (106–195)
Glucose (mmol/litre)	3·5 (2·7–6·0)	3·6 (2·9–4·8)	4·0 (3·2–6·3)	3·6 (3·2–4·1)	3·4 (2·8–3·9)	4·0 (2·8–5·3)
Creatinine (mmol/litre)	0·09 (0·06–0·12)	0·14 (0·13–0·16)	0·13 (0·11–0·14)	0·13 (0·11–0·16)	0·14 (0·12–0·15)	0·14 (0·13–0·16)
Total protein (g/litre)	55 (46–67)	65 (55–73)	63 (59–67)	64 (60–67)	64 (58–70)	63 (60–68)
Urea (mmol/litre)	4·6 (2·5–10·4)	5·2 (3·1–7·3)	4·6 (3·6–5·7)	4·7 (4·2–5·5)	4·9 (3·7–6·2)	5·3 (3·2–6·8)
K^+ (mmol/litre)	5·8 (4·0–7·4)	6·7 (6·2–7·5)	6·0 (4·3–7·5)	6·9 (5·9–7·8)	7·1 (6·0–8·3)	6·1 (5·4–7·0)
Na^+ (mmol/litre)	147 (135–162)	156 (152–164)	155 (148–162)	157 (145–168)	157 (152–164)	155 (147–164)
Ca^{2+} (mmol/litre)	2·7 (2·4–3·0)	3·1 (2·8–3·3)	3·2 (3·0–3·4)	3·1 (3·0–3·2)	3·2 (3·0–3·2)	3·2 (3·1–3·4)

*Values are the means for the numbers of animals (males and females) shown, with the range in parentheses.
†Mean pretreatment values for the 34 animals from all five groups. All other values are for samples taken on day 75.
‡Dry carrageenan in powdered diet.
§All determinations were on serum, except for glucose, which was estimated in whole blood.
‖Including data for the standard controls and an additional three pigs of similar age maintained under identical conditions.

Microbiology. Bacteriological examination by incubation on different agars at 37°C was performed on the flora of the jejunum, caecum, colon and ampulla of the rectum from all animals of the control group and the group given 500 mg carrageenan/kg/day as a jelly. Two samples of pus plugs from the ileo-caecal valve were examined.

RESULTS¶

The appearance and behaviour of the pigs remained normal throughout the experiment. In the group fed carrageenan mixed with the powdered diet, one animal died from acute shock during blood sampling (day 19) and another died in connexion with a herniotomy (day 42). One of the four pigs included in the control group died from acute shock during blood sampling (day 47). No effect on growth rate or on feed utilization in the weight range 30–60 kg was seen (Table 2). None of the parameters measured in blood and urine showed significant deviations from the control levels. Tables 3 and 4 show values before treatment and for day 75. The measurements on days 5, 19, and 47 revealed no change. No changes in relative organ weights were recorded. Table 5 gives the values from the control and highest dose groups.

Table 5. *Relative organ weights of pigs fed 0–500 mg undegraded carrageenan/kg/day for 83 days*

	Relative organ weight (g/kg terminal body weight) in animals given doses (mg/kg/day) of		
	0	500	500*
Organ / No. of pigs (M+F)	3+3	3+3	2+2
Brain	1·22 (1·06–1·33)	1·29 (1·15–1·37)	1·20 (1·16–1·26)
Heart	3·60 (3·22–4·05)	3·88 (3·42–4·77)	3·44 (3·20–3·55)
Liver	20·7 (18·2–22·6)	20·1 (18·3–22·0)	21·2 (20·9–21·8)
Kidneys	4·21 (3·67–4·86)	3·85 (3·43–4·27)	4·13 (3·88–4·49)
Spleen	1·39 (1·16–1·51)	1·45 (1·21–1·74)	1·33 (1·28–1·39)
Pancreas	1·71 (1·43–2·06)	1·85 (1·75–2·06)	1·90 (1·78–2·03)
Lungs	6·33 (5·28–7·65)	6·27 (5·30–7·02)	7·17 (5·59–8·93)
Thymus	1·38 (0·98–1·81)	1·35 (1·04–1·79)	1·23 (0·87–1·53)
Testes	3·33 (1·31–4·45)	3·12 (2·11–3·91)	3·56 (3·01–4·11)
Ovaries†	83·8 (69·3–95·9)	93·1 (83·1–100·4)	75·3 (69·3–81·3)
Adrenals†	53·6 (45·5–65·2)	56·7 (51·5–63·3)	58·4 (51·0–65·0)
Pituitary†	3·09 (2·13–4·02)	3·16 (1·97–3·83)	3·61 (3·31–3·80)
Thyroid†	50·5 (41·5–58·7)	51·9 (46·0–59·0)	45·3 (35·1–54·1)

*Dry carrageenan in powdered diet.
†Weights of this organ are expressed in mg/kg body weight.
The values are means for the numbers of pigs shown, with the range in parentheses.

The essential findings at autopsy are given in Table 6. No ulcerations or erosions were seen in the gastro-intestinal tract. In three pigs (one on 200 and two on 500 mg/kg/day), however, thick and uneven areas, 10–15 mm in diameter, were found in the colonic mucosa 1–2 m from the caecum (Fig. 1).

Histologically these areas showed infolding of intact epithelium. The lamina propria of the mucosa was thickened with diffuse infiltration of macrophages and lymphocytes. No

¶Details of the data summarized in this paper have been reviewed by the Editor. Space limitations preclude publication in full, but original data are available from the author on request.

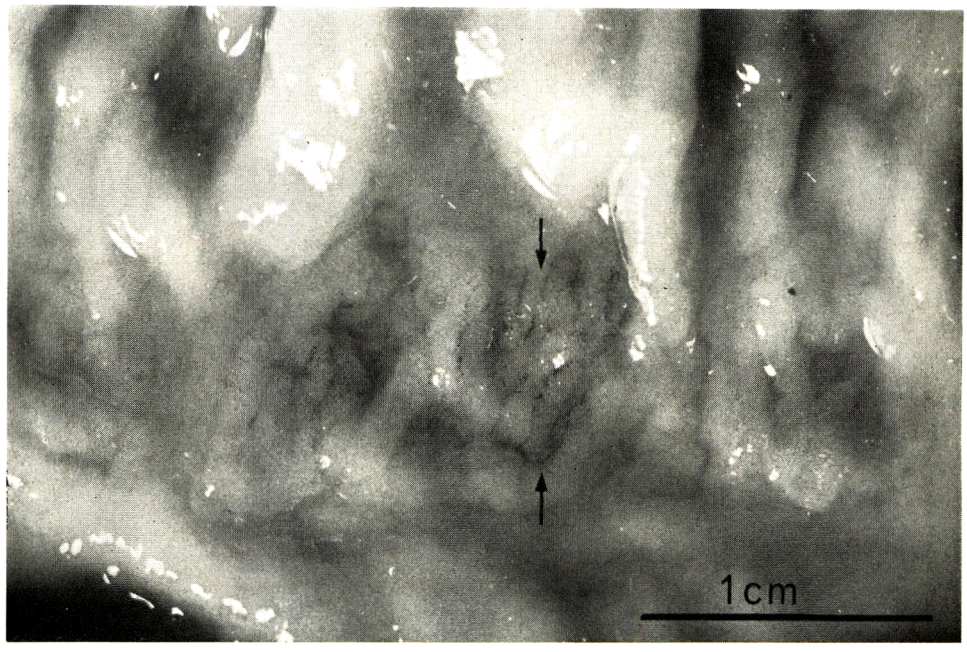

FIG. 1. Mucosa of colon from a pig fed 500 mg undegraded carrageenan/kg/day for 83 days, showing focal thickening (between the arrows) with numerous infoldings.

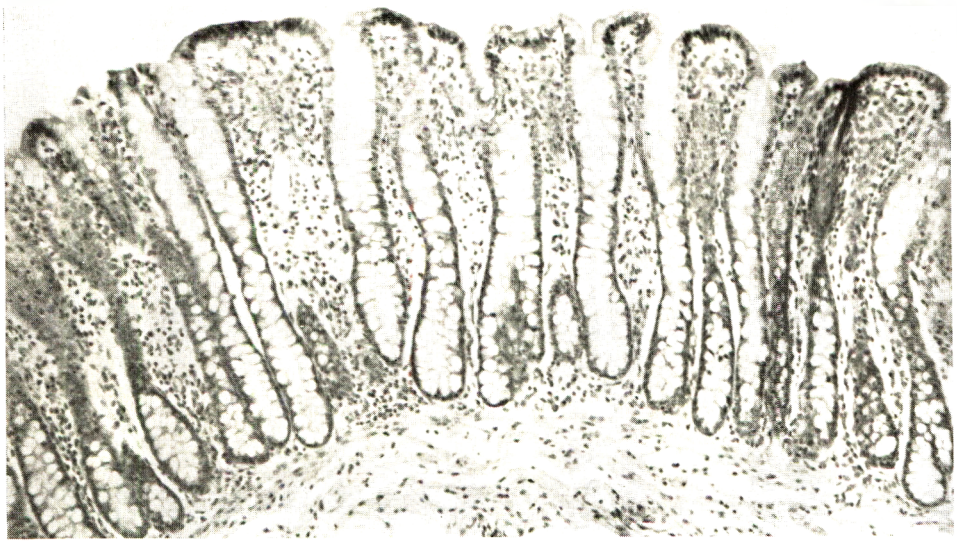

FIG. 2. Section of colon from a pig in the control group, showing small amounts of lymphocytes and macrophages in the mucosal interstitium. Iron haematoxylin–van Gieson (green filter) × 100.

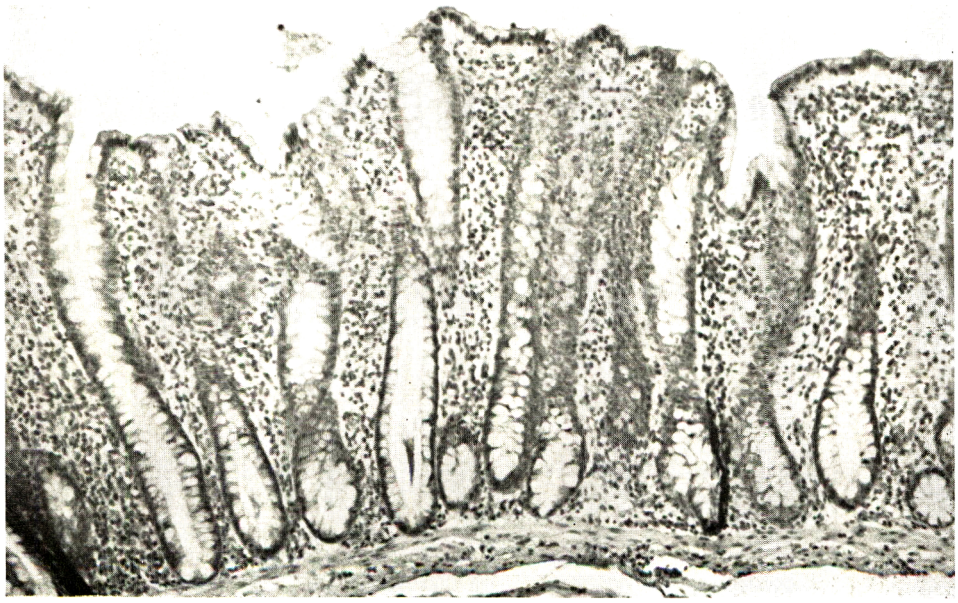

FIG. 3. Section of colon from a pig fed 500 mg undegraded carrageenan/kg/day for 83 days. The mucosal interstitium is enlarged and diffusely infiltrated with lymphocytes, macrophages and a few plasma cells. Iron haematoxylin–van Gieson (green filter) × 100.

Table 6. *Incidence of pathological findings in pigs fed 0–500 mg undegraded carrageenan/kg/day for 83 days*

Pathological findings	No. of pigs affected in groups given doses (mg/kg/day) of				
	0	50	200	500	500*
No. of pigs studied. . . .	6	6	6	6	4
Gross					
Focally thickened and uneven colonic mucosa	0	0	1	2	0
Hepatic cirrhosis	0	0	0	1	0
Reticulosarcoma invading mandibular gland	0	1	0	0	0
Plugs in lymphoid tissue in ileum and ileo-caecal valve	6	6	6	6	4
Gastric hyperkeratosis in oesophageal region	4	6	6	5	4
Ascaridosis	1	1	0	1	2
Histological					
Macrophages and lymphocytes in caecal mucosa:					
Focal infiltration	4	6	5	2	4
Diffuse infiltration	0	0	1	4	0
Macrophages and lymphocytes in colon:					
Focal infiltration	2	3	4	0	1
Diffuse infiltration	0	2	2	6	3

*Dry carrageenan in powdered diet.

granuloma formation of macrophages nor crypt abscesses were seen. Generally a shift from focal to more diffuse and increased cellular infiltration could be observed histologically in the mucosa of both caecum and colon in several pigs (Table 6; Figs 2 & 3). No metachromatic material was observed in caecum or colon.

In the large Peyer's patch of ileum descending to the ileo-caecal valve a large number of plugs could be observed in all animals. The plugs, 2–14 mm in diameter, consisted of mucus, pus and blood. They were located in epithelial crypts, always with intact epithelium but some with slight heterophilic infiltration into the adjacent mucosa. The two pigs that died during the experiment also showed these plugs.

The total count of aerobic bacteria/g intestinal contents was significantly reduced in the colon ($P < 0{\cdot}05$) and rectum ($P < 0{\cdot}01$) of the pigs given carrageenan compared with the control values. The number of Lactobacilli in the rectum was reduced in the group given 500 mg carrageenan/kg ($P < 0{\cdot}01$). No significant differences in the number of *Escherichia coli*, *Streptococcus faecalis*, sulphite-reducing Clostridia and anhaemolytic Sphaerophori were detected.

Str. faecalis was cultivated from the pus plug of the ileo-caecal valve of a pig in the control group, whereas from a pig in the highest dosage group, only *E. coli* was cultivated.

DISCUSSION

When fed to pigs, undegraded carrageenan was found to have no adverse effect on behaviour, growth or feed utilization. Neither were any significant changes in haematology or in blood and urine parameters detected during the 3-month feeding experiment.

The ulcerative colitis and erosions of the caecal and colonic mucosa found in some mammalian species (*Food and Cosmetics Toxicology*, 1971) were not observed in the present study in pigs. Another study in pigs was also reported to be negative, but no details have been published (Tournut, 1970).

The reticulosarcoma in one pig and the hepatic cirrhosis in another are not considered to be related to the carrageenan treatment. The plugs in the intestinal wall are a common finding of unknown aetiology in our experimental pigs, and the gastric hyperkeratosis, too, is commonly observed and is presumably related to the diet used.

The slight changes in the mucous membrane of the large intestine of the pigs fed undegraded carrageenan included a few focal areas with irregular surface and some shift in cellular infiltration pattern. These changes cannot be considered comparable to the erosions and colonic ulceration seen in carrageenan-treated guinea-pigs and rabbits (Table 1).

The changes observed in the present study are, in our opinion, of a reversible nature. The lack of metachromatic material and of macrophage granulomas in the intestinal wall of our pigs indicates that only small amounts of the carrageenan passed through the intestinal wall. It is possible that the changes observed are connected with the alterations of the intestinal flora in the pigs fed high doses of undegraded carrageenan.

Thus, this study in pigs provided no evidence to suggest that adverse effects might result in man from the use of undegraded carrageenan as a food additive.

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Toxicité perorale à court terme du carragène non dégradé chez le porc

Résumé—On a administré à des porcs, par voie orale et pendant 12 semaines, des doses journalières de carragène non dégradé, à raison de 0 (témoins), 50, 200 ou 500 mg/kg poids vif. On n'a observé aucun effet sur le comportement, le gain de poids, le rendement alimentaire, la composition et la chimie du sang, la composition de l'urine et le poids des organes, que l'on aurait pu imputer au carragène. Le traitement a cependant provoqué des modifications de la flore intestinale: le nombre total de bactéries aérobies du côlon et du rectum diminuait, de même que le nombre de lactobacilles du rectum. On n'a pas observé de colites ulcéreuses ni d'érosions de la membrane muqueuse du caecum et du côlon, mais décelé quelques zones focales à surface irrégulière, peu nombreuses, dans la muqueuse du côlon de certains des porcs qui avaient reçu 200 ou 500 mg de carragène/kg. Le profil d'infiltration cellulaire de la muqueuse du gros intestin présentait des altérations chez des porcs des différents groupes qui avaient consommé du carragène. Ces légères modifications de la muqueuse intestinale ne sont pas considérées comme l'indice d'un effet toxique significatif.

Kurzzeitige perorale Toxizität von unabgebautem Carrageen in Schweinen

Zusammenfassung—Unabgebautes Carrageen wurde Schweinen 12 Wochen lang oral in Dosen von 0 (Kontrolle), 50, 200 und 500 mg/kg Körpergewicht/Tag verabreicht. Es wurden keine der Carrageenverabreichung zuzuschreibende Wirkungen hinsichtlich des Verhaltens, der Körpergewichtszunahme, der Futterverwertung, Hämatologie, Blutchemie, Urinzusammensetzung oder der Organgewichte beobachtet. Die Verabreichung hatte jedoch eine Änderung der Darmflora zur Folge: die Gesamtzahlen der aeroben Bakterien wurde im Colon und im Rektum vermindert und die Zahl der Lactobazillen im Rektum vermindert. Es wurden keine ulcerative Colitis oder Erosionen der Schleimhäute von Caecum und Colon beobachtet. Einige fokale Gebiete mit unregelmässiger Oberfläche wurden in der Colon schleimhaut einiger der Schweine beobachtet, die 200 oder 500 mg Carrageen/kg erhielten. Eine Verschiebung der Zelleninfiltrationsverteilung der Schleimhaut des Mastdarms von Schweinen aller Gruppen, die Carrageen erhielten, wurde beobachtet. Die leichten Veränderungen in den Darmschleimhäuten werden nicht als signifikanter toxischer Effekt angesehen.

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Acute and Subacute Oral Toxicity of AHR-2438B, a Purified Sodium Lignosulphonate, in Rats

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Abstract—The acute oral LD_{50} of AHR-2438B in rats was found to be >40 g/kg. In subacute toxicity tests, rats were given AHR-2438B in the drinking-water at levels of 0 (control), 0·025, 0·25, 2·5 or 10 g/100 ml for 16 wk. No adverse effects on growth, organ weights, haematology, urine analysis or serum transaminase and alkaline-phosphatase activities were observed at the three lowest dose levels. Histological examination of tissues from animals at these dose levels revealed no abnormalities. On the other hand, at the highest dose level, male rats showed a decrease in weight gain and animals of both sexes showed skin lesions at the base of the tail, increased leucocyte counts, anaemia and an increase in the absolute and relative weights of the liver, kidneys and spleen. Histological changes in these animals were consistent with reticulo-endothelial activation in the liver and with vacuolar degeneration of the proximal convoluted tubules in the kidney.

The no-effect level of AHR-2438B in the drinking-water was found to be 2·5 g/100 ml, equivalent to a daily intake of 2·83 g/kg in males and 2·42 g/kg in females. These dose levels are approximately 50 times the estimated maximum intake for antipepsin activity in man.

INTRODUCTION

AHR-2438B is the purified sodium salt of a lignosulphonate isolated from the waste sulphite liquor obtained as a by-product in the manufacture of paper pulp from Norwegian spruce. It is a low molecular weight lignosulphonate, the average molecular weight being approximately 5000. Its chemical structure, although not fully elucidated, can be considered to be that of a sulphonated polymer in which the basic unit is a propylbenzene structure similar to that of coniferyl alcohol.

Although sodium lignosulphonate has been used for many years as an animal-feed additive (Code of Federal Regulations, 1962), recent attention has been focused on the antipepsin property of lignosulphonates. In common with other lignosulphonates (Fletcher, Dahl, Jesseph, Steinbock & Harkins, 1957; Vocac & Alphin, 1968 & 1969), AHR-2438B is a competitive inhibitor of pepsin proteolysis and protects against the development of experimental gastric ulcers when it is administered orally to pyloric-ligated rats. This report presents the results of acute and subacute toxicity studies carried out in rats as part of a programme concerned with the evaluation of the relative safety of AHR-2438B.

EXPERIMENTAL

Test material. AHR-2438B, a purified sodium lignosulphonate with a molecular weight of approximately 5000, was supplied by A. H. Robins Co. Ltd., Horsham, Sussex.

Animals. Male and female albino rats of the Wistar strain were purchased from A. Tuck & Son, Essex, England. Oxoid diet 41B and drinking-water were given *ad lib.*

Acute toxicity. Single doses of AHR-2438B were administered to groups of six male and six female rats by oral intubation of a 40% w/v solution in water. The animals (body weight 80–100 g) were fasted for 24 hr before dosing and were observed for 14 days after treatment for signs of toxicity. Autopsies were carried out on selected animals at day 14.

Subacute toxicity. Groups of 20 male and 20 female rats were housed five to a cage (with wire-mesh floor) in a room at controlled temperature and humidity. Four such groups were given AHR-2438B in the drinking-water in concentrations of 0·025, 0·25, 2·5 and 10 g/100 ml. A control group received untreated drinking-water. Administration of the test material was started after an initial conditioning period of 3 wk and continued for a further period of 16 wk. Body weight was recorded weekly and food and water consumption daily. From the overall mean daily fluid intake, the mean daily dose of AHR-2438B administered to each treatment group was calculated to be 0·017, 0·168, 2·83 and 10·02 g/kg in males and 0·026, 0·283, 2·42 and 9·99 g/kg in females for the groups given 0·025, 0·25, 2·5 and 10·0 g/100 ml drinking-water respectively.

Haematological investigations were carried out prior to treatment and during wk 6, 10 and 16. Blood was collected from the tail vein of groups of five animals and haemoglobin and haematocrit determinations, total erythrocyte and total and differential white cell counts and coagulation tests were carried out. In addition, blood samples were collected by cardiac puncture from rats under light ether anaesthesia for the determination of blood glucose, activities of serum glutamic–pyruvic and glutamic–oxalacetic transaminases and of serum alkaline phosphatase, serum urea and total serum protein content. Both control and treated animals were examined in wk 6, 10 and 16 for the presence of faecal occult blood, using the Hematest method.

Urine samples were collected in wk 6, 10 and 16 and tested for protein, ketone, glucose, blood and bile content. Spun-urine deposits were examined microscopically for epithelial cells, erythrocytes, mononuclear and polynuclear leucocytes, casts and other abnormal constituents.

The subacute toxicity test was terminated at the end of wk 16 when groups of ten animals from each of the treatment and control groups were killed by carbon dioxide euthanasia. The remaining animals were maintained on the established dosing schedules for further studies. At autopsy (wk 16) an examination was made for any gross abnormalities and the brain, pituitary, thyroid, spleen, heart, liver, kidneys, adrenals and sex organs were removed and weighed. Samples of these organs and of the pancreas, salivary gland, lung, thymus, oesophagus, stomach, duodenum, ileum, caecum, colon, rectum, lymph nodes and urinary bladder were preserved in 4% formaldehyde in normal saline and processed in the usual way for paraffin embedding. Sections of these tissues were stained with haematoxylin and eosin for microscopic examination.

RESULTS

Acute toxicity

Single oral doses of up to 40 g AHR-2438B/kg were without effect on rats previously fasted for 24 hr except that diarrhoea occurred in animals receiving dose levels of 10 g/kg or more. At autopsy, no macroscopic or microscopic abnormalities were seen. Oral LD_{50} values could not be determined because of the low toxicity of AHR-2438B.

Subacute toxicity

There were no differences from the controls in the general appearance or behaviour of rats treated with the three lowest dose levels of AHR-2438B. At the highest dose level, three male rats developed skin lesions at the base of the tail during wk 2 of treatment. Similar lesions developed in subsequent weeks in four more male and three female rats (Table 1). In addition to tail lesions, four male rats developed lesions of the scrotal sac. Animals presenting lesions showed signs of illness such as a reduction in body weight,

Table 1. *Development of tail lesions in rats receiving the highest dose level of AHR-2438B (10 g/100 ml drinking-water)*

Sex	No. of rats/group	Total no. of animals with tail lesions at wk				
		2	6	10	11	16
Male	20	3	5	6	6	7
Female	20	0	0	1	2	3

decreased locomotor activity and anaemia. Faeces of animals in the group on the highest dose were very soft, sticky and dark and were not segmented. The faecal changes became apparent between days 3 and 20 of treatment and continued throughout the period of administration. Severe diarrhoea was observed in two male rats, the stools being fluid in each case. With the three lowest dose levels of AHR-2438B, faecal consistency and colour were virtually unchanged, although in a few animals receiving 2·5 g/100 ml, the faeces were slightly softer and darker than those of controls. Despite the development of skin lesions in some animals, no faecal occult blood was detected in treated rats and in the terminal study no evidence of haemorrhage was found in the gastro-intestinal tract by low-power microscopical examination and histology.

Rates of growth were similar in treated and control animals with the exception of male rats receiving the highest dose of AHR-2438B (Table 2). The growth rate of this group of animals was significantly lower than that observed in other groups of male rats, from wk 6 onwards. Although female rats on the highest dose level showed a trend towards a reduced growth rate from wk 9 onwards, their weights did not differ significantly ($P > 0{\cdot}05$) from those of other female groups. Food and water consumption were similar in control and treated animals at each dose level throughout the test period (Table 3).

Haematological studies showed that only in the male rats receiving the highest dose level of AHR-2438B were there any consistent changes in the blood picture (Table 4). These changes were observed throughout the period of administration and consisted of reduced erythrocyte counts, reduced haemoglobin and haematocrit values and increased leucocyte counts. They appeared to be associated with the presence of lesions since rats that had not developed lesions possessed a blood picture similar to that of controls. On the other hand, rats with lesions presented marked anaemia with an increase in total leucocyte count. This is illustrated in Table 5, where haematological data are given for male rats both with and

Table 2. *Mean body weight of rats treated for 16 wk with AHR-2438B in the drinking-water*

Dose level (g/100 ml)	Mean body weight (g) at wk						Mean body weight gain (g) over 16 wk
	0†	3	6	9	12	16	
Males							
0·0	218	276	328	380	404	421	203
0·025	258**	270	352	409	429	439	181
0·25	229	300	347	384	408	432	203
2·5	235	297	348	377	409	426	191
10·0	222	252	287***	318***	341***	373*	151
Females							
0·0	187	216	224	241	241	243	56
0·025	198	230	240	248	250	253	55
0·25	189	221	232	245	241	249	60
2·5	190	221	232	243	243	250	60
10·0	190	213	225	237	234	233	43

†Day 1 of treatment.
Body weights are the means for groups of 20 rats and values marked with asterisks differ significantly (Student's *t* test) from those of controls: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

Table 3. *Mean values of food and water consumption of rats treated for 16 wk with AHR-2438B in the drinking-water*

Dose level (g/100 ml)	Mean daily food consumption (g/rat) at wk						Mean daily food consumption (g/rat) over 16 wk	Mean daily water consumption (ml/rat) at wk						Mean daily water consumption (ml/rat) over 16 wk
	0†	3	6	9	12	16		0†	3	6	9	12	16	
Males														
0·0	19·6	30·9	29·3	29·3	29·3	29·4	27·6	25·3	27·3	31·7	27·0	28·0	23·9	28·6
0·025	22·5	35·3	30·7	29·4	26·6	28·6	27·7	26·2	28·1	30·1	27·9	23·9	24·3	27·0
0·25	20·7	32·3	30·7	29·4	25·7	30·9	27·4	24·8	25·0	26·3	28·3	27·6	24·4	25·7
2·5	21·9	32·4	30·7	32·3	28·0	33·4	28·7	23·6	27·0	26·4	25·0	24·7	24·6	26·0
10·0	23·1	30·1	30·6	30·7	28·9	29·1	28·6	24·3	29·7	31·7	30·9	33·3	25·0	31·9
Females														
0·0	22·0	21·6	20·0	20·6	20·0	20·3	21·3	25·7	28·6	28·4	26·4	22·4	22·0	27·6
0·025	20·6	24·7	22·0	23·9	20·1	20·3	21·7	24·3	27·6	28·6	27·1	21·7	23·2	25·7
0·25	18·0	24·3	22·7	20·6	18·3	20·9	21·0	26·1	28·6	30·0	26·3	24·7	25·8	26·9
2·5	20·0	22·7	21·6	20·6	19·0	19·7	20·7	21·3	22·9	24·3	23·1	23·3	20·8	23·1
10·0	22·3	23·1	21·6	19·3	18·3	19·0	20·6	22·7	22·6	23·3	21·7	23·9	19·7	23·0

†Day 1 of treatment.

without lesions after the first 6 wk of the test period. Animals given lower dose levels showed no consistent changes in blood picture, although at wk 16, haemoglobin levels were reduced in male rats receiving 0·25 and 2·5 g/ml. However, it should be pointed out that after a

Table 4. *Haematological findings in rats treated for 6, 10 and 16 wk with AHR-2438B in the drinking-water*

Dose level (g/100 ml)	RBC (10^6/mm^3) at wk			Hb (g/100 ml) at wk			Haematocrit (%) at wk			Total leucocytes (10^3/mm^3) at wk		
	6	10	16	6	10	16	6	10	16	6	10	16
Males												
0·0	8·00	7·97	8·29	13·3	14·5	14·9	45	48	49	11·6	12·1	8·8
0·025	—	8·18	3·12	—	14·5	14·7	—	47	47	—	7·4	6·5
0·25	—	8·03	8·18	—	14·0	13·4**	—	46	44	—	21·1***	11·0
2·5	8·13	7·91	8·19	13·4	14·0	13·5**	46	44	42**	15·9	16·1	11·0
10·0	5·88*	6·93*	7·91	8·1**	11·6*	12·2***	35*	39**	42**	27·1*	21·7***	16·2*
Females												
0·0	7·79	7·96	7·62	12·5	14·1	14·0	43	45	45	7·3	5·8	5·0
0·025	—	7·64	7·77	—	14·1	13·9	—	46	45	—	7·6	5·6
0·25	—	7·95	7·62	—	14·2	13·9	—	46	40**	—	7·9	4·3
2·5	8·16	7·89	7·66	13·1	13·7	14·9	46	44	45	10·6	9·3	8·5
10·0	7·92	7·70	7·02	10·9	12·6*	12·7	38	42	42	13·8	11·2	10·4

RBC = Red blood cells Hb = Haemoglobin

Values are the means of groups of five animals and those marked with asterisks differ significantly (Student's *t* test) from control values: *$P < 0·05$; **$P < 0·01$; ***$P < 0·001$.

further 2 wk of treatment, the haemoglobin levels of these rats had returned to normal. These changes were not therefore considered to be caused by AHR-2438B. Blood coagulation times were unaffected by the treatment.

No significant differences ($P > 0·05$) were observed in the levels of blood glucose, serum urea and serum protein in the treated and control rats (Table 6). Likewise, the serum levels

Table 5. *Haematology of five male rats with lesions and two male rats with no lesions after treatment for 6 wk with the highest dose level of AHR-2438B*

Rat coding	Body weight (g)	RBC (10^6/mm^3)	Hb (g/100 ml)	Haematocrit (%)	Total leucocytes (10^3/mm^3)
Rats with lesions					
E 1 B	229	6·472	9·0	37	19·2
E 2 2L	282	6·552	10·3	40	18·6
E 3 2R	186	2·728	3·7	21	25·2
E 4 2R	256	7·664	10·2	42	17·6
E 4 1L	315	6·936	8·1	35	16·6
Means	254	6·070	8·3	35	19·4
Rats with no lesions					
E 3 B	316	7·976	9·8	41	11·8
E 3 1L	306	7·744	11·4	43	12·2
Means	311	7·860	10·6	42	12·0

RBC = Red blood cells Hb = Haemoglobin

of glutamic–oxalacetic transaminase, glutamic–pyruvic transaminase and alkaline phosphatase were similar at each dose level. Chemical analyses indicated the absence of any abnormal constituent from the urine of all animals.

At autopsy, all animals in the highest dose group and some in the group given 2·5 g/100 ml showed congested mesenteric lymph nodes. In addition, significant increases in the absolute and relative weights of the liver, spleen and kidneys were found in the males and females which had received the highest dose level (Table 7). Although the weights of these organs in the groups given lower doses showed some dose-dependent increase in absolute and relative weights, these weights were not significantly different ($P > 0{\cdot}05$) from control values. Treatment with AHR-2438B did not affect the weights of other organs in animals of either sex.

Histological studies revealed that treatment with AHR-2438B at the three lowest dose levels induced no changes in any of the tissues examined. At the highest dose level, however, histological changes were observed in the liver and kidneys of both sexes. The livers showed normal parenchymal cells but there were marked increases in the size and number of reticulo-endothelial and Kupffer cells. The kidney showed swelling and vacuolation of the epithelial cells of the proximal convoluted tubules in the outer cortex, but were otherwise normal.

DISCUSSION

Daily oral administration of AHR-2438B to rats did not alter growth rates except in males given the highest dose level, in which a significant retardation of weight gain occurred. A similar trend was observed in female rats on this highest dose level although the growth rates did not differ significantly ($P > 0{\cdot}05$) from those of controls. The reduced mean growth rate in males was not related to a lowered food intake but appeared to be related to the number of rats in the group with ulcerated lesions at the base of the tail. It can be seen from Table 5 that animals presenting tail lesions weighed less than unaffected animals and therefore the larger the proportion of affected animals in a group, the greater became the deviation from the mean body weight of unaffected animals. Thus from wk 6 onwards the number of males with lesions was sufficient to reduce the mean body weight of this group of rats to a level significantly different from control values. On the other hand, among the female rats of this group, the proportion of affected animals was smaller than in the corresponding group of males so that the mean body weight was not reduced to a level significantly different from that of the controls. In addition to the influence of tail lesions on growth rates, it is possible that weight gain may have been depressed to a limited extent as a result of a reduction in body fluid levels brought about by the continuous purgative action of very large doses of AHR-2438B.

The development of lesions in some animals appeared to be associated with the degree of adherence of sticky faeces to the affected areas. Lesions appeared in males earlier than in females since the scrotal sac trapped faeces between the base of the tail and the anus, and this aggravated the condition. The degree of faecal contamination varied from animal to animal and only in cases where the anal regions were continually very dirty did lesions develop. This suggested that the development of eroded areas occurred as a result of local irritation by adhering sticky faecal matter containing large quantities of the test material. It is possible that irritation was intensified by a wetting of the faeces with urine. The probability that lesions developed in this manner was supported by the fact that when five male

Table 6. *Blood and serum analyses of groups of five rats treated for 6, 10 and 16 wk with AHR-2438B in the drinking-water*

Dose level (g/100 ml)	Blood glucose (mg/100 ml) at wk			Serum protein (g/100 ml) at wk			Serum urea (mg/100 ml) at wk			GOT (IU) at wk			GPT (IU) at wk		
	6	10	16	6	10	16	6	10	16	6	10	16	6	10	16
Males															
0·0	87·4	108·9	100·3	6·2	6·5	6·5	15·9	17·6	16·3	44·3	65·4	39·9	8·7	12·7	12·2
2·5	91·8	114·3	89·4	5·9	6·5	6·5	15·6	19·3	14·2	42·6	54·9	39·9	5·1	7·1	11·9
10·0	97·4	110·8	97·4	5·2	5·9	6·1	21·7	23·7	20·2	45·2	63·2	33·9	10·8	14·3	6·7
Females															
0·0	86·8	107·0	86·5	5·9	6·7	7·0	17·3	16·1	19·5	50·5	59·7	40·9	11·5	8·1	7·2
2·5	87·6	107·8	90·1	5·5	6·4	6·9	22·7	22·7	22·0	48·0	54·1	25·9	5·5	6·3	1·9
10·0	100·0	114·8	100·7	5·4	5·9	6·4	19·7	20·8	18·1	44·2	55·5	35·4	6·4	8·1	1·6

GOT = Glutamic–oxalacetic transaminase GPT = Glutamic–pyruvic transaminase

Table 7. *Absolute and relative organ weights of rats treated for 16 wk with AHR-2438B in the drinking-water*

Sex	Dose level (g/100 ml)	Terminal body weight (g)	Brain	Heart	Liver	Spleen	Kidneys	Adrenals†	Pituitary†	Thyroid†	Testes or uterus	Seminal vesicles or ovaries
Absolute organ weight (g)												
Male	0·0	421	1·89	1·20	16·65	0·79	3·44	53·0	21·2	20·6	3·17	1·32
	0·025	439	1·91	1·20	17·39	0·67	3·07	47·4	43·3	20·2	3·68	1·38
	0·25	431	1·85	1·35	17·23	0·81	3·48	48·8	46·2	19·0	3·19	1·53
	2·5	426	1·86	1·27	19·22	0·92	3·89	48·1	22·4	23·9	3·60	1·62
	10·0	372	1·84	1·16	20·04**	0·97**	4·11**	57·4	35·0	17·8	3·98	1·36
Female	0·0	243	1·83	0·85	10·41	0·58	2·10	75·8	25·1	14·5	0·53	0·12
	0·025	253	1·81	0·78	9·97	0·50	1·77	63·5	38·7	13·1	0·58	0·11
	0·25	249	1·76	0·80	9·75	0·49	1·97	68·8	44·1	16·0	0·55	0·11
	2·5	250	1·82	0·77	10·48	0·56	2·19	69·2	32·5	12·5	0·48	0·11
	10·0	233	1·83	0·73	12·17**	0·70**	2·84**	89·0	26·3	16·3	0·46	0·10
Relative organ weight (g/100 g body weight)												
Male	0·0		0·45	0·29	3·98	0·19	0·83	0·012	0·005	0·004	0·76	0·31
	0·025		0·42	0·26	3·80	0·15	0·67	0·010	0·009	0·004	0·80	0·30
	0·25		0·43	0·32	4·05	0·19	0·82	0·011	0·010	0·004	0·75	0·36
	2·5		0·43	0·29	4·41	0·21	0·89	0·011	0·005	0·005	0·83	0·37
	10·0		0·48	0·30	5·19**	0·25**	1·06**	0·014	0·009	0·004	1·01	0·35
Female	0·0		0·70	0·32	3·96	0·22	0·80	0·028	0·009	0·005	0·20	0·05
	0·025		0·71	0·31	3·93	0·20	0·70	0·024	0·014	0·005	0·23	0·04
	0·25		0·70	0·32	3·87	0·19	0·78	0·026	0·017	0·006	0·22	0·04
	2·5		0·71	0·30	4·06	0·23	0·87	0·026	0·012	0·004	0·18	0·04
	10·0		0·81	0·32	5·38**	0·31**	1·26**	0·039	0·011	0·007	0·20	0·05

†Absolute weights of this organ are expressed in mg.

Values are the means for groups of ten rats. Those marked with asterisks differ significantly (Student's *t* test) from the control values: $^{**}P < 0{\cdot}01$.

and two female rats with lesions were placed in cages containing sawdust and cleansed twice daily in the anal region, the lesions healed or had substantially regressed within 5 wk of separation despite continued lignosulphonate treatment. At the same time an improvement in the blood picture was observed, suggesting that the anaemia and raised leucocyte levels in these animals was a result of the presence of the tail lesions.

It has recently been reported by Marcus & Watt (1969) that oral administration of carrageenans produces ulcerative lesions in the caecum, colon and rectum of some animal species, including the rat. Since AHR-2438B produced external ulcerative areas at the highest dose level and showed certain other similarities to the carrageenans, it was important in this study to determine whether AHR-2438B also caused ulcerative lesions of the rat intestinal mucosa. In fact, no faecal occult blood was found in animals receiving this material and since this is an early and reliable indication of the onset of ulcerative colitis, it was concluded that AHR-2438B does not produce such lesions in the rat. This was confirmed at autopsy, when no ulcerative lesions were found in any section of the gastro-intestinal tract.

It is well documented that sulphonated macromolecules such as sulphonated polysaccharides possess a powerful heparin-like anticoagulant property. Thus, it might be expected that AHR-2438B would also possess this property. However, this is not the case, since even at the highest dose level of AHR-2438B, blood coagulation times were unchanged throughout the period of administration. Furthermore, *in vitro* studies have indicated that the drug does not prolong blood-coagulation time in the rat at a concentration of 2·5 mg/ml. The explanation for this is no doubt connected with the molecular weight of AHR-2438B, which is approximately 5000. For example, Loomis & Beyer (1953) showed that the anticoagulant activity of sulphonated lignins was related to their molecular weight, high molecular weight fractions possessing greater anticoagulant activity than the lower molecular weight fractions. In addition, Vocac & Alphin (1968 & 1969) have reported that several of the low molecular weight lignosulphonates that they examined possessed very little heparin-like anticoagulant activity.

Biochemical and histological examination of the kidney and liver revealed no toxic effects induced by continuous treatment with AHR-2438B at the three lowest dose levels. However, histological findings in the group given the highest dose suggested that the material was absorbed and stored to some extent. For example, swelling and vacuolation of the epithelium of the proximal convoluted tubules of the kidney was consistent with the storage of material and so was the marked increase in the size and number of reticulo-endothelial and Kupffer cells in the liver. Likewise, the increase in weight of these organs compared with the controls at autopsy may indicate storage of AHR-2438B, since Golberg (1966) has reported that liver enlargement occurs following the administration of other macromolecular compounds, such as iron-dextran and methylcellulose.

The results of the acute oral toxicity tests indicate that AHR-2438B has a low acute toxicity, since it was impossible to produce death in rats by a single oral dose of up to 40 g/kg. The relative safety of the material is further supported by the lack of toxicity shown in the subacute toxicity tests. This study shows that the no-effect level of AHR-2438B administered daily in the drinking-water of rats for 16 wk is 2·5 g/100 ml, which is equivalent to a daily intake of 2·83 g/kg in males and 2·42 g/kg in females. These dose levels are approximately 50 times the estimated maximum intake by man.

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Toxicité orale aiguë et subaiguë d'un lignosulfonate de sodium purifié, l'AHR-2438B, chez le rat

Résumé—La DL_{50} orale aiguë de l'AHR-2438B est de > 40 g/kg chez le rat.

Pour déterminer la toxicité subaiguë du produit, on en a ajouté pendant 16 semaines 0 (témoins), 0,025, 0,25, 2,5 ou 10 g/100 ml à l'eau de boisson d'un groupe de rats. On n'a pas observé d'effets nuisibles à la croissance, aux poids des organes, à la composition du sang et de l'urine et aux activités de la transaminase et de la phosphatase alcaline sériques chez les animaux qui recevaient les trois doses inférieures et l'examen histologique des tissus de ces animaux n'a révélé aucune anomalie. Dans le groupe qui recevait la plus forte dose, par contre, on a constaté chez les mâles une diminution du gain de poids et chez les animaux des deux sexes des lésions cutanées à la base de la queue, des augmentations du nombre de leucocytes, de l'anémie et une augmentation des poids absolus et relatifs du foie, des reins et de la rate. Les modifications histologiques observées chez ces animaux concordaient avec une activation réticulo-endothéliale du foie et une dégénérescence vacuolaire des tubes contournés proximaux du rein.

Le seuil d'indifférence de l'AHR-2438B dans l'eau de boisson, déterminé à 2,5 g/100 ml; équivaut à une consommation journalière de 2,83 g/kg chez les mâles et de 2,42 g/kg chez les femelles. Ces taux correspondent à environ 50 fois la consommation maximale estimée nécessaire pour une activité antipepsique chez l'homme.

Akute und subakute orale Toxizität von AHR-2438B, einem gereinigten Lignosulfonat, in Ratten

Zusammenfassung—Die akute orale LD_{50} von AHR-2438B für Ratten wurde mit > 40 g/kg bestimmt.

Bei subakuten Toxizitätsversuchen erhielten Ratten AHR-2438B 16 Wochen lang im Trinkwasser in Konzentrationen von 0 (Kontrolle), 0,025, 0,25, 2,5 oder 10 g/100 ml. Bei den drei niedrigsten Dosierungen wurden keine nachteiligen Wirkungen auf Wachstum, Organgewichte, Hämatologie, Urinzusammensetzung und Serumtransaminase- und alkalische Phosphataseaktivität festgestellt. Die histologische Untersuchung von Geweben der Tiere, welche diese Dosierungen erhielten, zeigte keine Anomalien. Andererseits zeigten bei der höchsten Dosierung männliche Tiere eine Verminderung der Gewichtszunahme und Tiere beider Geschlechter Hautläsionen an der Schwanzwurzel, erhöhte Leukozytenzahlen, Anämie und eine Zunahme des absoluten und des relativen Gewichts von Leber, Nieren und Milz. Die histologischen Veränderungen bei diesen Tieren stimmten mit den Erscheinungen reticulo-endothelialer Aktivierung in der Leber und mit vacuolärer Degeneration der proximalen convoluten Nierenkanälchen überein.

Die wirkungsfreie Konzentration von AHR-2438B im Trinkwasser wurde mit 2,5 g/100 ml bestimmt, was einer täglichen Aufnahme von 2,83 g/kg durch männliche und von 2,42 g/kg durch weibliche Tiere entspricht. Diese Konzentrationen sind etwa das 50fache der geschätzten maximalen Aufnahme an Antipepsinaktivität beim Menschen.

Fd Cosmet. Toxicol. Vol. 11, pp. 239–244. Pergamon Press 1973. Printed in Great Britain

Assessment of the Teratogenic Activity of Dithiocarbamate Fungicides

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Abstract—The dithiocarbamate fungicides, maneb and zineb, were given in a single oral dose to groups of pregnant rats on day 11 or 13 of organogenesis. Single doses of 1–4 g maneb/kg and 2–8 g zineb/kg induced congenital anomalies in 12–100% of the foetuses. The maximum dose rate at which no teratogenic effect was observed was 0·5 g/kg for maneb and 1 g/kg for zineb. No adverse effect on the intrauterine development of the progeny was observed when the latter dose levels were given daily to groups of rats from day 2 to day 21 of pregnancy or when the rats were exposed in a dynamic inhalation chamber to a concentration of 100 mg zineb/m^3 for 4 hr/day from day 4 of pregnancy. The authors conclude that the present exposure to dithiocarbamate fungicides in agriculture is unlikely to present a hazard to the normal development of the human embryo.

INTRODUCTION

Pesticides are being used in increasing amounts in agriculture and are therefore potential environmental contaminants, which may have effects on a variety of biological systems. It may well be true to say that of all the toxicological hazards arising from chemicals in industry and agriculture, we know least about the risks to mammalian reproduction.

