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Research Section

Recherches sur l'Action Psychotrope de Quelques Substances Aromatiques Utilisées en Alimentation

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(*Reçu le 20 juin 1972*)

Résumé—Le *trans*-anéthole, la carvone, le limonène, le menthol et le citral exercent un effet psycholeptique chez la souris. La thuyone possède des propriétés convulsivantes et le thymol est sans action sur le système nerveux central. Cependant, l'activité psychotrope de ces arômes, en particulier dans le cas d'administration par voie orale, est faible. Dans le cas d'administration intrapéritonéale, les doses efficaces sont de l'ordre de 100–600 mg/kg selon le test envisagé et selon la substance étudiée. Seule la thuyone est active à dose relativement faible. Les actions psychotropes de ces différentes substances sont toujours très fugaces et il est peu probable qu'elles puissent avoir un effet quelconque chez l'homme aux concentrations auxquelles elles sont présentes dans les aliments ou les boissons.

INTRODUCTION

On a étudié depuis longtemps, les propriétés psychotropes de nombreuses substances aromatiques utilisées dans l'alimentation. Ces études ont été généralement effectuées sur des espèces animales et avec des techniques très variables, de telle sorte qu'il est difficile de comparer les résultats qu'elles ont fournis. Signalons par exemple, que Wesley-Hadzija et Bohinc (1956), étudiant l'action psychotrope de 32 huiles essentielles sur le cyprin doré, ont constaté que celles contenant des alcools ou des phénols exercent une action dépressive, tandis que les essences à cétones sont douées de propriétés spasmodiques. Seto et Keup (1969) ont constaté que l'anéthole, la safrole, l'asarone, l'eugénol et l'estragol exaltent l'action hypnotique du pentobarbital à l'égard de la souris. Eickholt et Box (1965) ont constaté que les essences de menthe étaient à l'égard du rat, à la fois convulsivantes et hypnotiques, tandis que Cadeac et Meunier (1891) avaient qualifié les essences de menthe d'excito-stupéfiantes. La thuyone a été l'objet de nombreux travaux (Liberman et Liebert, 1941; Pinto-Scognamiglio, 1967 & 1968; Wenzel et Ross, 1957), qui ont tous établi son activité convulsivante. La carvone serait, elle aussi, convulsivante à l'égard de la souris (Wesley-Hadzija et Bohinc, 1956).

Dans un précédent travail consacré à l'étude détaillée de l'activité psychotrope des anétholes (*cis* et *trans*), nous avons démontré que le *trans*-anéthole manifestait, à dose élevée, des propriétés psycholeptiques fugaces, tandis que l'isomère *cis* était totalement inactif à l'égard du système nerveux central (Boissier, Simon et Le Bourhis, 1967 & 1969). La fugacité de l'action du *trans*-anéthole doit être mise en parallèle avec la rapidité de sa destruction dans l'organisme (Le Bourhis, 1968, 1970 & 1972).

A la suite de cette étude, il nous a semblé intéressant de comparer, avec les mêmes techniques expérimentales, les activités psychotropes de quelques substances couramment utilisées dans l'alimentation—menthol, citral, limonène, thuyone et thymol; l'anéthole a été considéré comme témoin.

METHODES EXPERIMENTALES

Matériaux, animaux et méthode de traitement

Les substances aromatiques utilisées provenaient de différentes firmes: Carvone (+) *purum*, limonène (+) *purum* et menthol (−) *puriss*, Fluka AG, Buchs, Switzerland; thuyone (+), K and K Laboratories Inc., Plainview, N.Y.; thymol du Codex, E. Merck, Darmstadt, Germany; citral, Prolabo, Paris; *trans*-anéthole et estragol, Mane,

Les différents tests ont été réalisés sur des souris mâles (Swiss), pesant 18–22 g et provenant toutes du même élevage. L'anéthole, la carvone, le citral, l'estragol, le limonène et la thuyone ont été administrés aux animaux par voie intrapéritonéale ou orale sous forme d'émulsion dans une solution aqueuse de 1% de Tween 20. Le menthol et le thymol ont été solubilisés dans de l'huile d'olive. Les concentrations des émulsions ou des solutions étaient telles que les animaux recevaient toujours un volume de 20 ml/kg.

Description des différents tests de mise en évidence d'une action psychotrope

Parmi les substances psycholeptiques, Delay (1958) distingue les hypnotiques, les neuroleptiques et les tranquillisants mineurs. Pour mettre en évidence ces actions pharmacologiques on réalise divers tests. Certains mettent en jeu des réflexes et donnent la même réponse avec tous les psycholeptiques, les autres plus spécifiques fournissent des résultats permettant de préciser le type d'activité psycholeptique de la substance étudiée. Pour chaque test, nous avons étudié l'effet de diverses doses des substances étudiées, sur des lots de 10 ou 20 animaux.

Tests non spécifiques d'activité

Test de la traction (Courvoisier, 1956). On utilise une sorte de barre fixe constituée par un fil métallique de 2 mm de diamètre. Les souris témoins suspendues par les pattes antérieures à cette barre se rétablissent en moins de 5 sec. Les souris soumises à l'action d'un agent dépresseur n'en sont pas capables. Pour évaluer le degré de dépression, on note la proportion des animaux ayant perdu la faculté de se rétablir à des temps variables après le traitement.

Test de la cheminée (Boissier, Tardy et Diverres, 1960). Des souris normales, placées la tête en bas dans un tube de verre de 3 cm de diamètre, sont capables de remonter cette "cheminée" en marchant à reculons, Les substances psycholeptiques provoquent la perte de la faculté de remonter. On note la proportion d'animaux ayant perdu cette aptitude.

Test de la tige tournante (Dunham et Miya, 1957). Une souris normale peut se maintenir, pendant un temps supérieur à 5 min, sur un cylindre de bois de 2,5 cm de diamètre disposé horizontalement et tournant autour de son axe à une vitesse de 10 tours/min. Si on lui administre une substance dépressive en quantité suffisante, elle perd cette aptitude et se laisse tomber plus ou moins rapidement. On note, pour chaque souris, le temps pendant lequel elle se maintient sur le cylindre.

Tests d'étude du comportement

Ces tests permettent de mettre en evidence les possibilités de déplacements et la faculté d'exploration des animaux mis dans une enceinte nouvelle. Le test de la planche à trous et celui de l'évasion offrent la possibilité à l'expérimentateur d'observer le comportement des animaux; la mesure de la motilité ne le permet pas mais peut être poursuivie pendant un temps très long.

Test de la planche à trous (Boissier et Simon, 1964). On dispose une souris sur une planchette de bois de 40 × 40 cm, percée de 16 trous de 3 cm de diamètre. L'animal explore les trous en se déplaçant sur la planche et en plongeant sa tête dans chacun d'eux. On dénombre les trous ainsi explorés pendant une période de 5 min.

Test de l'évasion (Boissier, Simon, Lwoff et Guidicelli, 1965). Des souris sont placées dans une caissette dont elles peuvent "s'évader" par un plan incliné. On détermine, pour chaque animal, le nombre de sorties,

définies par le franchissement d'une ligne repère tracée sur le plan incliné et perpendiculairement à celui-ci.

Mesure de la motilité. Nous avons utilisé "l'actimètre" mis au point par Boissier et Simon (1965). Cet appareil est constitué par une boîte transparente traversée par deux faisceaux lumineux orthogonaux, agissant sur des cellules photo-électriques. Une souris emprisonnée dans la boite se déplace en tous sens et provoque donc des interruptions des faisceaux lumineux dont le nombre constitue une mesure de la motilité.

Mesure de l'activité analgésique

L'appréciation des propriétés analgésiques des substances étudiées a été effectuée avec la méthode proposée par Jacob et Blozovski (1959). Cette méthode consiste à chauffer la plante des pattes de la souris jusqu'à ce que la douleur qu'elle éprouve la conduise à se lécher les pattes; les analgésiques ont pour effet de retarder la perception de cette douleur. Le dispositif utilisé pour la réalisation de ce test est constitué par une sorte de marmite fermée, dont la partie supérieure plane est munie d'une cheminée terminée par un réfrigérant ascendant. Ce récipient est rempli de méthanol et posé sur une plaque chauffante. Le méthanol est porté à ébullition et par conduction les parois de la marmite sont portées à une température constante voisine de celle d'ébullition de l'alcool méthylique (65°C). Les souris sont placées individuellement sur la marmite et l'on note, pour chacune d'elles, le délai nécessaire à la perception de la douleur.

Mesure de l'effet hypnotique

Sous l'action de certains agents psycholeptiques, les souris subissent une léthargie qui se manifeste notamment par le fait que, placées sur le dos, elles ne tentent pas de reprendre une position normale.

Nous avons considéré, d'une part, le délai séparant le moment du traitement de celui de l'entrée des animaux en léthargie et, d'autre part, le "temps de sommeil".

Interaction avec des agents convulsivants

Cette interaction a été éprouvée sur des souris ayant préalablement reçu diverses doses d'anéthole, de limonène, de carvone, de citral, d'estragol, de thuyone ou de thymol et auxquelles on administrait ensuite des substances convulsivantes (pentétrazol, fluorothyl et strychnine) ou que l'on soumettait à des électrochocs. Pour chaque expérience on notait d'une part, la proportion des animaux manifestant des crises convulsives, d'autre part, la gravité de celles-ci (mortelles ou non).

Le pentétrazol était utilisé en solution aqueuse à la concentration de 5 mg/ml et les souris recevaient, par voie intraveineuse, 10 ml/kg de cette solution, soit 50 mg pentétrazol/kg. La strychnine était utilisée en solution aqueuse à la concentration de 0,1 mg/ml et les souris recevaient, par voie intraveineuse, 10 ml/kg de cette solution, soit 1 mg strychnine/kg. Le Fluorothyl était dilué au 1/75 dans du polyéthylène glycol 400 et les souris recevaient, par voie intraveineuse, 2,5 ml/kg de cette solution, soit 0,033 ml Fluorothyl/kg.