There are several reports of the deleterious effects of various thio- and dithiocarbamate fungicides, notably thiram, ziram, zineb, maneb and polycarbazin on reproduction in laboratory animals (Ivanova-Tchemishanska, 1969; Kaloyanova, Ivanova & Alexiev, 1967; Martson, 1967 & 1969; Martson & Martson, 1970; Ryasanova, 1967; Samosh, Pylinskaya, Martson & Kurinskii, 1968). There have, however, been few studies concerned specifically with embryotoxic or teratogenic effects, although Robens (1969) studied the embryotoxicity of thiram and disulfiram in the Golden Syrian hamster.

In a continuing study of the effect of dithiocarbamate fungicides on mammalian development, we have investigated the effect of single and repeated oral doses of maneb and of zineb on embryogenesis in the rat. We have also tried to assess the possible risks to the offspring of women exposed to these pesticides. For this reason, pregnant rats were exposed in an inhalation chamber to a concentration of zineb 100 times higher than the Maximum Allowable Concentration (MAC) of 1 mg/m^3.

EXPERIMENTAL

Technical grade maneb, manganese ethylenebis(dithiocarbamate), and zineb, zinc ethylenebis(dithiocarbamate) were used.

Virgin female white rats (body weight 120–160 g) were mated with proven males and the day on which spermatozoa were observed in the vaginal smears was designated day 1 of pregnancy. The pregnant animals were used for three experiments.

In the first, groups of up to nine animals were given a single oral dose of maneb or zineb on day 11 or 13 of pregnancy. The fungicide was given as a 20% suspension in water in volumes necessary to provide doses of 0·5–8 g/kg. A group of 14 untreated rats served as controls.

In the second experiment, groups of 9–12 rats were given maneb or zineb in a daily dose of 125, 250, 500 or 1000 mg/kg for 20 successive days, starting on day 2 of pregnancy.

Thirdly, four groups of rats were exposed in a dynamic inhalation chamber to an atmospheric concentration of 100 mg zineb/m^3 for 4 hr/day, from day 4, 5, 6 or 7 to the end of gestation. The animals exposed from day 4 were allowed to go to term and the embryo viability, the litter size, weight and survival and the weight gain of the pups in the first month after birth were recorded.

All the other rats in the three experiments were subjected to Caesarean section on day 21 of pregnancy and the numbers of corpora lutea, implantation sites, dead foetuses, viable foetuses and gross malformations were recorded. Approximately 65% of the offspring were preserved in alcohol and subsequently stained with alizarin red (Dawson, 1926) for skeletal examination.

RESULTS

In rats given a single dose of fungicide on day 11 or 13 of pregnancy, a high incidence of malformations was associated with doses of 2 or 4 g maneb/kg and 4 or 8 g zineb/kg, respectively equivalent to 25–50% and 33–66% of the LD_{50}s of the two materials for adult rats. Doses of 1 g maneb/kg and 2 g zineb/kg also caused some malformations. The dose–response relationship is readily seen in Table 1. The malformations were located mainly in the foetal brain, facial part of the skull, limbs and tail (Table 2). Gross effects are shown in Figs 1–5 and skeletal anomalies in Figs 6–10. This study established the no-effect level for a single oral dose as 0·5 g/kg for maneb and 1 g/kg for zineb. Skeletal examination of these foetuses revealed no defective ossification.

When these no-effect doses were administered daily throughout pregnancy they had no effect on the incidence of malformations (Table 3) and it was clear, therefore, that they did not exert a cumulative effect. No teratogenic or embryotoxic effects were detected in 15 litters from rats exposed for 4-hr periods to an atmosphere of 100 mg zineb/m^3 from day 5, 6 or 7 to the end of pregnancy (Table 4) and for the rats similarly exposed from day 4 to day 22 and allowed to deliver spontaneously, the embryo viability, the litter size, weight and survival, and the weight gain of the progeny were comparable to those for unexposed control rats.

DISCUSSION

The vulnerability of the early human embryo to chemicals that are tolerated by, or are perhaps even therapeutic to, the pregnant mother is well recognized. The continuing increase in air pollution and in the use of pesticides, food additives, drugs and radiation gives cause for anxiety about the eventual risk to human progeny, and while this is true for the general public, the problem may well be greater in women subjected to additional exposure to harmful substances while working in industry or agriculture. Apprehension would deepen

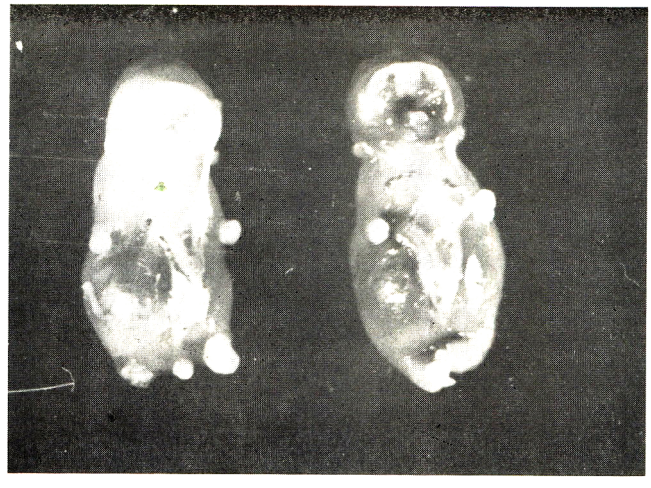

FIG. 1. Cleft face, phocomely and short tail in foetuses from rats given 4 g maneb/kg on day 11 of pregnancy.

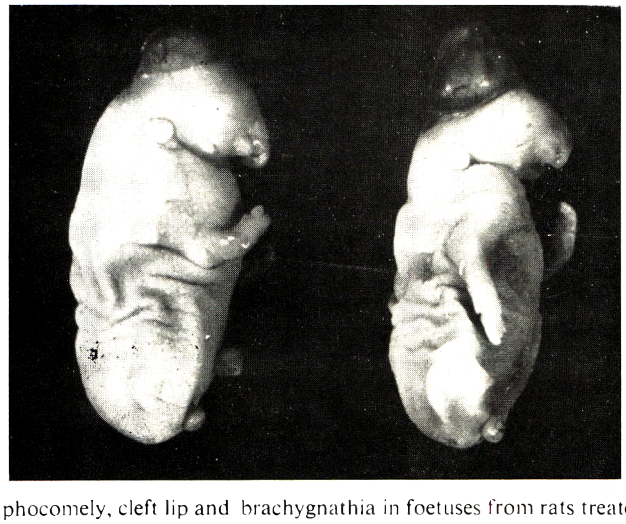

FIG. 2. Exencephaly, phocomely, cleft lip and brachygnathia in foetuses from rats treated with 2 g maneb/kg on day 11 of pregnancy.

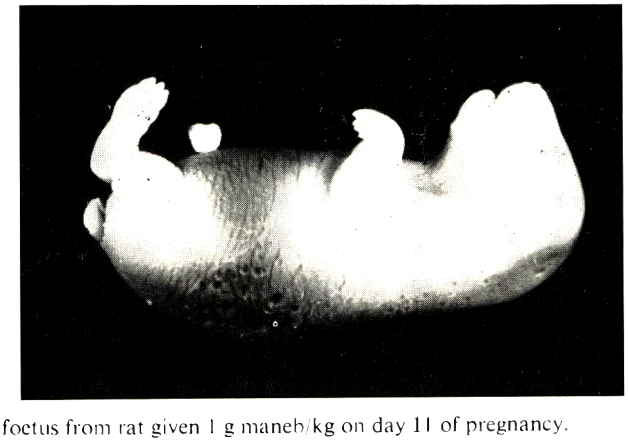

FIG. 3. Short tail in foetus from rat given 1 g maneb/kg on day 11 of pregnancy.

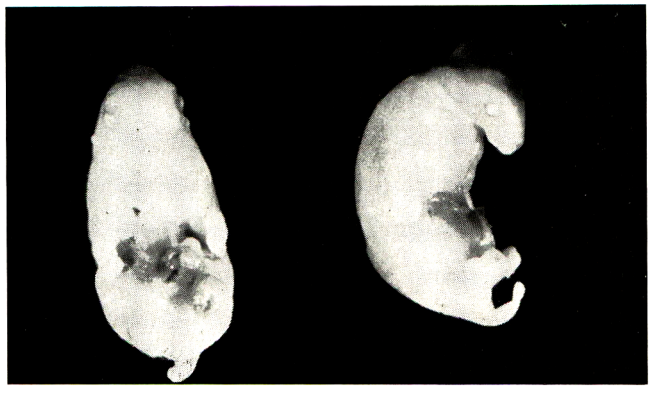

FIG. 4. Hydrocephalus, microcephaly, microphthalmia, brachygnathia and ectrodactyly in foetuses from rats given 4 g maneb/kg on day 13 of pregnancy.

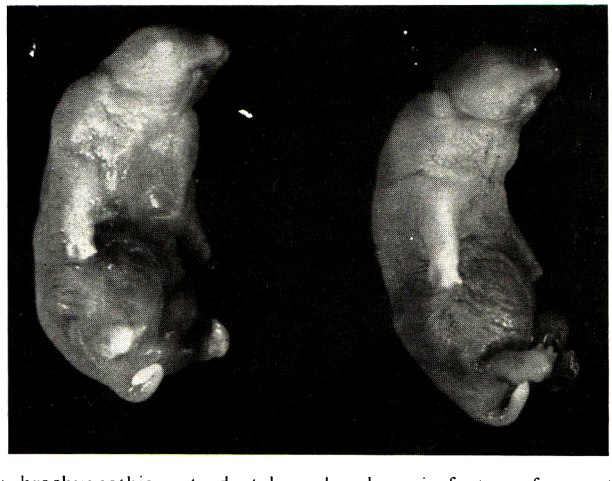

FIG. 5. Microcephaly, brachygnathia, ectrodactyly and oedema in foetuses from rats given 2 g maneb/kg on day 13 of pregnancy.

FIG. 6. Absence of centres of ossification in skull bones, vertebral column and long and short bones of the limbs together with wavy ribs in foetus from a rat given 4 g maneb/kg on day 13 of pregnancy.

FIG. 7. Absence of skull bones, ribs and some long and short bones of the limbs in foetus from rat given 2 g maneb/kg on day 13 of pregnancy.

FIG. 8. Absence of centres of ossification in the sacral and tail region of foetus from rat given 1 g maneb/kg on day 13 of pregnancy.

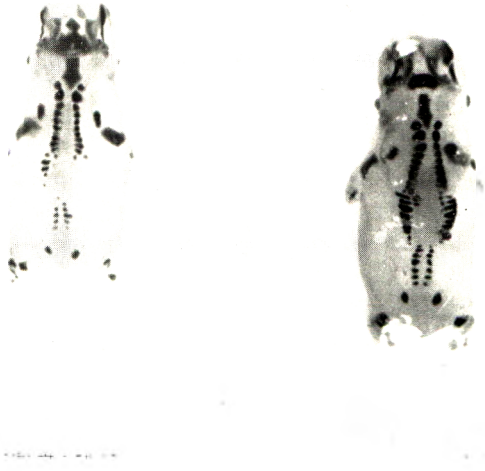

FIG. 9. Absence of upper part of skull bones, parts of vertebral column, and ribs and reduced long bones in foetuses from rats given 8 g zineb/kg on day 13 of pregnancy.

FIG. 10. Lack of ossification in skull bones, vertebral column and long bones together with wavy ribs in foetuses from rats given 4 g zineb/kg on day 13 of pregnancy.

Table 1. *Effect of a single oral dose of maneb or zineb administered to the rat on day 11 or 13 of pregnancy*

Day of treatment	Dose (g/kg)	No. of rats/group	Total no. of corpora lutea	Total implantations	Total no. of live foetuses†	Total no. of resorptions†	Total no. of late deaths†	Total no. of grossly malformed foetuses‡
—	0	14	132	114	113	1	0	0
				Maneb				
11	0·5	9	89	85	78*	5	2	0
	1·0	4	39	33	31	0	2	8***
	2·0	5	53	47	26***	0	21***	26***
	4·0	6	69	60	25***	17***	18***	25***
13	0·5	6	62	59	58	1	0	0
	1·0	8	67	51	51	0	0	33***
	2·0	3	31	29	27	1	1	27***
	4·0	6	71	66	63	1	2	63***
				Zineb				
11	1·0	6	57	55	55	0	0	0
	2·0	5	49	40	40	0	0	3*
	4·0	8	74	69	51***	11***	7 **	26***
	8·0	3	25	24	14***	0	10***	14***
13	1·0	6	61	59	58	1	0	0
	2·0	6	61	57	57	0	0	0
	4·0	4	33	33	33	0	0	15***
	8·0	5	49	45	31***	8***	6***	31***

The chi-squared test of significance was used for: †ratios of no. of events to no. of implantation sites; ‡ratios of no. of events to no. of live foetuses.

Values marked with asterisks differ significantly from those of controls: *$P < 0{\cdot}025$; **$P < 0{\cdot}005$; ***$P < 0{\cdot}001$.

were it better known that teratogens may do most harm during the earliest weeks of pregnancy, at a time when women seldom realize they are pregnant (Kalter, 1968).

It is therefore essential that sound procedures should be used for evaluating the safety of drugs, pesticides and food additives. The laboratory procedures currently used appear to be adequate for demonstrating the possession of teratogenic activity, but there still remain the problems of interpreting this experimental data in terms of human hazard and deciding what to do about substances unequivocally shown to be teratogenic in some animal species.

For several reasons the results of the studies reported here cannot be taken as an indication that the dithiocarbamate fungicides, maneb and zineb, may have a teratogenic effect on human embryos. First, the doses giving positive results in this study were appreciably higher than any likely to be ingested by man; making reasonable assumptions in the conversion of doses per unit of animal weight to human dietary levels, we have estimated that the effective doses are at least 1000 times higher than the daily human intake that could result from the consumption of foods containing the maximum permitted residues of these compounds. Secondly, the data obtained clearly demonstrate the existence of threshold

Table 2. *Incidence of gross malformations in the offspring of rats treated with maneb or zineb on day 11 or 13 of pregnancy*

	No. of foetuses affected following treatment with											
	Maneb on day						Zineb on day					
	11			13			11			13		
Malformation / Dose (g/kg)	1	2	4	1	2	4	2	4	8	2	4	8
Exencephaly	0	3	20	7	34	0	0	3	12	0	10	3
Encephalocele	0	4	0	0	16	61	0	0	2	0	0	38
Hydrocephalus	4	2	5	5	4	2	0	0	0	0	5	0
Exophthalmus	0	0	0	0	0	63	0	0	0	0	7	0
Cleft palate	0	0	25	0	54	63	0	0	14	0	15	41
Cleft lip	0	0	25	7	2	63	0	0	14	0	0	41
Micrognathia	0	0	25	0	34	63	0	0	14	0	15	41
Retarded/clubbed forelimb	0	0	25	0	54	63	0	0	14	0	15	41
Retarded/clubbed hind limb	0	8	25	5	54	63	0	4	14	0	15	41
Forelimb ectrodactyly/ oligodactyly	0	0	24	15	54	63	0	0	14	0	15	41
Hind limb: ectrodactyly	0	1	24	15	54	63	0	6	14	0	15	41
polydactyly	0	1	1	0	0	0	0	0	0	0	0	0
Short kinked tail	12	26	25	31	54	63	3	26	14	0	15	41
Umbilical hernia	0	0	0	0	0	11	0	0	0	0	0	0
Total malformed foetuses	12	26	25	31	54	63	3	26	14	0	15	41
Total viable foetuses	30	26	25	61	54	63	40	51	14	57	33	41

levels for teratogenic effect for single-dose administration of maneb and of zineb and show that at this threshold level neither compound has a cumulative effect. Thus, not only was a single dose of 1000 mg zineb/kg without effect, but so was the same dose given daily to provide a total intake of 20 g/kg. Finally it is particularly noteworthy that repeated maternal

Table 3. *Effect of daily administration of maneb or zineb to rats on days 2–21 of gestation*

Dose (mg/kg)	No. of rats/group	Total no. of corpora lutea	Total no. of implantations	Total no. of live foetuses	Total no. of resorptions	Total no. of late deaths	Total no. of malformed foetuses	Mean foetal weight (g)
0	10	88	81	73	8	0	0	4·57
				Maneb				
125	10	82	75	73	2	0	0	5·0
250	9	84	74	68	6	0	0	4·86
500	12	121	109	100	7	1	1	4·20
				Zineb				
250	9	84	70	69	1	0	0	4·84
500	11	110	83	82	0	1	0	5·18
1000	12	110	87	86	1	0	0	4·60

Table 4. *Effect of exposure of pregnant rats to an atmosphere of 100 mg zineb powder/m³ for 4 hr/day from days 5–7 to the end of pregnancy*

Days of exposure	No. of rats/group	Total no. of corpora lutea	Total implantations	Total no. of live foetuses	Total no. of dead foetuses	Total no. of foetuses with malformations
(Controls)	6	55	54	53	1	0
5–21	5	49	48	48	0	0
6–21	4	42	40	40	0	0
7–21	6	56	53	51	2	0

exposure to an atmospheric concentration of zineb 100 times in excess of the MAC had no adverse effect on the intrauterine development of the progeny. It seems unlikely, therefore, that present levels of exposure to these dithiocarbamate fungicides in agriculture present any hazard to the normal development of the human embryo.

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Détermination de l'activité tératogène de fongicides au dithiocarbamate

Résumé—On a administré à des groupes de rats femelles gravides, le 11e et le 13e jour de l'organogenèse, une dose unique orale de maneb ou de zineb, deux fongicides au dithiocarbamate. Des doses uniques de 1 à 4 g de maneb/kg et de 2 à 8 g de zineb/kg ont provoqué des anomalies congénitales chez 12 à 100% des foetus. Les dosages maximaux auxquels on n'a pas observé d'effets tératogènes étaient de 0,5 g/kg pour le maneb et de 1 g/kg pour le zineb. Aucun effet néfaste au développement intra-utérin de la progéniture n'a été observé quand on administrait journellement ces dernières doses aux femelles, du 2e au 21e jour de la portée, ou quand on exposait ces femelles dans une chambre d'inhalation dynamique, pendant 4 h par jour et à partir du 4e jour de portée, à une concentration de 100 mg de zineb/m^3. Les auteurs concluent que l'exposition aux fongicides au dithiocarbamate, telle qu'elle se produit actuellement dans l'agriculture, ne représente probablement aucune menace pour le développement normal de l'embryon humain.

Feststellung der teratogenen Aktivität von Dithiocarbamat-Fungiciden

Zusammenfassung—Die Dithiocarbamat-Fungicide Maneb und Zineb wurden in oralen Einzeldosen Gruppen trächtiger Ratten am 11. oder 13. Tag der Organogenese verabreicht. Einzeldosen von 1–4 g Maneb/kg und 2–8 g Zineb/kg induzierten congenitale Anomalien bei 12–100% der Foeten. Die maximale Dosis, bei der keine teratogene Wirkung zu beobachten war, betrug bei Maneb 0,5 g/kg und bei Zineb 1 g/kg. Es wurde keine nachteilige Wirkung auf die intrauterine Entwicklung der Nachkommen beobachtet, wenn die letztgenannten Dosen täglich Gruppen von Ratten vom 2. bis 21. Tag der Trächtigkeit verabreicht wurden oder wenn die Ratten in einer Inhalationskammer mit Luftumwälzung 4 Stunden/Tag vom 4. Tag der Trächtigkeit an einer Konzentration von 100 mg Zineb/m^3 ausgesetzt wurden. Die Autoren ziehen daraus den Schluss, dass die gegenwärtige Einwirkung von Dithiocarbamat-Fungiciden in der Landwirtschaft keine Gefahr für die normale Entwicklung des menschlichen Embryos darstellen dürfte.

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Effects of Methylmercuric Chloride on the Progeny of Mice and Rats Treated Before or During Gestation

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Abstract—Pregnant mice intubated with mercury as CH_3HgCl in doses of 0, 0·1, 1 or 5 mg Hg/kg/day from day 6 to day 17 of pregnancy, inclusive, were allowed to litter. Doses of 5 mg/kg/day caused 100% stillbirths or neonatal deaths but no apparent maternal toxicity. Doses of 1 mg/kg/day induced a transitory inhibition of cerebellar cellular migration from the external granular layer and depressed reactions of oxidative enzymes. These histological changes were not associated with any obvious adverse clinical effects and were not seen at lower doses.

Weanling female rats were fed diets containing levels of 0, 0·002, 0·01, 0·05 or 0·25 mg Hg (as CH_3HgCl)/kg/day and were mated at maturity with untreated males. Some dams were killed at term and others were allowed to litter. No adverse effect was apparent in foetuses taken at term from dams given up to 0·25 mg Hg/kg/day. Postnatally, developmental defects of undetermined aetiology were observed in the eyelids in animals from all test groups and the controls. These were associated with histological changes in the harderian gland, the exorbital lachrymal gland and the parotid salivary gland. The incidence of ocular defects was dose-related and increased significantly in the group on 0·25 mg Hg/kg/day. Rats with ocular defects were killed and at weaning the remaining offspring from each group were distributed into two sub-groups, one of which was given the appropriate methylmercury-containing diet while the other was reared on control feed. Adult mating within each sub-group revealed that continuation or cessation of treatment with methylmercury had no effect on the production of corpora lutea or on reproductive performance.

INTRODUCTION

Methylmercury has gained notoriety as an ubiquitous environmental pollutant. Investigations in the Minimata Bay area (Matsumoto, Kora & Takeuchi, 1965), at Niigata (Tsubaki, 1971) and in New Mexico (Snyder, 1971) have revealed the vulnerability of the human nervous system to methylmercury intoxication, particularly during perinatal development. The ability of methylmercury to cross the placenta, accumulate in foetal tissues and cause neuronal damage indicates that exposure to high concentrations of methylmercury constitutes a risk for the foetus. The effects of lower levels, such as those that might be encountered daily in the environment, are unknown.

There have been reports of teratogenic effects of a single or multiple parenteral injection of mercury compounds during gestation in rats (Matsumoto, Suzuki, Morita, Nakamura & Saeki, 1967), hamsters (Gale & Ferm, 1971) and mice (Spyker & Smithberg, 1972), and of postnatal effects in mice (Spyker, 1972). However, the long-term effects on foetal and postnatal development of small doses of orally administered methylmercuric chloride have not been investigated. The present study was designed to provide this information in the

*This work was carried out while one of us (S.A.T.) was the recipient of a Medical Research Council Fellowship tenable at the Food and Drug Directorate with Dr. W. P. McKinley.

rat. In addition, since the cerebellum is highly sensitive to methylmercury intoxication, a histological and histochemical study of postnatal cerebellar development was carried out in mice from mercury-treated dams.

EXPERIMENTAL

Mouse studies

Virgin Swiss–Webster female mice (from Canadian Breeding Farm Labs., St. Constant, Quebec) were paired overnight with males. The onset of pregnancy (day 1) was considered to be the first morning when a vaginal plug was observed. Methylmercuric chloride suspended in corn oil was administered orally as a single daily dose from days 6 to 17 of gestation inclusive. The doses (in mg/kg/day) and concentrations (%) of Hg (as CH_3HgCl) were: 0 (diluent alone), 0·001 (0·000025), 0·01 (0·00025), 0·1 (0·0025), 1 (0·025) and 5 (0·125) or 10 (0·25). Untreated controls were also included. The dams were allowed to litter. The day when litters were first noticed was designated postnatal day 1.

Two separate experiments were conducted: body weight, viability, appearance and behaviour of pups were observed in one experiment, and in the second, pups were killed to provide the cerebelli for histological studies. Pups were decapitated on postnatal day 2, 7, 14, 21 and 28 and the dorsal surface of the brain was exposed by removing the frontal bones. Some heads were frozen by immersion in isopentane at −78°C and stored at −20°C for periods not exceeding 3 months prior to histochemical testing. Others were fixed in 10% neutral buffered formalin for histology.

Only heads from groups given 0 (diluent only), 0·1 or 1 mg Hg/kg/day were processed further. Sections, 6–10 μ thick, taken from close to the mid-saggital plane of the cerebellum were stained with haematoxylin and eosin, with carbol-thionin and by Holzer's method (Holzer, 1929). Brains that had been frozen were used for histochemical studies, for which cryostat sections 10 μ thick were cut parallel to the mid-saggital plane of the cerebellum. The sections were gradually brought to room temperature by being placed in a freezer at −20°C for 1–2 hr, and then in the refrigerator at 4°C for 10–15 min. They were then dried under a fan for 3–5 min. The following enzymes were detected histochemically: alkaline phosphatase (Gomori, 1939), acid phosphatase (Barka & Anderson, 1962), 5′-nucleotidase (Barron & Boshes, 1961), adenosine triphosphatase (Padykula & Hermann, 1955), thiamine pyrophosphatase (Allen & Slater, 1961), esterase (Shnitka & Seligman, 1961), monoamine oxidase (Glenner, Burtner & Brown, 1957), lactic dehydrogenase (Hess, Scarpelli & Pearse, 1958), cytochrome oxidase (Burstone, 1961), aldolase (Lake, 1965), glucose 6-phosphatase (Chiquoine, 1955), succinic dehydrogenase (Nachlas, Tsou, DeSouza, Cheng & Seligman, 1957) and diphosphopyridine nucleotide diaphorase (DPND) (Scarpelli, Hess & Pearse, 1958). At least five pups at each dose level were examined at each developmental stage for each enzyme.

For all microscopic assessments, the anterior region (centralis and culmen) and posterior region (nodulus, uvula and pyramis) of the cerebelli from mercury-treated mice were compared with the corresponding anterior and posterior regions from treated control animals.

Rat study

Immature female rats (50 g body weight) of the Wistar strain (from Woodlyn Farms, Guelph, Ontario) were randomized into five groups, each consisting of 35 rats. One (control) group was fed a diet containing (in %): corn starch (64), casein (20), corn oil (Mazola, 6),

alphacel (5), mineral mix (4) (Jones & Foster, 1942) and vitamin mix AM (Becking & Morrison, 1970). (The corn oil was received from Canada Starch Co. Ltd., Montreal, and other ingredients from General Biochemicals, Laboratory Park, Ohio.) The remaining four groups were fed the same diet with CH_3HgCl added to provide 0·002, 0·01, 0·05 or 0·25 mg Hg/kg/day. The CH_3HgCl was dissolved in corn oil which was then thoroughly mixed in the diets. All animals were weighed at the start of the experiment and weekly thereafter. Dietary concentrations of mercury were adjusted weekly to maintain the dose levels indicated above. Diets were stored in air-tight containers at 6°C. All animals received diets and water *ad lib.*

When mature (weighing 175–200 g), the females were caged overnight with untreated Wistar males. The morning on which sperm were found in the vagina was considered as day 1 of pregnancy. From each dose group, 11–15 of the pregnant rats were killed at term. Foetuses from these and pups from the remaining dams that littered were examined. Foetal data such as survival, weight and skeletal anomalies were recorded, and histological sections of nervous and visceral tissues cut through the mig-saggital region were studied. Postnatal viability, body weight, behaviour and appearance were also noted. At weaning, pups with anomalous eyelids were either killed or given the control diet and then killed when 26 or 33 days old. The submaxillary, sublingual and parotid glands, as well as the harderian and exorbital lachrymal glands, were examined histologically. A representative number of the remaining weanlings from each test group was allowed to live, and of these, one-half were continued on the test diet appropriate to the group while the others received the control diet. When mature, the progeny from each sub-group were randomly paired for breeding. After 20 days, all pairs were killed and the females were examined for pregnancy. Live embryos, resorption sites and corpora lutea were noted.

Statistical methods

Standard parametric methods were used wherever possible. However, most of the data were nominal, so the binomial distribution was considered as a basis for making inter-group comparisons. It was found in each such case that intra-litter variation was significantly greater than that specified by the binomial variance. No suitable transformation or weighting procedure was found to permit inter-group comparisons by analysis of variance techniques on the nominal data, except in a few instances.

A litter formed the basic observational unit. The proportion of the litter having a particular attribute was calculated, and the proportions in different dose groups were compared by rank statistics. The Kruskal–Wallis one-way analysis of variance (Siegel, 1956) was used as a preliminary test of differences between the medians of groups. If a significance probability of less than 0·10 was obtained, two further comparisons were made: the control versus combined treated groups were compared by the Wilcoxon (Mann–Whitney) statistic and a test for dose response was made using Spearman's rank correlation coefficient. These were generally one-tailed tests. Differences significant at a 10% probability level are noted in the report of the results.

RESULTS

Postnatal effects in mice

Oral doses of 10 mg Hg/kg/day killed all the dams on test (Experiment I, Table 1). Six of the nine rats given 5 mg/kg/day in Experiment II (Table 1) failed to litter, and this dose

Table 1. *Effects on offspring of treating pregnant mice with methylmercuric chloride in daily doses of 0·001–10 mg Hg/kg/day on days 6–17 of pregnancy*

Parameter	Dose (mg Hg/kg/day)...	0 (untreated control)	0 (treated control*)	0·001	0·01	0·1	1	5	10
				Experiment I					
No. of pregnant mice		6	11	12	17	17	11	—	6†
Mean no./litter of:									
Live pups at birth		11·5	9·5	12·1	10·5	10·4	10·8		0
Live pups 2 days after birth		11·5	8·6	11·4	9·5	8·6	10·3		
Stillborns and deaths on day 1		0	0·9	0·7	1·0	1·8	0·5		
Mean pup weight (g):									
2 days after birth		1·62	1·72	1·71	1·74	1·70	1·75		
21 days after birth		10·6	10·2	12·0	12·3	10·5	11·9		
Postnatal survival (%)‡		83	92	99	95	92	83		
				Experiment II					
No. of pregnant mice		14	9	5	9	8	13	9§	—
Mean no./litter of:									
Live pups at birth		10·0	10·9	10·0	9·6	9·0	10·2	3·9	
Live pups 2 days after birth		10·0	10·8	9·2	9·6	8·9	9·6	0	
Stillborns and deaths on day 1		0	0·1	0·8	0	0·1	0·6	3·9	

*Given corn-oil vehicle.
†All died during pregnancy.
‡(No. of live pups on day 21/no. on day 1) × 100.
§Includes six dams that were unable to litter and had 56 dead foetuses.

also produced a high neonatal mortality, but no toxic effects were evident in the dams. In both experiments, the progeny from dams given doses up to 1 mg Hg/kg/day from days 6 to 17 of gestation appeared normal up to 28 days of age, as indicated by the number of live pups and stillbirths, body weight and postnatal survival.

Histologically, there was a low incidence of delayed cerebellar differentiation up to 14 days of age in offspring of mothers receiving a dose of 1 mg/kg/day (Table 2). Compared with the cerebellum from young mice in the control group (Fig. 1), there were fewer cells in the external granular layer (Fig. 2) and the molecular layer was poorly defined. The migration of Purkinje cells towards their ultimate sites was slower than normal.

In the cerebelli of 2-day-old control pups, histochemical reactions of DPND, succinic dehydrogenase and cytochrome oxidase were similar in appearance and distribution. Each enzyme was characterized by a finely granular intracytoplasmic reactivity of moderate

Table 2. *Changes in cerebellar histology in the progeny of mice given methylmercuric chloride in daily doses of 0·1 or 1·0 mg Hg/kg/day on days 6–17 of pregnancy*

Dose level (mg Hg/kg/day)	Incidence* of cerebellar change on postnatal day				
	2	7	14	21	28
0	0/27	0/22	0/21	0/20	0/21
0·1	0/5	0/2	0/2	0/2	0/5
1·0	2/16	3/11	3/12	0/11	0/15

*No. showing histological changes/total no. examined in the group at the stated time.

intensity in areas corresponding to the periphery of the future internal granular layer and lining cells of the choroid plexus. In these cerebellar areas there was diminished staining of the three enzymes in 2-day-old pups from the group treated with 1 mg Hg/kg/day. The difference in DPND reactivity in control and mercury-treated pups is demonstrated in Figs 3 and 4 respectively, and shown in greater detail in Figs 5 and 6. The corresponding areas also showed depressed adenosine triphosphatase staining in pups from this group treated with 1 mg/kg/day. Other enzymatic reactions were too faint to permit evaluation of mercury treatment in 2-day-old pups.

In 7-day-old mice, suppressed cerebellar activity was noted only in the cytochrome-oxidase reaction in pups from the 1 mg/kg/day group. At 14, 21 and 28 days of age, the cerebelli appeared normal.

Pre- and postnatal effects in rats

Long-term feeding of parent female rats with test diets containing up to 0·25 mg Hg/kg/day for 122 days had no discernible effect on body-weight gain, general behaviour or pregnancy. No histological changes indicative of mercury intoxication were seen in the maternal kidneys.

Prenatal values for corpora lutea, ratios of total implantations to corpora lutea, ratios of live to dead foetuses (including resorption sites), foetal weight and skeletal anomalies did not differ in treated groups from those in the controls. Gross and microscopic examination of foetal viscera, including the brain and spinal cord, showed no changes attributable to mercury treatment.

FIG. 1. Anterior cerebellar region from a 2-day-old mouse from the control group. External granular, molecular and Purkinje cell layers are well defined. Haematoxylin and eosin ×80.

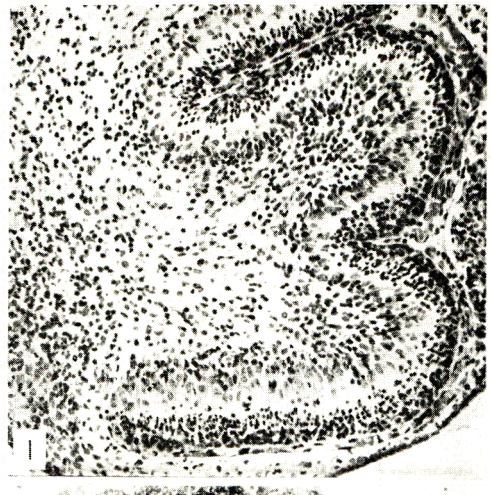
FIG. 2. Cerebellum (anterior region) of 2-day-old mouse from dam treated with methylmercuric chloride (1 mg Hg/kg/day), showing a reduction in the rows of cells at the external granular layer (arrowed). The Purkinje cells are not readily apparent. Haematoxylin and eosin ×80.

FIG. 3. DPND activity in the cerebellum of 2-day-old control mouse. The area corresponding to molecular and Purkinje cell layers shows moderate activity. ×40.

FIG. 4. Reduced DPND activity at areas of molecular and Purkinje cell layers in cerebellum of 2-day-old mouse from dam treated with methylmercuric chloride (1 mg Hg/kg/day). ×40.

FIG. 5. High-power photomicrograph of Fig. 3, showing granular distribution and staining intensity of the enzyme. ×512.

FIG. 6. High-power photomicrograph of Fig. 4 showing depressed enzyme activity. ×512.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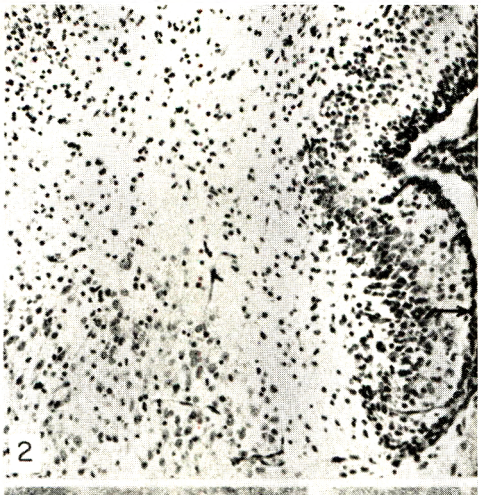
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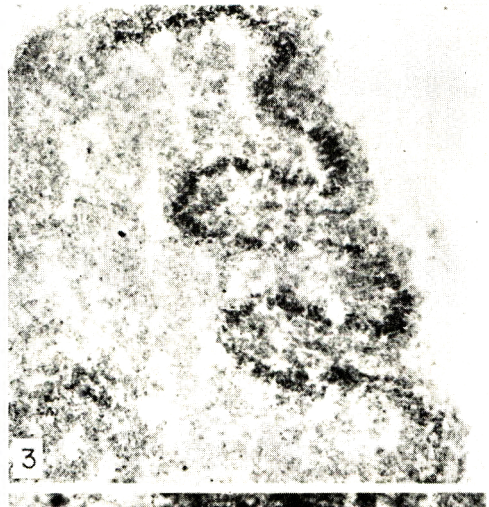
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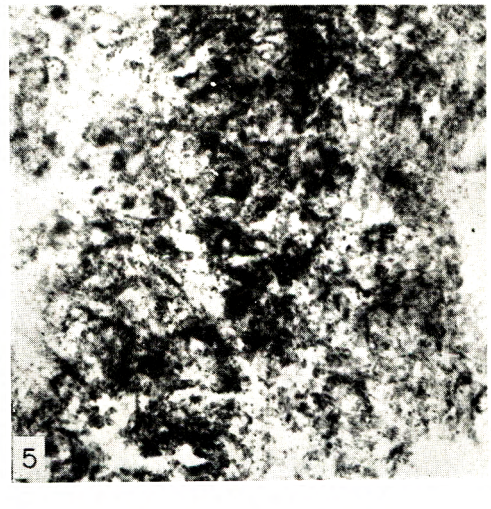
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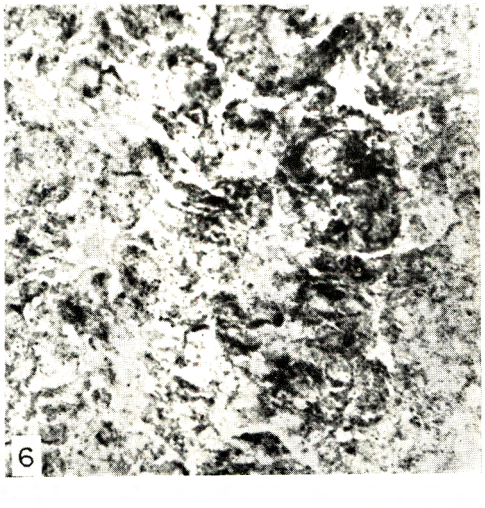
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The numbers of viable and dead (plus stillborn) pups as well as the body-weight gain and survival until weaning were comparable in all experimental groups.

A variable incidence of unilateral or bilateral ocular lesions characterized by a failure of the eyelids to separate and by suborbital oedema was seen. The sequela to the defect was either recovery in the majority of pups by 26–33 days of age or corneal opacity. These changes were seen in all experimental groups including controls (Table 3). The dose–response relationship was statistically significant ($P = 0{\cdot}01$).

Table 3. *Incidence of eyelid lesions and associated histological changes in the lachrymal and salivary glands of the progeny of rats given methylmercuric chloride at dietary levels providing intakes of 0·002–0·25 mg Hg/kg/day*

Parameter	Dose level (mg Hg/kg/day)...	0 (control)	0·002	0·01	0·05	0·25
No. of pups with eyelid and (or) eye lesions/total no. on test		23/167	25/170	35/129	28/139	51/184
No. of litters with one or more anomalous pups/no. of litters on test		9/18	6/17	5/17	10/16	15/19
No. of pups examined microscopically:						
Unilaterally affected		7	11	4	2	10
Bilaterally affected		1	1	7	2	6
No. of anomalous pups with lesions of:						
Harderian gland:						
Unilateral		3	5	3	0	3
Bilateral		1	7	7	4	13
Exorbital lachrymal gland:						
Unilateral		4	7	4	1	6
Bilateral		1	4	5	2	7
Parotid salivary gland:						
Unilateral		2	4	4	0	4
Bilateral		0	2	2	0	3

The ocular defects were associated with histological changes in the harderian and exorbital lachrymal glands and less often in the parotid salivary gland (Table 3). There was necrosis, cellular infiltration, metaplasia and keratinization of acinar cells in the harderian gland. Cytodifferentiation in the exorbital lachrymal gland was strikingly retarded. Immature acinar cells that contained oval or rounded nuclei surrounded by a relatively scarce cytoplasm were irregularly aggregated. Tubulo-alveolar arrangement was often poorly defined. In the parotid gland, acinar-cell size, cytoplasmic basophilia and secretory granules were markedly reduced. The interparenchymal stroma was increased. The incidence and severity of glandular lesions were greater in the mercury-treated groups than in the controls (Table 3).

No histopathological changes were seen in the harderian, exorbital and parotid glands of 18 weanlings in which the eyelids developed normally. The histology of these glands was similar to the normal histological appearance as described by Ball (1971), Lawson (1970), Redman & Sreebny (1971), Scott & Pease (1959), Teir (1949) and Walker (1958).

Progeny with no evident ocular defects were sub-divided at weaning. Parameters of reproductive performance were not altered in the test progeny raised to adulthood on control diet or on a mercury-containing diet at a level similar to the maternal level.

DISCUSSION

The changes in the intensity of enzyme staining in the cerebellum of 2-day-old mice suggested a focal transitory inhibition of energy metabolism. Cellular differentiation between 2 and 14 days of age was retarded in the areas of enzyme inhibition (Table 2). Since these are mitochondrial enzymes, it is noteworthy that ultrastructural defects have been reported in the mitochondria of cells treated with mercuric chloride in tissue culture (Kawahara, Yamagami & Takashima, 1971).

In the rat study reported here, continued exposure of up to 0·25 mg Hg/kg/day from weaning until the end of gestation had no adverse effect on prenatal development. In an unreported preliminary experiment, in which the protocol was similar to that of the present experiment, none of the offspring had ocular anomalies. During the present study a variable incidence of such anomalies was noted in all test groups and the control group. It was apparent that methylmercury treatment was not the direct cause of retarded eyelid development, but an increased incidence of eye defects and the marked severity of the histological lesions in the lachrymal and salivary glands following treatment with 0·25 mg/kg/day suggest that mercury was a contributory factor, in some unexplained way.

The corneal opacity observed may have been the sequela to destruction and consequently inhibited secretory activity of the harderian, exorbital and parotid glands. These glands produce a peroxidase (Essner, 1971; Herzog & Miller, 1970; Morrison & Allen, 1966), which probably functions together with lysozyme to control bacterial flora in the ocular area.

In other studies conducted in this laboratory (Khera, 1973), adult male rats continually dosed with mercury (as CH_3HgCl) were serially mated with untreated females, and preimplantation losses were recorded in all matings after 30 days of dosing with 1 mg/kg/day or 90 days with 0·5 mg/kg/day. Feeding of 0, 0·05 or 0·25 mg Hg/kg/day in the diet was initiated in 4-wk-old male rats and continued for 91 days. Four sequential trials using these males with untreated females revealed no effect on the incidence of pregnancies, deciduomata or viable embryos when compared with the control values (K. S. Khera, unpublished data, 1971). In the present study, there were no adverse effects on these reproductive parameters in two generations maintained on dose regimens of up to 0·25mg/kg/day for a maximum total of 192 days. Similarly, second-generation progeny derived from mercury-treated mothers showed no significant alterations in reproductive capacity when reared on control diets after weaning. In a recent study, no adverse effect on growth, on histology of brain or kidney or on reproductive performance was detected following chronic exposure of rats to methylmercury using a single-exposure level of about 12 μg/kg/wk (Newberne, Glaser, Friedman & Stillings, 1972). In the present investigation, somewhat similar results were obtained at a comparable dose level (0·002 mg/kg/day).

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Effets du chlorure méthylmercurique sur la descendance de souris et de rats traités avant ou pendant la gestation

Résumé—On a intubé du mercure, sous forme de CH_3HgCl et à raison de 0, 0,1, 1 et 5 mg Hg/kg/jour, du 6e au 17e jour de gestation à des souris, que l'on a ensuite laissé mettre bas. Les jeunes sont nés morts ou sont morts presque aussitôt dans 100% des cas où la mère avait reçu 5 mg/kg/jour, mais cette dose n'avait apparemment pas d'effets toxiques sur les mères. Les doses de 1 mg/kg/jour ont provoqué une inhibition passagère de la migration des cellules cérébelleuses de la couche granuleuse externe et ont affaibli les réactions des enzymes oxydatifs. Ces modifications histologiques n'étaient associées à aucun effet clinique néfaste évident et ne se sont pas manifestées à des doses plus faibles.

Des rats femelles ont été soumis à partir du sevrage à un régime alimentaire comportant 0, 0,002, 0,01, 0,05 ou 0,25 mg de Hg (sous forme de CH_3HgCl) par kg et par jour et ont été accouplés le moment venu avec des mâles non traités. Certaines femelles ont été sacrifiées au terme de la gestation et d'autres ont pu mettre bas. Aucun effet nocif n'a été observé sur les foetus prélevés à terme chez les femelles qui avaient reçu jusqu'à 0,25 mg Hg/kg/jour. Un développement défectueux des paupières, d'étiologie indéterminée, a été constaté après la naissance chez des animaux de tous les groupes, d'essai ou témoins. Il était associé à des modifications histologiques de la glande de Harder, la glande lacrymale exorbitale et la glande parotide salivaire. La fréquence des défauts oculaires était en relation avec le dosage et significativement plus élevée dans le groupe à 0,25 mg Hg/kg/jour. On a sacrifié les animaux qui présentaient ces défauts oculaires et réparti les survivants de chaque groupe de jeunes en deux sous-groupes, dont l'un a été soumis au régime comportant la dose appropriée de méthyle-mercure et l'autre l'aliment témoin. L'accouplement des adultes au sein de chaque sous-groupe a révélé que la poursuite ou l'arrêt du traitement auméthyle-mercure n'a vait aucun effet sur la production de corps jaunes ou sur les performances de reproduction.

Einfluss von Methylquecksilber(II)-chlorid auf die Nachkommenschaft von Mäusen und Ratten, die vor und während der Trächtigkeit damit behandelt wurden

Zusammenfassung—Trächtige Mäuse, die mit der Schlundsonde Quecksilber in Form von CH_3HgCl in Dosen von 0, 0,1, 1 oder 5 mg Hg/kg/Tag vom 6. bis 17. Tag der Trächtigkeit erhalten hatten, wurden werfen gelassen. Dosen von 5 mg/kg/Tag verursachten 100%iges Wegsterben der Foeten oder der Neugeborenen, aber keine offensichtliche Toxizität bei den Muttertieren. Dosen von 1 mg/kg/Tag induzierten eine vorübergehende Hemmung der cerebellaren Zellwanderung aus der äusseren granularen Schicht und unterdrückten Reaktionen oxydativer Enzyme. Diese histologischen Veränderungen waren nicht mit offensichtlich nachteiligen klinischen Effekten verbunden und konnten bei geringerer Dosierung nicht beobachtet werden.

Abgesetzte weibliche Ratten erhielten Futter mit 0, 0,002, 0,01, 0,05 oder 0,25 mg Hg (als CH_3HgCl)/kg/Tag und wurden bei Erreichung der Geschlechtsreife mit unbehandelten Männchen gepaart. Einige der weiblichen Tiere wurden bei Erreichung des Wurftermins getötet, andere werfen gelassen. Keine nachteiligen Wirkungen waren an Foeten zu beobachten, die am Ende der Trächtigkeitsperiode weiblichen Tieren entnommen wurden, die bis zu 0,25 mg Hg/kg/Tag erhalten hatten. Postnatal wurden Entwicklungsmängel unbestimmter Ätiologie an den Augenlidern von Tieren aller Versuchsgruppen und der Kontrolltiere gefunden. Diese waren mit histologischen Veränderungen der Harderschen Drüse, der exorbitalen Tränendrüse und der Ohrspeicheldrüse verbunden. Die Häufigkeit von Augendefekten war dosisabhängig und in der Gruppe der Dosierung 0,25 mg Hg/kg/Tag signifikant vermehrt. Ratten mit Augendefekten wurden getötet und beim Absetzen wurde die verbleibende Nachkommenschaft jeder Gruppe in zwei Untergruppen eingeteilt, von denen eine das entsprechende Futter mit Methylquecksilberchloridzusatz, die andere Kontrollfutter erhielt. Das Paaren der erwachsenen Tiere innerhalb jeder Untergruppe zeigte, dass die Fortsetzung oder die Einstellung der Behandlung mit Methylquecksilberchlorid keinen Einfluss auf die Gelbkörperproduktionoder auf die Fortpflanzungsleistung hatte.

Fd Cosmet. Toxicol. Vol. 11, pp. 255–263. Pergamon Press 1973. Printed in Great Britain

The Safety Testing of Medical Plastics. II. An Assessment of Lysosomal Changes as an Index of Toxicity in Cell Cultures

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Abstract—Four samples of PVC containing 0, 0·17, 0·5 and 1·4% dibutyltin diacetate but otherwise identical in every respect, were used to evaluate two tissue culture methods frequently used in the safety testing of plastics. In one method (agar overlay technique), primary neonatal rat-kidney cells were grown in petri dishes, covered with 1% agar and stained with neutral red. Pieces of the plastics to be tested were then placed on the agar and the plates were examined 24 hr later. The area of neutral-red loss underneath each piece of PVC was taken as an index of toxicity. In the other method, primary neonatal rat-kidney cells were maintained in a growth medium made with serum previously used to extract dibutyltin diacetate from the plastics. In this case, lysosomal acid-phosphatase activity was used in addition to loss of neutral red to indicate cytotoxicity. With both methods, the extent of cell necrosis was in direct proportion to the concentration of dibutyltin diacetate in the plastics. The agar overlay method proved to be the more sensitive in detecting low concentrations of the cytotoxic agent in the plastics. The histochemical method demonstrated cytotoxic changes in the cells much earlier than did the loss of neutral red, but did not improve the sensitivity of the serum extract technique.

INTRODUCTION

Tissue culture techniques have been advocated for use in toxicological evaluation because they are sensitive and relatively easy to carry out and results can be obtained with a minimum of delay. The criteria of toxicity most frequently used include estimations of cell viability, phagocytic activity and rates of mitosis (Guess, Haberman, Rowan, Bower & Autian, 1967; Rosenbluth, Guess & Autian, 1967). Histochemical techniques were used by Conning & Firth (1969) for the demonstration of cell damage in tissue culture but do not appear to have been extensively adopted.

An opportunity to investigate the usefulness of histochemistry in the safety testing of plastics presented itself during 'blind' trials of the cytotoxicity of four types of polyvinyl chloride (PVC) containing various concentrations of dibutyltin diacetate. Of the histochemical tests available (Farnes, 1967), we selected lysosomal acid-phosphatase activity because of the known participation of the lysosome in early cell damage (Abraham, Golberg & Grasso, 1967; de Duve & Wattiaux, 1966; Ericsson, 1969).

EXPERIMENTAL

Preparation of cell cultures

Culture medium. Growth medium consisted of Eagle's Minimum Essential Medium with Hanks' salts and 0·35 g sodium bicarbonate/litre, supplemented with 5% foetal calf serum, 0·2% glutamine, 100 units penicillin/ml, and 100 units streptomycin/ml.

Cultures in Leighton tubes. All experiments were performed on primary cultures of fibroblasts derived from the kidneys of 1–3-day-old rats (BRK cells). The kidneys were minced in a petri dish before being treated with 0·25% trypsin for 15 min to isolate cells. The supernatant, which contained the dispersed cells, was then pipetted into a centrifuge tube containing 10 ml of growth medium and was centrifuged at 106 *g* for 10 min. The resulting cell pellet was resuspended in growth medium and a viable cell count was carried out, using trypan blue as the vital stain. The cell suspension was diluted with growth medium to give a concentration of 4×10^5 cells/ml, and 1 ml aliquots were then placed in Leighton tubes containing glass coverslips. These tubes were incubated at 37°C for 48 hr, by which time confluent monolayers of cells were formed on the coverslips.

Cultures in petri dishes. The cells were prepared as described in the previous paragraph. The final cell suspension containing 4×10^5 cells/ml was dispensed in 7-ml aliquots into 60 mm glass petri dishes. These were sealed with either Sellotape or Parafilm and were placed in a polyethylene bag, which was heat sealed. This procedure was found to eliminate the need for a 5% carbon dioxide/air atmosphere in the incubator. The petri dishes were incubated at 37°C until a confluent monolayer of cells had formed. This took 48–72 hr.

Testing of plastics

Materials tested. The four PVC plastics used in these experiments (PVC A, B, C and D) were prepared by Imperial Chemical Industries Ltd. and were supplied as sterile sheets. They contained no plasticizer and differed from each other only in the quantity of dibutyltin diacetate they contained. At the time the tests were carried out the content of dibutyltin diacetate was unknown to us, but after the series of experiments reported here had been completed, it was disclosed that the levels were 0 in PVC A, 0·17% in B, 0·5% in C and 1·4% in D. Polyethylene, containing no tin stabilizer, was used as a negative control.

Agar overlay technique. The method described by Guess, Rosenbluth, Schmidt & Autian (1965) was employed. A 5 ml aliquot of Eagle's medium containing 1% agar and 2·5% foetal calf serum at 37°C was pipetted over the monolayer of cells in each petri dish to form an agar overlay about 4 mm thick. When this had set, approximately 5 ml of 0·01% aqueous neutral red was pipetted over the surface of the agar, and the plates were then left for 15 min to allow the neutral red solution to diffuse through the agar and stain the underlying cells. Excess neutral red solution was pipetted off. With this technique, viable cells are stained red while dead cells remain unstained. A piece of PVC, 1 cm^2, cut from each sheet by an aseptic technique was placed at the centre of a petri dish culture, which was then resealed with tape, sealed in a polyethylene bag and incubated for a further 24 hr. A transparent grid made up of 3 mm squares was used to measure the size of the zone of unstained cells produced.

Preparation of serum extracts of the plastics. Samples of the plastics measuring 5×3 cm were cut into pieces approximately $1{\cdot}5 \times 1$ cm, using an aseptic technique, and were placed in bijoux bottles containing 5 ml of foetal calf serum. These bottles were incubated at 37°C for 72 hr with occasional manual shaking. After this period the sera were pipetted off from the plastics pieces and were stored at 4°C until required. The foetal calf serum was assumed to have extracted some or all of the organotin compound from the plastics and is referred to as serum extract.

Treatment of cells with serum extracts. To investigate the toxicity of serum extracts the normal growth medium was poured off from Leighton tubes and replaced by a medium in which the foetal calf serum was replaced by one of the serum extracts. Cell sheets were

exposed to media containing the serum extract from polyethylene or from PVC A, B, C or D for 3, 7 and 24 hr and were then examined for viability using neutral red as the vital stain.

For lysosomal studies, three types of experiment were carried out. In the first, cell sheets in Leighton tubes were exposed to medium containing serum extract from each of the plastics for 3 hr and the cells on the coverslips were then stained to demonstrate lysosomal acid phosphatase activity. The procedure used was that of Gomori (1952) on cells fixed in citrate buffered 60% acetone at pH 4·6 for 15 sec (Kaplow & Burstone, 1963).

In the second experiment, the time-course of the lysosomal changes was investigated. Growth medium containing serum extract from PVC C was added to Leighton tubes for periods of 0·5, 1, 2, 3, 4, 6 and 7 hr and the cells were stained immediately afterwards for acid phosphatase. PVC C was chosen because preliminary tests showed that minimal cell necrosis occurred when monolayers were maintained for up to 7 hr in a medium containing serum extract from this material.

In the third experiment, the reversibility of lysosomal changes was investigated. Cells treated as in the second experiment were washed three times with warm phosphate buffered saline (pH 7·3) and allowed to recover in normal growth medium for periods of 3 and 24 hr before being stained for acid phosphatase.

Ultrastructural studies. BRK cells were prepared in Leighton tubes as described previously except that the cells were grown on 35 mm film-strip base instead of on glass coverslips. Once confluent monolayers of cells had been obtained, the Leighton tubes of cells were divided into three groups. Two groups were treated for 4 hr with growth medium made with the serum extract of PVC C. One of these was then allowed to recover for 24 hr in normal growth medium, before the cells were prepared for electron microscopy. The remaining group of treated cells was fixed and prepared for electron microscopy immediately after treatment, together with the third group containing untreated control cells.

Cells were prepared for electron microscopy by fixing them, still attached to the film strips, in 1% osmium tetroxide buffered with sodium cacodylate at pH 7·4 for 0·5 hr at 4°C. The cells were then dehydrated and the film strips with their attached monolayer of cells were placed in contact with Epon contained in gelatine capsules. The polymerization of the Epon was carried out overnight at 60°C. It was then possible to peel the film strips off the Epon leaving the cell monolayer embedded in the surface of the Epon (M.G. Wright, in preparation 1972). Ultra-thin sections were cut at 80 nm and stained with uranyl acetate and lead citrate before being examined in an AEI EM 6B electron microscope.

RESULTS

Agar overlay technique

The neutral red dye was taken up by the entire sheet of viable cells lying underneath the agar layer. Examined by the inverted microscope at ×50 magnification the dye appeared at first to be distributed irregularly throughout the cytoplasm, and later to be collected in discrete particles. A very few cells failed to take up the stain and these were probably necrotic.

Placing a specimen of the polyethylene control or of PVC A on top of the agar layer did not alter the staining properties of the monolayer of cells. On the other hand an area of unstained necrotic cells extended to 6 mm and 9 mm, respectively, from the edges of PVCs C and D (Table 1). PVC B produced an area not in which all cells were unstained but which consisted of a mixture of stained and unstained cells and extended up to 3–4 mm from the edge of the sample.

Table 1. *Comparison of results of agar overlay technique with those of the serum extract technique*

Type of PVC	Agar overlay: Radius of zone of cell necrosis (mm)	Serum extract: % cell death after	
		3 hr	24 hr
A	0	0	0
B	3–4*	0	0
C	6	0	20–30
D	9	5–10	90

*Mixture of live and dead cells.

Viability of cells in media containing serum extracts

Media containing serum extracts of the polyethylene control or of PVC A or PVC B had no effect on the neutral-red staining of cultured cells after contact for 3 or 24 hr. Serum extract from PVC C had no effect after 3 hr, but after 7 hr the cells were assuming bizarre shapes and about 5% had lost their neutral red stain. After exposure for 24 hr, the percentage of necrotic cells had increased to about 20–30%. Cells maintained for only 3 hr in media containing extracts of PVC D, however, showed bizarre morphological changes without appreciable decrease in neutral-red uptake. After 24 hr, most of the cells were dead or had disintegrated and were no longer stained (Table 1).

Lysosomal changes seen by light microscopy in cells treated with serum extracts of plastics

Few lysosomes could be demonstrated in cells from untreated 48-hr cultures from Leighton tubes. The number varied from 1–2 in some cells to 5–10 in others. These organelles were fairly uniform in size with a diameter of about 0·1 μm. They did not show any regular pattern of distribution (Fig. 1). This picture was not altered after the cells were exposed for 3 hr to a medium containing the serum extract from polyethylene, PVC A or PVC B.

Striking alterations occurred 3 hr after the addition of the medium containing the extract from PVC C or PVC D to the monolayer (Fig. 2). The number of lysosomes in the cells exposed to the extract from PVC C was greatly increased and the majority of lysosomes were clustered together close to the nucleus. Examined under oil immersion, the clumps appeared to be composed of discrete organelles most of which were of approximately normal size, though a few were noticeably larger than normal (Fig. 3). The number of viable cells was not reduced despite these profound lysosomal changes. The same lysosomal changes occurred in the cells exposed to the extract from PVC D for 3 hr but in addition some cell necrosis was also occurring and a number of enlarged acid phosphatase-positive organelles could be identified in a few of the cells. These enlarged organelles appeared to be about 5–10 times the size of the normal lysosomes.

Investigations into the time-sequence of these lysosomal changes revealed that the earliest changes could be detected after exposure to the extract from PVC C for 1 hr. The number of lysosomes per cell was increased and there was a distinct tendency for the organelles to aggregate close to the nucleus. After 3 hr there was a marked increase in the number of lysosomes, a perinuclear aggregation of the organelles, and the appearance of a few enlarged

FIG. 1. Control 48-hr culture of BRK cells, showing random distribution of lysosomes throughout the cytoplasm. Gomori–lead ×780.

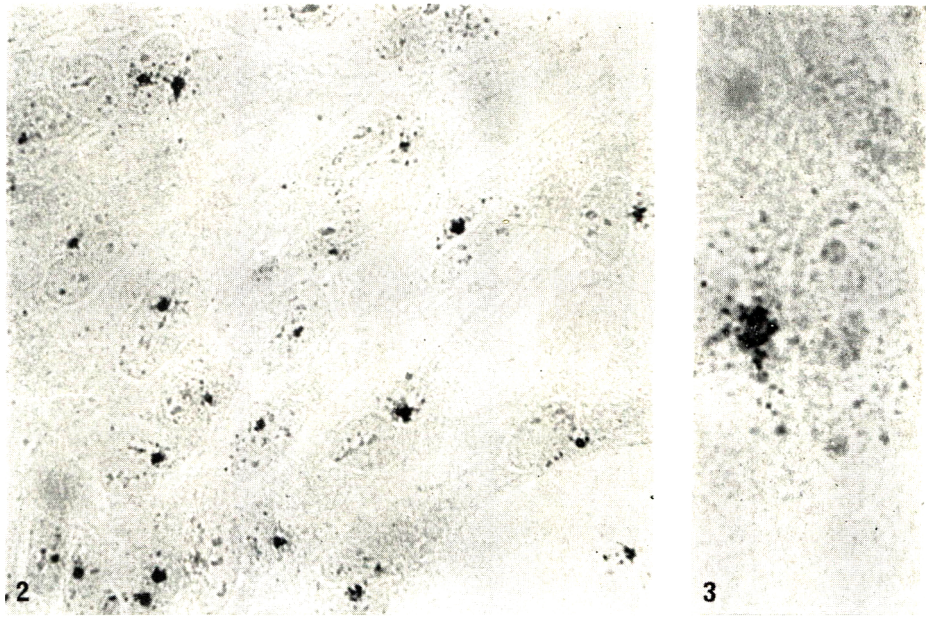

FIG. 2. A 48-hr culture of BRK cells after 3-hr exposure to growth medium containing the serum extract of PVC C, showing clumping of lysosomes in the perinuclear region. Gomori–lead. ×780.

FIG. 3. Higher magnification of a cell from the same preparation as in Fig. 2 showing clumping of lysosomes near the nucleus. Gomori–lead ×1930.

FIG. 4. Culture of BRK cells exposed for 7 hr to growth medium containing the serum extract of PVC C. Cells are assuming bizarre shapes and the majority contain enlarged lysosomes. Gomori–lead × 780.

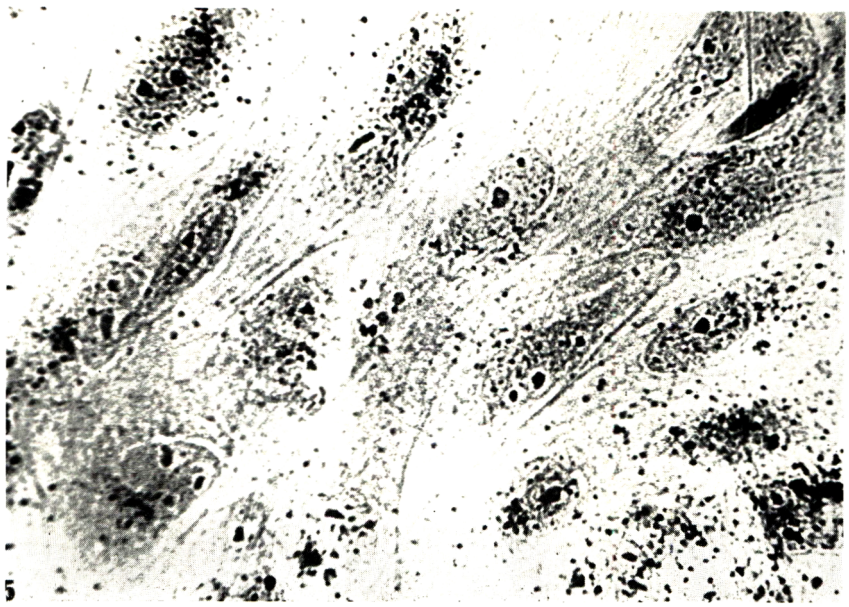

FIG. 5. After exposure of a 48-hr culture of BRK cells to growth medium containing the serum extract of PVC C for 4 hr and a subsequent recovery period of 24 hr, the number and distribution of lysosomes have returned to normal in some of the cells. Gomori–lead ×780.

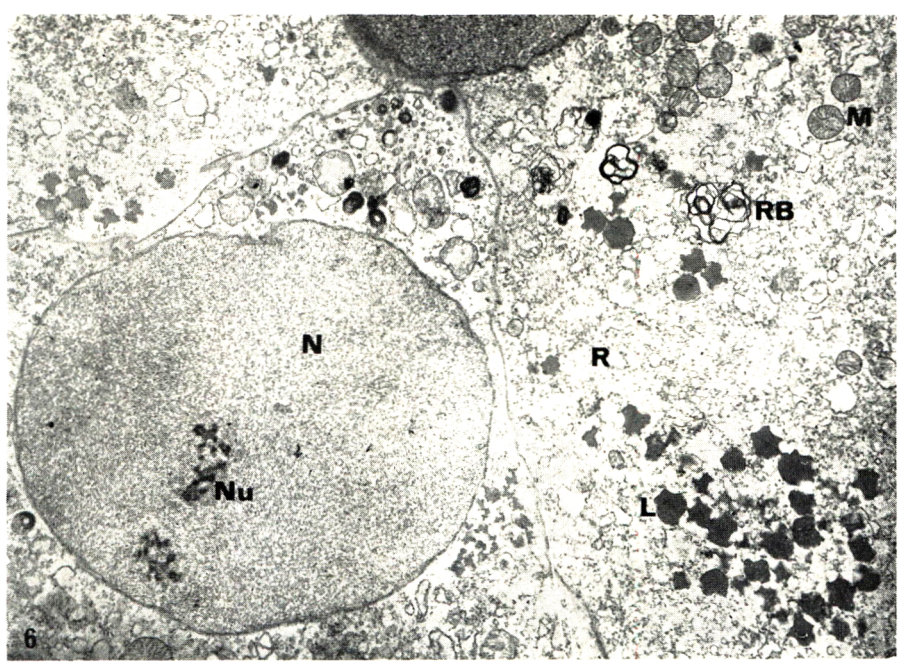

FIG. 6. Control 48-hr culture of BRK cells, with parts of two cells showing mitochondria (M) clusters of ribosomes (R), lipid droplets (L), large pale nuclei (N) with nucleoli (Nu), and loosely-whorled residual bodies (RB) enclosed by a single membrane. ×6500.

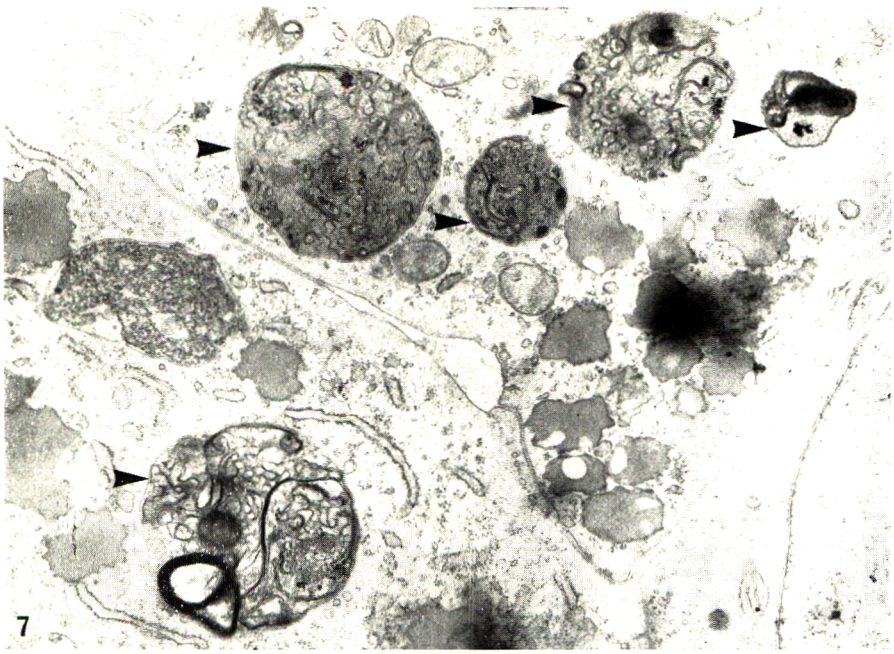

FIG. 7. BRK culture exposed for 4 hr to medium containing serum extract of PVC C. Parts of three cells are shown, with at least five autophagic vacuoles (arrowed) up to 2 μm in diameter containing material resembling mitochondria. × 17,000.

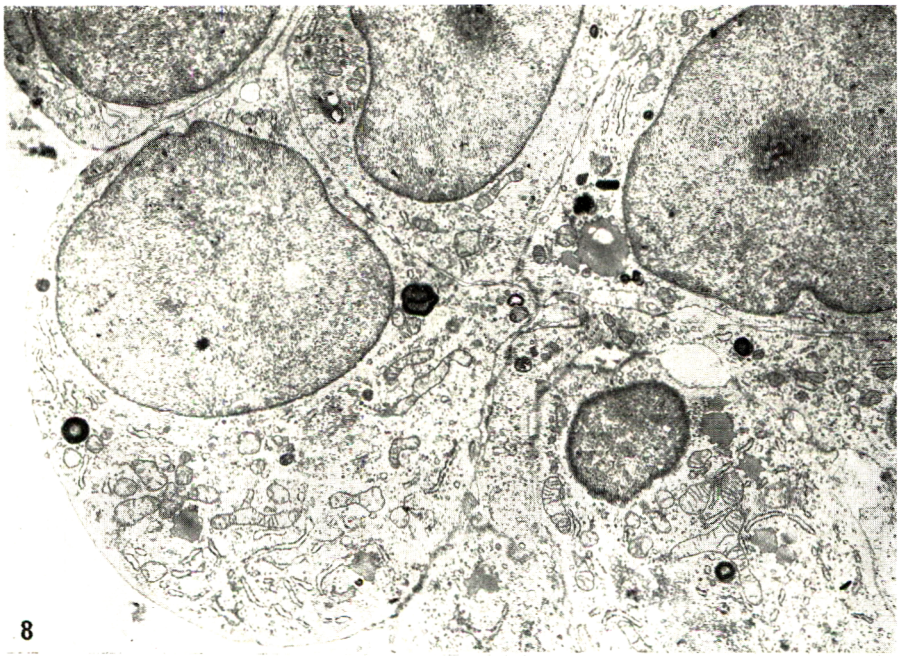

FIG. 8. In a 24-hr recovery period after 4-hr exposure of a BRK culture to growth medium containing serum-extract of PVC C, the cells have returned to normal except for numerous small myeloid bodies. × 7000.

lysosomes in this region. Enlargement of lysosomes was particularly prominent after exposure for 6 and 7 hr. The lysosomal picture at this time closely resembled that seen after exposure to the PVC D extract for 3 hr (Table 2; Fig. 4).

Table 2. *Increase in perinuclear lysosomes and formation of autophagic vacuoles in BRK cells maintained in media containing serum extracts of plastics for varying periods of time*

Duration of exposure (hr)	Reaction to serum extract from PVC			
	A	B	C	D
0·5	—	—	N	P
1	—	—	P	P
2	—	—	P	P
3	N	N	P	P/A
4	—	—	P	—
6	—	—	P/A	—
7	—	—	P/A	—

N = No reaction P = Perinuclear-lysosome increase
A = Autophagic-vacuole formation — = Not studied
Cells were examined immediately after exposure.

Some of the lysosomal changes were reversible. Thus after a 3-hr recovery period, the lysosomal pattern in cells treated with the serum extract of PVC C for 1 hr did not differ from that of untreated controls. In cells treated for 2, 3 or 4 hr, the lysosomal changes seen immediately after treatment were still present after a 3-hr recovery period but, after 24 hr, lysosomes had reverted to the control pattern (Fig. 5). In the case of cells treated for 6 or 7 hr, recovery was incomplete after a 24-hr recovery period (Table 3), and perinuclear clumping of lysosomes could still be detected in approximately 5–10% of cells.

Table 3. *Effect of duration of exposure to PVC C serum extract on rate of recovery of BRK cells from lysosomal damage, as indicated by persistence of perinuclear-lysosome increase and autophagic vacuoles*

Recovery period after treatment (hr)	Degree of recovery of cells after exposure to serum extract for					
	Hr...1	2	3	4	6	7
3	C	P	P	P	P & A	P & A
24	C	C	C	C	P	P

P = Perinuclear-lysosome increase maintained
A = Autophagic vacuoles persisting
C = Control pattern regained

Ultrastructural studies

The untreated cells from a 48-hr monolayer of BRK cells (Fig. 6) contained oval nuclei with large nucleoli in which the fibrillar and granular elements appeared to form discrete aggregates. The cells possessed a sparse rough and smooth endoplasmic reticulum which was

fairly evenly dispersed. The cisternae of the rough ER were dilated and filled with coarsely granular material. Numerous mitochondria were present, with well-defined cristae. There were few lysosomes. Occasionally larger structures were seen, consisting of loose whorls of membranes often bounded by a single outer membrane. These were interpreted as residual bodies, the end product of intralysosomal digestion.

After 4-hr exposure to serum extract from PVC C in the growth medium, the principal changes occurred in the lysosomes, which were increased in number in all the cells examined. Large autophagic vacuoles, containing objects tentatively identified as mitochondria, were locally numerous (Fig. 7). Some lysosomes resembled the myeloid bodies seen after the addition of chloroquine to leucocytes in culture (Fedorko, 1968). The mitochondria did not differ from those of untreated cells.

Not all the changes regressed if the cells were removed from the medium and allowed to recover for 24 hr. The numbers of lysosomes and autophagic vacuoles were considerably reduced, approaching the number and distribution seen in controls, but the number of myeloid bodies remained relatively high (Fig. 8).

DISCUSSION

Neutral red is readily taken up by living cells and stored in lysosomes (Allison & Paton, 1965). Stained cells lose their neutral red during the process of necrosis, so that dead cells appear unstained while living cells remain deep red in colour. Retention of neutral red by the cells in agar plates containing the negative control (polyethylene) samples or PVC A indicated that these plastics did not cause cell necrosis. On the other hand, in agar overlay cultures containing PVC B, C or D, unstained necrotic cells were present, indicating that some of the organotin additive contained in these plastics had diffused out. The cytotoxic effect of this organotin compound upon cells *in vitro* was demonstrated earlier by Conning & Firth (1969). In our experiment, the extent of the zone of necrosis surrounding the PVC B, C or D, was directly related to the concentration of the organotin compound present in the plastics. This clear concentration-related response in tissue culture was in strong contrast to the results obtained with the implantation of these plastics in the dorsal muscles of a number of rabbits. In this test system only PVC D produced an undoubted cytotoxic response, the other plastics producing a reaction comparable to that with the negative control (Pelling, Sharratt & Hardy, 1973).

Foetal calf serum appeared to elute the organotin compound from the PVC since necrosis was observed in about 5% of the cells in cultures maintained in serum extracts from PVCs C and D for 7 and 3 hr respectively. It seems likely that the lysosomal changes seen in the cells maintained in serum extracts from these plastics for shorter periods of time were also due to the organotin compound, since no lysosomal changes were seen in cultures maintained in serum extract from control plastics.

Lysosomal changes similar to those found by us have been described in macrophages and "L" cells exposed *in vitro* to toxic concentrations of chloroquine and in macrophages maintained in 50% calf serum. This high concentration of calf serum stimulates active pinocytosis in mouse macrophages, and this is accompanied by an increased lysosomal activity. Histochemical studies of these cells revealed that the first indication of an increased lysosomal acid-phosphate activity occurred in the Golgi region close to the nucleus and was detectable within 90 min (Cohn & Benson, 1965a; Cohn, Hirsch & Fedorko, 1966).

The Golgi complex was also the site at which morphological changes were first demonstrated in macrophages and "L" cells exposed to toxic concentrations of chloroquine in the medium (Fedorko, Hirsch & Cohn, 1968a,b). These changes consisted of the formation of large vacuoles which were shown to contain cytoplasmic debris and increased acid-phosphatase activity. Fluorescence studies revealed the presence of chloroquine in the Golgi region of the macrophages within a few minutes of its addition to the culture medium (Fedorko, 1968).

It is possible that a sequence of events resembling those observed on the addition of chloroquine takes place on the addition of the organotin compound, so that the increased acid-phosphatase activity and the enlarged lysosomes seen in the perinuclear area in our experiments represent early autophagic-vacuole formation. According to Arstila & Trump (1968), a considerable increase in autophagic vacuoles in response to the administration of a particular compound is evidence of chemical injury. It is therefore likely that the morphological and histochemical changes observed in the perinuclear areas of our tissue culture cells represent an early stage in cell damage.

Autophagic vacuoles are not confined to the perinuclear region, although they first appear in this area. Fedorko *et al.* (1968a) described numerous autophagic vacuoles in the peripheral areas of mouse macrophages exposed to toxic concentrations of chloroquine. Large autophagic vacuoles were also seen by us in the peripheral area of the cell after exposure to serum extract of PVC D at 3 hr and to serum extract of PVC C at 6 hr.

The perinuclear and peripheral lysosomal changes were more marked with PVC D than with PVC C, indicating that the lysosomal response was related to the concentration of the organotin compound in the medium. A threshold level of this compound appeared to be necessary for the production of cell damage, since no lysosomal changes were produced by serum extract from PVC B, although the plastics material itself produced a limited amount of necrosis in the cell monolayer by the agar overlay technique.

The increase in lysosomal acid-phosphatase activity produced by the organotin compound appeared to be reversible in the cells of the monolayer when it was confined to the perinuclear area. Reversibility occurred within 3 hr if the lysosomal changes were slight (Fig. 8), but took longer when the activity was marked. This suggested that the greater the degree of cell damage, the longer the cells took to recover. In fact, recovery was incomplete in about 20% of the cells of the monolayer when cell damage was severe enough to induce autophagy in areas distal as well as proximal to the nucleus.

The fate of the autophagic vacuoles, both perinuclear and distal to the nucleus, is uncertain. Progressive diminution in the size and in the enzyme activity of the lysosomes has been observed in macrophages removed from a medium containing a high concentration of calf serum to one with a low concentration (Cohn & Bensen, 1965b). A similar sequence of events was observed when macrophages were allowed to recover from the toxic effects of chloroquine (Fedorko *et al.* 1968a). Residual bodies could be identified by phase-contrast or electron microscopy after the return of lysosomal acid-phosphatase activity to the control level (Cohn & Fedorko, 1969). According to these authors, macrophages probably retain residual bodies during their lifespan. Our limited observations by electron microscopy identified residual bodies in 48-hr cultures, suggesting that BRK cells, like macrophages, retain residual bodies.

From our studies we conclude that the agar overlay technique is more sensitive than the serum extract method for the detection of cytotoxic agents that diffuse out of plastics. Increased lysosomal acid phosphatase in the perinuclear area provides an early means of detecting cell damage but does not increase the sensitivity of the serum extract method.

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Etude de l'innocuité de plastiques à usage médical. II. Détermination des modifications des lysosomes en tant qu'indices de toxicité dans les cultures de cellules

Résumé—Quatre échantillons de chlorure de polyvinyle (PVC) contenant respectivement 0, 0,17, 0,5 et 1,4% de diacétate de dibutyle-étain, mais sinon identiques sous tous les rapports, ont servi à évaluer deux méthodes de culture de tissus fréquemment appliquées pour vérifier l'innocuité de plastiques. Une de ces méthodes, la technique de la pellicule de gélose, consistait à cultiver dans des boîtes de Petri des cellules rénales primaires de rats nouveau-nés, à les couvrir de 1% de gélose et à les colorer de rouge neutre. On plaçait ensuite les fragments de plastique à étudier sur la gélose et examinait les boîtes 24 h plus tard. La zone de perte de rouge neutre sous chaque fragment de PVC a été considérée comme indice de toxicité. L'autre méthode consistait à garder les cellules rénales primaires de rats nouveau-nés dans un milieu de culture composé de sérum utilisé auparavant pour extraire le diacétate de dibutyle-étain des plastiques. Dans ce cas on considérait l'activité de la phosphatase acide des lysosomes, en plus de la perte de rouge neutre, comme indice de la cytotoxicité. Les deux méthodes ont révélé que l'ampleur de la nécrose des cellules était directement proportionnelle à la teneur en diacétate de dibutyle-étain des plastiques. La méthode de la pellicule de gélose s'est montrée plus sensible pour déceler de faibles taux de substance cytotoxique dans les plastiques. La méthode histochimique a dépisté beaucoup plus rapidement que la perte de rouge neutre les altérations cytotoxiques des cellules, mais elle n'a pas amélioré la sensibilité de la technique à l'extrait de sérum.

Die Sicherheitsprüfung von medizinisch verwendeten Kunststoffen. II.Die Bedeutung lysosomaler Veränderungen als Index der Toxizität in Zellkulturen

Zusammenfassung—Vier Proben von PVC, die 0, 0,17, 0,5 und 1,4% Dibutylzinndiacetat enthielten, sonst aber in jeder Hinsicht identisch waren, wurden zur Beurteilung von zwei Gewebekulturmethoden verwendet, die häufig für die Sicherheitsprüfung von Kunststoffen benutzt werden. Bei einer Methode (Agarbeschichtungsverfahren) wurden primäre neonatale Rattennierenzellen in Petrischalen gezüchtet, mit 1% Agar bedeckt und mit Neutralrot gefärbt. Stücke der zu prüfenden Kunststoffe wurden dann auf das Agar gelegt und die Schalen 24 Stunden später untersucht. Die Fläche des Neutralrotverlustes unter jedem PVC-Stück wurde als Toxizitätsindex verwendet. Bei der anderen Methode wurden primäre neonatale Rattennierenzellen in einem Nährmedium am Leben erhalten, das mit Serum hergestellt war, welches vorher zur Extraktion von Dibutylzinndiacetat aus den Kunststoffen gedient hatte. In diesem Fall wurde die Lysosomalsäurephosphataseaktivität zusätzlich zum Verlust an Neutralrot benutzt, um Cytotoxizität anzuzeigen. Bei beiden Methoden stand das Ausmass der Zellnekrose in direktem Verhältnis zur Konzentration von Dibutylzinndiacetat in den Kunststoffen. Das Agarbeschichtungsverfahren erwies sich als empfindlicher bei der Feststellung geringer Konzentrationen des cytotoxischen Mittels in den Kunststoffen. Die histochemische Methode liess cytotoxische Veränderungen in den Zellen viel eher erkennen als der Verlust von Neutralrot, verbesserte aber die Empfindlichkeit des Serumextraktverfahrens nicht.

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Electrocardiography and Blood Chemistry in the Detection of Myocardial Lesions in Dogs

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Abstract—Myocardial necrosis was induced in adult beagle dogs by a single sc injection of up to 1 mg isoprenaline/kg. Blood samples were taken at intervals up to 72 hr following injection, and electrocardiograms (ECGs) were recorded at the same times. Urine passed over the 12-hr periods before and after dosing was also collected. Clear dose- and time-related elevations of the serum enzymes, creatinine phosphokinase (CPK) and lactate dehydrogenase-1-isoenzyme (αHBDH) were seen within 12 hr of dosing, after which values gradually returned to normal. The remaining serum enzymes and all urinary enzymes examined showed no such changes. Myocardial damage, as evidenced in the ECG by S–T depression, increased T-wave amplitude and ventricular extrasystoles, correlated with the changes in the serum enzymes. Up to 8 days after dosing with isoprenaline, similar but less marked changes in the ECG pattern and in the same serum enzymes could be elicited by an iv challenge of 4 units vasopressin, indicating that the myocardial healing processes were incomplete. Concurrent recording of ECGs and assay of the serum enzymes, CPK and αHBDH, provided a sensitive monitor of isoprenaline-induced myocardial necrosis and of the subsequent recovery of the damaged tissues. Such a monitor might well be generally applicable to the detection of myocardial damage, obviating histological confirmation.

INTRODUCTION

The detection of cardiac damage in canine toxicity studies is frequently limited to the findings of terminal histopathology. Electrocardiograms recorded during the experiments may or may not show abnormal changes, while significant changes in diagnostically important serum enzymes are not commonly detected.

By sc injection of isoprenaline (2 mg/kg), Balazs, Ohtake, Cummings & Noble (1969) were able to produce myocardial damage in dogs, as evidenced by the ventricular extrasystoles evoked. Subsequent histological examination confirmed the presence of myocardial necrosis. These authors reported no significant increases in serum glutamic–oxalacetic transaminase (SGOT) or creatinine phosphokinase (CPK). The ectopic beats lasted for 1 or 2 days only. Up to 1 wk after the ECG changes caused by isoprenaline had ceased, paroxysmal ventricular extrasystoles were elicited by intravenous administration of various levels of vasopressin. Control animals showed no such changes.

This study was undertaken to determine whether any correlation existed between ECG changes and levels of certain serum and urinary enzymes. To detect these correlations, we

used the "isoprenaline-induced cardiac necrosis model" described above. Following a single sc injection of isoprenaline, frequent ECG recordings were made, and at the same intervals blood samples were collected. The total volumes of urine passed over the 12-hr periods preceding and following dosing were also collected. Blood samples were analysed for total lactate dehydrogenase (LDH), the heart-specific lactic dehydrogenase-1-isoenzyme (αHBDH), CPK and SGOT, while the urine was examined for pH, specific gravity, urinary lactate dehydrogenase (ULDH), urinary oxalacetic transaminase (UOT) and urinary alkaline phosphatase (UAP).

Between 2 and 8 days after isoprenaline administration, a single iv challenge dose of 4 units vasopressin was given. Resultant paroxysms of ectopic ventricular beats were taken as indicative of incomplete healing in the areas of myocardial necrosis induced by the isoprenaline. ECGs were recorded and blood samples were taken at about the same times as before.

EXPERIMENTAL

Animals and treatment. Nine pure-bred beagle dogs (five male and four female) varying in age from 12 to 14 months were used in the study. In an initial experiment, one animal (male A) was given a single dose of 2·5 mg isoprenaline/kg. Of the remaining eight animals, two males and one female were dosed with isoprenaline at 1 mg/kg, one male and two females were dosed at 0·5 mg/kg, and the remaining one male and one female were used as controls. A single sc dose of isoprenaline was given, the dose for each animal being made up in sterile saline to a total volume of 5 ml. Control animals received 5 ml sterile saline by the same route.

Two blood samples and two ECGs were taken before dosing. Subsequently, samples and recordings were obtained 0·25, 0·5, 1, 2, 4, 6, 12, 24, 48 and 72 hr after dosing. In all but the two females dosed at 0·5 mg/kg, the total amounts of urine passed in the 12-hr periods preceding and following dosing were also collected. When any isoprenaline-induced changes in ECG or serum enzymes had subsided, each animal was given a single iv injection of 4 units vasopressin. The times of the 'vasopressin challenge' varied from 2 to 8 days, as indicated in the appropriate tables. ECGs were recorded and blood samples were taken just prior to dosing with vasopressin, immediately after the injection, and then 0·5, 2, 6 and 12 hr after dosing. No urine analyses were performed.

ECG recordings. To obtain ECGs, the animals were restrained using an apparatus designed by Osborne (1970). This allowed the animals to sit comfortably in a "begging" position. Recordings were made in a quiet room maintained at 20°C using a Devices single-channel recorder. Three standard, three augmented, and one chest lead (V4) were taken on each occasion. A paper speed of 25 mm/sec and a standard of 1 mv = 1 cm were used throughout the study. Blood specimens were collected by puncture of the cephalic vein. To facilitate the collection of urine samples, the animals were housed in metabolism cages during the study. However, if occasion demanded, samples were collected by catheterization.

Serum-enzyme analyses. Blood specimens were centrifuged at 3000 rev/min for 15 min, and the serum was then removed for microanalysis of levels of the main enzymes affected by myocardial damage, namely, LDH, αHBDH, CPK and SGOT. Electrophoresis of the five isoenzymes of lactate dehydrogenase was performed, with subsequent qualitative and quantitative evaluation. The methods of analysis were as follows: LDH, Boehringer test kit no. 15948TLAC; αHBDH, Boehringer test kit no. 15953THAD; CPK, Boehringer test

kit no. 15926TCAF; SGOT, Sigma Bulletin 505. Electrophoresis of the five isoenzymes of LDH was carried out by a modification of the method of Farrow & Jones (1969). A solution of 1 % agar (Servac agarose) in barbitone buffer was poured over 3 × 1 in. glass slides. Two slots were cut in the agar enabling two 20 μl samples to be analysed on each slide. Electrophoresis for 1·25 hr at 60 mA was run inside a refrigerator at 4°C. Following staining, the preparations were incubated at 37°C in the dark for 1·5 hr ,washed in tap water and deionized water, and then allowed to air-dry overnight. The slides were left in contact with wet Whatman no. 1 filter-paper weighted from above to eliminate creasing of the gel. Visual estimation of the relative activity of the isoenzymes was supplemented by densitometry using a Millipore phoroscope with an appropriate filter.

Urine analyses. Urine samples were assayed for the following: volume; pH, using a Beckman veromatic pH meter; specific gravity, by refractometry; ULDH, Boehringer test kit no. 15948TLAT; UOT, Sigma Bulletin 505; UAP, Technicon method using 4-aminophenazone.

RESULTS

Within 5 min of the isoprenaline injection, vasodilation, as evidenced by bright pinkness of the abdominal skin, and increased respiration (panting) was seen in all animals. Occasionally excessive salivation or vomiting was also seen at this time. Although recovery occurred within 2 hr, the animals often preferred to remain in a lying position for up to 12 hr after dosing.

Initial study

Following a single dose of 2·5 mg isoprenaline/kg, male A showed the clinical signs described above, while ECG recordings revealed a doubling of the heart rate within 5 min of dosing (in this animal only, an ECG recording was made 5 min after dosing). The increase in heart rate persisted for 4 hr after dosing, and shortly after this the animal died. ECG changes observed included an increase in T wave amplitude (more obvious in leads I and V4), which was seen 5 min after dosing and persisted thereafter, and a few ventricular extrasystoles in the recordings made 1 and 2 hr after dosing. In addition, 2 hr after dosing complete S–A block was apparent, the rhythm being A–V junctional. The lack of inverted P waves in leads II, III and aVF indicated retrograde block. Ventricular tachycardia seen 4 hr after dosing was rapidly followed by death.

Analysis of blood specimens revealed abnormally high levels of total LDH 5 min and 2 hr after dosing. Levels of αHBDH and CPK were increased 1 hr after dosing, while 60 min later marked elevation of both parameters had occurred. Changes in SGOT were not considered significant, all values falling within the normal limits for beagle dogs. Electrophoresis of serum taken 2 hr after dosing showed clearly a marked shift of all isoenzymes to the myocardial band (LDH 1).

As a result of this initial study, three dogs were given a single isoprenaline dose of 1·0 mg/kg and a further three a dose of 0·5 mg/kg, while the remaining two animals were used as controls.

Main study

Electrocardiographic changes induced by dosing with isoprenaline

The ECG changes common to all dogs dosed with isoprenaline were an increase in heart rate, S–T depression, increased T-wave amplitude, inversion of T waves and subsequently

ectopic supraventricular or ventricular extrasystoles. No such changes were seen in control animals. Figure 1 illustrates some of these changes as seen in lead II.

Within 15 min of dosing there was a rapid increase in heart rate, and the change persisted for 12 hr after dosing. On average, an 81% increase in heart rate was seen in the dosed animals 15 min after treatment as compared to a 1% decrease in the controls over the same period.

As far as the ECG complexes are concerned, pathological changes were seen within 15 min of dosing. S–T depression, often associated with an increase in T-wave amplitude, was seen, being more obvious in leads I and V4. While the increased T-wave amplitude had subsided 2 hr after dosing, the S–T depression persisted up to 6 hr. Inverted T waves occurred within 15 min of dosing and persisted for up to 3 days thereafter. Ventricular extrasystoles were seen in all animals. They appeared as early as 1 hr after dosing and lasted up to 24 hr, except in one animal, in which they persisted for up to 3 days.

A few isolated abnormalities were seen, and these included a marked decrease in P-wave amplitude within 15 min of dosing, followed by A–V dissociation lasting up to 4 hr. This was seen in one animal given a dose of 1·0 mg isoprenaline/kg. A brief incidence of incomplete S–A block was seen in the other two animals dosed at this level, while in one male dog given a dose of 0·5 mg/kg, A–V junctional rhythm was seen over the period between 0·5 and 1 hr after dosing. In Table 1, group mean values for heart rate and the incidence of extrasystoles are listed with mean values of the serum enzymes, to demonstrate the obvious correlations that were found to exist between these parameters.