Les électrochocs ont été pratiqués en plaçant une électrode sur chaque globe oculaire d'une souris et en établissant entre les deux yeux une différence de potentiel de 55 v pendant 0,2 sec.

Mesure de la toxicité aigüe

La dose maximale non mortelle (DL_0) et la dose mortelle pour 50% des animaux (DL_{50}) ont été déterminées suivant la méthode proposée par Miller et Tainter et indiquée par Valette (1964).

RESULTATS

Nous considèrerons globalement les résultats fournis par l'anéthole, la carvone, le citral, l'estragol, le limonène et le thymol, car ils présentent de nombreux points communs. Par contre, nous examinerons séparément les cas du menthol et de la thuyone car ils se distinguent nettement des précédents.

Action psycholeptique de trans-*anéthole, de la carvone, du citral, de l'estragol, du limonene et du thymol*

Ces substances sont à des degrés variables des dépresseurs du système nerveux central. Elles diminuent l'aptitude des animaux à exécuter les tests mettant en jeu des réflexes: (tests de la traction, de la cheminée et de la tige tournante), elles provoquent une diminution de l'observation, de la curiosité et de la crainte mise en évidence par les tests de comportement (tests de l'évasion et de la planche à trous) et une réduction de la motilité.

En outre, le *trans*-anéthole et le citral ont des propriétés anticonvulsivantes et analgésiques. Seul le thymol est dépourvu d'activité mais manifeste une toxicité plus importante que les autres substances. Le tableau 1 résume les résultats fournis par les différents tests, après

administration intrapéritonéale ou orale des substances éprouvées. Les doses indiquées sont celles qui provoquent un effet psycholeptique net chez la moitié des animaux traités.

On constate que l'administration par voie orale est beaucoup moins efficace que l'administration par voie intrapéritonéale: les rapports des doses efficaces dans les deux cas varient de 3 à 7 selon la substance considérée. En outre, les doses efficaces sont élevées—généralement de 100 à 300 mg/kg dans le cas d'administration intrapéritonéale et de 500

Tableau 1. *Action psycholeptique, anticonvulsivante, analgésique et hypnotique de différentes substances aromatisantes*

Substances aromatiques	Mode d'administration	Actions psycholeptiques DE_{50} (mg/kg)* vis à vis de tests		Actions physiologiques diverses DE_{50} (mg/kg)* susceptibles de provoquer une action			DL_0§ (mg/kg)
		Non spécifiques†	De comportement‡	Anticonvulsivante	Analgésique	Hypnotique	
trans-Anéthole	Intrapéritonéale	200	100	140	300	330	420
	Orale	900	500	600	400	2400	2900
Carvone	Intrapéritonéale	200	50–500	–	–	330	440
	Orale	500–900	750–1000	–	–	–	1300
Citral	Intrapéritonéale	–	25–100	100	–	–	250
	Orale	–	400	800	800	–	900
Estragol	Intrapéritonéale	200	150–200	–	–	–	540
	Orale	1000–1500	1500		–	–	1600
Limonène	Intrapéritonéale		50–150	–	–	–	620
	Orale	inactif	3000	–	–	–	3500
Thymol	Intrapéritonéale	inactif	300	–	–	–	380
	Orale	inactif	400	–	–	–	480

*Pour chaque test on évalue la dose efficace 50 (DE_{50}) qui est la dose abaissant de 50% l'activité cérébrale des souris (action psycholeptique) ou protégeant 50% des souris contre l'action des agents convulsivants ou contre la douleur (action anticonvulsivante ou analgésique) ou endormant 50% des souris (action hypnotique).

†Tests de la traction, de la cheminée et de la tige tournante.

‡Tests de l'évasion, de la planche à trous et de l'actimétrie.

§La dose maximale non mortelle.

à 1500 mg/kg dans le cas d'ingestion. De ce fait, il est difficile de situer ces différentes substances dans la classification de Delay (1958). On peut cependant remarquer que le citral, l'estragol et le limonène ne provoquent pas d'hypnose ce qui élimine la possibilité de les classer parmi les "hypnotiques"; le *trans*-anéthole et la carvone peuvent au contraire être considérés comme des hypnotiques faibles.

Action psychotrope du menthol

Le menthol se présente comme une substance douée d'une activité psychotrope particulière.

Lors de l'exécution des tests de la traction, de la cheminée ou de la tige tournante, il manifeste par administration intrapéritonéale ou orale une faible activité psycholeptique. Une dose subtoxique provoque une dépression telle que une ou deux souris sur dix

demeurent encore capables de se rétablir sur la barre fixe, de remonter la cheminée ou de se maintenir 5 min sur le cylindre tournant, quel que soit le délai séparant le traitement du début du test.

Lors de la mesure de l'activité motrice ou de l'exécution des tests de la planche à trous et de l'évasion, on observe soit une action psychoanaleptique, soit une action psycholeptique, selon la dose administrée et le délai séparant le traitement du début du test. Les figures 1 et 2 indiquent les variations de l'activité psychotrope du menthol en fonction de la dose; les déterminations ont été réalisées 20 min après administration intrapéritonéale ou 30 min après administration orale du produit actif. On constate que le menthol, administré

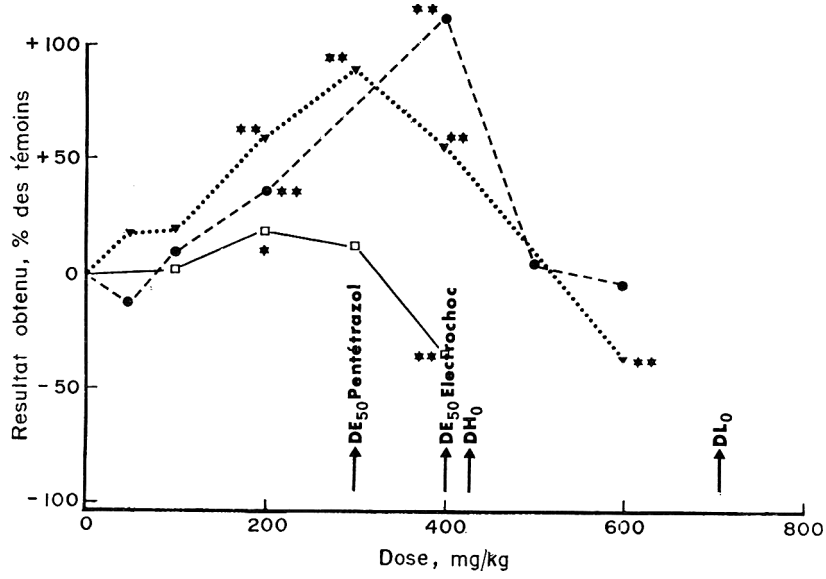

FIG. 1. Action du menthol administré par voie intrapéritonéale sur l'activité de la souris (▼·····▼) et sur la réalisation des tests de l'évasion (●– – –●) et de la planche à trous (□——□). On a également indiqué les doses de menthol susceptibles de manifester une interaction avec certains convulsivants ainsi que celles qui sont hypnotiques ou toxiques: les doses de menthol protégeants 50% des souris contre les crises convulsives provoquées par le pentétrazol (DE_{50} Pentétrazol) et par l'électrochoc (DE_{50} Electrochoc) et les doses maximales non hypnotique (DH_0) et non mortelle (DL_0). Test *t*: valeurs significativement différentes du témoin à la probabilité de 95% (*) et de 99% (**).

par voie intrapéritonéale (Fig. 1), provoque des phénomènes d'excitation dont l'importance croît avec la dose puis diminue au delà de doses respectivement égales à 200 mg/kg dans le cas du test de la planche à trous, 300 mg/kg pour l'activité des animaux et 400 mg/kg pour le test de l'évasion. Pour une dose au moins égale à 400 mg/kg, le test de la planche à trous permet de mettre en évidence une action dépressive du menthol. Enfin, pour les doses supérieures à 425 mg/kg, il provoque de la léthargie. Après administration orale, on constate (Fig. 2) que, de la même manière, le menthol provoque à des doses supérieures à 200 mg/kg une excitation dans l'intensité est maximale pour 500 mg/kg dans le cas du test de la planche à trous et 1000 mg/kg dans le cas de la mesure de l'activité des souris et dans celui du test de l'évasion. Pour les doses plus élevées, cette excitation est remplacée par de la léthargie qui apparaît pour une dose au moins égale à 1400 mg/kg.

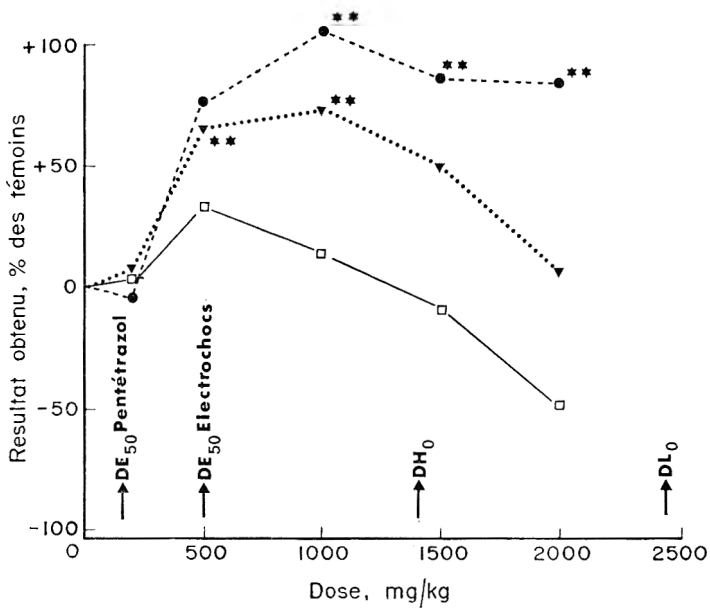

FIG. 2. Action du menthol administré par voie orale sur l'activité de la souris (▼····▼) et sur la réalisation des tests de l'évasion (●– – –●) et de la planche à trous (□——□). On a également indiqué les doses de menthol susceptibles de manifester une interaction avec certains convulsivants ainsi que celles qui sont hypnotiques ou toxiques: les doses de menthol protégeants 50% des souris contre les crises convulsives provoquées par le pentétrazol (DE_{50} Pentétrazol) et par l'electrochoc (DE_{50} Electrochoc) et les doses maximales non hypnotique (DH_0) et non mortelle (DL_0). Test *t*: valeurs significativement différentes du témoin à la probabilité de 99% (**).