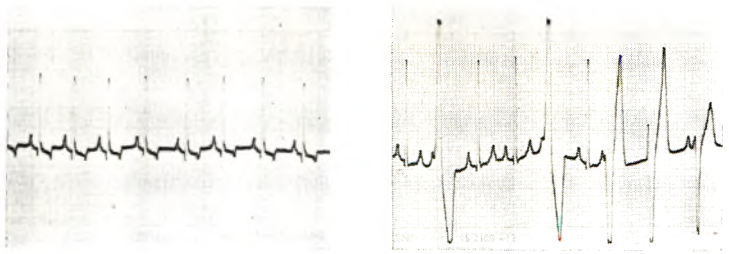
Table 1. *Mean values in ECG and serum-enzyme levels induced by a single sc dose of isoprenaline*

		Results of treatment with an isoprenaline (mg/kg) dose of								
		1·0			0·5			0 (control)		
Parameter	Time*...	P/D	6 hr	72 hr	P/D	12 hr	72 hr	P/D	6 hr	72 hr
Heart rate (beats/min)		100	174	101	127	141†	141	121	114	129
Ventricular extrasystoles‡		0	+++	0	0	+++	+	0	0	0
LDH (mIU)		100	347	160	109	324	162	113	129	124
αHBDH (mIU)		70	203	140	89	260	143	89	60	80
CPK (mIU)		33	324	55	47	390	55	25	37	29
SGOT (SF units)		25	87	42	29	82	30	25	27	23

*Hours after treatment except for initial predosing determinations (P/D).
†Although the increase in heart rate was subsiding at this time, serum enzyme levels were approaching a maximum.
‡Qualitative units for number of extrasystoles: 0 = none; + = single; +++ = many.

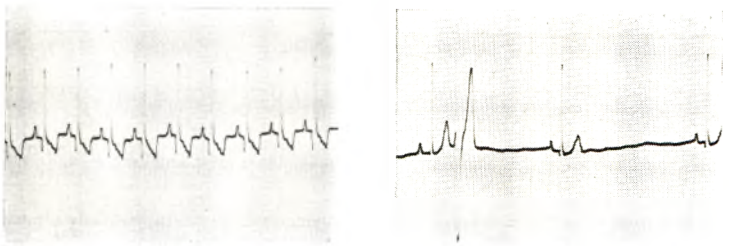
Changes in serum enzymes induced by dosing with isoprenaline

The results of the study have, for the sake of clarity, been summarized using mean values, which, in the absence of gross variations in individual results (as was the case in this study) are considered to be the more representative findings. Table 1 gives such values for each of the four enzymes studied before and after administration of isoprenaline. A clear dose-relationship emerged from the study, indicating a more rapid effect at the higher dose level.



(a) Before isoprenaline

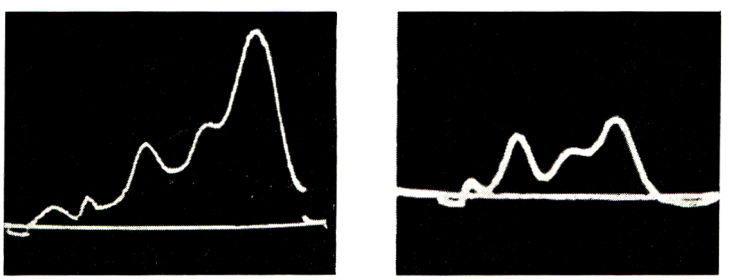
(b) +12 hr



(c) +48 hr

(d) 4U vasopressin

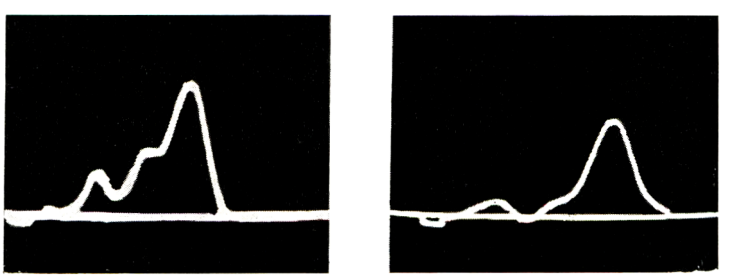
FIG. 1. ECG changes induced by a single sc dose of isoprenaline (0·5 mg/kg), and subsequently by an iv challenge dose of 4 units vasopressin.



LDH 1. LDH 2. LDH 3. LDH 4. LDH 5.

(a) 0 hr

(b) +4 hr



(c) +6 hr

(d) +12 hr

FIG. 3. Photographs of typical tracings obtained by densitometry of electrophoretic patterns obtained before and after sc injection of 1·0 mg isoprenaline/kg. Note the reduction of the LDH 5 and LDH4 isoenzymes 4–12 hr after dosing.

As such, values at 0, 6 and 72 hr after dosing are quoted for animals dosed at 1·0 mg/kg, elevations in serum enzymes approaching a maximum 6 hr after dosing, while for animals given 0·5 mg/kg, values at 0, 12 and 72 hr are given, the corresponding elevations occurring about 12 hr after dosing. Figure 2 indicates in histogram form the changes in serum enzymes induced by dosing with isoprenaline, along with the subsequent changes induced by an intravenous dose of 4 units vasopressin.

Based on values from a minimum of 500 beagle dogs, the accepted range of normality at our Research Centre is 80–150 mIU for LDH, 40–90 mIU for αHBDH, 24–46 mIU for CPK and 16–60 SF units for SGOT. In the present study, the predosing values of the above parameters were found to be normal in each of the nine animals.

Throughout the experiment, consistently normal values were found in the two control animals. In the animals dosed with isoprenaline, a transiently abnormal increase in total LDH was seen 0·25 hr after dosing with 1 mg/kg and 1 hr after dosing with 0·5 mg/kg. This is probably a flush of enzymes released by the initial action of the drug. Subsequently total LDH values rose steadily in all animals reaching a peak at about 6 hr in dogs given 1·0 mg/kg and 12 hr after dosing in those given 0·5 mg/kg. The values then began to subside.

Levels of αHBDH increased within half an hour of dosing, rising steadily thereafter and peaking at 6 hr in animals given 1·0 mg/kg and 12 hr in those given 0·5 mg/kg. Levels then began to fall. Over the period 2–72 hr after dosing, a steady total LDH/αHBDH ratio was obtained, indicating that the heart-specific enzyme was the cause of the increased total LDH values over this period.

CPK levels rose within 30 min of dosing in all animals and rapidly became abnormally elevated. Again the peak values were obtained 6 and 12 hr after dosing with isoprenaline at dose levels of 1·0 and 0·5 mg/kg respectively. The high levels did not subside until 72 hr after dosing, maximum enzyme activity occurring between 4 and 24 hr after dosing.

SGOT values increased gradually, peaking at the same time intervals as the other three enzymes. Peak values did exceed the upper limit of normality briefly, but the percentage change was less dramatic than that seen with CPK.

Electrophoresis of the five isoenzymes of LDH revealed quite clearly a cardiac shift of the enzyme pattern in all animals that had been given a single dose of isoprenaline. Figure 3 shows typical photographs of the tracings obtained by densitometry of the electrophoretic patterns. Quantitation of such tracings for a male and a female dosed with 1·0 mg/kg is given in Table 2. (Information relating to the effects of an iv dose of 4 units vasopressin is also given in Table 2, but this is described later.) Figure 3 demonstrates the reduction of the LDH5 (liver specific) and LDH4 bands after dosing with isoprenaline. In Table 2 it can be seen that as the percentages of the LDH5 and LDH4 bands were reduced, the LDH1-LDH3 bands were correspondingly increased, the increase being greatest in heart-specific LDH1. The electrophoretic pattern had almost returned to normal 24 hr after dosing.

Urinary changes induced by dosing with isoprenaline

In the early stages of the experiment, urine specimens were collected over the two 12-hr periods preceding and following dosing with isoprenaline. Samples were collected from all animals except two of those given 0·5 mg/kg. The results are given in Table 3.

No adverse changes that could be attributed to dosing with isoprenaline were seen in any of the parameters measured. However, it should be noted that after dosing with isoprenaline all urine samples showed a decrease in specific gravity.

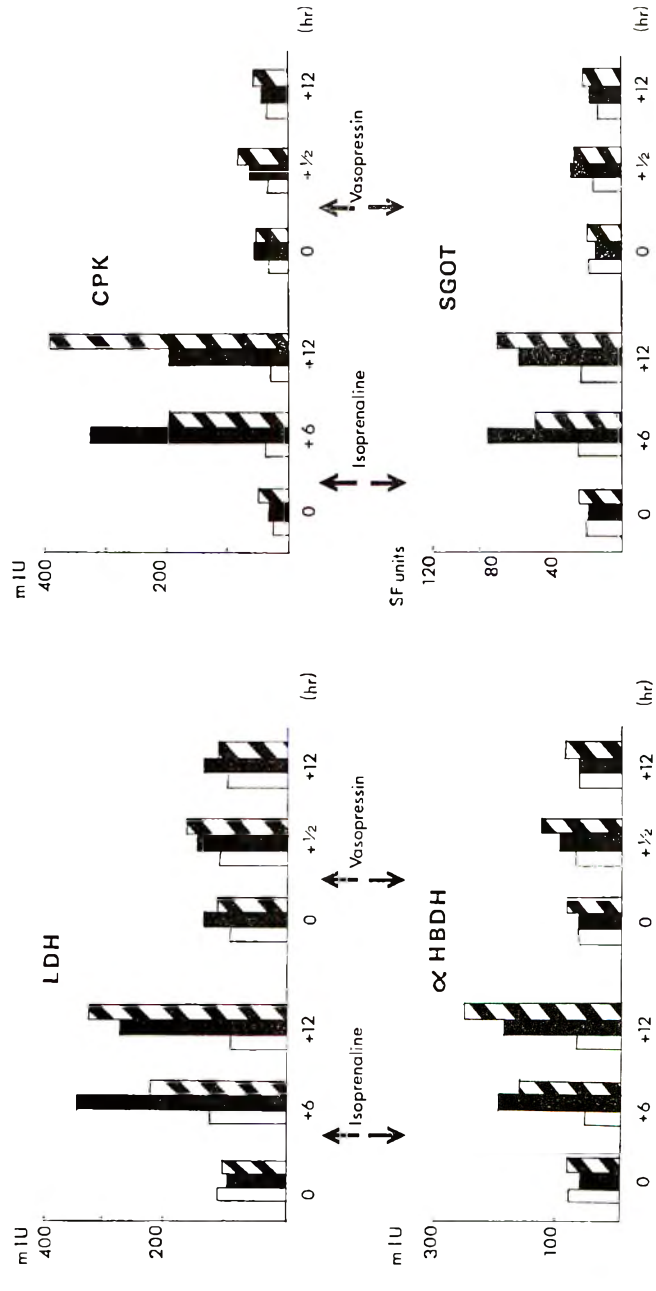


FIG. 2. Histogram of mean changes in serum enzymes in the first 12 hr following a single dose of isoprenaline (1 ▨ or 0·5 ■ mg/kg) and subsequently of vasopressin (4 units), compared with the control levels (□).

Table 2. *Quantitation of the five isoenzymes of LDH in two dogs each dosed once with 1·0 mg isoprenaline/kg sc and 7–8 days later with 4 units vasopressin iv*

Dog no. (sex)	Treatment	Sampling time* (hr)	Amounts of LDH isoenzymes (as % of total LDH)				
			LDH5 (liver)	LDH4	LDH3	LDH2	LDH1 (heart)
1 (M)	Isoprenaline	P/D	6	4	16	21	53
		1	4	6	25	23	42
		2	4	4	10	30	52
		4	—	5	15	26	54
		6	—	—	4	16	80
		12	4	14	30	14	38
		24	3	9	22	26	40
	Vasopressin	P/D	4	6	18	20	52
		0·5	—	4	54	14	28
		6	4	9	20	21	44
2 (F)	Isoprenaline	P/D	8	8	24	20	40
		1	4	6	25	23	42
		2	4	6	26	24	40
		4	—	—	15	25	60
		6	—	—	14	16	70
		12	—	3	10	15	72
		24	4	9	20	21	44
	Vasopressin	P/D	6	10	18	20	46
		0·5	—	8	42	12	38
		6	5	8	22	20	45

*Hours after treatments, except for initial predose sample (P/D).

Table 3. *Urinary enzymes in the 12-hr periods preceding and following a single dose of isoprenaline*

Isoprenaline dose (mg/kg)	Dog no. (sex)	Urine sample	Urine determinations					
			Volume (ml)	pH	SG	ULDH (mIU)	UOT (SFU)	UAP (KAU)
0 (control)	7 (M)	Predose	22	6·4	1040	67	12	17
		Postdose	120	6·9	1053	67	23	21
	8 (F)	Predose	10	6·6	1025	29	20	7
		Postdose	101	7·0	1040	48	18	19
0·5	5 (M)	Predose	25	6·4	1040	0	16	15
		Postdose	121	6·9	1021	29	13	13
1·0	1 (M)	Predose	23	7·2	1045	29	15	15
		Postdose	28	6·8	1018	67	12	17
	2 (F)	Predose	133	6·4	1035	29	10	14
		Postdose	55	6·9	1021	0	16	15
	3 (M)	Predose	144	6·7	1045	0	7	25
		Postdose	114	6·8	1026	29	12	9

SFU = Sigma Frankel units; KAU = King–Armstrong units

Electrocardiographic changes induced by vasopressin

Following the subsidence of any changes in ECGs and serum-enzyme levels induced by a single sc dose of isoprenaline, each animal was subject to a single iv injection of 4 units vasopressin, at the times indicated in Table 4. A brief return of ectopic beats of ventricular or supraventricular origin was seen in five of the six treated animals directly after the vasopressin challenge. The incidence of the extrasystoles was much lower than that seen when isoprenaline was administered. Table 4 indicates the incidence of the extrasystoles on a semi-quantitative basis. Additional changes, common to all six animals, included bradycardia (actual heart rate values are quoted in Table 5), and marked elevation and peaking of T waves, indicating myocardial anoxia. In three of the animals, S–T-segment depression was seen, being most obvious in lead III. The duration of the ectopic beats was only 2–3 min, while the bradycardia had usually disappeared 6 hr after dosing. Changes in S–T segments and T waves subsided within half an hour of dosing.

Table 4. *Effects of iv injection of 4 units vasopressin on the ECG of dogs previously dosed once sc with isoprenaline*

Isoprenaline dose (mg/kg)	Dog. no. (sex)	Time between isoprenaline dose and return of ECG to normal (days)	Time between isoprenaline and vasopressin injections (days)	Incidence of ventricular extrasystoles*
0	7 (M)	—	6	0
	8 (F)	—	7	0
0·5	4 (F)	2	3	+ (P)
	5 (M)	4	8	+ (P)
	6 (F)	2	3	+++ (R)
1·0	1 (M)	2	7	0
	2 (F)	1	8	+ (P)
	3 (M)	2	6	+ (P)

*Ventricular extrasystoles: 0 = None; + = single; +++ = many.
P = Paroxysmal ventricular premature contractions.
R = Run of ectopic ventricular beats.

Serum-enzyme changes following an iv challenge with vasopressin

The changes in serum-enzyme levels associated with a single iv dose of 4 units vasopressin have been summarized in Table 5. Because mean values are quoted, the predosing CPK values of dogs previously dosed with isoprenaline appear above the upper limits of normality (46 mIU). The reason for this is that the vasopressin challenge was given at different times following the return of the animals' ECG to normal (see Table 4), and some enzyme increases had not completely subsided.

Control animals showed no adverse changes in serum-enzyme levels following vasopressin administration, all values falling within normal limits. Within half an hour of dosing, abnormal increases in LDH, αHBDH and CPK occurred in all dogs previously dosed with isoprenaline. Transient but insignificant changes were seen in SGOT levels. Except for CPK values in dogs given 1·0 mg isoprenaline/kg, peak elevations occurred at this time. Of

Table 5. *Mean changes in serum-enzyme levels and heart rates produced by iv injection of vasopressin (4 units) in dogs previously dosed once with isoprenaline*

Sampling time*	Isoprenaline dose (mg/kg)...	Serum enzyme levels														
		LDH (MIU)			αHBDH (mIU)			CPK (mIU)			SGOT (SFU)			Heart rate (beats/min)		
		0	0·5	1·0	0	0·5	1·0	0	0·5	1·0	0	0·5	1·0	0	0·5	1·0
P/D		95	114	136	70	90	70	33	55	52	23	22	18	120	125	132
1 min		110	134	146	70	100	80	33	66	49	22	29	21	88	66	48
0·5 hr		110	169	146	75	133	100	33	80	57	20	32	33	102	73	64
2 hr		95	165	146	70	100	100	41	66	60	23	29	20	121	118	94
6 hr		110	124	146	75	100	83	37	55	47	20	23	22	113	125	96
12 hr		95	114	137	70	90	70	33	52	41	17	25	22	105	127	121

SFU = Sigma Frankel units

*Time after treatment, except for initial predosing determinations (P/D).

greater interest is the fact that dogs previously dosed once with 0·5 mg isoprenaline/kg showed a greater response than did animals dosed with 1·0 mg/kg.

Electrophoresis of the five isoenzymes of LDH isolated from dogs given vasopressin revealed at first glance a change similar to that produced by the previously given dose of isoprenaline (Table 2). However, with vasopressin the change was reduced both in duration and magnitude. Although detected within half an hour of dosing, it had subsided 5 hr later.

While disappearance of LDH5 indicated a cardiac shift, closer examination of the values revealed that LDH1 and LDH2 were also reduced, the main increase occurring in LDH3. The significance of this is not understood and warrants further investigation.

DISCUSSION

The results of this study indicate that sc administration of isoprenaline to beagle dogs in a dose of either 1·0 or 0·5 mg/kg induces myocardial damage, as indicated by the presence of extrasystoles in the animal's ECG within 1–4 hr of dosing. The incidence of aberrant wave forms reaches a maximum in the 6–24 hr after dosing, but after that it usually subsides. Associated with the ECG changes are abnormal elevations of heart-specific serum enzymes such as CPK and αHBDH. Corresponding, though less reliable, changes also occur in total serum LDH. A distinct relationship between dose level and the time of the peak enzyme levels was observed (6 hr with a dose of 1·0 mg/kg and 12 hr with 0·5 mg/kg). A single iv dose of 4 units vasopressin (approximating to 0·3 units/kg) given 3–8 days later re-elicited ventricular extrasystoles in five out of six animals, and also caused increases of lesser magnitude in the same serum enzymes over a period of 0·5–2 hr following dosing.

Unlike Balazs *et al.* (1969), we found definite increases in CPK and SGOT levels following sc administration of isoprenaline. While they found ventricular extrasystoles in the ECG, they reported no consistent increase in either of these enzymes 24 or 72 hr after two doses of isoprenaline. As a result of this study, we now know that at these times elevation in enzyme levels had subsided, having reached a maximum 6–12 hr after dosing depending on the dose level. A marked increase in SGOT 9–23 hr following myocardial infarction produced by bead embolization of the coronary tree, reported by Agress, Jacobs, Glassner, Lederer, Clark, Wroblewski, Karmen & LaDue (1955), did not appear to apply to this form of induced myocardial necrosis, the elevations in our experiments being much smaller. Siegel & Bing (1956) reported similarly timed increases in transaminase in their work with dogs.

Crawley & Swenson (1963) investigating the use of serum glutamic–pyruvic transaminase (SGPT), SGOT and LDH in detecting myocardial necrosis, concluded SGOT to be the most reliable enzyme. Values above 60 SF units were considered indicative of myocardial necrosis. Following coronary artery ligation, they encountered peak enzyme elevation 18–24 hr after infarction, as compared to 6–12 hr in our experiment. They also considered LDH to be a less reliable indicator because of erratic results and a wide range of normal values. We, too, have concluded that LDH is not an accurate index, since we encountered in control dogs values changing, for example, from 190 mIU down to 90 mIU in a 15-min period, thus making steady base-line figures unobtainable. Although two- to threefold increases in LDH values were obtained, we still consider that measurement of its heart specific isoenzyme αHBDH provides more reliable indications of myocardial damage. We have found CPK to be by far the most reliable and sensitive index of damage to cardiac tissue. It is interesting to note that in their evaluation of four different serum enzymes

(αHBDH, SGOT, CPK and LDH) in the diagnoses of acute myocardial infarction in man, Nissen, Ranløv & Weis-Fogh (1965) concluded that the combination of CPK and SGOT was the best suited for early diagnosis of myocardial damage.

The decrease in the specific gravity of the urine after isoprenaline treatment confirms the findings of Morimoto, Abe & Yamamoto (1971).

In considering the response to a vasopressin challenge, it is interesting to observe that we encountered greater changes both in ECGs and in isoenzyme levels in animals that had previously received the lower dose of isoprenaline. This may have been due to the fact that the challenge was given sooner to these animals (on average 5 days after the ECG appeared normal) whereas the animals dosed with 1·0 mg/kg received vasopressin somewhat later (on average 7 days after the normal ECGs were obtained). However, it would be logical to expect that the myocardial healing processes would be more advanced 5 days after a dose of 0·5 mg/kg than 7 days after administration of twice that level.

The pitressin test of coronary insufficiency as first described by Rusking (1947) was utilized by Balazs *et al.* (1969) and found to be a useful response for cardiotoxicity testing. We consider that this test can be further supplemented by estimation of CPK and αHBDH levels before and 30 min after the vasopressin challenge. Elevation of these heart-specific enzymes would confirm the presence of myocardial injuries.

As a result of our investigations, we suggest that by using electrocardiography in conjunction with appropriately timed estimations of CPK and αHBDH, the process of myocardial damage can be reasonably monitored. It would reveal when the myocardial healing processes are complete, and would thus obviate the need for histological confirmation of this stage.

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L'électrocardiographie et l'analyse chimique du sang dans la détection des lésions du myocarde chez le chien

Résumé—On a provoqué une nécrose du myocarde chez des chiens bigles adultes en leur administrant une injection sous-cutanée unique d'isoprénaline allant jusqu'à 1 mg/kg. On a prélevé des échantillons de sang à différents intervalles, jusqu'à 72 h après l'injection, et enregistré des électrocardiogrammes (ECG) aux mêmes moments. On a également recueilli l'urine excrétée dans les 12 h avant et les 12 h après l'injection. On a constaté que les enzymes sériques, la créatinine-phosphokinase (CPK) et l'isoenzyme 1 de la lactate-déshydrogénase (αHBDH) augmentaient nettement en corrélation avec la dose et le temps dans les 12 h suivant l'injection et que les valeurs revenaient ensuite graduellement à la normale. Les enzymes sériques restants et tous les enzymes urinaires examinés n'ont pas présenté de modification de ce genre. Les lésions du myocarde, mises en évidence dans l'ECG par la diminution de S–T, l'augmentation d'amplitude de l'onde T et des extrasystoles ventriculaires, étaient en corrélation avec les modifications des enzymes sériques. Des modifications similaires, mais moins marquées, des tracés de l'ECG et des mêmes enzymes sériques ont pu être mises en lumière jusqu'à 8 jours après l'injection d'isoprénaline par stimulation à l'aide de 4 unités de vasopressine intraveineuse; elles indiquaient que les processus de cicatrisation du myocarde n'étaient pas encore terminés. L'enregistrement d'ECG et la détermination des enzymes sériques, de la CPK et de l'αHBDH effectués en même temps ont fourni un moyen sensible de surveillance de la nécrose du myocarde provoquée par l'isoprénaline et de la guérison ultérieure des tissus lésés. Un moyen de surveillance de ce genre pourrait bien être utilisable d'une manière générale pour dépister les lésions du myocarde, tout en permettant l'économie d'une confirmation histologique.

Elektrokardiographie und Blutchemie bei der Feststellung von Myokardläsionen bei Hunden

Zusammenfassung—Myokardnekrose wurde bei erwachsenen Beaglehunden durch eine sc Einzelinjektion mit bis zu 1 mg Isoprenalin/kg herbeigeführt. Blutproben wurden in Abständen bis zu 72 Stunden nach der Injektion abgenommen, und Elektrokardiogramme wurden in den gleichen Zeitpunkten aufgenommen. Proben des während der 12 Stunden vor und nach der Injektion ausgeschiedenen Urins wurden ebenfalls gesammelt. Eindeutige dosis- und zeitabhängige Anstiege der Serumenzyme, der Creatininphosphokinase (CPK) und des Lactatdehydrogenase-1-isoenzyms (αHBDH), wurden innerhalb von 12 Stunden nach der Injektion beobachtet, wonach die Werte allmählich zur Normalität zurückkehrten. Die übrigen Serumenzyme und alle untersuchten Urinenzyme zeigten keine solchen Veränderungen. Myokardschäden, wie sie sich im EKG durch S-T-Depression, erhöhte T-Wellenamplitude und ventrikuläre Extrasystolen anzeigen, entsprachen den Änderungen der Serumenzyme. Bis zu 8 Tage nach der Injektion von Isoprenalin konnten ähnliche, aber weniger ausgeprägte Änderungen im EKG und bei den gleichen Serumenzymen durch eine iv Reizdosis von 4 Einheiten Vasopressin hervorgerufen worden, was anzeigte, dass die Myokardheilungsprozesse unvollständig waren. Die gleichzeitige Aufzeichnung von EKGs und die Auswertung der Serumenzyme, CPK und αHBDH, ergab eine empfindliche Anzeige für isoprenalininduzierte Myokardnekrose und der anschliessenden Wiederherstellung der beschädigten Gewebe. Eine derartige Überwachung könnte allgemein für die Feststellung von Myokardschäden anwendbar sein und eine histologische Bestätigung erübrigen.

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SHORT PAPERS

The Renal Toxicity of Folic Acid in Mice

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Introduction

Folic acid (pteroylglutamic acid) is widely used in the treatment of anaemias and in vitamin supplementation of the diet. There have been recent suggestions that it may have a toxic action in man (Blair, 1970; Hunter, Barnes, Curzon, Kantamaneni & Duncan, 1971; Hunter, Barnes, Oakley & Matthews, 1970) although these views have been extensively criticized (Hellström, 1971; Moir, Halliday & Williams, 1971; Richens, 1971). This communication describes some experiments which appear to lend support to the suggestion of possible toxic action in man.

During the course of experiments on the inhibition of methotrexate toxicity in mice, it became apparent that folic acid had a toxic action in this species. Unlike 5-methyltetrahydrofolic acid, small doses of which reverse the lethal action of methotrexate (Blair & Searle, 1970), folic acid would not be expected to show a protective action, since the drug blocks its conversion to the reduced derivatives essential for normal metabolism. However, under the conditions used before (Blair & Searle, 1970), it was found that folic acid in daily doses between 3 and 100 mg/kg did lead to some reduction in the mortality and weight loss due to methotrexate (25 mg/kg daily), but it was also noticed that mice treated with the higher doses of folic acid showed an increased loss in weight, indicative of an additional toxic effect attributable to folic acid. Further experiments which were then carried out with folic acid alone showed it to have a pronounced toxic action on the mouse kidney.

Preliminary experiments showed that a significant loss in weight occurred in mice treated daily with 50 mg folic acid/kg, and a severe loss in weight and of condition occurred with a dose of 100 mg/kg. Kidneys of treated animals showed marked tubular dilatation, which became more severe with increasing time of treatment. Two further series of tests were then carried out in which folic acid was administered at 75 mg/kg and the possible effects of diet and of 5-methyltetrahydrofolic acid administration were also investigated. The two experiments showed similar weight losses in the treated animals, and the results of the second rather more extensive trial are reported here.

Experimental

Male C57BL × IF F_1 hybrid mice were divided into six groups of ten, housed on sawdust in Perspex boxes each containing five animals. Groups were matched for weight, and were weighed at intervals for 2 wk before the start of treatment, at which time they weighed about 24 g. The dose regime is summarized in Table 1.

Table 1. *Scheme of treatment of groups of ten mice on days 1–5, 8–12 and 15–19 inclusive*

Group	Diet	Treatment* a.m.	Treatment* p.m.
I	Thompson	MTHF-Ca	Folic-Na
II	Oxoid	MTHF-Ca	Folic-Na
III	Thompson	Saline	Folic-Na
IV	Oxoid	Saline	Folic-Na
V	Thompson	Saline	Saline
VI	Oxoid	Saline	Saline

*MTHF-Ca = calcium salt of 5-methyltetrahydrofolic acid in normal saline, in a dose of 20 mg/kg.
Folic-Na = sodium salt of folic acid in normal saline, in a dose of 75 mg/kg.

From Monday to Friday inclusive for 3 consecutive weeks, four groups of mice were injected sc daily with folic acid, freshly dissolved in normal saline with the minimum amount of solid sodium bicarbonate (folic-Na). The solutions contained 75 mg folic acid/10 ml, and were given in an injection of 0·10 ml/10 g body weight, to provide a dose equivalent to 75 mg/kg. Two of the four groups were similarly injected 5 hr earlier with the calcium salt of 5-methyltetrahydrofolic acid (MTHF-Ca) freshly dissolved in normal saline and given in a dose of 20 mg/kg, while the other two groups were injected with normal saline only. The two remaining groups of the six received normal saline both morning and afternoon as controls.

Of each pair of groups, one was maintained throughout the experiment on a standard mouse diet containing 14–16% protein (Thompson cube diet 42; Heygates Ltd., Bugbrooke Mills, Northants.) and the other on Oxoid breeding diet (Oxo Ltd., London) containing approximately 21% protein.

Each group of mice was weighed 6 times weekly for the 3 wk of treatment and 3–4 times weekly for the following 5 wk. The day after the last injections the mouse nearest the average weight in each box was killed. The kidneys were weighed, and samples were fixed in formol-saline, sectioned and stained with Harris' haematoxylin and eosin for histological examination. At the end of the experiments, the pooled kidneys from the mice in each box were weighed, and samples for histological examination were taken as before from two mice in each group.

Results

Effects on body-weight. All mice treated with folic acid (groups I–IV) lost weight from the first injection (Fig. 1). Weight loss continued to the end of wk 2, after which some recovery

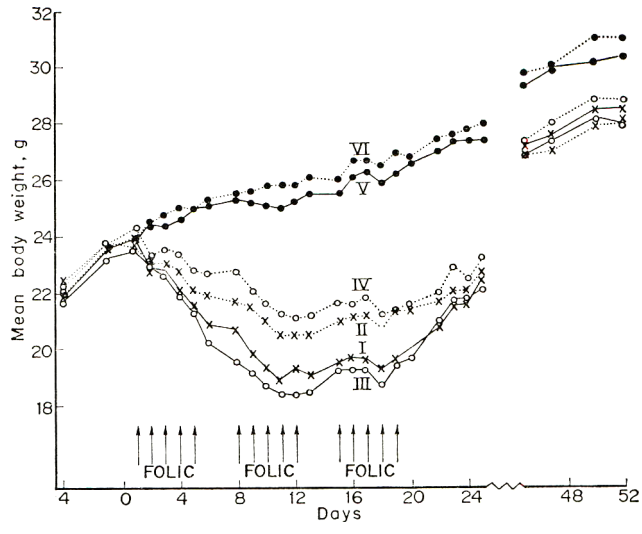

FIG. 1. Effect of folic acid injections (75 mg/kg, sc) on the body weight of male C57BL × IF mice. Experimental groups (I, ×——×; II, ×····×; III, ○——○; IV, ○····○) and control groups (V, ●——●; VI, ●····●) were treated as shown in Table 1.

occurred despite further treatment. After the last injection all these groups gained weight rapidly and after 17 days were within 2–3 g of the saline-treated controls (groups V and VI). They then remained 2–3 g lighter than the controls to the end of the experiment.

Treated mice fed the Oxoid diet (groups II and IV) lost rather less weight than those fed Thompson diet 42, but there was no indication that 5-methyltetrahydrofolic acid (groups I and II) exerted any protective action.

Effects on kidneys. Kidneys taken at the end of the last week of injections (day 20; two mice only from each group) were appreciably paler than control kidneys but did not show any consistent change in weight (treated, 131–167 mg; controls, 161–187 mg). After a further 34 days, during which no treatment was given, kidneys of the treated mice were very granular in appearance and mostly very pale. They had lost weight in the intervening period and now weighed over 40% less than the control kidneys, mean weights in the treated groups (I–IV) being 120–130 mg and in the control groups (V and VI) 211–219 mg.

At about day 30, a marked diuresis was noticed in the treated animals. The time of onset of this is not known, but subsequent measurement of water consumption over three periods of 3 days (Table 2) showed the intake of the treated mice to be increased by 81–94%, indicative of continuing derangement of kidney function.

Microscopic examination of kidney sections showed that by day 20 (Fig. 2a) there was a severe lesion in the treated animals which, though variable in extent, was constant in nature. This consisted of a widespread dilatation of tubules, which often appeared almost cystic with a single surrounding layer of columnar cells, while other tubules were shrunken or showed regeneration. There was no sign of any obstruction to account for the dilatation. The typical vacuolation of tubule cells seen in potassium deficiency was absent, and the glomeruli looked quite normal. A cellular infiltration around small blood vessels was seen in a few sections but did not appear to be related to the severity of the tubular changes. By day 54 (Fig. 2b) the changes had progressed to a characteristic irregular scarring of the

Table 2. *Water consumption of mice treated with folic acid compared with that of controls*

Time from start of experiment (days)	Mean water consumption (ml/mouse/3 days)	
	Treated groups (I–IV)	Control groups (V,VI)
33–36	28·0 ± 1·0	15·5 ± 1·0
37–40	30·7 ± 1·6	15·8 ± 0·1
44–47	24·1 ± 0·8	13·1 ± 2·0

Values are means ±SEM derived from two cages of four mice in each group.

kidney with crowding together of glomeruli. Sections then had an appearance similar to that of human material in the recovery stages of a tubular necrosis.

There was no indication that the Oxoid diet or injections of 5-methyltetrahydrofolic acid had exerted any protective effect on the kidneys. All kidney sections from control mice were normal.

Discussion

The view that folic acid administration may have toxic effects has been put forward by Hunter *et al.* (1970 & 1971) and by Blair (1970). Other authors (Hellström, 1971; Moir *et al.* 1971; Richens, 1971) have disagreed but have not obtained the high serum folate concentrations apparently necessary (Hunter *et al.* 1970 & 1971). However, in rats, a single administration of folic acid at much higher dose levels than those used in this study caused renal hypertrophy (Preuss, Weiss, Janicki & Goldin, 1972; Taylor, Threlfall & Buck, 1966; Threlfall & Taylor, 1969) and an initial anuresis followed by diuresis (Brade & Propping, 1970), while daily administration in doses up to 24 mg/kg was found to cause renal damage in guinea-pigs (Clark, Dodgen & Darby, 1953). Folic acid has also been shown to inhibit dihydrofolic acid reductase in the mouse kidney (Auletta, Mead & Waravdekar, 1970).

In our experiments, repeated administration of folic acid to mice at 75 mg/kg caused an immediate loss of weight, accompanied by severe renal tubular damage. Kidneys were still grossly abnormal 34 days after the last administration despite substantial recovery of bodyweight. The toxic effects, unlike those of methotrexate (Blair & Searle, 1970), were not reversed by 5-methyltetrahydrofolic acid, but early weight loss was marginally lower in animals on a high-protein diet.

Storage of folic acid in the tubular cells of the kidney has been demonstrated in the dog (Goresky, Watanabe & Johns, 1963), and this may be an important factor in the selective toxic effect on the kidney shown in the different studies. Preuss *et al.* (1972) showed that rat kidneys with folate-induced hypertrophy showed physiological characteristics similar to those of kidneys with hypertrophy following tubular obstruction, but in our studies neither tubular obstruction nor hypertrophy was observed in kidneys after folic acid administration. It is probably also relevant that the closely related antimetabolite methotrexate causes changes in the human kidney attributed to a direct toxic effect (Condit, Chanes & Joel, 1969).

The toxic effects we have observed in mice were obtained at dose levels of folic acid greatly in excess of those normally administered to man. However, kidney damage was

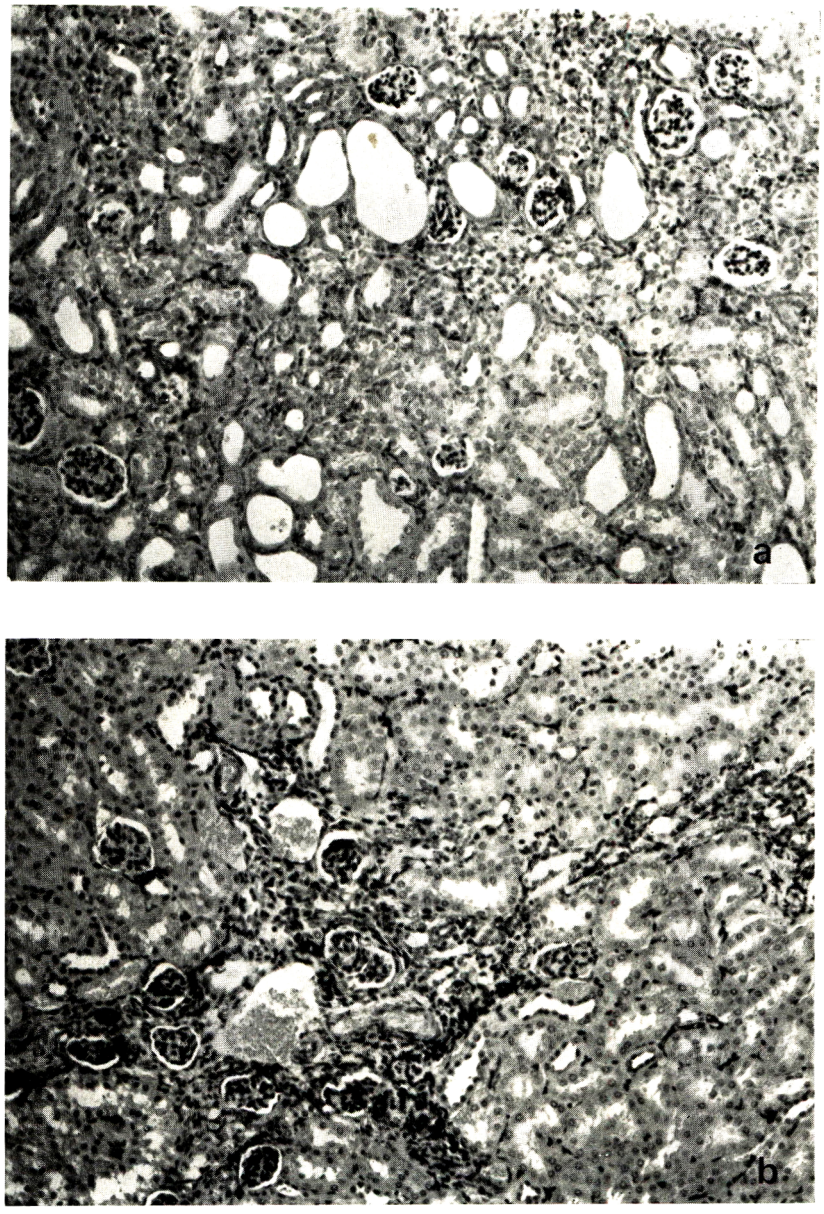

FIG. 2. Sections of kidneys from group III mice: (a) at end of folic acid treatment (day 20) showing marked dilatation of tubules; (b) after a further 34 days without treatment, showing severe scarring. Haematoxylin and eosin ×96.

severe and weight loss occurred immediately dosing began, giving reason to believe that adverse effects would still be found at dose levels considerably smaller than those employed here. As our observations are also consonant with other reports of renal effects of folic acid in experimental animals, we consider them to reinforce the view that folic acid is probably capable of exerting a toxic effect in man when given in sufficiently high doses.

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A Histochemical Study on Some Enzyme Changes in the Kidney, Liver and Brain after Chronic Mercury Intoxication in the Rat

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Summary—Adult male rats were injected sc with methylmercuric chloride (CH_3HgCl) or mercuric bichloride ($HgCl_2$) in a daily dosage of 1·0 mg Hg/kg body weight. Animals were killed after 4 wk. Histochemical studies of the enzymes glucose 6-phosphatase, alkaline phosphatase, acid phosphatase, adenosine triphosphatase (ATPase) and succinic dehydrogenase were performed on liver, kidney and brain. There were general decreases in glucose 6-phosphatase, alkaline phosphatase, ATPase and succinic dehydrogenase and a moderate increase in acid phosphatase in all the tissues examined. The extent of the enzyme changes appeared to be related to the amount of mercury distributed to each organ.

Introduction

Mercury is one of the most common causes of heavy-metal poisoning. It is also recognized as a hazardous environmental pollutant, since it may be passed on to man through contaminated food-chains. Kidneys, liver and brain are the organs most vulnerable to intoxication by mercury compounds (Klein, Herman, Brubaker, Lucier & Krigman, 1972; Takahashi, Kimura, Sato, Shiraki & Ukita, 1971). Morphological changes in these organs after mercury intoxication have been studied extensively by numerous investigators (Becker, Becker, Maher & Schreiner, 1962; Chang & Hartmann, 1972a,b,c; Fowler, 1972; Gritzka & Trump, 1968; Klein *et al.* 1972; Miyakawa & Deshimaru, 1969; Miyakawa, Deshimaru, Sumiyoshi, Teraoka, Udo, Hattori & Tatetsu, 1970; Sahaphong & Trump, 1971). However, histochemical studies on the enzyme changes following chronic mercury intoxication are few (Kosmider, 1965; Lapp & Schafé, 1960; Taylor, 1965; Timm & Arnold, 1960) and no correlative work concerning various enzyme changes in different organs has been carried out with organic and inorganic mercury compounds.

The present investigation was designed to provide information on the correlative changes of some enzymes in the kidney, liver and brain following prolonged intoxication by sublethal doses of an organic and an inorganic mercury compound.

Experimental

Animals and treatment. Three groups of four adult male Sprague–Dawley rats (mean body weight 200 g) were used. One group of animals served as controls, while the other two groups were given an aqueous solution of either mercuric bichloride ($HgCl_2$) or methylmercuric chloride (CH_3HgCl), by sc injection in a daily dose of 1·0 mg Hg/kg body weight. Animals were maintained on a standard diet and were killed by decapitation after treatment for 4 wk. Kidney, liver and cerebellum were sampled and frozen quickly for cryostat sectioning.

Enzyme studies. To demonstrate acid-phosphatase activity, sections were incubated in the glycerophosphate–lead medium of Gomori (1952) at pH 5·0 for 10 min at 37°C. Alkaline-phosphatase activity was demonstrated by incubation with a Gomori calcium–cobalt medium at pH 9·4 (Pearse, 1968) for 10 min at 37°C. Adenosine-triphosphatase activity was determined by the lead salt method (Wachstein & Meisel, 1957) at pH 7·2, with incubations of 15–30 min at 37°C. Succinic-dehydrogenase activity was demonstrated in fresh frozen sections incubated for 30 min at 37°C using the method of Nachlas (Burstone, 1962). Glucose-6-phosphatase activity was determined by the method of Zugibe (1970).

Results and Discussion

The activities of the enzymes were estimated from the intensities of the histochemical reactions. These findings are summarized in Table 1. Since the ratings of the enzyme activities were based on comparison of the same enzyme in the same organ under different experimental conditions, these ratings do not reflect valid comparisons of the activities of each enzyme in different organs.

Table 1. *Enzyme changes associated with organic (CH_3HgCl) and inorganic ($HgCl_2$) mercury intoxication*

Enzyme	Test group	Assessment of activity* in Liver	Kidney	Cerebellum
Glucose 6-phosphatase	Control	+++	+++	++
	$HgCl_2$	++	+	+
	CH_3HgCl	+	++	+
Alkaline phosphatase	Control	+++	+++	++
	$HgCl_2$	++	+	++
	CH_3HgCl	++	++	+
Acid phosphatase	Control	+	+	+
	$HgCl_2$	+	++	+
	CH_3HgCl	++	++	++
Adenosine triphosphatase	Control	++	+++	++
	$HgCl_2$	+	+	+
	CH_3HgCl	+	+	+
Succinic dehydrogenase	Control	+++	+++	++
	$HgCl_2$	++	+	++
	CH_3HgCl	+	+	+

*The activities of the enzymes in each organ were recorded as weakly positive (+), moderately positive (++) and strongly positive (+++). The rating of such histochemical reactions was based on comparison of the same enzyme in a particular organ under different experimental conditions.

The cerebellum has been shown to be extremely vulnerable to mercury intoxication (Chang & Hartmann, 1972b; Miyakawa & Deshimaru, 1969; Takeuchi, Morikawa, Matsumoto & Shiraiski, 1962). Degeneration of the granular cells as well as of the Purkinje neurons has been demonstrated. Because of its special sensitivity to mercury toxicity, the cerebellum was chosen for this study rather than any other part of the brain.

Except in the case of acid-phosphatase activity, mercury intoxication was associated with a general reduction of all the enzyme activities examined in the kidney (particularly in the

proximal convoluted tubules), in the liver (particularly near the portal area) and in the cerebellum (particularly in the granular layer). It is also apparent that methylmercury had a greater influence on the enzyme activities in the liver and brain, while mercuric bichloride exerted a stronger inhibition on those enzymes in the kidney.

It has been shown by autoradiographic study that, when given at low doses, mercury (^{203}Hg) from organic mercury compounds has a greater affinity for the liver and the nervous system, while inorganic mercury will concentrate to a greater extent in the kidneys (Berlin & Ullberg, 1963; Östlund, 1969). This may help to explain the fact that although both forms of mercury (organic and inorganic) influence enzyme activities, the enzymes in the brain and liver tend to be more affected by organic mercury and those in the kidney by inorganic mercury. It appears, therefore, that the extent of enzyme inhibition may have a direct correlation with the concentration of mercury compound in the organs involved.

The marked reduction in the activities of such enzymes as succinic dehydrogenase, adenosine triphosphatase, glucose 6-phosphatase and alkaline phosphatase reflects a disturbance in various cellular metabolisms leading to the eventual dysfunction of the organs (Chang & Hartmann, 1972d; McCreight & Witcofski, 1969; Munck & Nissen, 1956; Steinwall & Olsson, 1969). On the other hand, the increase in acid phosphatase activity is probably related to the increase in lysosomal activity in the injured cells occurring as part of the pre-necrotic changes (de Duve, 1963; Novikoff, 1961).

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Review Section

Metabolism as a Factor in Determining the Toxic Action of the Aflatoxins in Different Animal Species

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SUMMARY

Aflatoxin B_1 is a secondary metabolite of the fungus *Aspergillus flavus* and is one of the most powerful hepatotoxins known. This article reviews the present state of our knowledge of the metabolic fate of this and other aflatoxin molecules in the livers of avian and mammalian species and an attempt is made to relate metabolism to toxic action.

Introduction

Animals of different species vary in their susceptibility to acute aflatoxin poisoning, with LD_{50} values ranging from 0·3 to 17·9 mg/kg body weight (Table 1). Mature animals of a given species are generally more resistant than young ones, implying that aflatoxin-transforming enzymes develop in the liver with age. However, in a study of nine avian and mammalian species at this laboratory, no simple inverse correlation could be demonstrated between the ability of liver tissue to metabolize aflatoxin and an animal's susceptibility to aflatoxin poisoning. In fact, duckling liver metabolized aflatoxin very rapidly *in vitro*

Table 1. *A comparison of single dose LD_{50} values for aflatoxin B_1 in various species*

Species	LD_{50} (mg/kg body weight)	Reference†
Rabbit	0·3–0·5	Jones & Jones, 1969; Newberne & Butler, 1969
Duckling	0·34–0·56	Jones & Jones, 1969; Newberne & Butler, 1969
Cat	0·55	Jones & Jones, 1969
Pig	0·62	Jones & Jones, 1969
Rainbow trout	0·81*	Bauer, Lee & Sinnhuber, 1969
Dog	*c.* 1·0	Jones & Jones, 1969
Guinea-pig	1·4–2·0	Jones & Jones, 1969; Newberne & Butler, 1969; Rao & Gehring, 1971
Sheep	2·0	Armbrecht, Shalkop, Rollins, Pohland & Stoloff, 1970
Monkey	2·2	Rao & Gehring, 1971
Chick	6·5–16·5	Smith & Hamilton, 1970
Mouse	9·0	Jones & Jones, 1969
Hamster	10·2	Jones & Jones, 1969
Rat	5·5–17·9	Jones & Jones, 1969

*Intraperitoneal: other values refer to the oral route.
†Reviews and original papers.

(Patterson & Allcroft, 1970), although the species is sufficiently susceptible for day-old birds to be used widely in a sensitive bioassay for the toxin. The same data suggested that livers of different species transformed aflatoxin in different ways and although it has been held that aflatoxin is not metabolized before it exerts its toxic action (Barnes, 1967), the evidence seemed to indicate that, in many species, a metabolite was probably concerned with at least the acute toxic effects.

The *in vitro* assay of aflatoxin-metabolizing activity is intrinsically no less valid than countless similar procedures for studying hepatic metabolism generally, but special difficulties are associated with the use of aflatoxin as substrate. In particular, aflatoxin is unstable when exposed to light and because of its propensity for binding to protein, extraction from liver homogenates may be difficult. However, in all investigations carried out in this laboratory, and in many others conducted elsewhere, care has been taken to minimize errors that could arise in this way. For example, in our studies, incubations of liver tissues were carried out in stoppered flasks placed in a covered shaking incubator and, by making an initial extraction with 50% methanol, it was found that excellent recoveries of aflatoxin (measured spectrophotometrically) could be obtained from zero-time or heat-inactivated incubation mixtures. Some additional difficulties connected with the measurement of metabolic products are mentioned below. But, although rates of metabolite formation may be assayed only semi-quantitatively, the rates of overall aflatoxin metabolism (measured *in vitro*; Patterson & Allcroft, 1970; Patterson & Roberts, 1971a) are sufficiently reliable to permit the following comparisons of species differences.

Metabolism of an LD_{50} dose

Using crude liver-microsome preparations (9000 **g** supernatant fraction) in an *in vitro* assay, it was found that the overall rate of $NADPH_2$-dependent metabolism varied from 0·3 n-moles aflatoxin transformed/g liver tissue/min at 37°C in the rat to 65·7 n-moles/g/min in the duck (Patterson & Roberts, 1971a). Thus, the untransformed toxin survives long enough in rat liver for it to be regarded as the molecular form causing primary tissue damage, while in species like the duck, a high rate of metabolism probably indicates that a metabolite is involved. In order to make more valid comparisons across the species, theoretical time intervals have been calculated for the complete metabolism by the whole liver of an LD_{50} dose of toxin. This seemed a reasonable procedure because the LD_{50} value for each species is a measure of a quantity of the toxin which is capable *in vivo* of causing definite liver damage (Barnes, 1970). These time intervals may be regarded as crude reciprocal measures of the whole liver's capacity for metabolizing aflatoxin. Calculations for eight avian and mammalian species are given in Table 2. Rabbit, duckling and guinea-pig constitute a "fast-metabolizing" group being apparently capable of handling an LD_{50} dose in under 12 min. Chick, mouse, pig and sheep fall into an intermediate group, metabolizing an LD_{50} dose in a few hours. So far, the rat is the only example of a "slow-metabolizing" group in which an LD_{50} dose would probably disappear from the liver over a period of days.

Pathways of metabolism

In assessing these considerable species differences, it is important to know what metabolites are formed by the respective liver tissues, and a list of identifiable metabolites is given in Table 3. The metabolically produced changes in the structures of the aflatoxin molecules are defined in Fig. 1. In early *in vitro* studies here and elsewhere (Bassir & Emafo, 1970;

Table 2. *Liver metabolism and toxicity of aflatoxin B_1 in eight avian and mammalian species*

Species	Time required to metabolize one LD_{50} dose*	Typical hepatotoxic effect†	Aflatoxin metabolites‡			
			Microsomal			Cytoplasmic
			M_1	P_1	B_{2a}	F_1
	Fast					
Rabbit	39·6 sec	⊙	+	+	+	+
Duckling	49·8 sec	acute	+	⊙	+	+
Guinea-pig	11·8 min	acute	+	?	+	⊙
	Intermediate					
Chick	32·3 min–1·37§ hr	acute	+	⊙	+	+
Mouse	1·57 hr	⊙	+	+	+	⊙
Pig	2·50 hr	acute	+	⊙	⊙	⊙
Sheep	4·26 hr	(chronic)‖	+	⊙	⊙	⊙
	Slow					
Rat	0·8–2·6 days§	chronic	+	+	?	⊙

*Time for the disappearance of an LD_{50} dose from the whole liver calculated from *in vitro* metabolic data (Patterson & Allcroft, 1970; Patterson & Roberts, 1971a), published LD_{50} value sand liver weights (Patterson & Roberts, 1970b).

†Lancaster (1968): "acute" = acute necrosis with or without fibrosis and bile duct proliferation; "chronic" = liver tumours induced in chronic experiments; ⊙ = not stated.

‡For details of transformations see Fig. 1; + = metabolite formed; ? = doubtful; ⊙ = not detected.

§Using either of the two extreme LD_{50} values given by Smith & Hamilton (1970).

‖This is probably based on the observed occurrence of a liver tumour in only one of a small group of sheep fed contaminated groundnut for 5 years (Lewis, Markson & Allcroft, 1967).

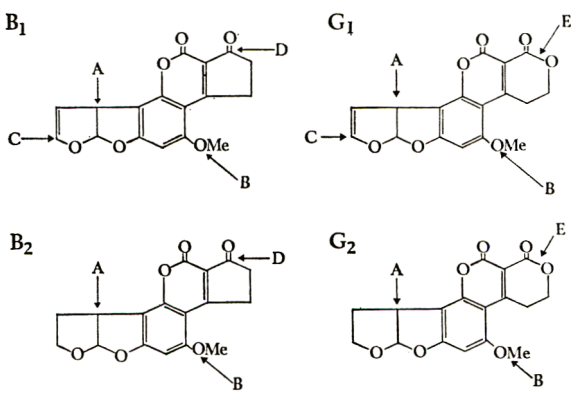

FIG. 1. Metabolic transformation of the aflatoxins by liver enzymes: A, 4-hydroxylation; B, *O*-demethylation to a phenolic derivative; C, hydration of the vinyl ether double bond to yield the 2-hydroxy derivative or hemiacetal; D, cyclopentenone reduction (aflatoxins B) to form a secondary alcohol; E, hydrolytic fission and decarboxylation of the δ-lactone ring of aflatoxins G (so far known only as a pathway of fungal metabolism). Known metabolites of aflatoxin B_1: aflatoxin M_1 (route A), aflatoxin P_1 (route B), aflatoxin B_{2a} or hemiacetal (route C), aflatoxicol or F_1 (route D). Known metabolites of B_2: aflatoxin M_2 (route A), dihydro-aflatoxicol or F_2 (route D). Known metabolites of G_1: aflatoxin GM_1 (route A), aflatoxin G_{2a} (route C), aflatoxin B_3 or parasiticol (route E). Aflatoxin G_2 is probably hydroxylated to GM_2.

Patterson & Allcroft, 1970; Schabort & Steyn, 1969) it was assumed that all metabolic transformations were mediated by the mixed function hydroxylases of the endoplasmic reticulum (microsomal enzymes). Indeed in the presence of $NADPH_2$, crude or isolated microsomal preparations from the livers of many species were found to be capable of transforming aflatoxin B_1 to its 4-hydroxy derivative (aflatoxin M_1) to some extent (Bassir & Emafo, 1970 & 1971; Patterson & Allcroft, 1970; Schabort & Steyn, 1969; Steyn, Pitout & Purchase, 1971), and the analogous product, aflatoxin GM_1 may be formed from aflatoxin G_1. However, it became apparent that some livers could also form aflatoxin hemiacetals *in vitro* (Patterson & Allcroft, 1970; Patterson & Roberts, 1970a). Both aflatoxins B_1 and G_1 were substrates for $NADPH_2$-dependent enzyme which hydrated the vinyl ether double bond of these toxins to form the 2-hydroxy derivatives or hemiacetals, also known as aflatoxins B_{2a} and G_{2a}, respectively. Rabbit, duckling, guinea-pig, chick and mouse livers have a marked ability to carry out this transformation and, by comparison, the formation of aflatoxin M is a minor pathway. It has also been suggested that rat liver has aflatoxin hydrase activity, but lengthy incubation periods were needed before the hemiacetal was demonstrable (Schabort & Steyn, 1969).

Table 3. *Comparative metabolism of aflatoxin B_1 in twelve species*

	Aflatoxin metabolites			
Species	M_1	P_1 (demethylated-B_1)	B_{2a}	Aflatoxicol
Mouse	+	+	+	⊙
Rat	+	+	?	○
Rabbit	+	+	+	+
Guinea-pig	+	?	+	○
Chick	+	○	+	+
Duck	+	⊙	+	+
Quail	+	○	+	+
Turkey	+	○	+	+
Cattle	+	○	○	–
Goat	+	○	○	–
Pig	+	○	○	–
Sheep	+	○	○	–

+ = Aflatoxin metabolite formed *in vitro* by liver preparation.
? = Doubtful.
○ = Metabolite not detected.
– = No observations made.

Another microsomal function of rat, mouse, guinea-pig and rabbit livers is the demethylation of the aflatoxins (Bassir & Emafo, 1970; Patterson & Roberts, 1971b). The phenolic product formed from aflatoxin B_1 and isolated from monkey urine has been named P_1 (Dalezios, Wogan & Weinreb, 1971). Presumptive evidence for this pathway based on the *in vivo* metabolism of ^{14}C-methoxy-labelled aflatoxin in rats and mice had been available for some time (Shank & Wogan, 1965; Wogan, 1969).

Microsomal-enzyme activity can be enhanced by phenobarbitone and DDT treatment and it has been shown that, by these means, the rat may be protected from the acute (McLean & McLean, 1967) and chronic (McLean & Marshall, 1971) effects of aflatoxin poisoning. In

stimulating the conversion of aflatoxin B_1 to M_1 and P_1, the elimination of the toxin is probably facilitated by its biliary excretion as taurocholic acid conjugates or as glucuronides (Bassir & Osiyemi, 1967).

When the effect of phenobarbitone pretreatment on the *in vitro* microsomal metabolism was compared in ducks and rats, it was found that while aniline-hydroxylating activity was stimulated in both species, the metabolism of aflatoxin was enhanced only in the rat, aflatoxins M_1 and P_1 being identified as the main metabolites. By contrast, the rate of overall metabolism in crude microsomes was unchanged in the duck by this treatment (Patterson & Roberts, 1971b). This led to the discovery of an alternative cytoplasmic pathway for aflatoxin metabolism (Patterson & Roberts, 1971a) which was well developed in the rabbit and in avian species (chick, duck, turkey and quail) (Patterson, 1971). Aflatoxin B_1 and B_2 are apparently reduced by a soluble $NADPH_2$-dependent enzyme (Fig. 1) to the corresponding cyclopentenols (Patterson & Roberts, 1971a), the former being already known as aflatoxicol, a product of fungal metabolism (Detroy & Hesseltine, 1970), and the latter, by analogy, as dihydro-aflatoxicol. As 17-ketosteroid sex hormones inhibited this cytoplasmic reduction of aflatoxin, it has been proposed that in these species a soluble $NADPH_2$-linked 17-hydroxysteroid dehydrogenase is involved in the transformation (Patterson & Roberts, 1972a).

Other microsomal transformations of aflatoxin may exist. Thus, it has been proposed by Schoental (1970) that the isolated vinyl ether double bond of aflatoxin is susceptible to metabolic oxidation in much the same way as is the K-region of polycyclic aromatic hydrocarbons and that this might account for the carcinogenic properties of the toxin. Indeed, the suggested aflatoxin epoxide could also be an intermediate in the formation of aflatoxin B_{2a} (hemiacetal) by liver microsomal enzymes in certain avian and mammalian species (Patterson & Roberts, 1970a).

The formation by microsomal enzymes of another but unidentified metabolite has been demonstrated by Garner, Miller, Miller & Garner (1971). This may or may not be related to Schoental's proposed epoxide and has been detected by its pronounced bactericidal activity.

Importance of different pathways

It is difficult in *in vitro* systems to quantify the relative importance of the soluble and microsomal pathways for aflatoxin metabolism where both exist, because of the protein-binding properties of aflatoxin hemiacetals (Patterson & Roberts, 1970a & 1972b). Early work was hampered because this fact was not appreciated. Thus, it was supposed that the production of a non-fluorescent metabolite accounted for a large portion of the total aflatoxin metabolism in livers of avian species (Patterson & Allcroft, 1970; Patterson, Roberts & Allcroft, 1969) when, in fact, a considerable proportion of the hemiacetal had been precipitated with protein after incubation of liver tissues with aflatoxin. It has recently been suggested that this metabolite in a dialdehydic resonance hybrid form (Fig. 2) binds to protein by forming Schiff bases with free amino-groups (Patterson & Roberts, 1972b) and this could play a fundamental part in the acute toxic action of aflatoxin, for example by the binding and inhibition of key enzymes of intermediary metabolism leading to hepatic cell necrosis. Some support for this proposal comes from the observations of Schabort & Pitout (1971) who found that aflatoxins B_{2a} and G_{2a} inhibit pancreatic DNAase probably by binding to the enzyme with energy greater than that of a hydrogen bond. None of this is necessarily at variance with the observation that aflatoxin hemiacetals are non-toxic

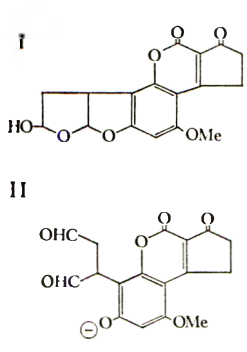

FIG. 2. Structure I represents the aflatoxin B_{2a} or hemiacetal molecule and II one of three dialdehydic resonance hybrids (Pohland *et al.* 1968) which may bind to proteins by forming Schiff bases.

(Dutton & Heathcote, 1968; Pohland, Cushmac & Andrellos, 1968). Binding to the epithelium of the gastro-intestinal tract would presumably occur when the material was given orally as it would remain unabsorbed, eventually to be eliminated with desquamated epithelial cells. Species belonging to the "fast-metabolizing" group and the chick and mouse from the intermediate group are all capable of hemiacetal formation. This seems to support the suggestion that the formation of the hemiacetal by microsomal enzymes within the hepatocyte is an important factor in producing acute toxic effects in species that metabolize aflatoxin rapidly (Table 2). The very active cytoplasmic reductive pathway in rabbit and avian livers (Patterson & Roberts, 1971a) almost certainly exerts a modifying effect, but as this pathway is reversible (Patterson & Roberts, 1972b) it may do no more than act as a reservoir for aflatoxin, which is subsequently converted to the hemiacetal or bound to intracellular structures and thus produces a chronic effect upon the liver (Fig. 3).

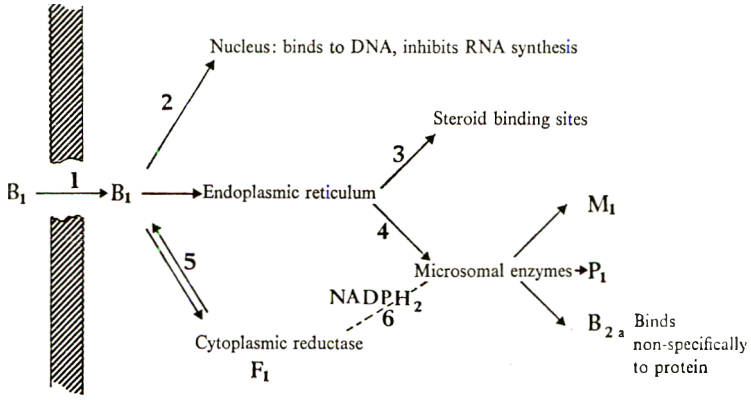

FIG. 3. Schematic representation of the factors controlling the fate of an aflatoxin B_1 molecule in a typical liver cell. Following transport across the cell membrane (1) the aflatoxin molecule may bind to nuclear DNA (2) or to sex-determined sites on the endoplasmic reticulum (3). Alternatively it may be metabolized by microsomal enzymes (4) or undergo reversible reduction by cytoplasmic enzymes (5). The co-factor $NADPH_2$ (6) is required for routes 4 and 5.

Factors determining the toxic action of aflatoxin

The carcinogenic action of aflatoxin is thought to depend upon its binding to DNA, on inhibition of RNA synthesis (Wogan & Pong, 1970) and possibly on its interaction with sex-related binding sites on the endoplasmic reticulum (Williams & Rabin, 1971). Animals, like the rat, capable only of slow metabolism would appear to be most vulnerable to this kind of chronic liver damage by the untransformed toxin. However, in any species, the possibility of chronic liver damage by unchanged aflatoxin is real when dosing or feeding is prolonged. Thus, hepatomas have been induced in the duck during a long-term feeding experiment (Carnaghan, 1967), although one more usually associates the acute effects of aflatoxicosis with this species.

Animals that actively metabolize aflatoxin to the hemiacetal seem to be particularly vulnerable to acute hepatotoxic effects and survivors from the effect of a single sub-lethal dose tend to escape chronic liver damage. However, those with an additional cytoplasmic reductive pathway are potentially liable to suffer protracted acute-type and/or even chronic effects if this pathway is considered to be an "aflatoxin reservoir" as suggested above (Fig. 3).

One factor not considered in the above discussion is the transport of aflatoxin into the liver cell. This may prove to be a decisive consideration in our eventual understanding of the relationship between the toxicity of aflatoxin and its metabolism. So far, comparative data are available only for the mouse and rat (Portman, Plowman & Campbell, 1970) and it has been shown that although mouse liver metabolizes aflatoxin much faster than rat liver (see also Patterson & Roberts, 1971a), it is probably less susceptible to acute poisoning because the toxin is less efficiently taken up by mouse hepatocytes than by those of the rat.

In summary, once the toxin has entered the liver cell, the agency causing tissue injury in a particular animal species is dictated by the rate and pattern of aflatoxin metabolism. When it is metabolized slowly, untransformed toxin is almost certainly the active molecular species with chronic liver damage the probable result. When it is metabolized rapidly, metabolites rather than the original toxin would seem to be involved. Acute liver damage may be caused by the intracellular formation of aflatoxin hemiacetal in many species. In view of the recent reports of an association between human liver disease and the presence of aflatoxin in body tissues (Becroft & Webster, 1972; Shank, Bourgeois, Keschamras & Chandavimol, 1971), it is urgently necessary to determine where man fits into this picture. The detection of aflatoxin at all in human liver may indicate a slow rate of metabolism, since our early experiments showed that in duckling, which is a "fast-metabolizing" species, an oral dose of 500 μg of the toxin was cleared from the liver in no more than 30 min (Patterson & Allcroft, 1969).

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REVIEWS OF RECENT PUBLICATIONS

Supplementary Report on the Review of the Emulsifiers and Stabilisers in Food Regulations, 1962. Food Additives and Contaminants Committee. Ministry of Agriculture, Fisheries and Food, London, 1972. pp. 34.

Following the 1970 Review of the Emulsifiers and Stabilisers in Food Regulations 1962 (*Cited in F.C.T.* 1970, **8,** 551), the Food Additives and Contaminants Committee (FACC) in conjunction with the Pharmacology Sub-Committee (PSC) of the Committee on Medical Aspects of Food Policy has published a supplementary report based upon representations made by industry. In making these further recommendations, the Committee has also considered the EEC's draft directive on emulsifiers, stabilizers and thickening and gelling agents.

The FACC recommends that starches (including modified starches) should continue to be exempt from future Emulsifiers and Stabilisers in Food Regulations and should be subject to a separate instrument, which may take into account certain EEC proposals. Sorbitan and polyoxyethylene sorbitan esters of fatty acids and dioctyl sodium sulphosuccinate, whose use in staple items of the diet would have been restricted by the recommendations of the 1970 report, have been reconsidered in the light of further toxicity data and no restrictions on their use are now proposed. Citroglycerides, propylene glycol esters of fatty acids and lactic acid, sucrose esters of fatty acids, sucroglycerides and the sodium and calcium salts of stearoyl-2-lactylic acid have been reclassified by the PSC from group E (substances on which the available evidence is insufficient to permit an adequate assessment) to group A (additives that the available evidence suggests are acceptable for use in food) or group B (those that on the available evidence may be regarded as provisionally acceptable for use in food, but about which further information is required within a specified time). The need for all these substances in food, other than bread, is accepted. Citroglycerides are recommended also for use in bread, but the sodium and calcium salts of stearoyl lactylic acid are only recommended for use in high-protein and gluten breads.

Polyglycerol esters of dimerized fatty acids of soya-bean oil, which have been reclassified by the PSC from group E to group B, are recommended by the FACC for inclusion in the permitted list for use as tin-greasing agents provided the carry-over into bread or other foods does not exceed 20 ppm. Other permitted emulsifiers or stabilizers may be used as tin-greasing agents provided the carry-over of oxidatively polymerized soya-bean oil does not exceed 50 ppm. Moreover, furcelleran and xanthan gums have been recommended for inclusion in the list of permitted edible gums, and the use of polyoxyethylene (20) sorbitan tristearate and mono-oleate and polyglycerol esters of polycondensed fatty acids of castor oil is to be allowed following up-grading of these materials by the PSC from group B to group A. Recommended specifications for permitted emulsifiers and stabilizers have been included as an Appendix to the Supplementary Report.

An Annex to the Supplementary Report reproduces the latest report of the PSC. The additional toxicological information submitted in support of representations on the earlier

report enabled the Sub-Committee to reclassify several substances in group A and also to include some previously unconsidered compounds in this category. These substances, on which no further work is urgently required, include carrageenan, polyoxyethylene sorbitan esters, polyoxyethylene (8) and (40) stearates, polyglycerol esters of polycondensed fatty acids of castor oil, furcelleran, xanthan gum and propylene glycol/glycerol esters of fatty acids and lactic acid. It is interesting to note that whereas the 1970 Report requested 18-month feeding studies in the mouse for several compounds, it is now recommended that such a study should be carried out on polyoxyethylene sorbitan mono-oleate, as a representative of the whole group of polyoxyethylene sorbitan esters.

Additives reclassified into group B include sucrose esters and sucroglycerides. Less than 10 ppm methyl alcohol and less than 50 ppm dimethylformamide are now considered acceptable as contaminants in these materials. Adequate short- and long-term studies on dimethylformamide are not available and studies of this contaminant in two species are considered essential before a higher classification can be considered for these food additives. Citroglycerides are hydrolysed *in vitro* to simple glycerides and citric acid but further data on the extent of *in vivo* hydrolysis by human and animal enzymes are required before the next review. A long-term feeding study on gum ghatti is also required but is not considered of high priority.

A representation that metabolic studies in man on Emulsifier YN could only be carried out using radioactive material, which in this case would not be justified, has been accepted by the PSC, but the results of long-term studies at present being carried out at BIBRA are required by the time of the next review. A single 18-month feeding study in mice on sorbitan monostearate is to be accepted as representative of the whole group of sorbitan esters of fatty acids. Long-term feeding studies in mice are not required on all individual compounds. Results of short-term feeding studies in the rat and mouse on the mono-oleate and mono-laurate esters are required before the next review. Also by that time, results of long-term studies on the stearoyl lactylates in two species other than the dog are required, together with investigations showing that man metabolizes these compounds in the same way as other species. While polyglycerol esters of dimerized fatty acids of soya-bean oil are acceptable for tin-greasing with a transfer limit of 20 ppm into the food, adequate metabolic studies and short-term studies in several species are still considered necessary. Finally, two compounds were classified in group E: insufficient data were available on tamarind-seed flour, and adequate long-term feeding tests on sodium lauryl sulphate were lacking.

Toxicological Evaluation of Some Enzymes, Modified Starches and Certain Other Substances. Joint FAO/WHO Expert Committee on Food Additives. WHO/Food Add. 72.1; *F.A.O. Nutr. Mtg Rep. Ser.* no. 50A, Rome 1972. pp. 109. £0.60.

Monographs have now been issued on the toxicological evaluation of some enzymes, modified starches and certain other substances considered in the report of the Fifteenth Meeting of the Joint FAO/WHO Expert Committee on Food Additives (*Tech. Rep. Ser. Wld Hlth Org.* 1972, **488**) held in Rome in June 1971, but the publication of specifications for the identity and purity of these materials is still awaited.

These supporting monographs have been drawn up for six enzymes derived from microbial sources, 11 modified starches, caramel colourings made by the ammonia process, esters of glycerol and thermally-oxidized soya-bean fatty acids, hexamethylenetetramine, micro-

crystalline cellulose, propylene glycol alginate, calcium and sodium salts of stearoyl lactylic acid and tin and stannous chloride.

It is interesting to note that the new data on esters of glycerol and thermally-oxidized soya-bean fatty acids and on calcium and sodium stearoyl lactylates may have been responsible for the recent up-grading of these two materials in the UK Food Additives and Contaminants Committee's recent supplementary report on emulsifiers and stabilizers (see previous review). Furthermore, it is understood that, both in the UK and within the EEC, a new instrument or directive will be drawn up for modified starches, which are now exempt from regulations covering emulsifiers and stabilizers in food. The toxicological data that have been evaluated will probably be used as a basis for permitted lists, and further data on metabolism in man may well be required in the near future.

The situation with regard to caramel colourings made by the ammonia process has been complicated by the discovery of imidazoles and pyrazines in these materials. Since 4-methylimidazole has been shown to be a convulsant when administered orally to rabbits, mice and chicks, long-term feeding studies on caramel colourings prepared by the ammonia or ammonium sulphate process and containing several levels of 4-methylimidazole are required by 1974.

The publication of adequate specifications for these and other materials considered at the Expert Committee's meeting in 1971 is eagerly awaited.

Phosphorus and Calcium Intakes by Dutch Diets. By M. I. El-Shaarawy, Doctoral Thesis, State University of Utrecht, 1971.

The idea of phosphorus poisoning immediately conjures up visions of Victorian match factories—lucifers and luminous paint rather than food additives. However Dr. El-Shaarawy expresses concern at the "growing application of phosphorus containing food additives". Nearly every foodstuff normally contains phosphorus and he feels this natural phosphorus content should be considered when assessing the risk associated with the use of these additives.

The metabolism of phosphorus is closely interlinked with that of calcium, so dietary calcium levels are important in any study of dietary phosphorus. This is especially so in assessments of the phosphorus intakes of underdeveloped countries where the calcium intake is likely to be low. In general, a higher phosphorus intake may be allowed for populations with a higher calcium intake.

Fortunately we are not threatened with the unpleasant consequences of ingesting pure phosphorus as were the match makers. Most of the phosphorus naturally in, or added to, food is in the form of organic or inorganic compounds. Overdosage with such compounds leads to metastatic calcification in rat kidneys, which may be more susceptible than human kidneys to damage by phosphates. At similar dose levels, guinea-pigs have shown calcification of the soft tissues. On the basis of these rat experiments the FAO/WHO Joint Expert Committee on Food Additives concluded in its seventh report that the lowest phosphorus level likely to be nephrocalcinic to man would be around 100 mg P/kg body weight/day. The Acceptable Daily Intake (ADI) was given as 30–70 mg P/kg body weight, depending on the diet. Dr. El-Shaarawy contends that the safety margin allowed by this ADI is insufficient, especially for groups with a low calcium intake. Old people and children may come into this category even in highly developed industrial communities.

The main part of this thesis consists of a detailed appraisal of methods for determining phosphorus and calcium in foods, on the basis of which the spectrophotometric vanado-molybdate method was chosen for phosphorus and atomic absorption for calcium determinations. Methods for ashing foodstuffs are described in detail, and the importance of estimating total phosphorus (rather than just phosphate) and total calcium is explained.

A selection of foodstuffs was analysed and the per capita phosphorus and calcium intakes for the Netherlands were calculated from published statistics on food consumption in 1968. The calcium and phosphorus intake was also calculated for some selected groups whose food intake had been previously studied in detail. These included men working in villages and towns, town inhabitants in summer and winter, nursing mothers and children.

Of the seven selected food groups two, dairy products and fruits/vegetables, have a low P/Ca ratio (0·8–1·1) and the five others (carbohydrate-, protein- and fat-rich foods, nuts and drinks) have a medium or high P/Ca ratio (1·9–8·5). Among foods or food products with a particularly high phosphorus content are cheese, pulses, cocoa, ham and nuts. Various items likely to be preferred by children, such as instant puddings, chocolate and cola drinks, have a high P/Ca ratio.

The National Research Council of the USA recommends a low P/Ca ratio for infants (0·66–0·8) and for all other age groups considers that calcium intake should balance phosphorus intake. This ideal is certainly not reached in the Netherlands with an average dietary P/Ca ratio of 1·6.

In summary, Dr. El-Shaarawy lists the P/Ca ratios and intakes of several groups. All adult diets exceeded the recommended P/Ca ratio of 1. Further, infants had an average daily intake of phosphorus of 60 mg/kg body weight at 3 years and 81 mg/kg at 1 year, the latter figure exceeding the conditional FAO/WHO ADI of 70 mg/kg.

This is an interesting thesis and the technical part is well written and detailed. It is a pity that the author had to rely so heavily on previously published food consumption tables in his calculations of calcium and phosphorus intakes. His direct analysis of the food intakes of two students could with profit have been extended to include a larger group.

No work on the long-term effects of high phosphorus intake has been carried out in man. This is an obvious gap in our knowledge which Dr. El-Shaarawy's study does not fill. He does clearly indicate, however, that certain consumer groups may be more at risk than others and controlled clinical investigation of such individuals might be profitable.

1970 Evaluations of Some Pesticide Residues in Food. The Monographs. Joint Meeting of the FAO Working Party of Experts and the WHO Expert Group on Pesticide Residues, Rome, 9–16 November 1970. AGP: 1970/M/12/1; WHO/Food Add./71.42, Rome, 1971. pp. vi + 571.

This set of monographs on some of the pesticides likely to appear in residual form in foodstuffs is the product of the 1970 FAO/WHO gathering of experts in this field. The general report of the meeting, which included the principles adopted for the assessments and a summary of the results of the evaluations of specific pesticide residues, has already been published and reviewed (*Cited in F.C.T.* 1972, **10,** 226).

The publication now under review is much larger than its predecessors, but its impressive size is due, at least in part, to the decision to use a much larger type than before. This certainly makes it easier to read, if not to carry and store. In all, it contains 23 monographs,

together with several appendices providing a tabulated summary of previous recommendations on acceptable daily intakes (ADIs), tolerances and practical residue limits (with dates of publication) for some 100 pesticides, a bibliography of relevant FAO/WHO publications, and a glossary defining terms used in this series of reports and associated monographs.

Each full monograph provides information on the nomenclature, chemical structure and properties of the pesticide, reviews of the biochemical and toxicological data on which evaluations were based, the toxicological evaluation and ADI estimation, the uses of the material, the occurrence, detection and fate of residues and an appraisal of the overall position with recommendations for tolerances and practical residue limits. Recommendations are also made regarding additional work or information considered to be essential (for the establishment of an ADI) or desirable. Unpublished data are cited in addition to the published literature.

Full monographs compiled along these lines are provided for chlormequat, 2,4-D, diquat, fentin compounds, mancozeb, paraquat, 2,4,5-T, thiabendazole and tricyclohexyltin hydroxide, which were considered for the first time at this meeting or for which considerable data had accumulated since earlier deliberations. For other previously reported pesticides, however, only a limited amount of additional information was made available during the intervening years and, for these, an addendum to the previously published evaluation summarizes the new data. Pesticides handled in this way are carbaryl, chlordane, diazinon, dichlorvos, dicofol, dieldrin, dimethoate, dithiocarbamate fungicides, endrin, ethion, heptachlor, malathion, parathion and pyrethrins.

The Group's decisions on ADI recommendations for these compounds have already been listed in our review of the report of the meeting. This publication now provides the factual background to those decisions and, at the same time, offers to those concerned with problems of pesticide residues and their possible toxicity a very useful reference book.

Pesticide Residues in Food. Report of the 1971 Joint Meeting of the FAO Working Party of Experts on Pesticide Residues and the WHO Expert Committee on Pesticide Residues, Geneva, 22–29 November 1971. *Tech. Rep. Ser. Wld Hlth Org.* 1972. **502.** pp. 46. £0.40.

The FAO and WHO experts on questions relating to pesticide residues met again in Geneva in 1971 to review use patterns and residue data for several food fumigants and pesticides, to estimate acceptable daily intakes (ADIs) for man for several pesticides, to consider available methods of analysis and to recommend tolerances for these pesticides on foods and crops. The Committees considered that all data used to generate ADIs and residue limits should be available in full to *bona fide* scientists on request and that long-term feeding studies in experimental animals were necessary in all but exceptional cases before an ADI could be set. Some ADIs have in the past been based on short-term and metabolic studies only, and in subsequent long-term studies adverse effects have been demonstrated. It was also considered essential that good agricultural practices should be employed by users of pesticides and that suitable analytical methods should be available to check tolerances.

The report deals first with seven compounds used as fumigants in food protection. Their presence in foods was first noted at the 1969 and 1970 meetings and the 1971 session considered further data on methyl bromide, 1,2-dibromoethane (ethylene dibromide), carbon

disulphide, carbon tetrachloride, 1,2-dichloroethane (ethylene dichloride), ethylene oxide and hydrogen phosphide, particular attention being paid to residues of bromide ions in foods. Residue levels recommended for raw cereals before milling, milled cereal products and bread and other cooked cereal products were, respectively, 10, 2 and 0·5 ppm for carbon disulphide, 50, 10 and 0·05 ppm for carbon tetrachloride, 20, 5 and 0·1 ppm for 1,2-dibromoethane, 50, 10 and 0·1 ppm for 1,2-dichloroethane, and 50, 10 and 0·5 ppm for methyl bromide. For hydrogen phosphide existing tolerances were confirmed, but no tolerances for ethylene oxide were set and it was recommended that further consideration should be given to this compound at a future meeting. The previously recommended tolerance of 50 ppm for bromide-ion residues, either from natural uptake of compounds from the soil (considered unlikely to exceed 10 ppm) or from treatment with bromine-containing fumigants, was confirmed for raw cereals and wholemeal flour. Bromide-ion tolerances for other foods will be considered at a future meeting.

Five organophosphorus compounds were considered for the first time. An ADI was set for chlorfenvinphos (0·002 mg/kg body weight) and temporary ADIs were given for fenthion, omethoate, and trichlorfon (0·005, 0·005 and 0·01 mg/kg). Explanations for the wide variation in species susceptibility to chlorfenvinphos, adequate long-term studies on fenthion and omethoate and further evidence of the non-carcinogenicity of trichlorfon were all considered necessary. Trichloronate was given no ADI, since although data showing its freedom from polychlorinated dibenzo-*p*-dioxins were available, data on its fate *in vivo* and reports of long-term feeding studies were lacking. Since the previous evaluation of 2,4-D, 2-year feeding studies in the rat and dog and a reproduction study in the rat had become available. Despite some statistical differences in tumour incidence between control and test rats, 2,4-D was not considered to be carcinogenic, an opinion supported by a feeding study in the mouse. Polychlorinated dibenzo-*p*-dioxins were not detectable in currently manufactured 2,4-D and its esters, and embryotoxicity was therefore considered unlikely. An ADI (0·3 mg/kg) was based on the 2-year feeding study in the rat. A temporary ADI (0·01 mg/kg) was established for chlordimeform, but no ADI could be established for chlormequat and no change was recommended in the present ADI for DDT. Tolerances were also recommended for each of these compounds.

A comparison of the potential daily intakes of several pesticides with their ADIs was also made. The results of this study indicated that there was not even a theoretical possibility that the ADIs of some 20 pesticides would be exceeded. These were binapacryl, bromide ion captafol, captan, chlordimeform, chlorfenvinphos, 2,4-D, diphenylamine, diquat, ethoxyquin, fenitrothion, folpet, formothion, mancozeb, paraquat, 2-phenylphenol, pyrethrins, thiabendazole, trichlorfon and tricyclohexyltin hydroxide. Several compounds were borderline cases and were considered to warrant further studies. These were chlordane, dichlorvos, endrin, fentin compounds, heptachlor, malathion, carbaryl, diazinon, hexachlorobenzene and quintozene. In addition, there was a significant theoretical possibility that the ADIs of DDT, dieldrin, fenthion, omethoate and piperonyl butoxide might be exceeded. Available information on the disappearance of residues during processing and cooking before consumption was taken into account for carbaryl, DDT and malathion. In this study, the effects of processing indicated that malathion could be considered to be in that group of compounds for which no further data need be developed because the quantity of residue reaching the consumer after processing would not exceed the ADI. In future pesticide evaluations, information on extent of use and on the effects of processing, storage and cooking before consumption should be considered. Where adequate information is not

available for this purpose, work should be initiated to obtain the necessary data on the disappearance of residues before consumption.

The first annex to the report tabulates the ADIs, tolerances and practical residue limits for compounds considered up to and including November 1971. Work required by June 1975 and other studies considered desirable are outlined in the second annex. The following items were suggested for consideration at future joint meetings: residues of benomyl, bromophos, bromophos ethyl, carbophenothion, chlorpyrifos, fensulfothion, methidathion, mevinphos, monocrotophos and phosalone and possibly the fungicide thiophanate methyl; evaluation of relevant toxicological and other related data on carbon disulphide, carbon tetrachloride, 1,2-dibromoethane, 1,2-dichloroethane, ethylene oxide, hydrogen phosphide and methyl bromide; re-evaluation of the temporary ADIs and/or temporary tolerances for azinphosmethyl, chlorobenzilate, chloropropylate, coumaphos, crufomate, dioxathion, ethion, fenchlorfos, parathion methyl, piperonyl butoxide, phosphamidon and pyrethrins, due to expire in 1972.

Finally it was reiterated that insufficient data were available on certain pesticides and that further research should be initiated by official bodies and supported by public funds. Full supporting monographs giving details of toxicological evaluations and comments on analytical methods are to be published separately.

Monks Wood Experimental Station. Report for 1969–1971. The Nature Conservancy, Natural Environment Research Council, Abbots Ripton, Huntingdon, 1972. pp. 118. £0.60.

The Toxic Chemicals and Wildlife Section of Monks Wood Experimental Station has in the past concentrated on the ecological effects of organochlorine pesticides (*Cited in F.C.T.* 1970, **8**, 302). Since voluntary measures were introduced under the Pesticides Safety Precaution Scheme the threat from these compounds has diminished, but research has been continued to assess their present impact. The experience gained has also been valuable in determining the significance of other fat-soluble organic pollutants such as the polychlorinated biphenyls (PCBs). Recently work has started on the determination of heavy metals in organisms from different habitats; although still in the reconnaissance stage, this study should eventually indicate which metals are likely to pose a hazard to man and his food supply. Indeed, the work of the Section is increasingly conceived as providing part of an early warning system for new types of pollution in addition to a monitoring service.

The hazards of leaded petrol have been much debated. A study now being prepared for publication has shown that the body levels of lead in mice and voles taken from roadside verges is significantly greater than in the same species from arable and woodland habitats. High lead levels have also been detected in grass and foliage from beside the M1, but have been found to fall off sharply to background levels within 8–16 m from the roadside.

PCBs were at first thought to have contributed to the mass deaths of seabirds in the Irish Sea in the autumn of 1969, but work by the Section later established that apparently healthy guillemots had a total body burden of PCBs and other organochlorines similar to that found in the dead birds. It was concluded that the primary cause of the mortality was malnutrition, and that the high PCB levels in the livers of many dead birds arose through mobilization of body fat. The results of this work and studies conducted at 12 other specialist laboratories have now been published in *The Seabird Wreck in the Irish Sea Autumn 1969*

(N.E.R.C. Publications Series C, no. 4, 1971). Research on the toxicity and sublethal effects of PCBs on seabirds is continuing, as is analysis for PCBs in wildlife. Mean levels of 36 ppm were detected in the livers of grebes, probably due to their habit of wintering on estuaries.

Analysis of dead badgers suggested that their deaths were due to the consumption of wood-pigeons poisoned by dieldrin used as a seed dressing, in spite of the voluntary ban on this use imposed some 10 years ago. However, the voluntary ban on dieldrin in sheep dips has probably been responsible for the steady decline in the dieldrin content of heron eggs, from an average of 4·5 ppm in 1966 to 0·6 ppm in 1970. Despite this, a third of the birds are still breaking their eggs, and the shells are abnormally thin. For the first time since the work started in 1964, some sparrowhawk eggs were found without detectable levels of dieldrin, but DDE residues remained relatively high and the shells were again thin. Other predatory bird populations, such as the peregrine falcon and golden eagle, have shown encouraging recoveries, and eggshell thickness in the latter species is now back to normal. However, in the eggs of the Sandwich tern, dieldrin and DDE levels rose slightly between 1967 and 1969, although since the species winters off West Africa the changes may not reflect contamination of British waters alone.

A study of the effects of 8 ppm *p,p'*-DDT and 4 ppm *p,p'*-DDE on the Bengalese finch revealed that at such levels, which have occurred in the field in the bodies of species forming the food of birds of prey, fertility, hatchability and fledging success were reduced. In addition there was a delay in egg-laying and a decrease in weight of both eggs and chicks. Results suggested that the birds were in a state of apparent hyperthyroidism, and this was confirmed in further work with *p,p'*-DDT on wood-pigeons, using a dose level of 3 mg/kg/day. At higher levels a hypothyroid condition resulted. Other studies, using Japanese quails, failed to confirm a suspected oestrogenic effect of *o,p'*-DDT and suggested that mobilization of body fat reserves, rather than yolk sac absorption, may be responsible for chick mortality from *p,p'*-DDT and *p,p'*-DDE during the first few days of life.

Several investigations have been carried out on amphibia, including studies of the effect of DDT, used as a mosquito larvicide, on frog tadpoles and the sensitivity of frogs, toads and newts to DDT, dieldrin and 2,4-D. Frog tadpoles were the most susceptible to the effects of DDT, becoming hyperactive and thus attracting greater attention from predators. Dieldrin had less effect on mortality and behaviour but, like DDT, produced distinct behavioural changes and morphological abnormalities. Only 2,4-D had no visible effect, and no tissue residues could be detected even after treatment in 50 ppm for 48 hours.

The apparently continuing use of dieldrin seed dressings, brought to light by the report, suggests that the present voluntary system of pesticide control is not working satisfactorily in all circumstances. There have been several proposals for a mandatory system and this may perhaps prove necessary. DDT and its metabolites are apparently still producing some adverse effects on bird populations, and it remains to be seen whether the partial restrictions announced in the UK in 1969 will be sufficient or whether more drastic curtailment of DDT use, such as has recently been proposed in the United States, will be considered advisable.

Fd Cosmet. Toxicol. Vol. 11, pp. 303–307. Pergamon Press 1973. Printed in Great Britain

BOOK REVIEWS

Food Chemicals Codex. Second Edition. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection, National Research Council. National Academy of Sciences, Washington, 1972. pp. xvi + 1039. $20.00.

This revised and enlarged version of the Food Chemicals Codex has been broadened in scope to include not only direct food additives, as before (*Cited in F.C.T.* 1971, **9**, 868), but also processing aids, such as extraction solvents and filter media, and substances that are not 'chemical additives' in the conventional sense, such as the modified food starches, the masticatory substances used in chewing-gum base and pectin. In all, 87 new monographs have been added to the 557 covered in the first edition and supplements, and only five of those previously published (calcium and sodium cyclamates, cyclohexylsulphamic acid, diethyl pyrocarbonate and nordihydroguaiaretic acid, no longer permitted in the USA for use as food additives) have been deleted. Many of the specifications and test procedures in existing monographs have been revised, with the approval of the Subcommittee on Toxicology of the Committee on Food Protection in cases where possible hazard was involved. The policy on limits of impurities has not been changed, except in the case of heavy metal limits for certain flavourings. Since experience has shown that the heavy-metal test is always negative when the flavouring is an organic liquid, is purified by distillation, is immiscible with water and does not dissolve inorganic substances, the specifications for flavourings meeting these criteria and used in foods at levels of 0·01 % or below no longer include limits for arsenic, heavy metals or lead.

The titles of a number of monographs have been changed in the new edition, and one title, 'poloxamer', has been specifically coined for two substances of the polyol series. Almost all the test procedures in the first edition have been retained, although many of the older ones have been substantially revised, and several new procedures have been added. Despite concerted attempts, no way of improving the heavy-metals test has been found, but a continuing effort is being made. Any amendments and additions to this and other parts of the Second Edition will be published in annual supplements, which will be sent without charge to all holders of the book. It is anticipated that a third edition of the Codex will be published in 1978.

The specifications in the Codex have been adopted as official not only by the FDA but also, under certain conditions, by the Canadian Food and Drug Directorate and the UK Food Additives and Contaminants Committee. These official acknowledgements of its authority seem well justified, and it is to be hoped that this new edition will enjoy even wider recognition than its predecessor.

Fibres, Films, Plastics and Rubbers. A Handbook of Common Polymers. By W. J. Roff and J. R. Scott. Butterworths, London, 1971. pp. xii + 688. £15.00.