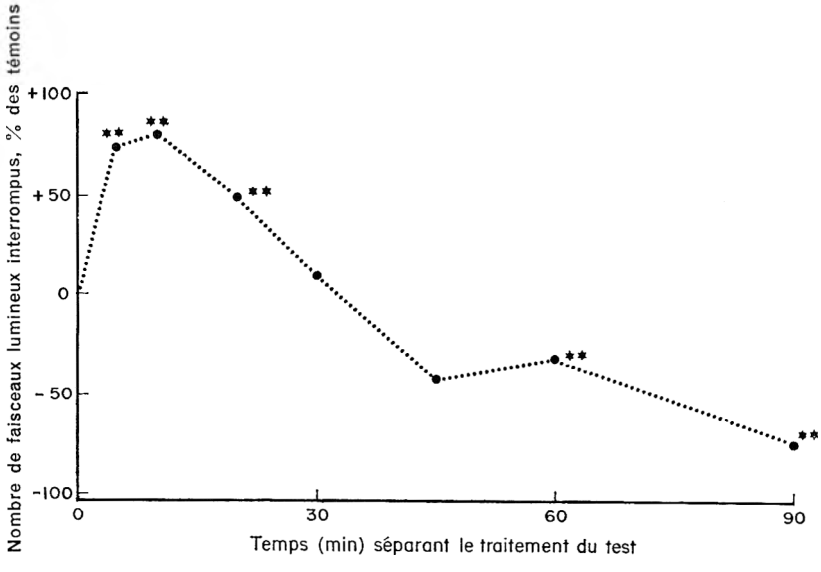

FIG. 3. Action d'une dose de 2000 mg menthol/kg, administrée par voie orale sur la motilité des souris. Test *t*: valeurs significativement différentes du témoin à la probabilité de 99% (**).

Enfin nous avons constaté qu'après administration par voie orale d'une dose hypnotique de menthol (2000 mg/kg), les souris étaient soumises à un état d'excitation mis en évidence par la mesure de leur motilité, avant d'entrer en léthargie (Fig. 3).

L'épreuve du chauffage des pattes de souris a établi que le menthol ne manifeste aucune activité analgésique. Par contre, le menthol possède une activité anticonvulsivante importante. Les doses de menthol protégeant 50% des souris contre les crises toniques sont, selon les convulsivants utilisés, de l'ordre de 300–400 mg/kg pour l'administration intrapéritonéale et de 150–500 mg/kg dans le cas d'ingestion.

En résumé, le menthol doit être considéré comme un psycholeptique; l'excitation précédant la dépression n'est d'ailleurs pas sans rappeler l'action de certains barbituriques et il se comporte donc comme un hypnotique faible, doué de propriétés anticonvulsivantes très nettes.

Action psychotrope de la thuyone

La thuyone possède des propriétés convulsivantes qui sont connues depuis fort longtemps. Les convulsions du type tonico-clonique maintes fois décrites, sont toujours mortelles et les doses convulsivantes correspondent donc aux doses léthales.

Le tableau 2 indique les valeurs de ces doses administrées par voie intrapéritonéale ou orale. L'administration intrapéritonéale de doses infraconvulsivantes de thuyone provoque une légère augmentation de la motilité pour une dose de 3 mg/kg et une dépression, mise en évidence par la mesure de la motilité et les tests de l'évasion et de la planche à trous, pour une dose de 24 mg/kg.

Tableau 2. *Doses léthales (et convulsivantes) de la thuyone pour la souris*

Voie d'administration	Solvant ou agent de suspension	DL_0 (mg/kg)	DL_{50} (mg/kg)	DL_{100} (mg/kg)
Intrapéritonéale	Huile d'olive	155	260 ± 8,0	440
	Eau + Tween 20	33	72 ± 3,0	155
Orale	Eau + Tween 20	75	250 ± 20	840

Cette dépression est due plutôt à des phénomènes comateux précédant les convulsions mortelles qu'à une véritable action psycholeptique. Bien qu'elle possède des propriétés convulsivantes importantes, la thuyone ne renforce pas celle du pentétrazol ou des électrochocs. D'autre part, elle n'empêche pas l'apparition du sommeil provoqué par le mébubarbital. Par contre, l'administration intrapéritonéale de 10 mg/kg de ce barbiturique protège toutes les souris contre l'action convulsivante de 150 mg thuyone/kg administrée par voie intrapéritonéale.

Le *trans*-anéthole s'oppose, lui aussi, aux convulsions provoquées par la thuyone (Tableau 3).

DISCUSSION

Notre étude a montré que toutes les substances étudiées, à l'exception du thymol, manifestent une activité psychotrope. La thuyone possède des propriétés psycho-analeptiques, tandis que les autres substances ont, au contraire, des propriétés dépressives. Nos résultats

Tableau 3. *Interaction thuyone*-trans-*anéthole, administrés par voie intrapéritonéale*

Doses (mg/kg)		Nombre des crises toniques (sur dix souris)	Mortalité (sur dix souris)
trans-Anéthole	Thuyone		
0	100	9	9
50	100	10	8
100	100	5	7
200	100	0	0
300	100	0	0

sont donc en accord avec ceux de nos devanciers, sauf en ce qui concerne la carvone, qui, selon Wenzel et Ross (1957) serait convulsivante à dose mortelle.

Il faut remarquer que toutes les substances éprouvées ont une activité plus faible après ingestion qu'après administration par voie parentérale. Certaines ont une activité analgésique (*trans*-anéthole et citral) mais cette activité n'est apparentée ni a une activité hypnotique importante ni à une activité du type "tranquillisant".

De même l'activité anticonvulsivante du citral, de l'anéthole, du menthol, ne semble pas en rapport avec une activité psychotrope quelconque. Il faut remarquer que le simple déplacement de la double liaison de la chaine latérale de l'anéthole pour donner l'estragol, supprime les propriétés anticonvulsivantes, analgésiques et hypnotiques. La carvone diffère du limonène par l'adjonction d'un groupe cétonique sur le cycle et cette adjonction ne modifie pas sensiblement les propriétés. Par contre, la réduction du menthol en thymol supprime presque complètement son activité psycholeptique.

En définitive, il semble bien que toutes ces substances aromatiques solubles dans les graisses, soient actives sur le système nerveux central. Il faut cependant remarquer, que, mise à part la thuyone, qui est active et toxique à dose relativement faible, tous ces composés n'agissent qu'à dose élevée et qu'il est peu probable qu'ils puissent avoir un effet quelconque chez l'homme, aux concentrations auxquelles ils sont présents dans les aliments ou les boissons.

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Studies on the psychotropic action of some aromatic compounds used in food

Abstract—*trans*-Anethole, carvone, limonene, menthol and citral all have a psycholeptic effect in the mouse. Thujone has convulsive properties and thymol is without action on the central nervous system. However, the psychotropic activity of these aromatic compounds, particularly when they are administered orally, is weak. When administered intraperitoneally, the effective doses are of the order of 100–600 mg/kg, according to the test used and the substance studied. Only thujone was active in a relatively weak dose. The psychotropic actions of these substances are always transient and it is unlikely that they would have any effects of this kind in man at the concentrations in which they occur in foods or drinks.

Untersuchungen der psychotropen Wirkung einiger in Lebensmitteln verwendeten aromatischen Verbindungen

Zusammenfassung—*trans*-Anethol, Carvon, Limonen, Menthol und Citral haben eine psycholeptische Wirkung bei der Maus. Thujon hat krampfauslösende Eigenschaften und Thymol zeigt keine Wirkung auf das Zentralnervensystem. Die psychotrope Aktivität dieser aromatischen Verbindungen ist jedoch, besonders bei oraler Anwendung, schwach. Bei intraperitonealer Anwendung betragen die wirksamen Dosen 100–600 mg/kg, je nach der angewendeten Untersuchungsmethode und der verwendeten Substanz. Nur Thujon war in einer relativ schwachen Dosis aktiv. Die psychotropen Wirkungen dieser Substanzen sind stets vorübergehend, und es ist nicht wahrscheinlich, dass sie Wirkungen dieser Art beim Menschen in den Konzentrationen, in denen sie in Lebensmitteln oder Getränken vorkommen, hervorrufen könnten.

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The Effect of Daily Ingestion of Caffeine on the Microsomal Enzymes of Rat Liver*

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Abstract—The effect of repeated ingestion of caffeine on rat-liver microsoma enzymes was studied. In the rat, ingestion of 20–24 mg caffeine/kg/day in the drinking water for more than 2 wk inhibited aminopyrine *N*-demethylase activity. This loss of activity approached 60% at the end of an 8-wk dosing schedule. Acetanilide-hydroxylating activity of rat-liver microsomal enzyme was initially stimulated, with a peak (145 ± 11% of control) at 2 wk, and returned to normal levels at the end of 8 wk. Caffeine-treated rats did not respond to the phenobarbitone stimulation of microsomal enzymes to the same extent as did rats treated with phenobarbitone alone.

INTRODUCTION

In addition to the caffeine in coffee, tea and other beverages, caffeine is present in many commercial preparations consumed by the general public (Gleason, Gosselin, Hodge & Smith, 1969), so that substantial amounts of caffeine are ingested by large numbers of people. Caffeine, a xanthine alkaloid, has several pharmacological and biochemical effects, including stimulation of the central nervous system, diuresis (Truitt, 1965), mutagenesis (Ostertag, Duisberg & Stürmann, 1965), inhibition of phosphodiesterase (Beavo, Rogers, Crofford, Baird, Hardman, Sutherland & Newman, 1971) and inhibition of DNA polymerase (Wragg, Carr & Ross, 1967). Psychotropic effects and tolerance to caffeine have also been reported (Colton, Gosselin & Smith, 1968; Goldstein, Kaizer & Whitby, 1969).