W. J. Roff, the author of *Fibres, Plastics and Rubbers*, published in 1956, has collaborated

with J. R. Scott of the Rubber and Plastics Research Association in completely revising and updating that well-known reference book. The present "bigger and better" volume has been extended to cover films—as is apparent from the revised title.

The authors' tentative claim that never before has so much information on fibres, films, plastics and rubbers been presented in a handy form in a single volume would seem to be more than justified. Part I contains data on individual polymers, divided into 42 sections. A brief synopsis of synonyms, trade names and general characteristics is followed in each case by information on structure, chemistry, physics, fabrication, serviceability, utilization, history and sometimes other important aspects, and finally by a list of further literature. Part II tackles the subject from another angle, the division in this case being into specific properties and methods of polymerization, identification and fabrication. These sections cover such areas as X-ray data, molecular weight and degree of polymerization, electrical resistivity, impact strength, water retention, plasticizers and solvents, and specific gravity and density. Additional sources of reference and author and subject indexes complete the volume.

This book is intended to provide essential information for industrial research and development laboratories and university and college laboratories concerned in any way with polymeric materials. It is also directed towards consultants and specialists in textiles, plastics, rubbers and similar materials and towards designers and engineers dealing with constructional and electrical materials. Certainly a wealth of general information on polymeric materials has been collected together in this volume, and all these classes of reader will appreciate the clarity of presentation and the ease with which data can be located.

Advances in Cancer Research. Vol. 12. Edited by G. Klein, S. Weinhouse and A. Haddow. Academic Press, New York, 1969. pp. xv + 317. $17.00.

This volume reflects a marked change in emphasis in cancer research. Until some 5 years ago, most experimental work in this field was concerned with the reactions between chemicals and cell constituents involved in the production of malignant tumours, the process of so-called "chemical carcinogenesis". Immunological reactions and oncogenic viruses were given much less consideration at that time, but in the past 5 years notable advances have been made in these two areas of cancer research and a voluminous literature has accumulated. The topics selected for review in the volume named above cover an important part of this work and deal principally with immunological phenomena in virus-induced tumours.

The first chapter reviews evidence for the existence of two types of antigen induced by the mouse leukaemia viruses. One of these is specific for cells carrying the virus whether they are transformed or normal, the other appears only in transformed cells. The techniques involved in work of this sort are laborious and time-consuming and require a considerable degree of expertise. There is little doubt, however, that they could be of considerable value in attempts to ascertain the degree to which human leukaemia is caused by viruses, a claim frequently expressed in the literature.

The following chapter deals with DNA tumour viruses, which are more difficult to detect in infected cells than the RNA viruses. This fact has caused special problems in the separation of tumour-specific antigens from those associated specifically with virus infection. The way in which these problems were approached *in vitro* and *in vivo* and the successes achieved are well reviewed here.

An important chapter is the one devoted to cellular immunity against tumour antigens. The information is laid before the reader in a clear and concise form, intelligible even to those unfamiliar with the subject. Both humoral and cell-mediated immunity have been demonstrated against tumour antigens. In this respect, the immunological processes at work against tumours resemble those that occur against foreign proteins in general. Immunological reactions are more easily detected if the tumour is induced by an oncogenic virus, but chemically induced tumours and 'spontaneous' tumours also elicit an immunological response. The reason why these reactions may fail to eliminate tumours is not known for certain, but an earlier chapter lists as possible reasons the inefficiency of a low level of immunity in the presence of a large number of cells, the emergence of immunoresistant tumour cells, the development of a state of 'tolerance' in the host animal and an impairment in the 'immunological competence' of the organism for various reasons.

In the remaining two chapters, the reader passes on from immunological reactions to two different fields. In one of these, the interactions of the oncogenic viruses of mice within host cells is discussed in detail. The subject is a difficult one and the information is, not surprisingly, fragmentary. However, there is evidence that tumour viruses interact with one another in the host cell, and malignant transformation is much more readily produced in tissue culture cells by such combinations than by each individual virus. The author of this chapter makes an interesting comment on this viral phenomenon. He thinks that frequent interactions may be one of the characteristics of the tumour viruses. Unlike "ordinary cytocidal viruses", the tumour viruses do not have a short life-span so that "there is ample opportunity for them to interact with a second virus either deliberately added or naturally present in the host cells." An interesting thought, but considering the complexity of virus-cell interactions in general, much more evidence is required before it can be considered scientifically acceptable.

The last chapter is devoted to a discussion of the epidemiology of leukaemia. Pointing to the two peaks of incidence of this disease, one in childhood and adolescence (5–14 years) and one in adults (40–70 years) the author suggests that two different aetiological factors may be in operation, the disease in the younger group having a genetic basis while that in adults is the responsibility of environmental factors. Surely there is something substantial here for the environmentalists to investigate in depth, with some hope of success!

Advances in Cancer Research. Vol. 13. Edited by G. Klein and S. Weinhouse. Academic Press, New York, 1970. pp. xiv + 440. £10.25.

Continuing the trend of the previous volume, reviewed above, this book pays considerable attention to immunological mechanisms and viruses in relation to cancer. The role of the immunoblast in cell-mediated immunological interactions is discussed at length in the first chapter. The evidence reviewed indicates that these cells are derived from lymphocytes and that they are involved in the rejection of histo-incompatible tissue grafts as well as tumours. They are far less effective in rejecting tumours than in rejecting foreign tissue.

This role of the lymphocyte receives further attention in the third chapter, which presents a review of experimental data, much hitherto unpublished, on the function of the lymphocyte in the delayed sensitivity reaction as revealed in tissue culture studies. The evidence obtained *in vitro* supports *in vivo* studies on the central role played in this reaction by the lymphocyte. Furthermore, the way in which the lymphocyte destroys the foreign tissue is elegantly

demonstrated by studies on rat lymphocytes sensitized to cells of a different genetic make-up. The rat lymphocyte enlarges and exhibits an enhanced degree of motility as soon as it is brought near the cells that trigger off the sensitivity reaction. Contact takes place, the lymphocyte subsequently moves away and the target cell undergoes lysis. This remarkable demonstration of cell lysis by lymphocytes is an important step forward in our understanding of the complex mechanism of tissue rejection.

Two less esoteric topics are given a fair degree of prominence. One of these is the important problem of cancer of the urinary tract, while the other is a review of the information available on Epstein-Barr (EB) virus. The former chapter has important sections on the comparative histology and ultrastructure of ureteric and bladder epithelium, on the permeability properties of the bladder epithelium and on the reaction of the epithelium to injury, all questions of fundamental importance to our understanding of the evolution of carcinoma in this organ. The rest of the chapter is a review of human and experimental bladder carcinogenesis—a well-ploughed field. The chapter on EB virus traces the history of the discovery of the virus and its association with Burkitt's lymphoma. Aspects of the morphology and biological activity of this virus, and in particular its resemblance to the herpes group of viruses, are discussed in some detail.

In the study of cancer, regulatory mechanisms are seldom given enough attention, and yet their importance to our understanding of the neoplastic process can hardly be overstressed. A lucid and comprehensive account of the regulation of tissue growth at intracellular and supercellular levels is given in a chapter on epigenetic processes and neoplasia. It appears that despite extensive work on organogenesis in embryonic tissue and stupendous advances in the field of molecular biology, the essence of the disturbance that leads to a loss of control in the evolution of neoplasia still eludes our comprehension.

Two chapters are devoted to the role of viruses in carcinogenesis. Evidence is presented in one of these to demonstrate that leukaemia in the cat, dog, pig and cattle is very probably due to viruses, thus bringing it into line with what we know of the aetiology of this disease in mice. A second chapter deals with virus induction or virus "rescue". The DNA viruses, including the oncogenic ones, integrate themselves with the nuclear DNA of the host cell but do not produce virus particles continually. Virus production is not only intermittent but also of fairly brief duration. The dormant virus can be induced or "rescued" by allowing uninfected cells to come into contact with the cells containing the virus genome. Considering the difficulties faced by earlier workers in identifying DNA viruses, this represents an important advance.

One chapter is devoted to characteristics of cells transformed *in vitro*. The chapter is lucid and informative but little real advance seems to have been effected in the last 5 years.

Free Radical Mechanisms in Tissue Injury. By T. F. Slater. Pion Limited, London, 1972. pp. 283. £4.80.

The effects that an increasingly complex chemical environment may have on biological activity constitute a major field of research. The extent to which free radicals are involved in the basic mechanisms of cell damage has long been a source of interest and experimental work for the author named here. An understanding of the subject demands an appreciation of various physical, chemical and biological concepts, and any effort to present these in a single volume is to be applauded. No author could attempt such a task without having to make personal selections from the extensive literature relevant to the subject.

The first quarter of the book is an introduction to the physical chemistry of free radicals, while the rest relates to probable free-radical involvement in a variety of effects exerted by chemicals on animal tissues. The latter section contains a preponderance of observations on liver damage, because the liver has been the organ most studied and the author himself has made significant contributions to this aspect of the problem.

The text, tables and figures are clear and precise but the few full-page reproductions of the histology and ultrastructure of damaged tissue are of doubtful value in the context of the need that this book is designed to meet. A number of references are appended to each chapter and the index is adequate.

To a very large number of biologists, particularly those having a special interest in the reaction of mammalian tissues to changes in the *milieu interieur*, this book will be most useful. It should be high on the toxicologist's reading list and the pure chemist should find it of interest principally because of its attempt to bridge some of the numerous gaps existing between chemistry and other sciences.

BOOKS RECEIVED FOR REVIEW

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 42. Edited by F. A. Gunther. Springer-Verlag, Berlin, 1972. pp. vii + 182. DM 46.70.

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 43. Edited by F. A. Gunther. Springer-Verlag, Berlin, 1972. pp. vii + 149. DM 44.80.

Mercury in the Environment. An Epidemiological and Toxicological Appraisal. Edited by L Friberg and J. Vostal. CRC Press, Ohio, 1972, pp. 215. $33.00.

A Guide to Marine Pollution. Seminar in Conjunction with the FAO Technical Conference on Marine Pollution and its Effects on Resources and Fishing. Edited by E. D. Goldberg. Gordon and Breach Science Publishers, New York, 1972. pp. x + 168. £4.15.

Biological Oxidation of Nitrogen in Organic Molecules. Proceedings of the Symposium held at Chelsea College, London, 19–22 December 1971. Edited by J. W. Bridges, J. W. Gorrod and D. V. Parke. Taylor & Francis Ltd., London, 1972. pp. xxi + 269. £6.00.

Food Allergy. Its Manifestations and Control and the Elimination Diets. A Compendium with Important Consideration of Inhalant (Especially Pollen), Drug, and Infectant Allergy. By A. H. Rowe and A. Rowe, Jr. Charles C. Thomas, Springfield, Ill., 1972. pp. xvi + 687. $20.00.

Fd Cosmet. Toxicol. Vol. 11, pp. 309–321. Pergamon Press 1973. Printed in Great Britain

Information Section

ARTICLES OF GENERAL INTEREST

MORE MEDITATIONS ON MSG

Ever since 'Kwok's quease' was reported among visitors to Chinese restaurants (*Cited in F.C.T.* 1968, **6,** 781), efforts have been directed towards finding a plausible basis for the neurotoxic effects of monosodium glutamate (MSG). At one stage, it was hoped that the quease might be discounted as an investigator's artefact, since it was not regularly reproducible (*ibid* 1971, **9,** 903), but this simple solution to a broadening problem did not prove universally acceptable. Even if it had done so, there remained a need to reconcile the wide variations in susceptibility to the toxic effects of MSG reported in animals of different ages and species, particularly with regard to the neuro-endocrine disturbances and retinal damage associated with this compound (*ibid* 1971, **9,** 717).

As far as chicks are concerned, the results of a study by Carew & Foss (*Poult. Sci.* 1971, **50,** 1501) appear to leave little room for doubt. The acute subcutaneous (sc) LD_{50} of MSG in day-old chicks was 3–4 g/kg, and 1000% mortality followed a dose of 5 g/kg. Daily MSG doses of 1 g/kg injected sc for 10 days neither increased mortality among chicks nor reduced weight-gain. No significant effect on weight-gain, food intake or mortality was observed during a 16-week observation of chicks given feeds containing 0·5–10% MSG for 10 days or 0·5–2·5% MSG for 28 days. Nevertheless, some toxic effects have been induced in the chick by MSG. In a study by Shimizu *et al.* (*Jap. J. med. Sci. Biol.* 1971, **24,** 271), the kidney fell victim to the administration of drinking-water containing 1·3 or 2·6% MSG. All the chicks given the higher level died within a few days, and the rapidly developing renal lesions in these animals were associated with marked deposition of uric acid salts. Some of the response could be ascribed to a high sodium intake and was avoided by the use of potassium glutamate rather than MSG, but it appeared that the glutamate moiety was to some extent responsible for the excessive synthesis of uric acid. It is important to remember, however, that the MSG intake in this experiment was relatively high, the daily intake possibly being between 3 and 4 g/kg/day in animals on the higher concentration. No neurotoxicity was reported in this study.

In contrast to the low level of toxicity in chicks, Burde *et al.* (*Nature, Lond.* 1971, **233,** 58) reported that infant mice given a single oral dose of 1 g MSG/kg developed microscopic lesions in the arcuate area of the hypothalamus and median eminence of the brain, as did rats given 2 or 4 g MSG/kg by the sc or oral route. Similar changes in the hypothalamus in newborn macaques have been described by Reynolds *et al.* (*Science, N.Y.* 1971, **172,** 1342) but ascribed to a different cause. When these infant monkeys were given orally intubated doses of 1, 2 or 4 g MSG/kg and killed 6 hours after treatment for examination of brain sections by light and electron microscopy, the morphology of the hypothalamus and arcuate nucleus did not differ from that of comparable sections taken from controls. It was

found, however, that in both treated and control animals, poorly fixed tissue, which tends to occur in scattered areas of the brain because of technical difficulties, was characterized by swollen dendrites lacking their normal cytoplasmic contents and by degenerative changes in neuronal perikarya. The ultrastructural appearance of such tissue was thus similar to that previously described in a newborn monkey treated with MSG.

Biochemical studies of the enzyme systems of liver and brain in animals given MSG possibly offer more enlightenment than has so far come from anatomical studies. Creasey & Malawista (*Biochem. Pharmac.* 1971, **20,** 2917) consider that MSG interference with glucose uptake by brain tissue may offer a plausible explanation of the symptoms of Kwok's quease. In their experiments, adult mice were given an intraperitoneal (ip) injection of 300 mg MSG/kg followed 15 minutes later by [2-^{14}C]glucose by the same route. This pretreatment with MSG reduced the uptake of glucose into the whole brain by up to 35·5%. With a dose of 600 mg MSG/kg, the brain uptake of glucose fell by 64%, indicating a dose-related response.

Observations on weanling rats fed diets containing 1–20% MSG for 16 weeks have been published by Prosky & O'Dell (*Proc. Soc. exp. Biol. Med.* 1971, **138,** 517). With a feed containing 20% MSG, growth was depressed by 15% (by week 16) and terminal liver weight by 9%, although the ratio of liver to body weight was not affected. A 1% MSG feed reduced brain levels of γ-aminobutyrate by 17%, and a 20% MSG feed by 20%. The weight of the brain and its protein and DNA levels were unaltered, and brain levels of glutamic acid decarboxylase, glutamine and aspartate remained roughly constant, whereas succinate levels in the brain rose with increases in the MSG content of the feed, reaching 20% above control values after a 20% MSG diet. All rats fed MSG became hyperirritable, an effect probably attributable to the decrease in brain levels of γ-aminobutyrate. The liver showed no change in protein, RNA-P or DNA-P, glutamate, lactate, malate or α-glycerophosphate, but the aspartate content increased by 25% at the 20% dietary level of MSG.

The same authors (*idem, Experientia* 1972, **28,** 260) showed that feeding rats a diet containing 10% MSG for 100 days before mating and then feeding the same diet to the offspring for a further 100 days had no marked effect on brain levels of protein, DNA, aspartate, glutamate and γ-aminobutyrate or liver levels of protein, RNA-P, DNA-P or glutamate, in the second generation animals. As was found with the adult rats in the earlier study, the activities of the transaminases and oxidases that metabolize glutamate in the liver were high enough to keep the hepatic glutamate level constant even with a high intake of MSG. A further look at the activity of these enzymes in the brains of fourth-generation rats fed 10% MSG in the diet (*idem, J. Neurochem.* 1972, **19,** 1405) showed that the activities of glutamic–oxalacetic and glutamic–pyruvic transaminases (GOT and GPT) and of glutamic dehydrogenase (GDH) all rose sharply during the first 21 days after birth, a 20-fold increase in GOT and GPT and a 15-fold increase in GDH being seen during this period, but at no stage did the levels in MSG-treated animals differ from those in the controls. The rapid development of these enzyme systems in the brain during this neonatal period (when their development proceeds at a rate 2–3 times that of total protein synthesis) fits in with the finding that MSG does not accumulate in the brain even in rats ingesting high levels of MSG in the diet.

It has also been reported (Mushahwar & Koeppe, *Biochim. biophys. Acta* 1971, **244,** 318) that in the first 20 days after birth, the glutamate level of the rat brain increased from an initial level of some 4 μmoles/g to the adult value of about 10 μmoles/g. While these authors confirmed that ip or intragastric treatment of young rats, 7 or 14 days old, with 4 g MSG/kg

did not interfere with this pattern in brain levels of glutamate, such treatment was found to increase the glutamine content of the brain, as did ip treatment with equimolar doses of monosodium L-aspartate or glycine. While the glutamate and aspartate treatments caused convulsions in the young rats, glycine did not, so it seemed unlikely that glutamate toxicity could be ascribed to ammonia, as had been suggested previously. It was therefore concluded that the toxicity of MSG was due to the amino acid anions.

THE CASE AGAINST THE BLIGHTED POTATO

Spina bifida cystica is now considered to be the commonest congenital malformation recorded in the UK; anencephaly (incomplete closure of the skull bones) lies a close second. For convenience, these two malformations have been considered by some physicians as a joint entity, designated ASB, and again for convenience, this strictly unjustified terminology is used in this article. Recently, Renwick (*Br. J. prev. soc. Med.* 1972, **26,** 67) considered such factors as parental occupation, geographical location, maternal age, and year and time of conception in relation to the reported incidence of ASB in various areas. Furthermore, he put forward the hypothesis that the avoidance of a specific (but unidentified) substance present in some potato tubers would serve as an effective means of preventing most cases of ASB in the Western world. Renwick (*loc. cit.*) found that the seasonal peak of ASB in Scotland and Wales, where the incidence of the condition is relatively high, occurred in February, and suggested that this might reflect the quality of potatoes in the preceding May, assuming that, as with other teratogenic agents, embryonic damage occurred early in pregnancy. The prevailing weather conditions prior to the harvesting of potato crops and the conditions under which the potatoes were stored during the following winter were therefore important factors to be considered in a search for the agent responsible for these malformations.

Changes in potatoes during storage

The presence of solanines and chaconines, which are glycosides of the steroid solanidine, under the skin of potatoes that have been damaged or exposed to light is one of the first points made by Renwick (*loc. cit.*) in considering the possible occurrence of a teratogenic agent in some potatoes. He also notes that solanidine has antifungal properties and that the incidence of *Synchytrium endobioticum*, the fungus responsible for potato wart disease, can be related to the geographical distribution of ASB. The severity of attack by *Phytophthora infestans*, the fungus causing late potato blight, can also be related to the occurrence of ASB in specific areas and seasons.

It has been shown that several steroid alkaloids are induced when certain varieties of potato are infected with *P. infestans*, but this may not occur in some varieties particularly sensitive to blight. Renwick (*loc. cit.*) points out that main or late crop varieties of potato tend to be relatively resistant to the blight fungus and total solanidine levels in such varieties are comparatively high. Furthermore, solanidine levels may rise further on storage, particularly in slightly blighted potatoes. It is these varieties that provide the old potatoes eaten in May, when injury to the embryo is most likely to occur, and it is, moreover, the tubers least

affected by the blight that are likely to survive storage well enough to be used at all for food.

Apart from the geographical and time factors correlating ASB with the incidence of potato blight, these malformations were found to occur more frequently among working-class children in a given area, and particularly among offspring of multiparous mothers. Such women may be particularly exposed to potatoes, which form the staple carbohydrate in the family diet and from which an unidentified teratogen could enter the body by percutaneous absorption during the peeling process and by inhalation in steam during cooking, as well as by ingestion.

Renwick (*loc. cit.*) considers these and other factors in considerable detail, and concludes that the correlations found reflect a teratogenic action by some potato-tuber constituent, which is found in above-average concentrations in some potatoes and particularly in blighted tubers from certain varieties after winter storage.

Teratogenicity of solanines and other antifungal compounds

The identification of this postulated teratogenic constituent is a major problem. Solanine was found to be highly lethal to chick embryos (*Cited in F.C.T.* **10,** 602) but no clear evidence of teratogenicity was reported, and mammalian data are apparently limited to a paper by Kline *et al.* (*Proc. Soc. exp. Biol. Med.* 1961, **107,** 807). Renwick (*loc. cit.*) suggests that the finding of intrauterine deaths but no foetal abnormalities in rats given solanine during pregnancy could have been due to the fact that the test diets were given only after pregnancy had been diagnosed by an increase in body weight, by which time the foetuses might have passed the suggested sensitive period. *In vivo*, solanine is thought to be split enzymatically by the microflora of the gut, and solanidine, a major product of this hydrolysis, is excreted in the urine and faeces. The mammalian toxicity of solanine varies with species, but in general solanines are more toxic than solanidine, particularly in man. Clarke (*Vet. Rec.* 1972, **91,** 184) claims to have some evidence that solanine-type alkaloids may have a teratogenic effect on the pig and has asked for information on the occurrence of deformities in litters born to sows that have been fed on sprouting potatoes.

Rishitin and phytuberin, two other antifungal compounds, are found only in trace quantities in uninfected tubers, but are synthesized in some potato varieties in response to *P. infestans* infection (Varns *et al. Phytopathology* 1971, **61,** 174). Little is known about the teratogenicity of these phytoalexins.

Another interesting compound that has been given some consideration is cytochalasin B. This toxic metabolite of certain fungi, possibly including *P. infestans*, can produce spina bifida in chicks (Linville & Shepard, *Nature New Biology* 1972, **236,** 246). However, while some fungal toxin cannot be discounted as the possible teratogen, the available data suggest that an antibiotic response of the tuber is the more likely villain. In areas in France where potatoes are mainly of a variety highly sensitive to blight, for example, the incidence of ASB is low.

Other epidemiological findings

Emanuel (*Lancet* 1972, **ii,** 879) has recently presented findings which conflict with the hypothesis put forward by Renwick (*loc. cit.*). Using 1966 figures, he reported that in Taiwan, where the annual consumption of potatoes per head of the population was only 1 lb, the incidence of neural-tube defects (anencephaly, spina bifida, encephalocele and iniencephaly) was 1·42/1000 total births. This figure was comparable with the incidence in Sweden (1·09/1000 births), where the annual potato consumption was some 390 lb/head.

In France, with a potato consumption of about 460 lb/head, the incidence of anencephaly was 0·54/1000 total births compared with 1·15 in Taiwan. Moreover, although the incidence of neural-tube defects is higher among the poorer social groups in Taiwan, as in Western countries, the consumption of potatoes is largely the prerogative of the affluent classes.

In response, Renwick (*Lancet* 1972, **ii,** 967) has pointed out that, elsewhere, Emanuel *et al.* (*Teratology* 1972, **5,** 159) drew attention to the very low incidence of spina bifida in Taiwan (0·16/1000). The incidence of anencephaly in Taiwan may thus be as much as seven times greater than the incidence of spina bifida, although the method of sampling may account for some of the difference. In Western countries, on the other hand, the incidence of spina bifida is consistently slightly higher than that of anencephaly. Regarding the higher risk in poor families in Taiwan, Renwick (*Lancet* 1972, **ii,** 967) suggests that the malformations may be associated with poor-quality rice and soya beans as well as poor-quality potatoes, on the assumption that these plants may also produce phytoalexins in response to infection.

Investigations in laboratory animals

While controversy continues and epidemiological data are assembled, the problem is not being ignored in the laboratory. Recently Poswillo *et al.* (*Nature, Lond.* 1972, **239,** 460) carried out studies on the use of the cotton-eared marmoset, *Callithrix jacchus*, in order to determine whether this species would be useful as an animal model for teratological research. This small omnivorous primate is similar in size to the laboratory rat and breeds readily in captivity in all seasons, with a high incidence of multiple births. Regular recordings made in a colony of 30 conjugal pairs over 2 years have demonstrated that uterine diameter gives an indication (accurate to within 2 days) of the stage of pregnancy up to 50 days. The use of gonadotrophin to stimulate ovulation on withdrawal of the drug has enabled the timing of pregnancy to be predicted within 48 hours. Experimental data obtained in preliminary studies have shown that after the early somite stage, embryogenesis in the marmoset is similar to that in man, at least up to the period of closure of the posterior palate. Furthermore, irradiation and thalidomide at doses known to produce embryonic damage in man produce similar lesions in marmosets. Although further data are required on placental transfer of drugs and metabolic similarities between the marmoset and man, this species shows promise as a suitable animal for teratological research.

The reason for mentioning work on this species here is that Poswillo *et al.* (*ibid* 1972, **239,** 462) have fed boiled blighted-potato concentrates to both Wistar rats and the cotton-eared marmoset. The solanine content was enhanced by covering the potatoes with brown paper and illuminating them under fluorescent light for 48 hours. In six rats given 10 g potato concentrate daily from 2 days before mating to day 20 of pregnancy, when hysterectomy was performed, no resorptions or abnormal offspring were seen, whereas in four rats given 10 g of a commercial potato starch daily over the same period, a total of 12 resorptions and one offspring with exencephaly were seen. One resorption and no abnormal offspring were recorded among the controls. In marmosets given freeze-dried blighted-potato concentrate as a daily dietary supplement equivalent to 10 g/kg, in addition to the usual diet of bread, banana, Vionate and hard-boiled eggs, four out of 11 foetuses showed gross abnormalities broadly classified as cranial osseous defects. No such abnormalities were seen in the eleven foetuses from control animals. The oldest foetuses (120 days), which were born to conjugal pairs given the shortest preconception exposure to blighted-potato concentrate, showed no abnormalities. The authors consider that since the teratogenicity of the

blighted-potato concentrate has been demonstrated in the marmoset, it should be no more difficult to carry out selective testing of individual components of the potato in this species, in an attempt to identify the causal agent. They sound a note of caution, however, about attempts to extrapolate the results so far obtained to man, at least until it can be shown that the defect is similar in the marmoset and man and follows a common developmental pathway.

Thus, while many questions remain unanswered, there seems to be a fair amount of evidence in favour of a relationship between the consumption of blighted potatoes and congenital malformations of the ASB type.

POLYMERS IN SURGICAL REPAIR

The promise and problems of cyanoacrylates

Surgeons have for some years taken a keen interest in the tissue-adhesive and haemostatic properties of certain 2-cyanoacrylates (CAs). When applied to bleeding tissues, alkyl CAs polymerize in contact with water and form a powerful seal (*Cited in F.C.T.* 1969, **7,** 75), and they have therefore been used to arrest nasal bleeding or severe and intractable haemorrhage from solid organs such as the liver. Butyl CA applied to pulpal and periapical lesions in rats has produced only a mild inflammatory reaction, and in a dental cement has proved no more irritant than the conventional zinc oxide/eugenol filling (*ibid* 1971, **9,** 146). Isobutyl CA has been reported to show promise in animal experiments as a pulp-capping material (*ibid* 1971, **9,** 146), although a more recent study did not support the suitability of *n*-butyl CA for pulp capping (Nixon & Hannah, *Br. dent. J.* 1972, **133,** 14). The failure rate was found to be high, with unsatisfactory formation of dentine barriers and unfavourable pulp reactions.

One of the problems of the CAs lies in choosing a compound that the surgeon can handle without too much hazard and without undue prolongation of operating time. Matsumoto *et al.* (*Milit. Med.* 1969, **134,** 247) have drawn attention to two specific hazards associated with the movement of monomer away from the immediate site of application. One of these is the risk of thrombosis, which occurs if CA monomer enters the blood vessels and polymerizes on the intima. The *n*-butyl, isobutyl and possibly the isoamyl CA monomers seem relatively satisfactory in this respect, whereas the methyl ester, which is slow to polymerize, is difficult to control during application. If CA enters the renal calyces it predisposes to calculus formation there, the higher homologues with a more prolonged biological half-life being more likely to have this effect. Watts (*Br. med. J.* 1970, **1,** 501) has commented that the handling of CAs may create problems which increase operating time. Moreover, he claimed that wounds closed by sealing the skin with CA initially hold but later tend to break down to expel the foreign material. On a similar note of caution, Capperauld (*ibid* 1970, **1,** 819) asserted that CAs are less attractive as adhesives for skin closure than as reinforcements for sutures, as seals for leaks in hollow organs or as means for controlling bleeding from severe liver injuries.

Effect of CAs on blood coagulation and liver and kidney function

Gollub *et al.* (*Surgery Gynec. Obstet.* 1970, **130,** 3) have determined the *in vitro* effects of isobutyl CA on the morphology and coagulation of human and canine blood, and its *in vivo*

effects on coagulation. Whole blood, plasma, platelet and erythrocyte concentrates, fibrinogen and thrombin produced a slight exothermic reaction when the monomer was added dropwise, but not when the CA was applied in an aerosol spray (probably because of the refrigerant effect of the propellant). Polymerization appeared to be complete within 10 seconds of contact, with production of convoluted lamellae. A distinct irregular zone appeared between the external polymer film and the internal blood preparation, the cells adjacent to the contact zone remaining morphologically intact. The *in vitro* Lee-White clotting time of CA-treated blood was normal or only slightly raised, and thromboelastogram values were unaffected. When isobutyl CA was applied topically to operated canine kidneys the only observed change in the systemic blood coagulability of the animal was a slight fall in platelet count.

Liver function tests were carried out for 6 months after the subcutaneous implantation of 400 mg *n*-butyl CA/kg in dogs (Houston *et al. Biomed. Mater. Res.* 1970, **4,** 25). This treatment was followed by decreases in serum levels of lactic dehydrogenase (LDH), isocitric dehydrogenase and bilirubin and increases in partial thromboplastin time and glutamic-pyruvic transaminase activity, but although statistically the mean values differed significantly from the means of the pretreatment controls, all the values fell within the ranges considered normal for the dog. At autopsy, gross and histological examination of vital organs revealed no adverse effects of the implant.

Truss (*Urologe* 1969, **8,** 355) studied the metabolism of underlying tissue after application of methyl CA to the decapsulated surface of the rat kidney. Oxygen uptake, *p*-aminohippuric acid transport, anaerobic glycolysis and tissue activities of LDH, glutamic–oxalactic transaminase, aldolase and alkaline phosphatase did not differ significantly from those of the control kidney on which decapsulation alone had been performed.

In a comparative study of methyl, ethyl, *n*-propyl, isopropyl, *n*-butyl, isobutyl, *n*-pentyl, *n*-hexyl, *n*-heptyl, *n*-octyl and *n*-decyl CAs, McTernan *et al.* (*Toxic. appl. Pharmac.* 1970, **16,** 227) determined the tissue respiration of preparations of rat-liver cytoplasm after addition of the monomer. They used ^{14}C-labelled acetate as the source of respiratory $^{14}CO_2$. The greatest respiratory inhibition (45%) was produced by the methyl monomer, and in general, an increase in the length of the alkyl chain was associated with a decrease in capacity to depress respiration. These results were in accordance with earlier reports associating the CAs possessing longer alkyl chains with some reduction in tissue toxicity (*Cited in F.C.T.* 1966, **4,** 94).

Toxicological studies on CAs

A similar trend was established in *in vitro* and *in vivo* toxicity studies on these esters. DeRenzis & Aleo (*Oral Surg.* 1970, **30,** 803) incubated monolayers of mouse fibroblast cells with filter paper discs saturated with methyl, isobutyl or *n*-octyl CA and measured the zones of cytotoxicity produced. Within 1 hour, zones of toxicity appeared round the methyl-CA discs, while 6–12 hours elapsed before any sign of toxicity appeared around the other discs. The indications were that isobutyl and *n*-octyl CAs exerted a moderate and delayed cytotoxic effect compared with methyl CA, and that it was among the higher homologues that surgeons would have to seek for a safe and effective tissue adhesive. There was no appreciable difference in the effects of discs applied to the cell culture before or after polymerization of the CA.

A direct experiment to determine the contact toxicity of isobutyl CA applied to the oral cavity as a spray has been reported by Bhaskar *et al.* (*ibid* 1970, **29,** 313), who examined the

trachea, oesophagus, lungs, liver, stomach and kidneys of rats after the oral spraying, using Oil Red O stain to detect penetration of the polymer. When the animal's throat was protected from the spray no penetration of the compound into the trachea or oesophagus occurred, and no histological alteration in the respiratory or gastro-intestinal systems, or in vital organs, was discernible at various intervals from 12 hours to 21 days after spraying. A further study of 109 patients in whom butyl and isobutyl CA spray was applied on 296 occasions as a surface dressing for the labial gingival area or deeper parts of the oral cavity (with gauze protection of the back of the throat) revealed no irritation of the throat or respiratory tree (Bhaskar *et al. loc. cit.*).

Open surgical wounds of the liver in dogs were treated with methyl, butyl, heptyl, isobutyl or isoamyl CA or with mixtures (95:5 and 50:50) of heptyl and methyl CAs (Collins *et al. Surgery, St. Louis* 1969, **65,** 256). In three of ten dogs treated with methyl CA, abscesses developed at the site, and histological examination showed severe necrosis underlying the polymer layer during the first week. In contrast, the early tissue reactions to butyl, heptyl and isobutyl CAs were mild. In none of the 13 dogs examined 12–21 months after treatment of the liver with long-lasting cyanoacrylates were any signs of local or distant malignant change observed. This lack of tumorigenicity was confirmed by a report by Matsumoto & Heisterkamp (*Am. Surg.* 1969, **35,** 825), who found no signs of tumour formation in dogs studied for up to 2 years, in mice, or in first- or second-generation rats, after *n*-butyl or isobutyl CA had been sprayed inside the abdominal cavity.

The application of isobutyl CA to bilateral experimental ulcers of the rat tongue has been reported to reduce the intensity of the inflammatory response (Giunta & Shklar, *Oral Surg.* 1971, **31,** 140). Some 6 hours after application, the adhesive lay over a narrow zone of moderate oedema and an inflammatory exudate of neutrophils and macrophages, while the control side of the ulcer showed an oedematous zone of infiltration surmounted by scab formation. At 18 hours, the adhesive covered a narrow band of intense surface necrosis with neutrophil infiltration but little oedema, contrasting with a fibrin and neutrophil clot with moderate surface necrosis and oedema and inflammatory infiltration in the control ulcer. By day 4 the cyanoacrylate film had disappeared.

Gyurkó & Szalóki (*Archs Surg., Chicago* 1971, **102,** 503) applied butyl CA adhesive to intestinal loops of dogs in an experimental Noble operation and examined the animals at intervals up to 250 days after the operation. Early inspection showed the infiltration of round cells around the adhesive particles. The process advanced so that granulation tissue bridges linked the intestinal loops from 100 days onwards. By 250 days, the adhesive was almost completely replaced by connective tissue. Egry & Somogyvari (*Chirurg* 1971, **42,** 22) who performed side-to-side anastomoses of the small intestine and gastro-intestinal anastomoses in dogs using butyl CA as adhesive, reported less tissue reaction and more rapid healing than with the usual suturing process.

An evaluation of β,β,β-trifluoroisopropyl CA in comparison with methyl and *n*-butyl CAs as an adhesive for skin wounds in the rat and a comparative estimate of the toxicity of these compounds applied to the exposed liver surface in rats are reported by Nelson *et al.* (*Archs Surg., Chicago* 1970, **100,** 295). Trifluoroisopropyl CA (MBR 4197) produced a stronger skin bond than the other adhesives. Discs of the polymer implanted subcutaneously in mice lost some 18% of their initial weight by 16 weeks, while in the same time discs of polymerized *n*-butyl CA were virtually not absorbed at all. None of the three monomers injected subcutaneously into mice in a dose of 5, 10 or 20 g/kg caused any deaths or signs of systemic toxicity over 14 days. At autopsy, the inflammatory changes induced locally by

MBR 4197 and *n*-butyl CA were far less severe than those with methyl CA. Similarly, application to the exposed liver surface in rats induced less extensive inflammation with MBR 4197 and *n*-butyl CA than with methyl CA. With each of the adhesives, the initial fibropurulent response was replaced at 7–28 days by granulation tissue and a local foreign-body reaction, but this replacement was relatively slow in the case of the methyl CA adhesive.

In view of the favourable aspects of *n*-butyl CA, it was used by Collins *et al.* (*Surgery, St. Louis* 1969, **65,** 260) to control severe haemorrhage in seven Vietnam casualties in whom routine surgical attempts to arrest bleeding had failed. Haemostatis was achieved in each patient, and the polymer did not contribute to the three deaths that subsequently occurred. The monomer was used on five liver wounds, one kidney wound and two retroperitoneal haemorrhages. Nevertheless, Collins *et al.* (*loc. cit.*) recommend that the use of *n*-butyl CA should be restricted to life-threatening situations, since it is bound to result in a permanently indwelling foreign body which might lead to complications.

CAs and the eye

Webster *et al.* (*Archs Ophthal., N. Y.* 1968, **80,** 705) have described the successful use of *n*-heptyl CA in sealing leaking corneal perforations in two patients. The adhesive promptly and permanently restored the anterior chamber of the eye. After an initial vascular engorgement of the treated area there was a minimal inflammatory response to the adhesive. On the other hand, Ferry & Barnert (*Am. J. Ophthal.* 1971, **72,** 538) have more recently reported the development of a granulomatous keratitis in the eye of a patient in whom isobutyl CA was applied to close a perforation in an extensively necrotic cornea. Examination of the excised corneal button showed marked stromal irregularity, vascularization and a heavy infiltration of inflammatory cells. The same material injected into rabbit corneas did not have this effect and it was felt that the extensive necrosis in the patient's eye permitted relatively large amounts of CA to enter the stroma.

Adverse effects in rabbits were described, however, by Seelenfreund *et al.* (*Archs Ophthal., N. Y.* 1970, **83,** 619), who used isobutyl CA to seal experimental choroid perforations in this species. When the monomer was placed on the sclera a mild foreign-body reaction ensued, whereas application to the choroid induced severe localized inflammation accompanied by disorganization of the choroid and degeneration of the rods and cones of the iris.

The effects of isobutyl, hexyl, octyl and decyl CAs after intracorneal, subconjunctival or intraocular injection in rabbits have been reported by Gasset *et al.* (*Investve Ophthal.* 1970, **9,** 3). All injections induced an inert-body reaction in the cornea and conjunctiva, but this was less than that induced by silk or catgut sutures. When introduced into the anterior chamber of the eye, the CAs induced iritis, which lasted about 1 week and then spontaneously subsided except in the case of hexyl CA, which caused permanent clouding and vascularization of the cornea. Abrasions of the cornea in rabbits were treated with octyl CA, and similar abrasions in germ-free guinea-pigs were treated with isobutyl, octyl or decyl CA by Aronson *et al.* (*Archs Ophthal., N. Y.* 1970, **84,** 342). Injections of octyl CA were also made into the corneal stroma of guinea-pigs. There was a marked initial inflammatory reaction to all the adhesives and this was followed by granuloma in both animal species. The onset of severe inflammation after intrastromal injection of octyl CA was much more rapid than after surface application. Corneal vascularization was apparent by day 2 and was most severe by day 5. In the intrastromally-injected guinea-pigs killed 5 days after treatment, a diffuse polymorphonuclear leucocyte reaction was found throughout the corneal stroma. *In vitro* studies of protein binding by the compounds indicated that the inflammatory

activity of the CA adhesives might be related to their binding to γ-globulin, their complement-fixing potential and polymorphonuclear leucocyte chemotaxis.

While the most promising clinical applications for adhesives in ophthalmology seem to be concerned with the sealing of perforated corneas or scleral incisions, another interesting use is in the removal of foreign bodies from the vitreous cavity. A technique recently described (deGuillebon *et al. ibid* 1972, **87,** 407) uses a fine glass tube connected to a microsyringe to inject a small quantity of *n*-butyl CA (1 μl) on to the foreign particle, which thus becomes firmly stuck to the tip of the tube as the CA polymerizes on contact with the moist surface. Not only the foreign particle but also all the injected adhesive may be removed when the tubing is withdrawn.

An alternative adhesive

CAs are not the only solution to the problem of surgical adhesives. Koehnlein & Lemperle (*Surgery, St. Louis* 1969, **66,** 377) have used a preparation (GRF) containing gelatine (45%), resorcinol (15%) and water (40%). This is warmed and applied to the wound site on top of an initial application of 37% formaldehyde solution. Very little heat was evolved during the condensation reaction between the gelatine, resorcinol and formaldehyde, and most of the formaldehyde was bound and therefore not liable to induce deeper tissue damage. In 212 experiments, in which GRF was applied to the skin, kidney, liver, intestines and blood vessels of rats and to the dura, brain and blood vessels of rabbits, the typical coagulation necrosis caused by CAs was not seen. A method of minimizing tissue trauma involved adjusting a solution of gelatine and resorcinol to pH 5, stirring in formaldehyde solution and then applying very rapidly, as condensation commenced within some 30 seconds of mixing. Further work on tissue tolerance to preparations of this type is in progress.

Polyglycollic acid sutures

Finally, a word about the use in surgical repair operations of a form of suture other than the traditional catgut or unabsorbable plastics material. Absorbable sutures made of a braided high-molecular-weight linear homopolymer of glycollic acid (PGA, Dexon) have been reported to induce only a mild inflammatory and fibrotic reaction in man (Myllärniemi, *Annls Chirurg. Gynaec. Fenn.* 1971, **60,** 123). Confirmation of the lack of antigenicity of such sutures comes from Miln *et al.* (*Scott. med. J.* 1972, **17,** 108), who have used PGA sutures for surgical gastro-intestinal anastomoses and abdominal-wall closures in 134 patients. Dardik *et al.* (*Am. J. Surg.* 1971, **121,** 656) described the characteristics of PGA sutures, which are digested by hydrolysis rather than by proteolytic enzymes, induce a foreign-body reaction in tissues, and promote only slight oedema and exudate formation when compared with catgut, the tissue reactions to which tend to be marked. When PGA was used in ten patients for initial assessment, no effects on twelve biochemical parameters were detected. Further use in 126 operations on 118 unselected patients revealed no clinical complications from PGA. Herrmann *et al.* (*Archs Surg., Chicago* 1970, **100,** 486) have reported that PGA sutures, implanted for periods of up to 28 days in rats, produced only minimal inflammation, and in surgical procedures carried out in dogs, PGA suture lines were again somewhat less oedematous and less indurated than catgut lines. Some dissolution of the implanted material was observed in rats after 7 days, and after 21 and 28 days the site of the implants was difficult to find.

ORGANOSILOXANES AND REPRODUCTION

Organosiloxanes varying in their degree of polymerization and ranging in form from rubbers and resins to fluids have been widely used in industry for many years, and for some time they have also been used in the manufacture of food-contact materials and cosmetics and in medicine and surgery (for tissue implants and augmentation and pharmaceutical preparations as well as for medical equipment). During this time and in various experimental studies, they have established a reputation for being biologically inert both when injected and when administered orally (*Cited in F.C.T.* 1966, **4,** 189; *ibid,* 1967, **5,** 263; Joint FAO/WHO Expert Committee on Food Additives, *F.A.O. Nutr. Mtg Rep. Ser.* no. 46A, WHO/Fd Add./70.36, 1970, p. 151). It is true that there have been some reports of adverse reactions to the injection of large quantities of silicone fluid for mammary augmentation, but the evidence suggested that these cases were due to contaminants or additives in the preparation rather than to the silicone fluids themselves (*Cited in F.C.T.* 1968, **6,** 659).

Because of their blandness and their capacity to impart water-repellancy and lubricity to treated surfaces, the silicones are popular ingredients of topical cosmetics formulations. It must, therefore, have come as something of a shock when an experimental hand-cream formulation containing linear and cyclic siloxanes was found to cause spermatogenic depression in rabbits after application of 500–2000 mg/kg to about 10% of the body surface for 20 days (Palazzolo *et al. Toxic. appl. Pharmac.* 1972, **21,** 15). This finding prompted a series of further tests, the results of which were reported in a group of papers published together earlier in the year.

Separate testing of the various components of the hand-cream revealed that the responsible agent was an equilibrated copolymer of mixed cyclosiloxanes (I) conforming to the general formula:

$$\text{cyclic } [(C_6H_5CH_3SiO)_x.([CH_3]_2SiO)_y], \text{ where } x + y = 3\text{–}8$$

Dermal application of only 5 mg I/kg was sufficient to produce testicular atrophy, while even at 2·5 mg/kg spermatogenesis was mildly depressed. Rabbits given an oral dose of 200 mg I/kg/day for 28 days showed a similar testicular response, which like that resulting from dermal application was at least partially reversible after discontinuation of treatment. The material also affected the female reproductive system at sufficiently high dose levels; dermal or subcutaneous administration of 500 mg/kg or more to pregnant rabbits resulted in maternal weight loss, increased resorptions and decreased viability of the young, but at the 200 mg/kg level such effects were not statistically significant. Monkeys, too, were sensitive to the compound when it was given orally, a daily dose of 50 mg/kg producing clinically apparent testicular atrophy after 8–10 weeks, although successful mating was again possible after an 8-month recovery period. However, unlike rabbits, monkeys showed no response to dermal application of up to 2000 mg/kg/day for 145 weeks, and were able to breed successfully; no significant increase in their urinary silicon concentration was detected even after 1 year of dosing. Fortunately, tests in man suggested conformity with the monkey rather than with the rabbit pattern, no increase in the silicon concentrations of blood or urine being apparent after dermal application of 50 mg/kg/day for 10 days.