Caffeine is readily absorbed following ingestion and is distributed in the body according to the water content of the tissues (Bertoli, Dragoni & Rodari, 1968). It is metabolized in mammals by demethylation to methylxanthines and to methyluric acids by oxidation at the C-8 position (Cornish & Christman, 1957; Khanna, Rao & Cornish, 1972; Parke, 1968).

Acute doses of caffeine have been shown to stimulate hepatic, drug-metabolizing microsomal enzymes in the rat (Mitoma, Sorich & Neubauer, 1968). It has been suggested (Lombrozo & Mitoma, 1970) that the effect of caffeine on cytochrome P_{450} is similar to that of 3-methylcholanthrene. Other than a single reference (Cornish, Wilson & Abar, 1970), reporting that in rats treated with caffeine for 6 wk *N*-dealkylation activity was 66% of that in controls, no information was evident in the literature concerning the effect of repeated ingestion of caffeine on drug-metabolizing microsomal enzymes. Since caffeine is consumed by the public in large quantities, the present study on the effect of prolonged ingestion of caffeine on microsomal enzymes was undertaken.

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EXPERIMENTAL

Animals and maintenance. Male rats of the Sprague–Dawley strain, weighing 250–300 g, were used for these studies. They were fed Rockland Rat Chow *ad lib.* and housed in stainless-steel cages.

Conduct of experiments. Caffeine was administered in the drinking-water (0·2 mg/ml), the daily dose being calculated on the basis of a daily consumption of 25–30 ml water/rat. In the first experiment, groups of rats (six treated animals and two controls) were killed at intervals between 4 and 56 days from the start of caffeine administration for hepatic microsomal-enzyme determinations. In the second study, caffeine was administered for 6 wk, on the last 3 days of which sodium phenobarbitone (25 mg/kg/day) was injected ip into some of the treated and control animals. Enzyme induction of the liver microsomal enzymes was determined by measurement of acetanilide-hydroxylation and aminopyrine-*N*-demethylation activities. Values in the caffeine/phenobarbitone-treated rats were compared with those in rats treated only with caffeine or only with phenobarbitone and with those in untreated rats of the same age. In the third study, caffeine was administered for 14 wk, and phenobarbitone (25 mg/kg) was injected ip on the last 3 days of treatment. Animals were killed 24 hr after the last dose for the determination of hepatic microsomal levels of cytochrome P_{450} and cytochrome b_5.

Fractionation of liver samples. The rats were anaesthetized with ether and killed by open-chest surgery. They were bled by heart puncture with the aid of vacutainers. Liver samples (5 g) were immediately frozen on dry ice and stored at −20°C before use. Each 5 g sample was homogenized in 10 ml 0·05 M-tris buffer (pH 7·4) containing 1·15% KCl by means of glass homogenizers and a teflon pestle fitted to a mechanical drill press. The homogenate was centrifuged at 13,000 **g** in a Sorvall refrigerated high-speed centrifuge. This supernatant was used for microsomal-enzyme analysis.

Enzyme and other anlyses. The incubation mixture (total volume 5 ml) contained supernatant (1 ml), substrates (17·8 μM acetanilide for hydroxylation and 3·5 μM aminopyrine for demethylation) and the following co-factors: glucose 6-phosphate (25 μM), NADP (2·25 μM), nicotinamide (20 μM), NAD (2·25 μM) and magnesium sulphate (12 μM) in 4 ml 0·1 M-phosphate buffer (pH 7·4). This mixture was incubated under oxygen for 20 min in 25 ml Erlenmeyer flasks using a Dubnoff shaking incubator at 37°C. For *N*-demethylation studies, aminopyrine was used as a substrate and 4-aminoantipyrine was measured as the reaction product by the method outlined by Brodie & Axelrod (1950). Hydroxylation activity was determined essentially by the method of Brodie & Axelrod (1948), using acetanilide as a substrate. The enzyme activity was expressed as μg product formed/g liver/20 min. Cytochrome P_{450} and cytochrome b_5 values were determined by the method of Omura & Sato (1964) and expressed as nmoles/mg protein. A Beckman DK-2A Spectrophotometer was used for absorbancy measurements. Protein concentrations were measured as described by Lowry, Rosebrough, Farr & Randall (1951). The Student's *t* test was used for statistical evaluation of the results.

RESULTS

It was established that the average increase (233 ± 24 g) in the body weight of rats given caffeine (20–24 mg/kg/day) orally for a period of 12 wk was essentially the same as that of controls (202 ± 49 g). The liver weight/100 g body weight (3·58 ± 0·11 g) of rats fed caffeine was also comparable with that of untreated rats (3·67 ± 0·12 g).

Table 1 shows the effect of daily caffeine ingestion (20–24 mg/kg) on the mixed-function drug-metabolizing microsomal enzymes of the livers of rats killed at intervals of 4–56 days

Table 1. *Hepatic microsomal-enzyme activity in rats given 20–24 mg caffeine/kg/day in the drinking-water*

Duration of dosing (days)	Acetanilide hydroxylation (% of control)*	Aminopyrine *N*-demethylation (% of control)*
4	123 ± 4	115 ± 7
8	127 ± 12	104 ± 11
14	145 ± 11	90 ± 6
28	139 ± 7	73 ± 3
56	93 ± 9	38 ± 5

*Values are means for six treated rats at each sacrifice period ±SEM. Two control rats were killed at each time.

from the start of treatment. Acetanilide hydroxylation and aminopyrine *N*-demethylation activities of these enzymes appeared to be minimally stimulated after a period of 4 days to approximately the same level (hydroxylation 123 ± 4% and *N*-demethylation 115 ± 7% of controls). The hydroxylation activity of these enzymes increased gradually with time, having a peak (145 ± 11% of control) at 2 wk and returning essentially to control level at 8 wk (93 ± 9%). In contrast, the mean activity of the *N*-demethylating enzyme showed only a slight initial elevation, with values declining over the subsequent study period. A marked decrease in *N*-demethylation activity was observed after 4 wk (73 ± 3% of control, $P < 0.05$). This loss of activity progressed as treatment continued, the *N*-demethylation enzyme levels of caffeine-treated animals being only 38 ± 5% of that of controls at the end of the 8-wk period.

In the second study, the phenobarbitone-treated animals showed the highest levels of acetanilide-hydroxylating activity (200 ± 13%) and aminopyrine *N*-demethylating activity (186 ± 19%), whereas animals treated with caffeine plus phenobarbitone had enzyme activities of 157 ± 12 and 140 ± 9% respectively (Table 2). This suggests that caffeine-treated animals were not able to respond to the phenobarbitone treatment to the same extent as did animals treated only with phenobarbitone.

Table 2. *Effect of phenobarbitone on the hepatic microsomal enzymes of rats given 20–24 mg caffeine/kg/day in the drinking-water for 6 wk*

Treatment	No. of animals/group	Acetanilide hydroxylation (% of control)*	Aminopyrine *N*-demethylation (% of control)*
None	3	100 ± 8	100 ± 5
Caffeine	6	110 ± 12	80 ± 11
Phenobarbitone†	5	200 ± 13	186 ± 19
Phenobarbitone† + caffeine	6	157 ± 12	140 ± 9

*Values are means for the numbers of animals shown ±SEM.
†Phenobarbitone (25 mg/kg) was injected ip on each of the last 3 days of the caffeine treatment.

Cytochrome P_{450} and cytochrome b_5 values of rats fed caffeine for 14 wk are given in Table 3. Rats dosed orally with caffeine (20–24 mg/kg) daily for 14 wk showed no change

Table 3. *Effect of phenobarbitone administration on cytochrome P_{450} and b_5 levels of hepatic microsomes of rats given 20–24 mg caffeine/kg/day for 14 wk*

Treatment	No. of animals/group	Cytochrome P_{450} (nmoles/mg protein)*	Cytochrome b_5 (nmoles/mg protein)*
None	4	1·10 ± 0·09	1·09 ± 0·04
Caffeine	6	1·13 ± 0·05	0·98 ± 0·06
Caffeine + phenobarbitone†	6	1·60 ± 0·11	0·74 ± 0·05

*Values are means for the numbers of animals shown ±SEM.
†Phenobarbitone (25 mg/kg) was injected ip on each of the last 3 days of caffeine treatment. The animals were sacrificed 24 hr after the last dose.

in their mean cytochrome P_{450} or cytochrome b_5 values when compared with the controls. However, caffeine-treated rats subsequently administered sodium phenobarbitone (25 mg/kg, ip) for the last 3 days of the treatment showed an increase in P_{450} level in comparison with both controls and caffeine-dosed animals. Cytochrome b_5 levels were essentially the same in caffeine-treated animals and in controls. In contrast to cytochrome P_{450} values, however, cytochrome b_5 levels were decreased in rats given both caffeine and phenobarbitone. The decrease was statistically significant ($P < 0{\cdot}01$) when compared with caffeine-treated rats.

DISCUSSION

The prolonged ingestion of 20–24 mg caffeine/kg/day by normal, healthy rats does not seem to affect their growth rate. However, alterations in environmental temperature and the amount and quality of the diet have been reported to affect the toxicity of caffeine. Muller & Vernikos-Danellis (1970) showed that a slight increase or decrease in the normal environmental temperature (22 ± 7°C) of mice enhanced the toxicity of caffeine, and when food intake was less than half the voluntary intake, an increase in the toxicity of caffeine was noted (Peters, 1966).

The effect of short-term exposure to caffeine on the drug-metabolizing liver microsomal enzymes has been previously reported (Mitoma *et al.* 1968; Mitoma, Lombrozo, Le Valley & Dehn, 1969). Mitoma *et al.* (1968) reported that caffeine, tea and coffee in acute doses induced the *O*-demethylation of *O*-nitroanisole and the hydroxylation of acetanilide by rat-liver microsomes. Similarly, the present study shows some initial elevations of the microsomal-enzyme activity. However, our results (Table 1) show that the prolonged ingestion of caffeine by the rat markedly reduced the aminopyrine *N*-demethylation activity of rat liver. In contrast to *N*-demethylation, acetanilide-hydroxylating activity of the liver, although initially stimulated, was nearly normal after 8 wk of caffeine ingestion.

The mechanism of the stimulation of the drug-metabolizing enzymes of rat-liver microsomes by acute dosing with caffeine has not been satisfactorily elucidated. Stimulation of these microsomal enzymes by caffeine was shown to be partially inhibited by actinomycin D (Mitoma *et al.* 1969). Proof of enzyme induction, as measured by the increase in the incorporation of labelled amino acids into the microsomal proteins, was inconclusive. This

suggests that caffeine may stimulate microsomal-enzyme activity without causing an increase in enzyme synthesis. Caffeine and phenobarbitone in acute doses were shown by Mitoma *et al.* (1968) to have an additive effect with respect to the stimulation of the hydroxylation of acetanilide by the microsomal enzymes. On the other hand, this effect was not additive when *O*-nitroanisole or aminopyrine was used as the substrate. In the present study, phenobarbitone stimulated hepatic microsomal enzmyes in rats that had been fed caffeine for 6 wk. This stimulatory effect of phenobarbitone on both the hydroxylation of acetanilide and the *N*-demethylation of aminopyrine was not additive (Table 2). In fact, it appears that, on prolonged ingestion, caffeine hinders the induction of microsomal enzymes by phenobarbitone.

Cytochrome P_{450} and the cytochrome b_5 contents of rat hepatic microsomes were evaluated in order to study the effects of caffeine on the electron transport system of mixed-function oxygenases. Cytochrome P_{450} levels in the liver microsomes of rats dosed with caffeine were essentially the same as those in the untreated rats. Similar results on cytochrome P_{450} contents of hepatic microsomes from rats given caffeine in acute doses had been reported by Lombrozo & Mitoma (1970). However, these workers suggested a possible alteration in the nature of the cytochrome P_{450} of liver microsomes from caffeine-treated rats. Recently, cytochrome b_5 of hepatic microsomes has been shown to be involved in the hepatic drug-metabolizing microsomal-enzyme system (Estabrook, Franklin, Cohen, Shigamatzu & Hildebrandt, 1971). Cytochrome b_5 is postulated as being responsible for the transfer of an electron to cytochrome P_{450} during the metabolism of substrates. It is rate-limiting only when the NADPH levels are low in the reaction mixture. Interestingly enough, cytochrome b_5 values are shown (Table 3) to be significantly decreased in the rats given caffeine and phenobarbitone. Studies in our laboratories (K. L. Khanna & H. H. Cornish, unpublished data, 1971) have shown that rats treated with phenobarbitone alone have increased levels of cytochrome P_{450} but not of cytochrome b_5. The mechanism by which caffeine and phenobarbitone-treated rats inhibit the synthesis or estimation of cytochrome b_5 is not clear.

In conclusion, rats fed caffeine for more than 2 wk begin to show a diminution of aminopyrine-*N*-demethylating activity in the liver. This loss of activity approaches 60% at the end of an 8-wk dosing schedule. Acetanilide-hydroxylating activity was not similarly affected. Although phenobarbitone stimulated microsomal-enzyme activity in caffeine-treated animals, the extent of induction was considerably below that found in control animals receiving only phenobarbitone. In addition, phenobarbitone stimulated production of cytochrome P_{450} in caffeine-treated animals, but cytochrome b_5 levels in the same animals were considerably below normal.

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Effet de l'ingestion quotidienne de caféine sur les enzymes microsomiques du foie chez le rat

Résumé—On a étudié chez le rat l'effet de l'ingestion répétée de caféine sur les enzymes microsomiques du foie. L'ingestion quotidienne, pendant plus de 2 semaines, de 20–24 mg/kg de caféine ajoutée à l'eau de boisson a inhibé l'activité de l'aminopyrine-*N*-déméthylase. Cette diminution d'activité s'est élevée à près de 60% à la fin d'un traitement de 8 semaines. L'activité d'hydroxylation de l'acétanilide de l'enzyme microsomique du foie du rat était d'abord stimulée, atteignait un maximum (145 ± 11% par rapport aux animaux témoins) à 2 semaines et revenait à des niveaux normaux à la fin des 8 semaines. Les rats traités à la caféine ne réagissaient pas au même degré à la stimulation par le phénobarbital des enzymes microsomiques que ceux traités uniquement au phénobarbital.

Der Einfluss täglicher Ingestion von Koffein auf die mikrosomalen Enzyme der Rattenleber

Zusammenfassung—Der Einfluss wiederholter Ingestion von Koffein auf mikrosomale Enzyme der Rattenleber wurde untersucht. Bei der Ratte hemmte die Aufnahme von 20–24 mg Koffein/kg/Tag im Trinkwasser für die Dauer von über 2 Wochen die Aktivität von Aminopyrin-*N*-demethylase. Dieser Aktivitätsverlust kam gegen das Ende einer 8-Wochen-Verabreichungsperiode 60% nahe. Die Acetanilidhydroxylierungsaktivität des mikrosomalen Rattenleberenzyms wurde anfänglich angeregt, wobei nach 2 Wochen ein Maximum (145 ± 11% der Kontrolle) erreicht wurde, und kehrte nach 8 Wochen zu normalen Werten zurück. Koffeinbehandelte Ratten reagierten nicht auf die Phenobarbitone-Stimulierung von mikrosomalen Enzymen im gleichen Ausmass wie Ratten, die nur mit Phenobarbitone allein behandelt worden waren.

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Short-term Toxicity Studies on Some Salts and Oxides of Tin in Rats

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Abstract—Rats were fed on diets containing 0·0 (control), 0·03, 0·10, 0·30 or 1·00% of various salts or oxides of tin for periods of either 4 or 13 wk. The criteria examined included mortality, body-weight change, diet utilization, measurements of blood, urine and biochemical parameters, organ weights and gross and micropathology. No adverse effects were noted with any levels of stannous sulphide or oleate or of stannous or stannic oxides. Severe growth retardation, decreased food efficiency, slight anaemia and slight histological changes in the liver were observed with 0·3% or more of stannous chloride, orthophosphate, sulphate, oxalate and tartrate. The findings showed a marked difference in toxicity within the range of compounds studied. The signs of anaemia induced by certain cationic tin compounds included distinctly depressed haemoglobin concentrations, slight decreases in red cell counts and haematocrit value and a decreased level of serum iron. Dietary supplements of iron had a markedly protective effect against tin-induced anaemia, whereas a decrease of dietary iron aggravated the condition. These data suggest that some tin compounds may inhibit haematopoiesis, possibly by interfering with the intestinal absorption of iron. The no-effect level of the active tin salts examined was 0·1%, or 22–33 mg tin/kg/day, in a diet containing a liberal amount of iron. The level may be lower on diets marginal in iron.

INTRODUCTION

Tin occurs in traces in most natural foods but much larger amounts may be present in processed foods and drinks as a result either of corrosion of the metal from plain (unlacquered) cans and tin foil used for packaging or of the addition of tin to foods as a colour preservative. Although the tin content of canned foods and drinks is usually below 100 ppm, much higher levels may be present in certain products after prolonged storage in closed plain cans or after some days of storage in open plain cans (Monier-Williams, 1949). Tin is poorly absorbed from the alimentary tract of man (Calloway & McMullen, 1966) and animals, but small amounts may pass into the tissues (Barnes & Stoner, 1959). Absorption and accumulation of tin from canned foods might explain the relatively high levels of tin in various human tissues in wealthy countries (Schroeder, Balassa & Tipton, 1964).

The toxicology of tin has been extensively reviewed (Barnes & Stoner, 1959; Browning, 1969; Cheftel, 1967). Metallic tin and its salts are considered to be of low oral toxicity, whereas alkyl derivatives are highly toxic. The oral LD_{50}s of stannous chloride in rats and mice were reported to be 700 and 1200 mg/kg respectively (Calvary, 1942). Soluble salts of tin are gastric irritants. This property is held responsible for the acutely toxic effects occasionally observed in man, cats and dogs after the consumption of canned foods containing high levels of tin. The literature on the amounts of tin required to elicit an acute gastro-intestinal response is controversial (Cheftel, 1967). In a recent study in several animal species and human volunteers, signs of illness were observed only after the drinking of fruit

juices containing 1400 ppm tin in amounts of 4–5 ml/kg body weight (Benoy, Hooper & Schneider, 1971).

Canned foods and drinks containing much lower levels of tin (250–700 ppm) have been held responsible for outbreaks of nausea, vomiting and diarrhoea in large numbers of people in several countries (Benoy *et al.* 1971; Cheftel, 1967).

Very little information is available on the toxicity of tin oxides and their salts upon repeated oral administration. When stannous chloride, tartrate or acetate was given to rabbits in amounts of 1 g/animal or more once every 6–10 days, the animals died after 1–2 months with gastritis, degeneration of the liver and kidneys and paralysis of the hind legs (Eckardt, 1909). Rats fed 1% stannous 2-ethyl hexoate in the diet showed severe growth retardation and anaemia, whereas 2% sodium chlorostannate was inactive (Roe, Boyland & Millican, 1965).

The level of 250 ppm tin which is often considered a permissible upper limit in canned foods is not based on toxicological evidence of safety but on the fact that higher levels are rarely found under normal conditions of processing and storage. In view of the daily exposure of man to tin in canned foods and drinks at levels much higher than those naturally occurring in animals and plants, it was deemed desirable to conduct feeding studies in rats with various oxides and salts of tin. Since the corrosion of tin-plate involves the solution of bivalent tin ions which remain in the bivalent state (Cheftel, 1967; Monier-Williams, 1949), stannous compounds were chosen for most of the experiments. The present paper summarizes the results obtained in feeding studies carried out on eight tin compounds for 28 days and on two tin compounds for 90 days.