A more specific investigation of the effect on reproduction was then conducted by Le Fevre *et al.* (*ibid* 1972, **21,** 29), using I and a relatively pure preparation of monophenylheptamethylcyclotetrasiloxane (II). In mature female rats, I given orally at a dose level of 110 mg/kg/day for 30 days from the first day of oestrus caused the animals to progress no

further than dioestrus. Normal cycles and pregnancies occurred after withdrawal of treatment, but failures of implantation and embryo deaths were common. When 220 mg I/kg was given daily, starting at various intervals after mating, no pregnancies occurred in rats treated before day 7 and embryotoxic effects were common in animals treated on days 7–12 of pregnancy. Offspring of mothers treated from days 12 to 16 were normal, but treatment on days 16–21 resulted in urogenital malformation and urinary incontinence in the female offspring. Dissection and histopathological studies suggested possible neurological involvement. At oral dose levels of 22–220 mg/kg/day II had very similar effects, except that only dead pups were born to mothers treated from day 16 onwards with 100 mg/kg or more and there was no evidence of any urogenital defect. With doses of 22 mg/kg, the effects were marginal, but a minor degree of urinary incontinence was present in surviving pups. In rabbit offspring, the urogenital system appeared unaffected by I, although earlier findings of embryotoxicity (Palazzolo *et al. loc. cit.*) were confirmed. Mature male rats given I at an oral dose level of 100 or 1000 mg/kg/day for 25 weeks showed a decrease in the weight of seminal vesicles and prostate, and after treatment with the higher level for 6 months there was evidence of reduced fertility on mating. Topical application of a level of 2 g I/kg caused a significant reduction in testicular weight and an inhibition of spermatogenesis, although only some animals were affected in the latter respect. Male rabbits proved more susceptible than rats, spermatogenesis being completely inhibited by repeated oral or topical application of 1 or 2 g I/kg, respectively.

Not surprisingly these findings led to concern that other organosiloxanes, in particular those already widely used in cosmetics, might have hitherto unsuspected reproductive effects. Hobbs *et al.* (*ibid* 1972, **21,** 45) therefore compared the effects of dimethylpolysiloxane (III), tris(trimethylsiloxy)phenylsilane (IV), trifluoropropylmethylpolysiloxane (V), trimethyl end-block dimethylphenylmethylpolysiloxane, 1,1,3,3-tetraphenyl-1,2,2,3-tetramethyltrisiloxane and methylhydrogenpolysiloxane with those of I when given orally to mature male rats at dose levels of 3·3–5·0 ml/kg/day for 5–7 days. The first four compounds were also applied dermally to rabbits, each at a dose level of 200 mg/kg/day for 28 days. It must have caused some relief when none of the rats except those treated with I (which produced significant atrophy of the seminal vesicles) showed any depression of seminal vesicle weight, and in the rabbits there was no evidence that spermatogenesis was impaired by the treatment. Because all production lots of I were not equally potent in effect, 54 different batches of IV were tested orally in rats, and five lots were applied dermally to rabbits; again, however, no adverse effects were seen. Finally, III, IV and V were applied to the entire back area of five male human volunteers at a level of 50 mg/kg for 10 days, any excess material being removed by washing after 20 hours each day. In no case did silicon concentrations of blood or urine show any significant increase.

An attempt was then made to identify the active components of I (Bennett *et al. ibid* 1972, **21,** 55). The compounds were given orally to mature male rats, and reductions in the weights of seminal fluid, seminal vesicles and prostate were used as criteria of activity. A phenyl group proved to be an essential prerequisite and of the phenyl-substituted linear disiloxanes, trisiloxanes and tetrasiloxanes, only the last-named appeared inactive. Cyclic tri- and tetrasiloxanes were generally more potent than their linear analogues, but cyclic pentasiloxanes were inert in this respect. The most active compounds were II and another cyclic tetrasiloxane, cyclic 2,6-*cis*-diphenylhexamethylcyclotetrasiloxane (VI), which showed roughly equal potency in the rat. The mouse, however, showed a much greater sensitivity to VI. When given orally II was much more active than when given parenterally, but the reason

for this was not clear; its potency was unaffected by bile-duct ligation or by the simultaneous administration in drinking water of three non-absorbable antibiotics, ruling out the possibility of conversion of II to an active material by the gastro-intestinal bacteria. An oleaginous vehicle, however, greatly enhanced activity. In addition to causing reductions in the weight of the seminal fluid and vesicles and the prostate, in testicular size and in plasma testosterone level, II at high dose levels (e.g. 100 mg/kg/day for 7 days) caused adrenal hyperplasia, hepatomegaly, and a decrease in body weight and in serum levels of cholesterol, phospholipids and alkaline phosphatase. Both II and VI shortened hexobarbitone sleeping time in mice, but not in rats, when given orally in doses of 33–100 mg/kg/day for 4 days, indicating that some stimulation of the microsomal enzymes occurred.

The next step was to examine the effects of this same series of organosiloxane compounds on the female reproductive system, and in this connexion Hayden & Barlow (*ibid* 1972, **21,** 68) studied changes in uterine weight and histology and the production of uterine hyperaemia after oral administration of the compounds to immature, bilaterally ovariectomized rats. Again cyclotetrasiloxanes were the most potent compounds tested and two phenylmethylsiloxy units conferred greater activity than one, VI being some 100 times more potent than II at the same dose level. Replacement of the first methyl group in VI with hydrogen greatly reduced its activity. The degree of hyperaemia and the histology of the uterus after administration of II and VI were very similar to the effects produced by diethylstilboestrol and oestradiol benzoate. Adrenalectomy did not affect the response, indicating that it was due to intrinsic oestrogenicity rather than to an indirect effect on the adrenal oestrogen-synthesizing mechanism. As in the male rat (Bennett *et al. loc. cit.*), the parenteral route was markedly less effective than the oral route.

The precise effects of VI in the mature male rat were defined in a further investigation (Le Vier & Jankowiak, *ibid* 1972, **21,** 80). When given orally, VI was similar in activity to known oestrogens, and the reduction it induced in plasma testosterone levels and in the weight of seminal vesicles and ventral prostate could be counteracted by replacement therapy with testosterone propionate or gonadotropins. Within the first 24 hours there was also a rapid increase in pituitary weight and in prolactin, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) activity, followed by a slow decline in FSH and LH activity, which in the light of the developing accessory-tissue atrophy was indicative of an anti-release mechanism. Testicular weight was reduced only after more prolonged treatment (14 or more days). In support of previous findings with II and VI (Bennett *et al. loc. cit.*), VI was found to depress serum cholesterol and drug-metabolizing activity, as shown by reductions in hexobarbitone sleeping time in mice and antipyrine toxicity in rats. On these grounds, it was considered possible that enhanced steroid catabolism might contribute to the anti-androgenic effect of VI, although the mechanism of its action could not be more precisely defined.

This impressive series of papers has drawn attention to a novel group of compounds with potent pharmacological effects on reproductive physiology. Obviously a considerable amount of work still needs to be done before their mode of action is clearly understood, but there are some interesting indications that some of the cyclic siloxanes act directly on gonadal tissue while others appear to interfere with the pituitary control of gonadal function. An important feature of this work is the indication that the linear polysiloxanes, used extensively in the food and cosmetics industries, do not have these adverse effects on the gonads. The cyclosiloxanes of higher molecular weight also appear to be inert in this respect.

Fd Cosmet. Toxicol. Vol. 11, pp. 323–349. Pergamon Press 1973. Printed in Great Britain

TOXICOLOGY: ABSTRACTS AND COMMENTS

COLOURING MATTERS

2484. Hepatotoxicity of amaranth. . .

Gales, V., Preda, N., Popa, Letitia, Sendrea, Doina et Simu, G. (1972). Recherches toxicologiques sur le colorant amaranthe. *Eur. J. Toxicol.* **5,** 167.

Readers will be familiar with the problems of amaranth (C.I. (1956) no. 16185; FD & C Red No. 2; EEC no. E123) and the cloud that hangs over this red food colouring as a result of recent allegations of carcinogenicity and teratogenicity (Andrianova, *Vop. Pitan.* 1970, **29** (5), 61; Shtenberg & Gavrilenko, *ibid* 1970, **29** (2), 66). The present study describes the effect of the long-term administration of amaranth on rat liver.

When amaranth was fed to rats for periods of up to 18 months at a dietary level of 0·12% (giving an intake of approximately 30 mg/rat/day), growth was markedly depressed and mortality increased. Both biochemical and morphological evidence of liver damage were obtained. The level of vitamin A was decreased by about 50% within a few days and continued to decrease progressively throughout the study. No marked increase in serum or liver glutamic–oxalacetic and glutamic–pyruvic transaminases was noted, however, such as resulted from the administration for 10 days of a dose of amaranth approaching the LD_{50}. A rise in serum albumin and β-globulin was comparable with that produced by 4-dimethylaminoazobenzene (DAB). Pathological damage to the liver included vacuolization of the hepatocytes and evidence of fatty degeneration.

The similarity of the liver changes obtained to those induced by DAB (butter yellow), a known hepatocarcinogen, led the authors to suggest that the permitted level of amaranth in food should be re-assessed.

2485. . . . and its biliary excretion

O'Reilly, W. J., Coates, P. E., McMahon, Kerry A., Ting, Ellen & Priestly, B. G. (1972). Pharmacokinetic models for the biliary excretion of amaranth in the rat. *Life Sci.* **11,** Part I, 197.

The faeces are the major route of elimination of amaranth following its administration orally (*Cited in F.C.T.* 1972, **10,** 875) or intravenously (*ibid* 1963, **1,** 102). A correlation has been established between the rate of biliary excretion of this and other water-soluble azo dyes and the ratio of protein-binding in the liver to that in the blood (*ibid* 1966, **4,** 623).

O'Reilly *et al.* (cited above) used amaranth as a model compound to evaluate the utility of several possible pharmacokinetic models designed to describe the blood level and biliary excretion rate of anionic compounds in the rat. When amaranth in aqueous solution was injected intravenously into anaesthetized rats at dose levels of 1–100 μmoles, blood levels of the dye fell rapidly to below 10% of their initial value in the first 10 min, following first

order (linear) kinetics. Some 54–80% of the administered dose was rapidly recovered in the bile, first-order kinetics again being followed at dose levels up to 20 μmoles, although at higher levels non-linear behaviour was observed. Application of digital and analogue curve-fitting techniques suggested that at low doses amaranth uptake from blood by the liver was a first-order reaction, with amaranth remaining temporarily bound to liver tissues before first-order excretion into the bile. However, a small amount of reflux from liver to blood could not be excluded from the model.

2486. Io-dye-d

Vought, R. L., Brown, F. A. & Wolff, J. (1972). Erythrosine: An adventitious source of iodide. *J. clin. Endocr. Metab.* **34,** 747.

Studies of the possible biochemical repercussions following the breakdown of the food colouring, erythrosine, and the consequent release of iodine (I) have suggested no cause for concern at current levels of use. The present work was carried out to establish by the use of a refined radio-tracer method the extent to which iodide released from this important food and drug colouring may contribute to the dietary intake of I.

The ^{127}I and ^{131}I levels in the thyroid, serum and urine were measured in groups of rats that had been fed on diets containing an erythrosine-coloured cereal for 3 or 5 wk and then given an intraperitoneal injection of ^{131}I as sodium iodide. Both ^{131}I uptake by the thyroid and protein-bound radioactivity in the serum were reduced significantly in these rats, but there was an increase in serum radioactivity not bound to protein. The strongest evidence for erythrosine deiodination was, however, the marked rise in ^{127}I levels in the urine and thyroid. When iodine-free [^{131}I]erythrosine was administered to rats, 25–33% was metabolized to iodide. Most of the free iodide appeared in the urine, but it was also found to a small extent in the thyroids.

[Extrapolation of the results of such work to man is difficult, since it is still not known how closely human metabolism of erythrosine resembles that in the rat, although some release of iodine from erythrosine has been demonstrated in man (Andersen *et al. J. clin. Lab. Invest.* 1964, **16,** 249). We recently commented briefly on the possible significance of a high dietary intake of iodine (*Cited in F.C.T.* 1972, **10,** 591).]

2487. Competitive attacks on azo dyes

Du Plooy, Marianne & Dijkstra, J. (1971/72). Early stage in the metabolism of aminoazo dyes in the liver of rats. *Chemico-Biol. Interactions* **4,** 163.

Thorough examination of the metabolism of the azo dye, butter yellow (*N*,*N*-dimethyl-aminoazobenzene; DAB), has shown it to consist basically of three detoxication reactions (azo reduction, *C*-hydroxylation and *N*-demethylation) followed by steps that have been postulated to be involved in the carcinogenic action of the dye, namely *N*-hydroxylation and binding to cellular macromolecules (nucleic acids and proteins). The present paper examines the structure and function of the early DAB metabolites.

In the livers of rats given 250 mg DAB/kg in a single dose by gastric intubation, TCA-soluble metabolites reached their maximum level 4 hr after treatment. These metabolites were fractionated into six components using high-voltage paper electrophoresis, the two

most important (accounting together for 90% of the total) being 4′-hydroxyDAB and 4′-hydroxy-monomethylaminoazobenzene, both in sulphated form. The other four, also present as ethereal sulphates, were not fully identified. It was found that the amount of 3-hydroxylation that occurred was inversely proportional to the extent of 4′-conjugation and of protein binding, leading to the postulation that competition exists between the respective binding sites. The fact that at least one *N*-methyl group is essential if an aminoazo dye is to bind with rat-liver proteins may be due to stereochemical considerations, since the activity of rat-liver *o*-hydroxylase may be sterically hindered by the presence of the methyl group in the adjacent 4-position, thus leaving the 3-position free for protein binding.

FLAVOURINGS, SOLVENTS AND SWEETENERS

2488. The hepatotoxicity of allyl alcohol

Serafini-Cessi, Franca (1972). Conversion of allyl alcohol into acrolein by rat liver. *Biochem. J.* **128,** 1103.

Both allyl alcohol and allyl formate, which is hydrolysed to the alcohol *in vivo*, cause marked liver damage, which is localized in the periportal region of the lobules (*Cited in F.C.T.* 1964, **2,** 135; Popper, *Virchows Arch. Path. Anat. Physiol.* 1937, **298,** 574). Rees & Tarlow (*Biochem. J.* 1967, **104,** 757) suggested that the damage might be due to the conversion of allyl alcohol to acrolein by the alcohol dehydrogenase which was also found primarily in the periportal region, but they did not identify acrolein in rat-liver suspensions incubated with allyl alcohol. This deficiency has now been rectified.

Rat-liver homogenates, mitochondria, microsomal preparations and post-microsomal supernatants were incubated with allyl alcohol at 37°C, with or without addition of NAD^+ or $NADP^+$, and acrolein was determined by spectrophotometry and paper chromatography after conversion to a semicarbazone. The presence of NAD^+, which acts as a cofactor for liver alcohol dehydrogenase, led in all cases to the formation of acrolein, and some acrolein was formed with whole homogenate and the mitochondrial fraction even in the absence of NAD^+. Inclusion of $NADP^+$ in the incubation mixture, however, failed to affect the formation of the aldehyde. The amount of acrolein formed increased with increasing levels of allyl alcohol up to 2 mM, and with time up to 40 min, although the proportion of allyl alcohol converted was never large (a 5% conversion in the case of 2 μmoles allyl alcohol). Conversion was prevented or markedly decreased by the alcohol-dehydrogenase inhibitors *p*-chloromercuribenzoate and hydroxylamine, depending on their concentration. Allyl formate incubated with NAD^+ in the same manner as allyl alcohol also gave rise to acrolein, but allylamine, which does not cause periportal damage, was inactive in this respect.

2489. Further thoughts on safrole metabolism

McKinney, J. D., Oswald, E., Fishbein, L. & Walker, M. (1972). On the mechanism of

formation of Mannich bases as safrole metabolites. *Bull. env. contam. & Toxicol.* (*U.S.*) **7,** 305.

Tertiary aminomethylenedioxypropiophenones (Mannich bases) are products of safrole metabolism in the rat and guinea-pig (*Cited in F.C.T.* 1972, **10,** 257). The possible mechanism of their formation has now been investigated.

When safrole was heated in dimethylsulphoxide in the presence of anhydrous chromium trioxide at 100°C, the major product of the reaction was piperonyl acrolein, with smaller amounts of a vinyl ketone and piperonal. Isosafrole under the same conditions gave rise to the same products, although this time piperonal predominated and a minor amount of a fourth (unidentified) compound was also present. Heating of the vinyl ketone in piperidine at 80°C for 3 hr produced a Mannich base, 3-piperidyl-1-(3′,4′-methylenedioxyphenyl)-1-propanone, which resisted decomposition at elevated temperatures and reduced pressures. Other reactions of the vinyl ketone included the quantitative formation of a keto alcohol on heating an aqueous solution from steam distillation in a closed container, dimer and polymer formation in protic solvents and acetate formation on solvolysis in glacial acetic acid in the presence of sodium acetate; its reactivity thus approached that of a strong alkylating agent.

It is suggested that, on oxidation, both safrole and isosafrole give rise initially to an allyl alcohol and another conjugated alcohol, which are further oxidized to the vinyl ketone and piperonyl acrolein respectively. Piperonal could be formed from either of the intermediate metabolites and could also arise from oxidative cleavage of the double bond of isosafrole, which would account for the higher yield of piperonal obtained from this starting material. Condensation of the vinyl ketone with an amine would then lead to the formation of a Mannich base.

2490. Cyclamate conversion in the guinea-pig

Asahina, M., Yamaha, T., Sarrazin, Ginette & Watanabe, K. (1972). Conversion of cyclamate to cyclohexylamine in guinea pig. *Chem. pharm. Bull., Tokyo* **20,** 102.

Urinary metabolites of cyclamate have been shown to include cyclohexylamine (CHA) in both animals and man (*Cited in F.C.T.* 1967, **5,** 398). Some workers have suggested that the conversion occurs in animal tissues, whereas others have indicated that enzyme systems in the gut flora of animals and man may adapt to the presence of cyclamate (*ibid* 1970, **8,** 694; *ibid* 1971, **9,** 293). The present report substantiates the latter argument.

Following oral administration of sodium cyclamate to guinea-pigs, urinary CHA concentrations increased markedly after treatment had continued for several days. However, when the cyclamate was injected intraperitoneally or given orally with antibiotics very little CHA was excreted in the urine. The pattern of CHA excretion in the faeces, as well as its level in the caecum, mirrored the urinary situation, with high levels only after cyclamate had been given orally without antibiotics. In *in vitro* tissue studies, in which cyclamate was incubated with homogenates of liver, kidney and small intestine, no accumulation of CHA was detected.

It was concluded that the microflora present in the guinea-pig gut are responsible for the conversion of cyclamate to CHA and that, as in other species, this activity can be induced by the presence of ingested cyclamate.

EMULSIFIERS AND STABILIZERS

2491. Polyglycerol esters: Short-term toxicity and metabolism

King, W. R., Michael, W. R. & Coots, R. H. (1971). Subacute oral toxicity of polyglycerol ester. *Toxic. appl. Pharmac.* **20,** 327.

Michael, W. R. & Coots, R. H. (1971). Metabolism of polyglycerol and polyglycerol esters. *Toxic. appl. Pharmac.* **20,** 334.

Tests on polyglyceryl esters (PGEs) have shown that these compounds lack carcinogenic activity and can be absorbed and utilized as well as natural fat with no deposition in body tissues (*Cited in F.C.T.* 1966, **4,** 216).

In the first study cited above, rats were fed 0, 2·5, 5·0 or 10% PGE (a decaglyceryl decaoleate preparation) for 90 days. Growth, haematology, survival, organ weights and pathology were all within normal limits. Total fat absorption, as measured by faecal fatty acids, decreased with increasing PGE level. Males on 10% PGE showed a reduction in efficiency of feed utilization and females on 10% PGE showed an increase in urinary nitrogen excretion. The increased faecal excretion of fatty acids in general, and oleic acid in particular, with increasing dose indicated that absorption of dietary PGE was incomplete. Some 56% of the fatty acid moiety of the PGE tested was accounted for by oleic acid.

Radiotracer studies provide an accurate means of following the absorption and elimination of both the polyglycerol and fatty acid moieties of PGE. To this end, monoglycerol, triglycerol, polyglycerol, polyglyceryl monooleate and polyglyceryl decaoleate were each labelled with ^{14}C in the glycerol moiety, while triglyceryl monooleate, triglyceryl tetraoleate, polyglyceryl monooleate, polyglyceryl decaoleate and polyglyceryl monoeicosanoate were labelled in the fatty acid moiety. The polyglycerol used contained ten glycerol units. Rats were dosed orally with each compound, and the ^{14}C in expired air, urine, faeces, gastrointestinal contents and carcass were determined up to 51 hr after treatment. In addition, rats with cannulated thoracic ducts were similarly dosed and ^{14}C was assayed in the lymph as well as in expired air, urine and faeces.

It was found that the ester bonds were hydrolysed mainly before absorption occurred. The free fatty acids were absorbed via the thoracic lymph duct, with the oleic acid moiety being more effectively absorbed than the eicosanoic acid moiety. Free or partially esterified polyglycerols were less readily absorbed and absorption was probably via the portal vein. Thus about 90% of the monoglycerol or triglycerol administered was absorbed, compared with only 40% of the polyglycerol moiety (following dosing either in free or esterified form). The absorbed polyglycerols were excreted unchanged, primarily in the urine, with less than 7% of the dose appearing in the expired air and carcass. Unpolymerized glycerol present as an impurity in the polyglycerol could account for most of the ^{14}C in the expired air. The distribution of the fatty acid moiety from the polyglyceryl esters into lymph glycerides, phospholipids and sterol esters showed a pattern similar to that obtained previously with a triglyceride, glyceryl trioleate. Exhaled carbon dioxide was the major end-product of fatty acid catabolism.

When tri- and polyglyceryl esters labelled in the fatty acid moiety were hydrolysed *in vitro* with pancreatic juice plus bile, the oleate bond was split as readily as the oleate bond in glyceryl trioleate. The eicosanoate bond, however, was more resistant to cleavage than the oleate bond.

In summary, this well designed and executed metabolic study shows that PGEs are readily hydrolysed to polyglycerol and fatty acids. About 40% of the polyglycerol moiety is absorbed and this is rapidly excreted unchanged in the urine. The fatty acid moiety of PGE is absorbed and utilized as well as that of natural fats. However, the polyglycerol moiety of these esters is not utilized as an energy source, as was reported previously, since no catabolism of the glycerol ethers took place.

ANTIOXIDANTS

2492. BHT in the lung

Marino, A. A. & Mitchell, J. T. (1972). Lung damage in mice following intraperitoneal injection of butylated hydroxytoluene. *Proc. Soc. exp. Biol. Med.* **140,** 122.

The oral toxicology of butylated hydroxytoluene (BHT) has been well documented in recent years (Gaunt *et al. Fd Cosmet. Toxicol.* 1965, **3,** 445; Botham *et al. ibid* 1970, **8,** 1; *Cited in F.C.T.* 1971, **9,** 583) and the effects of BHT on liver weight have been studied extensively. Recently it has been reported that BHT injected intraperitoneally (ip) into mice produced histologically identifiable damage in the lung.

BHT was administered ip in olive oil to two strains of mice using doses ranging from 0·004 to 2·5 g/kg. Swiss Ha/ICR non-inbred mice given a dose of 2·5 g BHT/kg showed signs of respiratory distress during the first day after treatment and died within 1 wk. Animals receiving a single dose of 0·83 g BHT/kg showed no signs of ill-health during the 5-day post-injection period, but histological damage in the lung, characterized by hyperplasia, hypertrophy and general disorganization of the cellular components, was evident at autopsy. In DBA/2 mice given doses of 0·004–0·4 g/kg, similar histological damage was seen after 3 and 5 days at the 0·4 g/kg level. Mice given 0·04 g/kg showed minimal changes which were reversible, in that the lungs returned to normal by day 7 after injection, while after a dose of 0·004 g/kg no histopathological effects were seen.

[No data on the blood levels attained after intraperitoneal injection are available, but it is unlikely that concentrations of the same order could occur in man following oral or skin exposure to BHT.]

2493. Antitumorigenic antioxidants

Wattenberg, L. W. (1972). Inhibition of carcinogenic and toxic effects of polycyclic hydrocarbons by phenolic antioxidants and ethoxyquin. *J. natn. Cancer Inst.* **48,** 1425.

Conflicting data exist on the effect of phenolic antioxidants and α-tocopherol on the carcinogenicity of polycyclic aromatic hydrocarbons. Jaffe (*Expl Med. Surg.* 1946, **4,** 278) claimed that adding wheat-germ oil, rich in tocopherols, to the diet of rats reduced the number of a mixed group of tumours resulting from intraperitoneal injection of 3-methylcholanthrene, while Haber & Wissler (*Proc. Soc. exp. Biol. Med.* 1962, **111,** 774) indicated that the addition of α-tocopherol to the diet of mice inhibited the induction of subcutaneous

(sc) sarcomas by 3-methylcholanthrene injection. Epstein *et al.* (*Life Sci.* 1967, **6,** 225) failed to confirm such inhibition by α-tocopherol and phenolic antioxidants in the case of tumour induction by sc injection of dibenzo[*a,l*]pyrene in mice. Other evidence of an inconclusive nature has been published by Shamberger (*J. natn. Cancer Inst.* 1970, **44,** 931) and by Harman (*Clin. Res.* 1969, **17,** 125).

The antitumorigenic and antitoxic effects of butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ethoxyquin and α-tocopherol have now been re-investigated in mice and rats. Addition of BHA or BHT to the diet at a level of 10,000 ppm reduced tumour induction in the forestomach of HA/ICR mice by benzo[*a*]pyrene (BP) given at a dietary level of 300 ppm, the levels of incidence being 68% without antioxidant treatment, 22% with BHA and 50% with BHT. At a dietary level of 50 ppm, 7,12-dimethylbenz[*a*]anthracene induced forestomach tumours in all treated mice, but addition of 10,000 or 2000 ppm BHA to the diet reduced this tumour incidence to 58 and 75%, respectively. Ethoxyquin at a dietary level of 10,000 ppm reduced the tumour incidence to 41%, but addition of α-tocopherol at 10,000 ppm caused no significant reduction. A reduction in the incidence of forestomach tumours induced by 1000 ppm BP in the diet was found in the A/HeJ mice when BHA or BHT was added to the diet at a level of 5000 ppm.

A similar inhibition was found in the induction of mammary gland tumours in Sprague–Dawley rats by DMBA administered by oral intubation in a single dose of 12 mg in 1 ml olive oil. Pretreatment with 200 mg BHA, BHT or ethoxyquin in olive oil reduced the tumour incidence after 18 wk from 80% in the unprotected rats to 39, 28 and 12%, respectively. α-Tocopherol reduced the incidence to 56%, but this reduction was not statistically significant.

In studies on the capacity of these antioxidants to counteract certain toxic effects, simultaneous addition of BHA to the diet at a level of 10,000 ppm reduced the death rate in mice given 200 ppm DMBA in the diet, mortality being reduced from 100 to 65% in a 35-day experiment and from 88 to 10% in a 28-day experiment. Adrenal necrosis was taken as a measure of toxicity in the rat and was produced within 72 hr of treatment in 29 of 30 female Sprague–Dawley rats treated with 30 mg DMBA by oral intubation. Pretreatment with BHA, BHT and ethoxyquin protected all rats similarly treated with DMBA against the development of adrenal necrosis. The antioxidants were given at a level of 200 mg, 1 hr before DMBA administration. BHA also protected three out of ten rats when given at a dose of 100 mg, while ethoxyquin afforded complete protection down to a dose level of 25 mg (equimolar to the dose of DMBA) and was partially effective at lower dose levels. α-Tocopherol in a dose of 200 mg failed to protect any of the rats against adrenal necrosis.

A single topical application of BHA (18 μg–1·8 mg) or α-tocopherol (43 μg) did not prevent the process of tumour initiation in mouse epidermis when DMBA (25 μg) was applied once only at the same site 5 min later.

The mechanism of action of the antioxidants in these experiments is not fully understood. Protection may result from the antioxidant properties of the compounds, but other mechanisms cannot be ruled out since these compounds enter into a number of other types of biochemical reaction.

PRESERVATIVES

2494. Ethylene chlorohydrin in the skin

Bruckner, J. V. & Guess, W. L. (1972). Morphological skin reactions to 2-chloroethanol. *Toxic. appl. Pharmac.* **22,** 29.

In the rabbit, ethylene chlorohydrin (EC) administered intraocularly is irritant at concentrations above 0·5% (*Cited in F.C.T.* 1972, **10,** 719), while topical application caused irritation with concentrations of 2% or more. Following subcutaneous or intramuscular injection or mucosal application, EC elicited reactions in concentrations above 1% (*ibid* 1971, **9,** 150). The present study was designed to assess more accurately the nature of the damage caused to dermal and epidermal structures by EC and by ethanol.

EC and ethanol at concentrations of 0·1–10 and 1–20%, respectively, were injected intracutaneously into the shaved skin of the rabbit back and the skin was examined by light and electron microscopy after 12 hr. An inflammatory response was provoked by all the concentrations administered and this response increased with increasing concentration, striking reactions being elicited by EC at 5% or more and by ethanol at 10% or more. At these higher levels a central zone of necrotic collagen containing inflammatory cells and degenerated fibroblasts was surrounded by a variety of polymorphonuclear leucocytes and oedematous tissue. Subcellular changes included the aggregation of ribosomes and tonofibrils (which together comprise a large proportion of the volume of both epidermal and hair-follicle cells), plasmalemmal retraction, swelling of the endoplasmic reticulum and mitochondrial degeneration. Intracellular inclusion bodies of multilamellar myelin and unusually shaped nuclei and nucleoli were also present. There was little qualitative difference between the effects of EC and ethanol, although EC was always the more potent. The changes seen were attributed to the lipophilicity of the alcohol molecule and its enhancement by halogenation, which thus increased the possibility of protein denaturation and lipid solvation.

MISCELLANEOUS DIRECT ADDITIVES

2495. Dihydroxyacetone not for lunar picnics

Shapira, J. (1972). Effect of large amounts of dihydroxyacetone in the diet of rats. *Proc. West. pharmac. Soc.* **15,** 65.

The possibility of using waste products for the regeneration of food materials has been enthusiastically explored in recent years. One such scheme involves the utilization of carbon dioxide and water as a carbohydrate source during space travel, a conversion that includes the condensation of formaldehyde to produce monosaccharides, which may contain a significant amount of dihydroxyacetone (DHA). With this in mind, the author cited above attempted to assess the potential of DHA as a large-scale nutrient. Earlier work had shown that DHA was absorbed rapidly in the rat and that about half of that absorbed was oxidized, while much of the rest was deposited as liver or body glycogen, generally without prior conversion to glucose.

Male rats were fed diets containing 40% glucose for 23 days, after which the glucose was replaced by DHA. Such replacement caused a reversal of the increase in weight-gain, and food and water consumption was reduced. After 21 days on the DHA diet, 75% of the animals had died. In a further experiment, in which the rats were given a diet containing 20% glucose and 20% DHA, growth was slower than with the 40% glucose diet, and a marked deterioration of the quality of the food was noticeable during the feeding period.

Thus, in the light of this work, ingestion of substantial quantities of DHA in the diet, either during prolonged space missions or in other circumstances, cannot be considered advisable.

2496. Bacterial irony

Nutrition Reviews (1972). Iron and zinc absorption and metabolism in germfree rats. *ibid* **30,** 148.

The idea that intestinal micro-organisms affect the absorption of iron, and possibly zinc, has derived some support from experiments with germ-free animals, in which biochemical signs of iron deficiency in vital organs have often been observed. The review cited above investigates such claims by considering certain balance studies on germ-free rats fed diets adequate in iron and zinc.

In a recent study (Reddy *et al. J. Nutr.* 1972, **102,** 101), the urine and faeces of adult conventional and germ-free rats were collected over a period of 10 days immediately preceding sacrifice. Absorption of iron was about 25% lower in germ-free than in conventional rats, and net retention of iron also decreased, although this was not reflected in any change in the level of urinary excretion. Growth was comparable in both types of rat and there was no significant difference in the degree of absorption or retention of zinc in the two groups. The activities of hepatic xanthine oxidase and renal catalase, both of which contain iron, were markedly lower in the germ-free group, but levels of zinc-containing alkaline phosphatase in the liver, kidney and serum and of catalase in the liver did not differ from those of controls. The zinc levels in the liver, kidneys and femur were the same in both groups, but when calculated per unit of body weight that of the femur was significantly higher in the germ-free group because the femur weight and ash-values were greater. This finding was consistent with the greater calcium and magnesium deposition in the bones of germ-free animals compared with conventional groups and probably reflected the necessary maintenance of a given ratio of mineral components in bone.

It may be that the iron requirement is lower in germ-free than in conventional animals and that this results in a corresponding decrease in absorption. In any event, these results serve to emphasize the important part played by the intestinal flora in normal animal nutrition.

AGRICULTURAL CHEMICALS

2497. Carbaryl toxicity testing criticized

Weil, C. S., Woodside, M. D., Carpenter, C. P. & Smyth, H. F., Jr. (1972). Current status of tests of carbaryl for reproductive and teratogenic effect. *Toxic. appl. Pharmac.* **21,** 390.

Carbaryl (1-naphthyl methylcarbamate) has been shown to reduce the female fertility index when fed at 10,000 ppm in the diet to rats and gerbils, and to have a dose-related adverse effect on litter size (*Cited in F.C.T.* 1972, **10,** 261). It has also been reported to have teratogenic effects in beagles (*ibid* 1969, **7,** 686) and guinea-pigs (*ibid* 1970, **8,** 102).

The authors of the paper cited above report some discrepancies between their own results and other published and unpublished reports on the effect of carbaryl on reproduction and foetal growth. A three-generation study in rats given feed additions calculated to provide a daily intake of up to 10 mg/kg body weight failed to demonstrate any significant dose-related effect of carbaryl on fertility, gestation, viability of pups or lactation. In a later study, pregnant rats were given carbaryl at dietary levels equivalent to doses of up to 500 mg/kg/day without any effect on fertility, gestation or incidence of teratogenic abnormalities. Weight gain was unaffected in rats given 100 mg/kg/day but was reduced in the 500 mg/kg group, in which many of the pups died before being weaned from the carbaryl-fed mothers. Neither teratogenic effects nor adverse effects on fertility or gestation were seen in another rat study in which animals were fed up to 200 mg carbaryl/kg/day, although 100 mg/kg/day given by intubation caused significant mortality, cholinesterase inhibition and a reduction in fertility. Growth was reduced to a comparable extent in both these groups.

These results from US tests are in marked contrast to findings reported from the USSR, where effective doses were found to be much lower and where daily doses were given in a single intubation rather than in the diet. Greater uniformity of results would be expected to result from the universal adoption of guidelines for test protocols. One such set of guidelines (Weil, *Toxic. appl. Pharmac.* 1970, **17** (2), i) involves using a species which metabolizes the test substance in a way similar to man, giving several dose levels (relying on high doses only to define the mechanism of action and on low doses to define the no-effect threshold), applying statistics only to randomly distributed units and administering the substance by a route relevant to the mode of human exposure.

Apart from the three studies reported in this paper, only one study out of the many discussed conformed to all these guidelines and the results showed that over three generations of rats a dietary level of 5000 ppm carbaryl (about 250 mg/kg/day) affected reproduction, whereas a level of 2000 ppm (100 mg/kg/day) did not. The much lower no-effect levels established in the Russian studies were attributed to the method of dosing, which placed the whole daily dose in the stomach at one time unaccompanied by any food and would thus be expected to result in much higher peak levels of carbaryl in the blood than would ever be attained with the more relevant dietary administration.

2498. Chlorodibenzodioxins in the liver

Cunningham, H. M. & Williams, D. T. (1972). Effect of tetrachlorodibenzo-*p*-dioxin on growth rate and the synthesis of lipids and proteins in rats. *Bull. env. contam. & Toxicol. (U.S.)* **7,** 45.

Williams, D. T., Cunningham, H. M. & Blanchfield, B. J. (1972). Distribution and excretion studies of octachlorodibenzo-p-dioxin in the rat. *Bull. env. contam. & Toxicol. (U.S.)* **7,** 57.

The chlorodibenzo-*p*-dioxins have been shown to be causative agents in the chick oedema syndrome (*Cited in F.C.T.* 1967, **5,** 584; *ibid* 1969, **7,** 394). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (I), which has been shown to be a teratogenic contaminant of the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (*ibid* 1970, **8,** 596; Emerson *et al. Fd Cosmet. Toxicol.*

1971, **9**, 395) is one of the most toxic of the group, inducing hydroperitoneum and hydropericardium in chicks and liver enlargement and fatty infiltration in mammals. It has also been implicated in neurological and psychopathological disorders affecting industrial workers handling 2,4,5-T or 2,4,5-trichlorophenol, in which I also occurs as a contaminant.

In order to determine the effect of I on lipid and protein synthesis, the authors of the first paper cited above administered 0–10 μg I/kg in corn oil by stomach tube to rats, each of which was afterwards (1–7 days later) given an intraperitoneal injection of 1 μc [1-^{14}C]-L-leucine and 10 μc sodium [^{3}H]acetate and killed exactly 1 hr after receiving the tracer dose. I significantly reduced body-weight gain and the incorporation of labelled acetate into free fatty acids and other lipid fractions of the liver. In contrast, the radioactivity of liver protein was increased by prior dioxin treatment, showing an increase in the incorporation of labelled leucine. The effects of I on growth and radioacetate incorporation into liver lipids were generally greater with a dose of 10 μg I/kg than with 1 μg/kg, but the differences were generally not large. Incorporation of leucine into the heart was unaffected by administration of I but that of acetate into epididymal adipose tissue was significantly reduced. Incorporation of [^{3}H]acetate into liver lipids was slightly reduced 2 days after the dose of I was given and significantly reduced 7 days after. Liver weight was increased with doses of 0·1 μg I/kg or more, an increase of 21 % being found at the 10 μg/kg level. Part of the apparent reduction in the incorporation of [^{3}H]acetate/g liver following I administration could have been due to this increase in the size of the liver. An increased synthesis of liver proteins, which was detected 3 days after treatment, may have been the result of liver-enzyme induction by I.

In the second paper cited above, octachlorodibenzo-*p*-dioxin (II) was selected for metabolic studies on account of its relatively low toxicity and the consequent fact that it could be fed to rats at higher levels than other related dioxins. A feed containing 0, 0·1 or 0·5 ppm II was fed for 2 wk to male rats and produced no alteration in feed consumption or weight gain. The only gross pathological change observed was liver congestion in rats given the higher dietary level of II. Faeces, liver and adipose tissue all contained II, but none was detected in the heart, kidney, spleen, lung, skeletal muscle, testes or urine.

Other rats with cannulated bile ducts were given a single intragastric dose of 58 μg II in corn oil, and their faeces, urine and bile were collected separately for analysis. Large quantities of II appeared in faeces but generally only traces in the bile, so it appeared that gastro-intestinal absorption was very limited. This was supported by the absence of any detectable II in the urine. Thus the small amount of II absorbed appeared to be located mainly in the liver, where it induced little toxic reaction. Further studies are being directed to the possible detection and identification of metabolites of II in the liver.

2499. Filling in the dieldrin picture

Uzoukwu, M. & Sleight, S. D. (1972). Dieldrin toxicosis: Fetotoxicosis, tissue concentrations, and microscopic and ultrastructural changes in guinea pigs. *Am. J. vet. Res.* **33**, 579.

Storage and excretion studies of dieldrin have been published in abundance (*Cited in F.C.T.* 1970, **8**, 566), and its effects on reproductive processes and its two-way transfer across the placenta have been described (*ibid* 1969, **7**, 536). A recent study, cited above, assesses the toxicity of dieldrin alone and in combination with sodium nitrite in guinea-pigs.

Guinea-pigs (males and pregnant and non-pregnant females) were given dieldrin in cottonseed oil orally every 4–5 days for 1–15 treatments, the initial dose being 15 or 30

mg/kg and the subsequent ones 15 mg/kg. After completion of the treatment, the animals were killed and concentrations of dieldrin were determined in body fat, liver, kidney, brain and foetuses. Other groups of pregnant and non-pregnant females were given a diet containing 100 ppm dieldrin with or without 0·5% sodium nitrite in their drinking-water, while a third group was given sodium nitrite in the drinking-water and a dieldrin-free diet.

Of the 42 animals given oral doses of dieldrin, 30 died after severe dyspnoea and convulsions. They showed variable degrees of fatty change in the liver, vacuolation of the renal tubular epithelium, and hyperaemia in the lungs, with emphysema in some areas. Pregnant females did not abort, and foetuses appeared grossly normal and showed no liver lesions microscopically. No lesions were observed in the brain tissue of the treated animals. However in male guinea-pigs aged 3 months given a single intraperitoneal injection of 75 mg dieldrin (about 140 mg/kg), electron microscopy showed swelling of the mitochondria of the cerebral cortex and cerebellum, with disintegration of the associated cristae.

Feeding of 100 ppm dieldrin alone killed two out of five females and induced abortion in two out of three, while nitrite given alone in the drinking-water induced abortion in all three pregnant females so treated. A combination of both factors killed three and induced abortion in nine of the 14 pregnant females, and killed three of the five non-pregnant ones. In the animals that aborted, the uterus showed focal epithelial vacuolation and necrosis.

The studies confirm that dieldrin is stored in body fat in proportion to its intake and that it accumulates in brain tissue, and add emphasis to previous findings that it passes both the placental and the blood-brain barriers. No evidence was obtained that nitrite potentiates the toxicity of dieldrin.

2500. No diphenylamine like old diphenylamine

Crocker, J. F. S., Brown, D. M., Borch, R. F. & Vernier, R. L. (1972). Renal cystic disease induced in newborn rats by diphenylamine derivatives. *Am. J. Path.* **66,** 343.

We have previously reported 2-yr feeding studies in beagles in which 0·1% diphenylamine caused growth retardation and a mild anaemia, which became marked at the 1% dietary level and was accompanied by fatty changes in the liver, haemosiderosis of spleen, kidney and bone marrow and a slight increase in kidney weight (*Cited in F.C.T.* 1968, **6,** 412). The study cited above indicates that earlier reports may have oversimplified the possible toxic effects of diphenylamine, and that ageing of individual samples may affect their toxicity.

Pregnant rats were fed 1·5 or 2·5% diphenylamine in the diet for the last 7 days of gestation, and the kidneys of their viable newborn were examined. A crude sample of diphenylamine which had been kept for some 2 yr produced a high incidence of cystic tubular dilatation in the kidneys of offspring, whereas a freshly manufactured sample of the compound had a much less severe effect. The histological changes observed were similar to those observed in certain forms of cystic kidney disease in man.

Thin-layer chromatographic examination of several samples of diphenylamine demonstrated three unidentified contaminants of aged samples. One of these contaminants when given to pregnant rats by intubation in daily doses of 50 μg induced moderate cystic changes in the kidney tubules of the offspring similar to those seen after daily administration of 20 mg of the aged diphenylamine. These lesions did not appear when the chromatographically pure compound was given.

2501. Microsomal enzyme induction by piperonyl butoxide

Wagstaff, D. J. & Short, C. R. (1971). Induction of hepatic microsomal hydroxylating enzymes by technical piperonyl butoxide and some of its analogs. *Toxic. appl. Pharmac.* **19,** 54.

The insecticide synergist piperonyl butoxide (PB) has been thought to act by inhibiting the hepatic microsomal enzymes responsible for detoxication of foreign metabolites (*Cited in F.C.T.* 1970, **8,** 122). However, reports which appear to contradict this view have been published (Falk & Kotin, *Ann. N.Y. Acad. Sci.* 1969, **160,** 299), stating that under certain conditions PB may in fact stimulate these enzyme activities. The present report assesses the validity and general applicability of this inductive effect to PB and its analogues.

Female rats were administered technical-grade PB at various dietary levels for 15 days. Induction was considerable with 10,000 ppm PB, as monitored by cytochrome P_{450} levels, *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothioate (EPN) detoxication, *O*-demethylase activity and hexobarbitone sleeping time, but the increases in activity were marginal with a diet containing 100 ppm. Using the same parameters, greater inductive activity was shown to be associated with the methylenedioxyphenyl group in PB analogues than with the dimethoxyphenyl group. The PB analogues safrole, isosafrole, eugenol methyl ether and isoeugenol methyl ether all caused a significant induction at 10,000 ppm in the diet. PB together with safrole or phenobarbitone had an additive effect on induction in most of the parameters measured, with the notable exception of cytochrome P_{450}. The fact that the effects of safrole and phenobarbitone given together were not additive suggested that the two compounds probably acted through two different mechanisms.

It was concluded that these so-called inhibitors may in fact act as alternative substrates for microsomal enzymes, though the kinetics of the reaction may vary widely with the specific inhibitor used. The inductive effect resulting from chronic exposure of an animal to these compounds may be directly proportional to the inhibitory effect resulting from acute exposure.

[The validity of this work must remain doubtful, as the piperonyl butoxide used was of technical grade and of only 80% purity. Thus, as the authors point out, contaminants could conceivably have been involved in the inductive effect.]

2502. Detoxication in the rat

Mehendale, H. M. & Dorough, H. W. (1971). Glucuronidation mechanisms in the rat and their significance in the metabolism of insecticides. *Pestic. Biochem. Physiol.* **1,** 307.

Conjugation is a major metabolic process for the elimination of potentially toxic compounds in animals. Insecticides, particularly those with a carbamate structure, are notable examples of substances that are detoxified by this type of reaction. In order to define more clearly the conditions under which carbamate conjugation occurs in animals, an *in vitro* system was set up using 1-naphthol, which is produced by hydrolysis of carbaryl, as the substrate for the glucuronidation step.

Enzyme preparations from homogenates of rat intestine were used to study the glucuronidation of labelled 1-naphthol, while a liver-microsomal system was used to study both conjugation and its relation to oxidative metabolism. In both systems, glucuronidation required the presence of the cofactor, uridine-5-diphosphoglucuronic acid (UDPGA), and

magnesium was found to be the only metal to enhance this step. A number of insecticide synergists, including piperonyl butoxide and safrole, were shown to inhibit the conjugation of 1-naphthol, but a series of insecticides (including DDT and parathion) did not cause inhibition when used at 10^{-3} M concentration.

Conjugation of carbaryl and of banol, another *N*-methylcarbamate, depended on the presence of $NADPH_2$, and hence an oxidative step was clearly an essential preliminary to conjugation. Restriction of conjugative metabolism by a decrease in the concentration of available UDPGA was found to produce an overall slowing-down of carbamate degradation; no accumulation of oxidative metabolites occurred, although $NADPH_2$ was present in excess and other conditions were appropriate for this reaction. It appears therefore that oxidative and hydrolytic metabolism of carbamates only continues when the intermediate metabolites are conjugated. Examination of the substrate specificity of the microsomal system showed that, although a number of hydroxylated carbaryl metabolites could act as substrates, the 5,6-dihydro-5,6-dihydroxy compound did not form a glucuronide *in vitro*.