EXPERIMENTAL

Materials. The tin compounds examined were stannic oxide (pure, E. Merck AG, Darmstadt, Germany), stannous oxide (J. T. Baker Chemical Co., Phillipsburg, N.Y.), stannous orthophosphate, oxalate and sulphide (laboratory reagents, British Drug Houses Ltd., Poole, Dorset), stannous chloride 2 aq. (Analar, BDH Chemicals Ltd., Poole, Dorset), stannous sulphate (Schuchardt AG, Munich, Germany), stannous oleate (K and K Laboratories Inc., Plainview, N.Y.) and stannous tartrate (Sigma Chemical Co., St. Louis, Mo.).

Animals and diets. Male and female weanling rats from the Institute's Wistar-derived colony were housed in groups of five in stainless steel cages with screen bottoms. The diet used for both control and treated groups was the Institute's stock diet, with the following percentage composition: soyabean-oil meal, 10; fish meal, 8; meat scraps, 4; dried whey, 2; yellow maize, 29·05; wheat, 36; grass meal, 3; brewer's yeast, 3; complete B-vitamin mixture, 0·1; vitamin-ADEK preparation, 0·6; bone meal, 0·75; trace mineral salt, 0·5; soyabean oil, 3. This diet was found to contain calcium (0·98%), phosphorus (0·80%), iron (205 ppm), copper (23 ppm), manganese (85 ppm) and zinc (38 ppm). Test diets were prepared by blending the stock diet and the tin compounds in a Stephan cutter. Diets and tap water were provided *ad lib.*

Experimental design and conduct

Subacute (4-wk) feeding studies. The various tin compounds examined were fed to groups of ten male and ten female rats at dietary levels of 0·0 (control), 0·03, 0·10, 0·30 and 1·00% for 28 days. Body weight and food intake were recorded weekly. Haematological examinations were made on all rats on day 27 using blood from the tip of the tail. Measurements were made of haemoglobin concentration, packed cell volume and counts of erythrocytes

and leucocytes. At autopsy the liver, kidneys, heart and spleen were weighed and samples of these organs were processed in the usual way for histological examination.

Subacute (13-wk) feeding studies. Two tin compounds, stannous oxide (SnO) and stannous chloride ($SnCl_2 \cdot 2H_2O$) were examined in 13-wk feeding studies. Each of these compounds was fed to groups of ten male and ten female rats at dietary levels of 0·0 (control), 0·03, 0·10, 0·30 and 1·00% for 90 days. Individual body weights were recorded weekly. The food intake of each group was measured at weekly intervals up to wk 4 and in wk 11–12. Haematological studies were carried out at wk 12 and provided measurements of haemoglobin concentration and haematocrit value, counts of red blood cells and total and differential counts of white blood cells. Additional haematological observations were made in the study on tin chloride. These consisted of haemoglobin readings at wk 2, 4, 6 and 9, and terminal determinations of haptoglobin concentration (Van Ros & Van Sande, 1964), numbers of reticulocytes and the osmotic resistance of the erythrocytes. Serum activities of glutamic–oxalacetic and glutamic–pyruvic transaminases (Reitman & Frankel, 1957) and of alkaline phosphatase (Bessey, Lowry & Brock, 1946) were determined terminally in both experiments. Bilirubin concentrations (Jendrasik & Gròf, 1938) were measured terminally only in the study with tin chloride.

Urine examinations, including appearance, pH, glucose, protein, occult blood, ketones and microscopy of the sediment were conducted upon pooled samples from each group in wk 13.

The rats fed the highest level of tin chloride were sacrificed after 8 wk because of poor condition and high mortality. Organs and tissues were fixed in buffered formalin. In wk 14, the remaining rats were killed by decapitation and examined for gross changes. The heart, kidneys, liver, spleen, brain, gonads, thymus, thyroid and adrenals were weighed and paraffin-wax sections of these and a wide range of other organs were stained with haematoxylin and eosin. Detailed microscopic examinations were performed on all rats fed 1% tin oxide, on those fed the two highest levels of tin chloride and on the controls. In the rats fed the intermediate levels of tin chloride, only the liver, kidneys and stomach were examined.

RESULTS

Subacute (4-wk) feeding studies

Results obtained on eight different tin compounds are summarized in Table 1.

Rats fed stannic oxide, stannous sulphide and stannous oleate at dietary levels up to 1·0% gained weight at a normal rate and showed no abnormalities in the haematological data examined, apart from a statistically significant increase in the haematocrit in male rats fed the highest level of stannous sulphide. The absolute and relative weights and the gross and microscopic appearance of the liver, kidneys, heart and spleen were not altered. There was no evidence of any deleterious effects of these tin compounds at dietary levels up to 7900 ppm tin.

The feeding of tin as the chloride, orthophosphate, sulphate, oxalate and tartrate, however, resulted in considerable growth retardation and distinct indications of anaemia at dietary levels of 0·3 and 1·0% in both sexes. The reduced gain in body weight was accompanied by a decrease in food intake, but food efficiency was also decreased, at least at the 1·0% feeding level. The signs of anaemia included decreased haemoglobin levels, haematocrit values and erythrocyte counts. The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC)

Table 1. *Mean values for weight gain, food efficiency and haematological parameters of rats fed on a stock diet supplemented with various tin compounds at levels of 0–1·0% for 4 wk*

	Males						Females					
Dietary level (%)	Weight gain (g/rat)	Total food intake (g/rat)	Food efficiency†	Hb (g/100 ml)	HC (%)	RBC (10^6/mm^3)	Weight gain (g/rat)	Total food intake (g/rat)	Food efficiency†	Hb (g/100 ml)	HC (%)	RBC (10^6/mm^3)
Stannic oxide												
0·00	125	358	0·35	14·5	46·2	6·2	77	293	0·26	15·2	48·1	7·3
0·03	128	367	0·35	14·3	45·8	6·0	77	293	0·26	15·1	49·7	7·5
0·10	127	361	0·35	14·5	46·6	6·2	76	295	0·26	15·5	49·7	7·6
0·30	125	358	0·35	14·5	46·5	6·2	73	281	0·26	15·3	50·4	7·0
1·00	129	362	0·36	14·2	45·8	6·0	75	286	0·26	15·8	50·4	6·9
Stannous chloride												
0·00	132	369	0·36	14·2	–	6·0	78	294	0·26	15·5	–	7·0
0·03	134	376	0·36	13·9	–	5·9	81	295	0·28	15·4	–	6·9
0·10	127	361	0·35	13·5	–	6·1	76	284	0·27	15·2	–	6·9
0·30	93***	278	0·34	12·8*	–	5·7	68*	254	0·27	14·2**	–	6·6
1·00	47***	190	0·25	12·2**	–	5·9	43***	181	0·24	14·0*	–	6·6
Stannous orthophosphate												
0·00	125	371	0·34	14·4	46·7	6·9	63	271	0·23	15·1	50·6	7·3
0·03	124	367	0·34	14·5	45·3	7·0	73	295	0·27	14·5	49·6	7·3
0·10	121	353	0·34	14·3	45·9	7·0	76	297	0·25	14·6	50·1	7·3
0·30	87***	299	0·29	12·8**	42·4***	6·6	63	260	0·24	14·7	49·3	6·9
1·00	46***	212	0·22	12·3***	40·8***	6·4	37***	183	0·20	11·9***	42·4***	5·9***
Stannous sulphate												
0·00	129	365	0·35	14·3	45·5	6·3	82	293	0·28	14·4	46·8	7·9
0·03	135	381	0·35	14·5	45·9	6·3	87	293	0·30	14·1	48·4*	7·4
0·10	125	361	0·35	14·7	45·9	6·5	88	289	0·30	14·0	47·4	7·3
0·30	108***	312	0·35	13·8	43·6*	6·5	79	255	0·31	13·6*	44·2*	7·1*
1·00	38***	153	0·25	12·1***	38·6***	6·1	46***	182	0·25	12·1***	42·3***	6·9**
Stannous sulphide												
0·00	126	326	0·39	14·3	45·8	6·2	75	251	0·30	14·8	48·2	6·6
0·03	123	326	0·38	14·1	46·0	6·1	75	258	0·29	14·8	47·6	6·5
0·10	113	306	0·37	14·1	45·5	5·8	75	254	0·30	14·2	47·2	6·2
0·30	125	324	0·39	14·3	46·1	6·1	83	274	0·30	14·1	48·8	6·6
1·00	131	344	0·38	14·3	47·4*	6·2	84	279	0·30	14·9	48·0	6·4
Stannous oleate												
0·00	127	373	0·34	14·2	45·7	6·8	84	315	0·27	15·5	48·0	7·0
0·03	134	384	0·34	14·1	44·8	6·9	81	305	0·27	14·8	46·4	6·9
0·10	129	364	0·35	14·3	45·9	6·7	82	302	0·27	14·7*	47·1	7·2
0·30	129	364	0·35	14·1	45·1	6·4	81	306	0·26	15·5	48·5	7·2
1·00	129	366	0·35	14·7	47·4	6·7	81	300	0·27	15·2	48·0	7·1
Stannous oxalate												
0·00	123	343	0·36	14·6	47·7	6·8	80	285	0·28	15·5	49·6	7·2
0·03	133	368	0·36	14·6	48·0	6·6	83	288	0·29	14·8	48·1	7·1
0·10	118	333	0·35	14·5	47·0	6·8	77	274	0·28	15·2	49·7	7·5
0·30	92***	285	0·32	12·4***	42·1***	5·8***	66***	245	0·27	13·0***	42·7***	6·3*
1·00	36***	160	0·22	11·1***	37·9***	5·5***	32***	155	0·21	11·7***	39·7***	6·2**
Stannous tartrate												
0·00	128	351	0·36	13·4	44·6	6·5	74	266	0·28	14·1	46·9	7·3
0·03	132	372	0·35	13·7	45·3	6·5	82	289	0·28	14·1	48·3	7·1
0·10	134	358	0·37	13·6	44·8	6·4	87*	298	0·29	14·2	48·2	7·2
0·30	114**	321	0·36	12·7*	42·5**	6·0	73	262	0·28	13·6	46·2	7·2
1·00	60***	207	0·29	12·0**	41·0***	6·4	50***	199	0·25	11·0**	38·7***	6·4

Hb = Haemoglobin HC = Haematocrit RBC = Red blood cells

†Food efficiency = weight gained (g)/food consumed (g).

Values are the means for groups of ten rats. Those marked with asterisks differ significantly (Wilcoxon's test) from those of controls: *$P<0·05$; **$P<0·01$; ***$P<0·001$.

of the rats fed the highest level of the active tin compounds were generally slightly lower than in the corresponding controls. Although the differences were not considerable (less than 12%) most of them were statistically significant ($P<0·05$).

The white blood cell counts showed the usual wide variation, both among individual rats and among the various groups, but there was no evidence that tin compounds affected the number of leucocytes.

None of the organs weighed (liver, kidneys, heart and spleen) was distinctly enlarged, except the livers of females ingesting tin orthophosphate, the relative weights of which showed a dose-related increase at 0·1 % and above. Slightly decreased liver-to-body weight ratios occurred in groups exhibiting growth retardation.

At autopsy, signs of anaemia (pale eyes and viscera) were observed in animals fed the 1 % dose level of tin chloride, tin oxalate or tin sulphate. In addition, rats fed on diets containing 1 % tin chloride or 1 % tin orthophosphate showed slightly distended small and large intestines.

Microscopic examination revealed distinct changes in the livers of males and females fed 1 % of each of the various tin compounds found to be capable of inducing anaemia and growth retardation. The changes consisted of clearly homogeneous liver cell cytoplasm and a slight but definite oval cell type hyperplasia of bile ducts. Similar hepatic alterations, though of a lesser degree and frequency, were found in rats fed 0·3 % dietary levels of tin chloride, tin oxalate or tin orthophosphate. The histological appearance of the kidneys, heart and spleen was unremarkable in all cases.

Subacute (13-wk) feeding studies

Stannous oxide. The feeding of stannous oxide at various dietary levels up to 1·0 % did not cause any noticeable changes in appearance or behaviour, nor in body-weight gain and food intake. The haematological values and serum-enzyme activities were not altered and there were no significant changes in the composition of the urine. No treatment-related differences were observed in the weights of the organs or in the gross and microscopic appearance of the organs examined. Since none of the criteria examined was affected, no details of this study are presented. Obviously the oxide of bivalent tin is a relatively harmless substance when ingested by rats in their diet (Til, Seinen & de Groot, 1972).

Stannous chloride. The rats fed the diet containing 1 % stannous chloride ate little food and already showed abdominal distension during wk 1. Growth was slow in the first few weeks and stopped completely in males after 4 wk and in females after 6 wk. At wk 8, loss of body weight occurred in seven males and four females and one male died. At wk 9, another three males died. Since several other males were moribund it was decided to discontinue this group and autopsy was performed (see below).

Poor appetite and abdominal distension were observed also at the 0·3 % feeding level during the first 2 wk. However, all rats kept growing, except for one female which lost weight in wk 10 and died in wk 11.

Table 2 summarizes some of the data on mean body weights, consumption of food and water and food efficiency. The growth retardation that occurred at 1·0 and at 0·3 % during the first 2 wk was associated with a decrease in food consumption. Thereafter body-weight gain and food-intake figures at the 0·3 % level returned to normal. Food intake was relatively low also at the 0·1 % level, but only during wk 1. Water intake per unit of food consumed was slightly increased in the 0·3 and 1·0 % groups during wk 1.

The haemoglobin values determined at various stages (Table 3) showed decreased levels in the 1·0 and 0·3 % groups in both sexes from wk 4 onwards. At 1·0 % there was a gradual decrease in haemoglobin content, and at 0·3 %, although a gradual rise did occur, it was distinctly slower in the initial stages than that in the controls.

The terminal haematological findings are presented in Table 4. The mean values of haemoglobin content and cell volume were decreased in both sexes in the group given the 0·3 % diet but the differences from the controls were statistically significant only in males.

Table 2. *Mean body weights, food consumption, food efficiency and water intake of rats fed stannous chloride at dietary levels of 0–0·3% for 13 wk and 1·0% for 8 wk*

Dietary level (%)	Body weight (g) at end of wk					Food consumption (g/rat/day) at wk				Food efficiency† during wk 1–4	Water consumption at wk 1 (ml/g food)
	0	2	4	8	12	1	2	4	12		
Males											
0·00	55	119	189	259	305	9·1	12·6	16·0	16·6	0·37	1·3
0·03	55	121	190	270	315	9·4	12·7	16·0	16·5	0·37	1·4
0·10	54	116	182	253	301	8·4	12·2	15·5	16·4	0·37	1·4
0·30	54	104***	161***	242	291	6·8	10·8	15·0	17·6	0·34	1·7
1·00	54	78***	108***	105***	–‡	4·0	7·6	9·5	–	0·26	1·7
Females											
0·00	53	101	131	163	184	8·3	10·7	10·8	11·2	0·28	1·5
0·03	53	101	134	167	189	8·5	10·6	11·6	11·8	0·28	1·5
0·10	52	101	133	166	186	7·9	10·8	11·5	11·6	0·28	1·4
0·30	53	91**	125	162	189	6·0	9·2	11·5	12·5	0·28	1·9
1·00	52	72***	98***	115***	–‡	3·6	7·1	9·0	–	0·23	2·3

†Food efficiency = weight gained (g)/food consumed (g).
‡All rats sacrificed at wk 9.
Values are the means for groups of ten rats, except for the group of females fed 0·3% which consisted of nine rats at wk 12. Values marked with asterisks differ significantly (Wilcoxon's test) from those of controls: *$P<0·05$; **$P<0·01$; ***$P<0·001$.

Table 3. *Changes in mean haemoglobin levels of rats fed stannous chloride at dietary levels of 0–0·3% for 13 wk and 1·0% for 8 wk*

Dietary level (%)	Haemoglobin content (g/100 ml)							
	In males at end of wk				In females at end of wk			
	2	4	6	9	2	4	6	9
0·00	13·0	14·8	15·8	16·4	14·3	16·1	15·3	16·6
0·03	13·2	14·7	15·6	16·2	14·7	15·9	15·4	17·2*
0·10	13·1	14·4	15·6	16·2	14·1	15·2*	15·7	16·9
0·30	12·4	13·0***	13·7***	15·0**	13·4**	14·2***	14·3**	16·4
1·00	12·8	11·5***	10·8***	–†	14·3	12·9***	12·1***	–†

†Surviving rats were sacrificed in wk 9.
Values are the means for groups of ten rats. Those marked with asterisks differ significantly (Wilcoxon's test) from those of controls: *$P<0·05$; **$P<0·01$; ***$P<0·001$.

The calculated MCV and MCH of males and females in the 0·3% group were only slightly lower than those of the other groups. The feeding of tin had no noticeable effect on the osmotic resistance of erythrocytes or on the numbers of reticulocytes.

Table 4. *Haematological response in rats fed stannous chloride at dietary levels of 0–0·3% for 13 wk*

Dietary level (%)	Hb (g/100 ml)	HC (%)	RBC (10^6/mm^3)	Leucocytes: Total (10^3/mm^3)	Differential (%): L	N	E	M
Males								
0·00	16·3	49·5	8·5	12·0	82·9	15·0	2·1	0·0
0·03	16·1	49·6	8·5	12·7	85·6	11·8	2·6	0·0
0·10	15·9	48·5	8·3	10·7	82·2	14·5	3·3	0·0
0·30	15·3*	47·5*	8·3	11·6	85·2	13·9	0·9	0·0
Females								
0·00	15·9	47·9	8·1	9·1	83·9	13·7	2·4	0·0
0·03	16·3	48·0	8·1	9·5	88·3	10·3	1·4	0·0
0·10	15·9	48·6	7·9	9·5	85·1	12·4	2·5	0·0
0·30	15 2	46·2	7·9	10·3	86·7	11·0	2·3	0·0

Hb = Haemoglobin HC = Haematocrit RBC = Red blood cells
L = Lymphocytes N = Neutrophils E = Eosinophils
M = Monocytes

Values are the means for groups of ten rats except for the group of females fed 0·3%, which consisted of nine rats. Values marked with an asterisk differ significantly (Wilcoxon's test) from those of controls: *$P < 0·05$.

The biochemical studies of terminal blood samples (Table 5) showed a tendency of the alkaline-phosphatase activities to decrease with increasing levels of the tin compound but the difference from the controls was statistically significant ($P < 0·05$) only at the 0·3% feeding level in both sexes. The activities of glutamic–oxalacetic transaminase and glutamic–pyruvic transaminase were comparable in all groups. Terminal blood samples from the 0·3% group and the controls did not differ significantly in bilirubin or in haptoglobin content.

Table 5. *Terminal estimations of blood biochemistry in rats fed stannous chloride at dietary levels of 0–0·3% for 13 wk*

Dietary level (%)	Males: GOT (ImU/ml)	GPT (ImU/ml)	AP (BLU)	Bilirubin (mg %)	Females: GOT (ImU/ml)	GPT (ImU/ml)	AP (BLU)	Bilirubin (mg %)
0·00	165	49·8	7·1	0·12	163	29·9	4·3	0·10
0·03	169	39·1	6·1	–†	164	30·9	3·9	–
0·10	154	37·5	5·9	–	172	30·8	3·7	–
0·30	153	42·5	5·3*	0·13	160	29·1	3·1*	0·12

GOT = Glutamic–oxalacetic transaminase GPT = Glutamic–pyruvic transaminase
AP = Alkaline phosphatase BLU = Bessey–Lowry units

†Not determined.

Values are the means for groups of ten rats, except for the group of females fed 0·3%, which consisted of nine rats. Values marked with an asterisk differ significantly (Wilcoxon's test) from those of controls: *$P < 0·05$.