FEED ADDITIVES

2503. Assessing feed-additive hazards to man

Ferrando, R. et Truhaut, R. (1972). La toxicité de relais. Nouvelle approche pour la méthodologie d'évaluation toxicologique des additifs aux aliments des animaux d'élevage. *Cr. hebd. Séanc. Acad. Sci., Paris* **275,** 279.

This paper is concerned with the assessment of the safety-in-use of compounds that are not deliberately added to food but could find their way into the diet indirectly, from their use, for example, as growth-promoting agents in the feed of animals intended for human consumption. Such compounds are of course dealt with in various ways by the animal system before the animal ever becomes food for man. This makes toxicological evaluation more complex than in the case of a direct food additive, since in the latter case the initial concentration in the food and the identity of the compound are known. It is possible that man could ingest potentially toxic substances over a large part of his lifespan as a result of the use of feed additives, and the authors of this paper feel that the present method of testing for the safety of such compounds by routine oral toxicity tests on the feed additive and its principal metabolites is not only a long and complicated procedure but is also far from satisfactory.

They suggest that, instead, the food suspected of containing feed-additive residues should itself be fed to laboratory animals. Provided only foods that constitute a relatively limited percentage of the average diet for man are involved, it should be possible to introduce a safety factor of the same order as the 100-fold factor widely accepted for extrapolating data from animals to man. For example, instead of feeding the laboratory animals on diets containing tissues of the treated animals at levels comparable to the probable proportion of this particular type of food in the human diet, a higher proportion could generally be incorporated in the toxicity-test diets without upsetting the nutritional pattern, and thus a safety factor would be incorporated. Controls would have to be fed the same higher level of the specific food in their diet but in their case it would come from animals fed diet free of the

feed additive in question. The safety factor could also be increased by obtaining test tissues from animals fed higher levels of feed additives than are required in practice, and by continuing to administer the feed additive right up to the time the animals are killed for testing, instead of returning the animals to an unsupplemented feed before slaughter as is often the case under normal conditions of animal husbandry.

It is suggested that this method would offer a more realistic means of assessing the safety-in-use of these indirect food additives than the procedures currently used.

THE CHEMICAL ENVIRONMENT

2504. Pre-war nickel pollution

Ashton, W. M. (1972). Nickel pollution. *Nature, Lond.* **237,** 46.

This short communication indicates that at least some progress has been made in the protection of the environment from the airborne effluent from nickel (Ni) refineries in the past 30 yr. The author compares Ni levels found in soil and herbage collected in 1971 from a site 1 mile from a large Ni works with those that he found in 1934. The 1934 levels (1700–3200 ppm in grass and 500–600 ppm in soil) were high enough to permit simple gravimetric estimation by the familiar dimethylglyoxime method. In 1940, a cleaner Ni-extraction process was put into operation at the plant and, by 1971, levels in the vegetation had fallen to 1·0–4·5 ppm, requiring the use of the sophisticated and sensitive technique of atomic absorption spectrophotometry for their determination. The pre-war study demonstrated that the Ni content of wheat and oats occurred mainly in the leaves and was largely superficial, being considerably reduced by washing.

The Ni concentrations found in soil samples in 1934 and 1971 are not strictly comparable, since in 1971 care was taken to remove only the top 2 in., whereas in 1934 no attempt was made to select surface soil. The author suggests that further attention should be given to the high levels still found in the topsoil, the figures for 1971 being 350–1150 ppm in the first inch and 200–350 ppm in the second.

2505. Tellurium on the brain

Duckett, S. (1972). Teratogenesis caused by tellurium. *Ann. N.Y. Acad. Sci.* **192,** 220.

We have recently discussed the long-term effects of tellurium (Te) included at a level of 2 ppm in the drinking-water of the mouse (*Cited in F.C.T.* 1972, **10,** 870) and the neurotoxicity of high dose levels of Te in weanling rats (*ibid* 1971, **9,** 596). The present paper reviews work showing that Te can induce hydrocephalus in the offspring and even in the foetus when fed to pregnant rats over days 10–15 of gestation at dietary levels of 500–3300 ppm.

It was at first thought that Te did not cross the placental barrier, but radioactive Te injected into the tails of pregnant rats was subsequently found to reach all embryonic or

foetal organs within 1–2 min. Te can also cross the blood-brain and cerebrospinal fluid (CSF)-ependymal barriers, the latter route enabling it to reach the parenchyma of the telencephalon (forerunner of the cerebrum) before blood vessels appear in the telencephalic wall. The hydrocephalus is non-obstructive for the first few days after birth but then stenosis of the aqueduct, usually associated with closure of the subarachnoid space due to the increasing volume of ventricular CSF, leads to death within 2 wk unless a spontaneous ventriculostomy appears. In the newborn hydrocephalic rat, lesions can be detected histologically only in the ependyma. The severity of these lesions increases until no ependyma remains. The pathological mechanisms that result in the hydrocephalus have still to be investigated.

2506. A breath of dust

Davis, J. M. G. (1972). The fibrogenic effects of mineral dusts injected into the pleural cavity of mice. *Br. J. exp. Path.* **53,** 190.

The way in which mineral dusts irritate lung tissue has been the subject of a number of reports (*Cited in F.C.T.* 1971, **9,** 594). In a recent study, various mineral dusts were tested by the injection of aqueous suspensions into the pleural cavity of mice. The materials included chlorite, chromite, magnetite, olivine, pyroxene, serpentine, talc, short-fibre chrysotile and synthetic chrysotile (all 'non-fibrous' samples), and brucite, forsterite, glass fibre, silica fibre, man-made insulation fibres and normal chrysotile asbestos ('fibrous' minerals). The effect of the physical size and shape of the fibrous dusts was also investigated by grinding and sieving them so that no long fibres remained. Injection into the pleural space has the advantage that a large population of macrophages is available to react to the irritant, leading to granuloma formation with or without adhesions.

The smallest response was elicited by magnetite and chromite. Since the dose was calculated by weight and these were the two most dense minerals studied, only a small volume of dust was administered. This aggregated into large clumps, which were poorly penetrated by the macrophages, and the resulting granulomas were small and loosely anchored in the chest cavity. Some non-fibrous dusts (talc, serpentine, pyroxene, olivine and finely-ground brucite), which were less closely packed, elicited a marked cellular response, producing large granulomas generally loosely fixed in the chest cavity, although with talc and sometimes with pyroxene they tended to be more firmly attached. The long-fibre dusts (glass, silica, long brucite and the insulation fibres) caused large, highly cellular granulomas with firm adhesions between diaphragm, lungs and chest wall. When finely ground to non-fibrous powder, however, the same dusts generally produced small granulomas and few adhesions.

Clearly the physical shape and size, and to a lesser extent the chemistry, of mineral dusts are crucial factors in determining the severity of the tissue response. It is a pity that the author does not comment on the work of Gross *et al.* reviewed earlier (*ibid* 1971, **9,** 594), since these workers instilled almost the same range of mineral dusts in suspension via the trachea of rats. The resulting lesions showed some features which the authors attributed to the method of administration, since they were not found in animals inhaling high concentrations of the same dusts. Airborne fibrous particles larger than 3 μm in diameter rarely reach the lung alveoli and hence pose little hazard, but as the author of the present paper warns, it is as well to remember that if industrial use is found for any fibrous substances much less than 3 μm in diameter, such substances will need very careful handling.

2507. Carbon disulphide and ANIT

Gibson, J. D. & Roberts, R. J. (1972). Effect of carbon disulfide on liver function *in vivo* and in the isolated perfused liver. *J. Pharmac. exp. Ther.* **181,** 176.

We have previously discussed the hepatotoxic effects of carbon disulphide (CS_2) in terms of a marked depression of microsomal and related enzyme activities (*Cited in F.C.T.* 1970, **8,** 231). There are, of course, numerous other manifestations of CS_2 toxicity at biochemical and morphological levels, and some of these effects mirror those produced by the insecticide, α-naphthylisothiocyanate (ANIT). The present work was designed to investigate these similarities, and hence the underlying mechanism of CS_2 toxicity.

Following administration of CS_2 to mice and rats, either intraperitoneally, orally or by inhalation of the vapour in air, loss of intravenously administered bromsulphthalein (BSP) from blood plasma was reduced considerably over a period of 4 hr. Subsequently, BSP retention decreased, returning to control levels some 12 hr after CS_2 treatment. The effect of CS_2 on BSP retention was markedly decreased by pretreatment with phenobarbitone. A similar impairment of liver function was noted in respect of the clearance of exogenous bilirubin from the plasma. Hepatic cell damage was monitored by following the activities of serum glutamic–pyruvic transaminase (SGPT) and alkaline phosphatase. The activities of these enzymes were not affected in mice following 60-min exposures to 110 ppm CS_2 over 5 days. *In vitro*, a notable reduction in BSP loss from the perfusing fluid and impairment of blood and bile flow were observed during the perfusion of livers isolated from CS_2-treated animals.

Comparison of these effects with those induced by ANIT revealed similar responses in respect of BSP and bilirubin retention and impairment of bile flow. On the other hand, ANIT administration has been shown to increase SGPT activity and its effect on BSP retention has been reported to be potentiated by phenobarbitone, so that it was not possible on the basis of this study to postulate any parallel mechanism for the liver toxicity of these two compounds.

2508. Handling dimethylamides

Tanaka, K.-I. (1971). Toxicity of dimethylformamide (DMF) to the young female rat. *Int. Arch. Arbeitsmed.* **28,** 95.

Wiles, J. S. & Narcisse, J. K., Jr. (1971). The acute toxicity of dimethylamides in several animal species. *Am. ind. Hyg. Ass. J.* **32,** 539.

The toxicity of dimethylformamide (DMF) and other mono- and dialkyl formamides following single and repeated administration by intraperitoneal (ip) injection to rats was reported recently (*Cited in F.C.T.* 1972, **10,** 599). The first paper cited above describes the short-term inhalation toxicity of DMF in young female rats.

Five experimental groups were used each containing ten rats aged 3, 4, 5, 8 or 12 wk. Five rats in each group were exposed to concentrations of 200 ppm DMF for a period of 8 hr/day for 4 wk, while the remaining rats were used as controls. In all rats exposed to DMF, histopathological changes were observed in the liver, but not in any of the other major organs examined. Significant increases in serum glutamic–oxalacetic and glutamic–pyruvic transaminases were found only in the group of rats first exposed to DMF at the age

of 3 wk. No significant changes were detected in serum alkaline phosphatase (AP) and lactic dehydrogenase (LDH) activities. The principal histological change in the liver was cloudy swelling, together with some fatty degeneration, around the central vein of the liver lobules. The younger the rats, the more severe were the liver changes, which appeared to reach a peak after wk 1 of exposure, with some regeneration occurring after this time. Increases in serum enzymes were also seen after 1 wk in rats exposed to 200 ppm DMF for 1 hr/day. Rats similarly exposed to concentrations of 200 ppm carbon tetrachloride showed peripheral fatty change in the liver lobules within 1 wk and greater increases in serum enzymes, including AP and LDH. The author concludes that DMF is not only less toxic than carbon tetrachloride but causes a different type of liver injury. Moreover he suggests that changes in serum enzymes may be useful in detecting DMF poisoning in man.

The second paper cited above reports toxicity studies on several *N*,*N*-dimethylamides (Hallcomids). LD_{50} values for 12 Hallcomids were determined by the intravenous (iv), ip, oral and percutaneous routes in mice and rabbits. The compounds tested were a homologous series ranging from dimethylpropionamide to dimethyloleamide. Some additional dermal studies were carried out in mice to evaluate the potential of these materials for enhancing the skin penetration of an organophosphorus anticholinesterase. The acute iv LD_{50}s in both mice and rabbits showed a biphasic response related to molecular weight. The compounds in the dimethylcaproamide to dimethyllauramide range were the most toxic (with iv LD_{50}s between 30 and 90 mg/kg in both species compared with 820 and > 1000 mg/kg for dimethylpropionamide, 300 and 70 mg/kg for dimethyloleamide and 2800 and 1000 mg/kg for DMF in mice and rabbits, respectively). Toxic signs observed included weakness, anaesthesia, dyspnoea, collapse and convulsions. A similar response was seen in mice given ip injections. In rabbits given Hallcomids orally, the compounds from dimethyllauramide upwards were similar in toxicity to DMF, while lower members of the group were more toxic. The percutaneous absorption studies showed that the caproamide, caprylamide and capramide compounds were absorbed in lethal concentrations when applied to mice in a dose of 5000 mg/kg and also enhanced the skin penetration of an anticholinesterase. In rabbits, only the higher molecular-weight materials (dimethylpalmitamide and oleamide) significantly enhanced the penetration of the anticholinesterase. Some reddening and irritation of the skin occurred with all the compounds in both species.

The authors also refer to eye and dermal irritation studies carried out under contract and conclude that the compounds of medium molecular weight, particularly those with carbon chain lengths of 6–12 are the most toxic and are somewhat more toxic than DMF. They stress that for the safe use of these compounds, precautions should be taken to prevent skin contact.

2509. Tube transport disrupted by maleic acid

Schärer, K., Yoshida, T., Voyer, L., Berlow, S., Pietra, G. & Metcoff, J. (1972). Impaired renal gluconeogenesis and energy metabolism in maleic acid-induced nephropathy in rats. *Res. exp. Med.* **157,** 136.

We have previously considered maleic acid as a cause of defective reabsorption in the renal tubules of the rat, a condition that simulates the human Fanconi syndrome (*Cited in F.C.T.* 1964, **2,** 436). A direct physiological effect on absorption was considered unlikely, however, since succinic dehydrogenase activity was markedly depressed, suggesting a

possible inhibition of glucose production by way of the Krebs cycle. In the work cited above, subcutaneous injection of 100–400 mg maleic acid/kg body weight into rats was followed by a dose-dependent inhibition of gluconeogenic capacity in the renal cortex, and the cortical levels of adenine nucleotides and the activity of adenylate kinase were also markedly reduced. *In vitro* incubation of slices of renal cortex with 0·25–16·0 μmoles maleic acid/ml also inhibited glucose production from a variety of substrates. These effects were associated with an increase in urinary volume, and disturbances in the renal transport of certain amino acids. The findings were indicative of an impairment of energy generation, as were the dose-related ultrastructural changes, notably vesiculation of the mitochondrial cristae found in both proximal and distal tubules.

It was concluded that the inhibition of renal gluconeogenesis was the cause of the disruption of tubular transport. Two possible mechanisms of inhibition were suggested, an overall depression of energy production and a more specific binding of maleic acid to coenzyme sulphydryl groups. Support for the latter was provided by the observation of some decrease in the acetyl coenzyme A content of the kidney cortex following maleic acid injection, and by the differing degrees of inhibition of gluconeogenesis associated with the use of different substrates.

[This paper illustrates the type of approach required in modern toxicology—a co-ordinated interpretation of the changes produced by a toxic substance at the metabolic, physiological and ultrastructural levels.]

2510. Encouragement for hexachlorophene from down under

Plueckhahn, V. D. & Banks, Joan (1972). Hexachlorophene toxicity, the new-born child and the staphylococcus. *Med. J. Aust.* **1,** 897.

Hexachlorophene (HC) is readily absorbed percutaneously (*Cited in F.C.T.* 1972, **10,** 114) and has produced neurotoxic effects in animals after dermal and oral application (*ibid* 1972, **10,** 275; FDA Notice to Physicians, 6 December 1971). These findings have led to restrictions on its use in Britain and the United States, and in the latter country use of 3% HC solutions for total-body bathing of infants is no longer recommended.

The paper cited above reports on the use of HC on all babies born at Geelong Hospital between 1960 and 1971. As a routine measure during this period, 2 ml of a 3% solution of HC was applied to the entire body surface, lathered with water and wiped off with dry cotton wool; a further 2 ml was then applied and allowed to dry on the skin. This procedure was carried out within 2 hr of birth and every second day thereafter for the duration of the baby's stay in hospital. In none of the 24,322 babies so treated were any adverse reactions observed, nor was there any gross or microscopic evidence of cystic vesiculation of the myelin in 109 samples of cerebrum, 28 samples of cerebellum and three samples of spinal cord taken from the 226 babies who died within a year of birth. Of 2400 infants born after April 1970, 56 suffered convulsive seizures, but a clinical reason for this was found in 52 of these cases and the remaining four were not thought to be related to HC exposure. Furthermore, although a circular from the Australian Drug Evaluation Committee in July 1971 asked the major obstetric and paediatric hospitals in the country for reports of suspected HC toxicity, no such reports were received in the following 6 months.

The paper also presents data in support of the recently questioned value of HC in reducing staphylococcal skin disease, and reviews existing literature on HC toxicity and efficacy. In

the light of their reported findings, the authors of this paper recommend that the neonatal use of HC in maternity hospitals should continue, the skin being subsequently rinsed with water as a precautionary measure, but they consider that use of HC in the home and extended use in hospital should be avoided. There is considered to be little scientific basis for the use of HC in cosmetics, toothpastes, mouthwashes or vaginal deodorants (see following abstract) since *Staphylococcus aureus* is rarely found in either mouth or vagina.

2511. But no support for vaginal deodorants

Gowdy, J. M. (1972). Feminine deodorant sprays. *New Engl. J. Med.* **287,** 203.

Adverse skin reactions may occasionally be caused by a variety of commonly-used ingredients of cosmetics and toiletries, including ethanol (*Cited in F.C.T.* 1970, **8,** 438), isopropyl myristate (*ibid* 1970, **8,** 239), fluorocarbons such as dichlorodifluoromethane (*ibid* 1966, **4,** 467), perfumes (*ibid* 1970, **8,** 240), talc (*ibid* 1967, **5,** 590) and hexachlorophene (HC) (Gump, *J. Soc. cosmet. Chem.* 1969, **20,** 173). All these ingredients are frequently used in vaginal deodorants, which up to May 1972 had been the subject of 28 reports of injury received by the FDA. Reactions included burning, rash (ranging from simple itching to a herpetiform reaction), vaginal discharge, urinary infection, abdominal cramping (in one case with threatened abortion), recurrent rash in the absence of further contact with the product, and balanitis in the male partner. Some patients recovered after ceasing to use the sprays, but in most cases therapy with systemic steroids was required; the average recovery time was 30 days but in nine cases it was greater than this.

The reactions could not be attributed to any single cause. HC, typically used at 0·1% in such formulations, was the principal suspect, since evaporation of the propellant can raise its concentration on the skin to as high as 95% and it is known to be irritant above 5% (Gump, *loc. cit.*). However, some reactions occurred with HC-free products, and there was no correlation between the HC concentration and the incidence of reactions. Nor was there a correlation with the concentration of alcohol, which can also irritate mucous membranes, although there was some indication of a connexion between a high perfume content and the number of reactions. Pressure from propellants was thought to be an important factor in urethral reactions, and their cooling or even freezing effect on the skin may have caused local damage. A similar report of adverse reactions to vaginal deodorants was published earlier (Kaye, *J. Am. med. Ass.* 1970, **212,** 2121), and there have been other reports in the lay press.

[In view of the possibility of adverse effects, the need for such products must be seriously questioned, particularly in view of the ready availability of soap and water.]

NATURAL PRODUCTS

2512. Galactose and the blind eye

Keiding, S. & Mellemgaard, L. (1972). Dose dependence of galactose cataract in the rat. *Acta ophthal.* **50,** 174.

Galactose-induced cataract formation appears to be linked to an accumulation of this sugar, or its 1-phosphate derivative, in the lens of the eye (*Cited in F.C.T.* 1964, **2,** 76). The current work was undertaken to establish whether there was a quantitative relation between galactose intake and the extent of cataract formation.

Female rats were fed varying amounts of galactose in the diet, and the rate and extent of subcortical, and later nuclear, cataract formation were found to be directly proportional to the galactose concentration in the diet and also to the blood levels of the sugar. Thus, when galactose made up 15% of the dietary calories, only cortical cataracts developed. Nuclear cataract was associated with diets in which galactose accounted for 20% or more of the calories and took at least 45 days to develop, compared with the minimum of 4 days required for the development of cortical cataract. After the test diets had been fed for about 1 wk, there was a marked fall in the ATP concentration in the lens. Levels of ADP and AMP remained constant, so it was clear that the ATP change was not merely a relative decrease due to water accumulation.

These results are compatible with the view that cataract formation can result from the overloading of the pathway for galactose metabolism (*ibid* 1971, **9,** 602) and the consequent accumulation of the sugar in the plasma.

2513. The heady effects of red wine

Trethewie, E. R. & Khaled, Laila (1972). Wine and migrainous neuralgia. *Br. med. J.* **3,** 290.

High levels of tyramine and histamine often occur in foodstuffs such as wine, beer and cheese (*Cited in F.C.T.* 1964, **2,** 761; *ibid* 1965, **3,** 508) and may lead to severe reactions in patients also receiving monoamine oxidase inhibitors. Even in the absence of such drugs, it has long been recognized that these foods may provoke migraine attacks in susceptible individuals, and in one case, ripe cheese containing 850 ppm histamine was held responsible for flushing, headache, palpitations and hypotension (*ibid* 1968, **6,** 812).

The authors cited above described an Australian who had suffered severe attacks of migrainous neuralgia at least once a month for many years, principally in response to strong beer and red wine, but who was symptom-free for 18 months after avoiding these beverages and also cheese, chocolate and oranges. Fortunately he was able to drink white wine with impunity. Analysis of the red Australian wine that provoked such attacks showed it to contain no detectable tyramine and only 2–3 ppm histamine, but many other unidentified amines were present. The white wine, on the other hand, contained no histamine but large quantities of bradykinin and the presumed kinin SRS. In some migraine sufferers the mechanism of tyramine conjugation has been found to be defective (Youdim *et al. Nature, Lond.* 1971, **230,** 127) and in the present case it would appear that deficient conjugation of amines other than tyramine or histamine may have been responsible for the attacks.

2514. There's poison in them there herbs

Schoental, R. (1972). Herbal medicines to avoid. *Nature, Lond.* **238,** 106.

In spite of the undoubted hazard of some modern synthetic drugs, that associated with traditional herbal remedies may be even greater. Nitrosamines present in self-administered

medicines and beverages have been suggested as a possible explanation for the high incidence of certain cancers common in East Africa (*Cited in F.C.T.* 1971, **9,** 897), and pyrrolizidine alkaloids, known to cause centrilobular necrosis and cirrhosis and suspected of involvement in some liver tumours in man, have also come under close scrutiny (*ibid* 1971, **9,** 893).

Poisonous plants are usually recognized as such by local people provided cause and effect are clearly related. Those that cause insidious disease and delayed morbidity, however, go undetected in the absence of sophisticated surveys. The author of this short contribution has made a detailed on-the-spot study of this problem. She points out that there are more than 200 species of Crotalaria and many species of Senecio, Heliotropium, Cynoglossum and Trichodesma in East Africa and that, between them, these plants contain more than 100 pyrrolizidine alkaloids. Apart from their known toxic effects on the liver, these alkaloids have been shown to damage the lungs, heart, vascular system, pancreas, kidneys and brain in experimental animals. It is surely significant that in countries that are poorly developed industrially and rely on natural products for their traditional medicines, liver disease and primary liver tumours are common. Documentation of the use of herbal remedies by mothers during pregnancy and lactation, or for the treatment of childhood ailments, would pinpoint the particularly dangerous suspects, but records are not readily collected and may prove unreliable when they are. The pharmacological and toxicological properties of traditional herbal remedies are being investigated on an increasing scale in several East African countries and such studies seem to offer the best hope of controlling dangerous local traditions in medication.

2515. Sheep unsafely graze on locoweed

James, Lynn F. (1972). Effect of locoweed on fetal development: Preliminary study in sheep. *Am. J. vet. Res.* **33,** 835.

Congenital malformations and abortions have followed the consumption of locoweeds (species of Astragalus and Oxytropis) by livestock in the USA. In the investigation cited here, pregnant ewes fed 400 g dried and pelleted *Astragalus lentiginosus* daily for periods of 30 days at different stages of gestation were examined at the end of their feeding period.

Ewes fed the test material from 0 to 30 days of gestation showed inhibition of placentation, a reduction in the vascular development of the uterine walls and some anaemia of the uterus. The foetuses from animals treated on days 30–60 were oedematous and haemorrhagic. Those from ewes treated on days 60–90 showed enlarged and pale thyroids and hearts and enlarged spleens, together with marked ascites and haemorrhage. The foetuses appeared to be most susceptible to the locoweed treatment at this stage. Two of three foetuses from ewes fed locoweed from 90–120 days had ascites, in one case with omental oedema, while the third was dead and oedematous. Foetuses from locoweed-fed dams showed increased serum-magnesium and alkaline-phosphatase levels, and some bone fragility compared with controls.

The overall findings indicate that the so far unidentified toxic constituents of locoweed, or the toxic metabolites of locoweed components, are able to cross the placental barrier and exert marked toxic effects on the foetus and foetal membranes of the sheep.

2516. Trichotoxin among the corn

Hou, C. T., Ciegler, A. & Hesseltine, C. W. (1972). New mycotoxin, trichotoxin A, from

Trichoderma viride isolated from southern leaf blight-infected corn. *Appl. Microbiol.* **23,** 183.

Corn infections, such as that which recently plagued areas of the USA, owe their effects to toxic compounds present in the mycelia of parasitic fungi. To add to the list of such compounds that have been identified is the mycotoxin, trichotoxin A, isolated by solvent extraction from the mycelium of *Trichoderma viride*. Its physicochemical properties were found to be in keeping with those of a cyclic peptide with no carbohydrate moiety. The amino-acid composition of the peptide was: $(glu)_3$, $(pro)_2$, $(gly)_1$, $(ala)_3$, $(leu)_3$ and a less common amino acid deduced by chromatographic techniques to be 2-methylalanine. The compound proved to be far less toxic when administered orally than by the intraperitoneal (ip) route, the acute ip LD_{50} in the mouse being 4·4 mg/kg, while a single dose of 600 mg/kg given orally in a commercial mouse ration had no detectable effect over an observation period of 4 months. This marked lack of oral toxicity suggests either that the compound is detoxified in the gut or that it is not absorbed through the intestinal wall.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

2517. Living in a cloud—of talc!

Nam, K. & Gracey, D. R. (1972). Pulmonary talcosis from cosmetic talcum powder. *J. Am. med. Ass. J.* **221,** 492.

Talc pneumoconiosis has been reported occasionally in workers employed in talc mills and mines and in those using talc in production processes, such as are encountered in the rubber industry. Despite the extensive use of talc as a dusting powder, however, there seem to be no reports of talcosis resulting from the cosmetic use of talcum powder. The case of the newspaper truck driver who died at the age of 39 from diffuse peritonitis is therefore of some interest.

The cause of his peritonitis was not identified, but during the preceding 6 yr, he had had multiple abdominal operations for peptic ulcer disease and small-bowel obstruction from adhesions. At autopsy, the peritonitis was confirmed. The lungs were heavy and stiff and contained multiple firm yellow nodules, a finding in line with the diffuse nodular opacities observed radiologically before death. Histologically the nodules consisted of granulomas with numerous needle-like birefringent crystals. Similar crystals were observed within alveolar macrophages. X-ray diffraction of the crystals obtained after ashing pieces of lung identified them as talc. The occupation of this patient could not account for any exposure to talc severe enough to produce this type of effect, but a study of his history revealed an extraordinary use of cosmetic talcum powder. He had dusted himself liberally at least three times a day following frequent baths, which were taken to relieve his recurrent abdominal pain, and he had also dusted his bed sheets nightly with large quantities of talcum powder.

[Despite its clinico-pathological interest this case is so extreme that it is difficult to appraise its relevance to any question of hazard from the inhalation of talc used for normal cosmetic or toiletry purposes. In view of the extensive use of talc for these purposes, the

most important point in this report is perhaps the statement that the authors have been "unable to find a case of pulmonary talcosis associated with the use of cosmetic dusting powder".]

2518. Metabolism of alkyl sulphates

Burke, Barbara, Olavesen, A. H., Curtis, C. G. & Powell, Gillian M. (1972). The metabolism of some anionic surface-active compounds in the rat. *Biochem. J.* **128,** 139P.

Previous studies have shown that dodecyl [^{35}S]sulphate given orally or intraperitoneally (ip) to rats is metabolized in the liver to butyric acid 4-[^{35}S]sulphate, which may then be desulphated to γ-butyrolactone (*Cited in F.C.T.* 1971, **9,** 760). Similar studies have now been extended to include the potassium salts of decyl sulphate (DS), octadecyl sulphate (ODS) and undecyl sulphate (UDS).

Rats were given these three compounds labelled with sulphur-35 in doses of 5 mg/kg either ip, intravenously (iv) or orally, and urine and faeces were collected over a period of 48 hr. In each case, over 60% of the radioactivity was recovered in the urine as the ester sulphate. After iv administration of DS or ODS to anaesthetized animals with a cannulated bile duct and ureter, less than 2% of the injected radioactivity was found in the bile. The dosage of ODS administered iv to the latter animals was 0·1 mg/rat. Chromatography and electrophoresis were carried out on the urine samples obtained from animals receiving DS or ODS, and butyric acid 4-[^{35}S]sulphate was identified as the major radioactive component in these samples. Further studies, in which UDS was administered by injection in a dose of 5 or 2·5 mg/kg to rats cannulated for urine collection, showed that 30 and 48% of the administered radioactivity was excreted in the urine from doses of 5 and 2·5 mg/kg respectively. At both dose levels, less than 2% of the radioactivity was found in the bile in male rats, whereas in female rats 6–14% of the ^{35}S-label was recovered from the bile. The chromatographic and electrophoretic properties of three metabolites associated with the sulphate recovered from the urine suggested that they were short-chain sulphate esters structurally related to butyric acid 4-sulphate. Whole-body autoradiography located the liver as the site of metabolism of all the three sulphates administered.

The authors conclude that, like DS and ODS, odd-numbered alkyl sulphates are probably metabolized in the rat by a mechanism similar to that of dodecyl sulphate, which is degraded in the liver by ω-oxidation and subsequent β-oxidation.

METHODS FOR ASSESSING TOXICITY

2519. Onward from Draize

Phillips, L., II, Steinberg, M., Maibach, H. I. & Akers, W. A. (1972). A comparison of rabbit and human skin response to certain irritants. *Toxic. appl. Pharmac.* **21,** 369.

Davies, R. E., Harper, K. H. & Kynoch, S. R. (1972). Inter-species variation in dermal reactivity. *J. Soc. cosmet. Chem.* **23,** 371.

Predictive tests for irritancy on rabbit skin using the Draize technique do not always

correlate well with the results of tests on human skin. The difference may be particularly important for materials intended for cosmetics formulations but is also relevant in the case of materials liable to be handled industrially.

In the first study cited above, the results of modified Draize skin tests in the rabbit, single-application tests to the human forearm and 21-day open and occlusive patch tests were compared, using a wide range of different types of chemical compound in aqueous, ethanolic or acetone solution. The test substances included Dowco 214 (*O*,*O*-dimethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate), a carbamate (Hercules 9007), dibrom (dimethyl 1,2-dibromo-2,2-dichloroethylphosphate), 2-[(*p*-methoxybenzyl)oxy]-*N*,*N*-dipropylacetamide, ethylmercaptophenylacetate *O*,*O*-dimethyl phosphorodithioate, diethyltoluamide, undecenoic acid, *O*,*O*,*O*′,*O*′-tetramethyl *O*,*O*′-thiodi-*p*-phenylene phosphorothioate, sodium lauryl sulphate, 10% formaldehyde and propylene glycol.

The modified Draize test showed Dowco 214, Hercules 9007 and dibrom to be moderate irritants, but in the human tests significant irritancy occurred only with the two latter compounds. The 21-day occlusive patch test indicated that these two were in fact the most potent irritants in the test series, and this test discriminated best between the irritancy of the remainder of the compounds. Draize testing in rabbits was thus shown to be able to predict those compounds likely to produce severe irritation or to be non-irritant on application to human skin, but did not distinguish adequately between the mild and moderate skin irritants. Where the Draize test was inconclusive, however, the moderate to mild irritants could only be separated by direct testing on human skin.

The differing degrees of irritation produced by a given compound in these tests are probably largely attributable to differences in the permeability, and perhaps also the metabolism, of rabbit and human skin. The second paper cited above questions the established popularity of the rabbit for predicting the potential dermal irritancy of cosmetics ingredients and reports a study which compared the relative reactivity of the skins of a variety of species to a series of test materials applied under occlusion for 24 hr. Results were evaluated at 24 and 72 hr, and the animals used were mice, guinea-pigs, rabbits, beagles, piglets (Large White), miniature pigs, baboons and man.

Lanolin caused a minimal reaction in piglets, but none in any other species. Propylene glycol caused minimal reactions in rabbits, piglets and man only. Aluminium chlorhydrate induced hyperkeratinization in rabbits, mice, guinea-pigs, dogs and man, but not in the pig or baboon. Thioglycollate paste (an aqueous mixture of 15% calcium thioglycollate with calcium carbonate and hydroxide, having a pH of 11) was much more irritant to abraded than intact skin in the rabbit, dog and guinea-pig, all of which seemed to be more sensitive than the primates, in which, however, only intact skin was tested. Predictably, sodium lauryl sulphate was more reactive at 5% than at 1%, with the mouse, rabbit and piglet showing the greatest reaction and the dog and guinea-pig being slightly less sensitive. Man and the miniature pig showed only a minimal response and none was detected in the baboon. *p*-Phenylenediamine proved most reactive in the rabbit and piglet. Responses were similar in type but less intense in other animals with the exception of the baboon which gave no observable reactions. In tests on a commercial cream shampoo, rabbits and guinea-pigs showed by far the greatest sensitivity, although there were moderately severe reactions in the mouse and dog, and mild ones in man and the piglet. Response was minimal in the miniature pig and baboon.

In general, the variability between the test species was greatest with the more irritant materials tested, and there was evidence that too much reliance on a single test species might

be misleading. The authors suggest that phased studies of irritancy seem to offer most promise for the cosmetic chemist, with initial tests on an animal at least as sensitive as man and generally somewhat more so, followed by further tests on a species known to react to a degree more comparable with that seen in man. In such a scheme, the rabbit offers a reliable preliminary step, since with none of the compounds tested was the irritation in the rabbit less than that on human skin. The selection of a species for the second test phase is more difficult, however, and would probably have to take into account the type of product to be evaluated.

CANCER RESEARCH

2520. Lead effects in the rat kidney

Choie, D. D. & Richter, G. W. (1972). Cell proliferation in rat kidneys after prolonged treatment with lead. *Am. J. Path.* **68,** 359.

Intranuclear inclusions in the proximal epithelial cells of the kidneys and induction of renal neoplasms are characteristic features of prolonged treatment of rats with lead salts. In a study designed to investigate the state of cell proliferation after repeated treatment with lead, one group of rats was given weekly intraperitoneal (ip) injections of an aqueous solution of lead acetate for 6 months. The doses given varied from 1 to 7 mg/rat. A second group of similarly treated rats was uninephrectomized at the start of the experiment. Groups of untreated intact or uninephrectomized rats served as controls. At the end of the 6 months, each of the rats was given an ip injection of ^{3}H-thymidine and killed after an interval of 1 hr.

Approximately 40% of the proximal tubular epithelial cells in the lead-treated rats contained recognizable intranuclear inclusions. The uptake of ^{3}H-thymidine was approximately 15 times greater in the proximal tubules of treated rats than in those of controls and mitotic activity was roughly doubled. Occasionally, foci of epithelial hyperplasia were found in segments of the proximal tubules but there was no evidence of renal carcinoma or frank tubular cell necrosis at the end of the treatment period. Nephrectomy did not influence the extent or severity of the pathological process.

These findings indicate that prolonged administration of lead stimulates renal cell replication. The induction of renal neoplasms is thought to be related to this proliferative activity. The same authors have also reported a proliferation of the proximal tubular epithelium in rats given a single dose of 40 mg lead/kg (Choie & Richter, *Am. J. Path.* 1972, **66,** 265).

[The findings reported here are not in accord with those reported by Van Esch *et al.* (*Br. J. Cancer* 1962, **16,** 289), who reported an inhibition of renal tubular epithelial proliferation by the prolonged administration of basic lead acetate and linked this inhibition with the carcinogenic effect of lead. This discrepancy is not readily explained, but perhaps the application of the technique involving ^{3}H-thymidine uptake provides a more sensitive index than does a mitotic count.

The data reported in this paper are important to our understanding of the carcinogenic effect of lead on the rat kidney. The proliferative activity preceding tumour development recalls the problem of interpreting the experimental induction of subcutaneous sarcoma in

rats and of skin carcinoma in mice. In both these instances a sustained proliferative activity of the local cells was thought to be a major factor in the induction of malignant tumours (Grasso & Golberg, *Fd Cosmet. Toxicol.* 1966, **4,** 297; Grasso & Crampton, *Fd Cosmet. Toxicol.* 1972, **10,** 419). It would seem that we may have to look critically in future at claims of carcinogenic activity based on the induction of renal tumours in the rodent.]

2521. Microsomal enzymes and DMBA

Kinoshita, N. & Gelboin, H. V. (1972). The role of aryl hydrocarbon hydroxylase in 7,12-dimethylbenz(a)anthracene skin tumorigenesis: On the mechanism of 7,8-benzoflavone inhibition of tumorigenesis. *Cancer Res.* **32,** 1329.

The enzyme system, aryl hydrocarbon hydroxylase (AHH), is part of the mixed-function oxidase system, which converts polycyclic aromatic hydrocarbons (PAH) to phenols, dihydrodiols and epoxides. It has been suggested that this enzyme system may convert PAH to other intermediates with a toxic and carcinogenic potential. In tissue culture, the amount of AHH in a variety of mammalian cells is directly related to the susceptibility of the cells to benzo[*a*]pyrene (BP) cytotoxicity. A powerful inhibitor of the enzyme, 7,8-benzoflavone (BF), prevents PAH cytotoxicity and inhibits the tumorigenicity of 7,12-dimethylbenz[*a*]anthracene (DMBA) in the mouse skin. The enzyme system catalyses the formation of covalently bound complexes of hydrocarbon with DNA and protein and also the formation of epoxides of naphthalene and dibenz[*a,c*]anthracene, the latter epoxide being a more potent transforming agent *in vitro* than the parent hydrocarbon.

The role of AHH in the induction of skin tumours by DMBA has now been explored further in mice. It was found that the AHH of mouse skin could be induced by benz[*a*]-anthracene or DMBA and was inhibited by BF. Formation of covalent complexes of DMBA with DNA, RNA and protein was also inhibited by BF. Furthermore, the tumour formation that normally follows a single application of DMBA followed by repeated weekly application of croton oil, or repeated application of DMBA, was prevented by the application of BF within 12 hr of DMBA administration.

These studies are consistent with the view that DMBA requires metabolic activation by aryl hydrocarbon hydroxylase before it can exert its carcinogenic potential.

Fd Cosmet. Toxicol. Vol. 11, pp. 351–352. Pergamon Press 1973. Printed in Great Britain

LETTERS TO THE EDITOR

TOXICOLOGY OF NISIN

Sir,—As the UK manufacturer of the food preservative nisin, my company has an obvious interest in the toxicological studies that add to our knowledge of the safety-in-use of this substance. We have devoted much of our activity towards ensuring that information on nisin usage, its toxicological properties and so on is available to those who need it. Furthermore, to be certain of our facts we scrutinize closely any new reports on nisin.

On studying an article entitled "Toxicological evaluation of some combinations of food preservatives" by Shtenberg & Ignat'ev (*Fd Cosmet. Toxicol.* 1970, **8,** 369), we have discovered some criticism of our nisin preparation which we believe to be unfounded and we feel justified in asking you to publicize our comments in your journal.

In the discussion on p. 378 of the above article, there is the statement: "As the data obtained for nisin were not convincing, it was decided to carry out a further study with nisin manufactured in the Soviet Union. This was recently completed (Shillinger, Bogoroditskaya & Osipova, 1969) and the home-produced nisin was found to be less toxic than the imported." In this context the imported nisin refers to the preparation manufactured by Aplin & Barrett Ltd. However, when we refer to the article by Shillinger *et al.* (*Vop. Pitan.* 1969, **22,** 44), we find that the English summary states, and is confirmed by the Russian text: "A comparative investigation into the toxic properties of the English and Soviet-made preparations of nisin conducted on animals did not reveal any essential difference in their action on the organism of laboratory mice." It is regrettable that confusing statements should enter into the important field of food additives.

The statement on p. 370 of the Shtenberg & Ignat'ev article, that nisin purchased from Aplin & Barrett Ltd. was of largely unknown composition, also requires some comment. Aplin & Barrett Ltd. manufacture a commercial nisin preparation having standardized activity of 1×10^6 IU/g to suit the needs of the user. Since pure nisin has an activity of approximately 45×10^6 IU/g, our commercial nisin preparation contains a high proportion of non-nisin substances. These consist almost entirely of denatured milk protein derived from the process of manufacture and sodium chloride, which is added to standardize the nisin activity of the preparation. Compositional details of the Aplin & Barrett Ltd. nisin preparation are freely available to anyone who requires them, and attention may also be drawn to the informative chapter on nisin in the report: "Specifications for Identity and Purity of Some Antibiotics" (F.A.O. Nutr. Mtg Rep. Ser. no. 45A; WHO/Food Add./69.34, Rome, 1969).

Turning to the actual results presented in the article by Shtenberg & Ignat'ev, we do not feel that any firm toxicological conclusions can be reached in view of the following.

In experiments involving many groups of animals (some of which are very small in number) and a variety of treatments, it is necessary to offer explanations for apparently contradictory results. No explanation has been given for the fact that quite high mortalities

occurred in control animals or for the first line of Table 3, where the mortality of male controls was higher at 2·5 months than at 8 months. In Table 2, mortalities of 28·6, 62·5 and 45% are given for groups of ten mice.

It is extremely difficult to accept the survival rates given in Table 3 as 12 and 32% at 2·5 months for male and female mice, respectively, receiving nisin at 4 mg/kg, when it is stated under Results (p. 372) that for the 2-month study on mice "Survival and food consumption in the test groups did not differ significantly from those of the controls."

We regret that the article "Toxicological evaluation of some combinations of food preservatives" does not add to the present state of knowledge on nisin toxicology.

G. G. FOWLER,
Aplin & Barrett Ltd.,
Newton Road,
Yeovil,
Somerset, England

Sir,—We have studied very carefully the letter [printed above] from Mr. G. G. Fowler, have looked again at the publication to which the author of the letter refers and would like to reply as follows:

The paper we published in your journal (Shtenberg & Ignat'ev, *Fd Cosmet. Toxicol.* 1970, **8,** 369) showed that no conclusive data were obtained in the study of the activity of nisin of English manufacture and, in consequence, work was later carried out on our recommendation by other authors (Shillinger *et al. Vop. Pitan.* 1969, **28** (2), 44). Like ourselves, these authors, too, failed to discover any definite signs of toxicity in adult rats or mice treated with nisin, either home-produced or of English manufacture. However, in our experiments, the animals that appeared to be most sensitive to nisin were weanling mice with a starting weight of 8–10 g, on which no-one has worked either before or after us. In these same animals, we established for the first time that nisin possessed the ability to stimulate their growth, with a simultaneous reduction in their overall resistance (Ignat'ev, *Vop. Pitan.* 1965, **24** (3), 61). On this basis, one can explain the higher mortality of the treated animals. The phenomenon of a stimulation of weight gain is a well-known characteristic of antibiotics.

In Mr. Fowler's letter, we did not find any valid and significant objections to our studies on nisin and we regretfully conclude that the author of the letter has considered our data with insufficient care.

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Fd Cosmet. Toxicol. Vol. 11, p. 353. Pergamon Press 1973. Printed in Great Britain

FORTHCOMING PAPERS

It is hoped to publish the following research papers and other communications in the next issue of *Food and Cosmetics Toxicology:*

Short-term toxicity of Orange G in pigs. By I. F. Gaunt, Ida S. Kiss, P. Grasso and S. D. Gangolli.

Long-term toxicity studies of Chocolate Brown FB in mice. By I. F. Gaunt, P. G. Brantom, P. Grasso and Ida S. Kiss.

Influence de la teneur en protéines du régime sur quelques effets du Bordeaux S et du Jaune de Beurre chez le rat. Par R. Albrecht, Ph. Manchon et R. Lowy.

The metabolism of saccharin in laboratory animals. By J. L. Byard and L. Golberg.

The excretion and metabolism of saccharin in man. I. Methods of investigation and preliminary results. By E. W. McChesney and L. Golberg.

The toxicology of dieldrin (HEOD). I. Long-term oral toxicity studies in mice. By A. I. T. Walker, E. Thorpe and D. E. Stevenson.

The toxicology of dieldrin (HEOD). II. Comparative long-term oral toxicity studies in mice with dieldrin, DDT, phenobarbitone, β-BHC and γ-BHC. By E. Thorpe and A. I. T. Walker.

Polychlorinated biphenyls: Evidence of transplacental passage in the Sherman rat. By August Curley, V. W. Burse and Mary E. Grim.

Sensitivity of feeding tests in detecting carcinogenic properties in chemicals: Examination of 7,12-dimethylbenz[*a*]anthracene and oxidized linoleate. By Margaret G. Cutler and R. Schneider.

Reproduction study with formaldehyde and hexamethylenetetramine in beagle dogs. By H. Hurni and H. Ohder. (Short Paper)

Metabolism of aflatoxin B_1 by the guinea-pig. By Mary T. Koes, L. J. Forrester and H. D. Brown. (Short Paper)

Susceptibility of vitamin A-deficient rats to aflatoxin. By G. Sriranga Reddy, T. B. G. Tilak and D. Krishnamurthi. (Short Paper)

Monographs on fragrance raw materials. By D. L. J. Opdyke.

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Aims and Scope

The Journal provides a wide-ranging service of general articles and abstracts on food and cosmetics toxicology. The journal is primarily intended for the use of food scientists and technologists, manufacturers and administrators who neither read nor have access to the medical, pharmacological or toxicological literature. In addition, the journal will constitute a medium for the publication of reviews and original articles relating to the fields of interest covered by the British Industrial Biological Research Association.

Instructions to Authors

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Submission of a paper to the Editor will be held to imply that it reports unpublished original research, that it is not under consideration for publication elsewhere and that if accepted for the Journal, *Food and Cosmetics Toxicology*, it will not be published again, either in English or in any other language without the consent of the Editor.

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