Terminal urine samples showed normal colour and pH in all the test groups. Tests for reducing substances, ketones and occult blood were negative, and proteinuria was no more marked in the tin groups than in the controls. Microscopic examination of the sediment did not reveal any treatment-related abnormalities.

Mean values of relative organ weights are shown in Table 6. The relative heart weights of males on the tin diets were higher than those in controls but showed no tendency to increase with increasing dietary levels of the tin compound. The relative thymus weight was significantly increased at 0·3% only in females. None of the other organ weights showed statistically significant differences between groups.

Table 6. *Relative organ weights of rats fed stannous chloride at dietary levels of 0–0·3% for 13 wk*

Dietary level (%)	Terminal body weight (g)	Relative organ weight (g organ weight/100 g body weight)								
		Heart	Kidneys	Liver	Spleen	Brain	Gonads	Thymus	Thyroid	Adrenals
					Males					
0·00	317	0·335	0·67	3·53	0·166	0·57	0·86	0·090	0·0048	0·0122
0·03	327	0·360*	0·69	3·38	0·166	0·55	0·86	0·098	0·0053	0·0116
0·10	313	0·350	0·68	3·38	0·154	0·57	0·87	0·095	0·0054	0·0125
0·30	303	0·363*	0·70	3·57	0·188	0·59	0·94	0·093	0·0053	0·0125
					Females					
0·00	189	0·375	0·69	3·17	0·202	0·88	0·030	0·134	0·0073	0·0224
0·03	195	0·378	0·69	3·09	0·183	0·86	0·028	0·140	0·0067	0·0228
0·10	194	0·379	0·67	3·02	0·191	0·87	0·030	0·135	0·0071	0·0250
0·30	196	0·358	0·64	3·09	0·209	0·86	0·030	0·164*	0·0076	0·0220

Values are the means for groups of ten rats, except for the group of females fed 0·3%, which consisted of nine rats. Values marked with an asterisk differ significantly (Student's *t* test) from those of controls: $*P < 0·05$.

At autopsy, the rats of the 1% group, in which treatment had to be terminated prematurely, showed distended intestines, slight ascites, small oedematous pancreases and greyish-brown livers. Histopathological study of these animals revealed moderate testicular degeneration, severe pancreatic atrophy, a spongy state of the white matter of the brain, acute bronchopneumonia, enteritis and distinct liver changes, mainly characterized by a homogeneous appearance of the liver cell cytoplasm and a mild proliferation (oval cell type) of the bile-duct epithelium.

Terminal gross autopsy findings were essentially negative. Minor histopathological alterations that could be related to treatment were noticed only in the livers of a few males and females of the 0·3% group, in which the cytoplasm of the hepatocytes was homogeneous and the bile-duct epithelium proliferated. Additional randomly distributed microscopic pathology unrelated to treatment included early signs of chronic respiratory disease, slight focal myocarditis, foci of reticulo-endothelial cells in the liver, "morphological activation" of the thyroid, Trichosomoides parasites in the urinary tract and large proteinaceous droplets within the cytoplasm of renal tubular epithelial cells. Calcareous deposits in the intercorticomedullary area of the kidneys were not found at all in females at dietary levels of 0·1% and above.

DISCUSSION

The results obtained in these studies show a marked difference in the toxicity of the various tin compounds examined. The feeding of stannous sulphide and stannous oleate, as well as of the oxides of bi- and tetravalent tin at levels up to 1% in the diet did not evoke any noticeable effects, whereas stannous chloride, orthophosphate, sulphate, oxalate and tartrate at levels of 0·3 and 1·0% resulted in growth retardation, anaemia and histological changes in the liver. Roe *et al.* (1965) observed poor growth and anaemia in rats fed 1·0% stannous 2-ethyl hexoate, but not in those fed 2·0% sodium chlorostannate. These differences in response to different tin compounds suggest that insoluble tin compounds are relatively harmless substances whereas cationic tin compounds soluble in water or dilute acid may be toxic at dietary levels above 0·1%.

The controversial reports in the literature on the levels of tin in canned foods that may cause acute intoxication in man may be due to the presence of different tin compounds in the various products concerned.

The growth-retarding effect of some tin salts, which was associated with a decrease in food consumption, cannot be explained completely by poor acceptability of the test diets because food efficiency was also impaired. This conclusion was supported by the results of a 4-wk paired-feeding experiment, which showed lower body weights and haemoglobin levels in animals on a 1% stannous chloride diet than in those on the control diet, in spite of equal food consumption (M. Th. Spanjers and A. P. de Groot, unpublished observations, 1971).

Some of the marked pathological changes noticed at the 1% dose level in the 13-wk study on tin chloride may have been at least partly due to the concomitant reduction of food intake and retardation of growth or even loss of body weight. This may also hold true for the liver changes (homogeneous cytoplasm of hepatocytes and bile-duct hyperplasia) observed with 0·3 and 1% dietary levels of several tin compounds, particularly since compact cytoplasm of hepatic parenchymal cells is a well-known feature of partial starvation (Herdson, Garvin & Jennings, 1964).

The absence of calcareous deposits in the kidneys of female rats fed high levels of stannous chloride for 13 wk was remarkable. Renal calcification not accompanied by impairment of renal function has been described in female Wistar rats (Cousins & Geary, 1966) and is a relatively common finding in the Wistar-derived female rats from which those used in the present study were derived. Both hormones and dietary factors are known to be implicated in the aetiology of nephrocalcinosis (Komárková, Záhoř & Czabanová, 1969; MacKay & Oliver, 1935; Meyer & Forbes, 1967). A decrease in the severity of renal calcinosis has been observed repeatedly in our rats upon dietary administration of test compounds varying widely in chemical structure.

The slight anaemia observed upon feeding certain tin salts was characterized by a distinct decrease in haemoglobin levels and haematocrit values and a slight decrease in erythrocyte counts. The MCV, MCH and MCHC were generally slightly decreased only at 1·0%.

A determination of serum iron and iron-binding capacity (by a micromodification of the method of Schade, Oyama, Reinhart & Miller, 1954) in decapitation-blood of rats which had been fed on stock diet with or without a supplement of 0·3% stannous chloride for 6 wk, revealed a decreased iron content (42 and 9% respectively) in males and females of the test group, whereas the total iron-binding capacity was not affected (N. A. Pikaar and H. P. Til, unpublished observations, 1971). These haematological findings are indicative of an iron-deficiency anaemia. It may be mentioned, however, that the amount of iron in

the basal diet (205 ppm) was far above the 25 ppm required by the growing rat (National Academy of Sciences-National Research Council, 1962). Nevertheless, distinctly improved haemoglobin levels occurred at 1·0% stannous chloride by further enrichment of the test diet with 250 ppm iron as 0·1% $FeSO_4 \cdot 4H_2O$. However, growth rate was not improved and the haemoglobin levels remained below normal (A. P. de Groot and H. P. Til, unpublished observations, 1971). These results suggest that cationic tin may interfere with absorption and utilization of dietary iron.

Subsequent observations have shown that feeding of stannous chloride caused growth depression and decreased haemoglobin contents, not only in animals on a stock diet, but also in those on a semi-purified casein diet and on a modified stock diet containing skim-milk powder instead of the high-iron ingredients, fish meal and meat scraps. The results suggest an inverse relationship between the extent of the tin effects and the amount of iron in the diets. In animals on a semi-purified casein diet containing only 25 ppm iron, mainly as ferrous sulphate, decreased growth and haemoglobin levels occurred even with 0·1% stannous chloride after a feeding period of 4 wk (A. P. de Groot and H. P. Til, unpublished observations, 1971).

The percentage difference in haemoglobin levels between the group fed 0·3% stannous chloride and the controls in the 13-wk study was greater at wk 4 and 6 than at wk 9 and 13. No increased haematopoietic activity was observed, however, in the microscopic examination of the spleen at wk 13. Since the food intake per unit of body weight in young rats decreases with increasing age, the less severe anaemia in animals on the tin diet in the later stages of the feeding period is attributed to a decrease in tin consumption per unit of body weight rather than to an adaptive increase in haemoglobin formation.

The highest level of the active tin compounds that did not affect any of the criteria examined in the present studies was 0·1% in diets containing liberal amounts of iron. Therefore, 0·1% could be considered a no-effect level of these compounds, a level which is equivalent to 450–650 ppm tin in the diet. This range is equivalent to an intake of 22–33 mg tin/kg body weight/day in a 90-day study. The results obtained on a diet marginal in iron suggest, however, that the no-effect level may be lower in animals maintained on diets containing less iron.

The safe level of tin is remarkably low for a substance known to be poorly absorbed. This might be explained by the assumption that tin exerts its effect in the intestinal lumen by inhibiting the absorption of dietary iron.

The significance of the present results for human health cannot be assessed owing to a lack of sufficient knowledge of the type and toxicity of the tin compounds present in canned foods and drinks. The aggravation of the tin effects by restricted intake of iron is another complicating factor.

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Etude sur le rat de la toxicité à court terme de certains sels et oxydes d'étain

Résumé—On a soumis des rats pendant des périodes de 4 ou de 13 semaines à des régimes comportant 0,0 (témoins), 0,03, 0,10, 0,30 ou 1,00% de différents sels ou oxydes d'étain et on a étudié les critères suivants: mortalité, modifications du poids vif, utilisation alimentaire, données hématologiques, paramètres urologiques et biochimiques, poids des organes, pathologie générale et micropathologie. On n'a observé aucun effet nocif aux différents taux de sulfure, d'oléate et d'oxyde stanneux et d'oxyde stannique. De graves retards de croissance, une diminution du rendement alimentaire, une légère anémie et des altérations histologiques mineures du foie ont été observés chez les animaux dont le régime comportait 0,3% ou plus de chlorure, d'orthophosphate, de sulfate, d'oxalate ou de tartrate stanneux. Ces constatations ont révélé de nettes différences de toxicité dans la gamme des composés étudiés. Parmi les indices d'anémie provoquée par certains composés cationiques de l'étain figuraient de nettes diminutions du taux d'hémoglobine, de légères diminutions du nombre de globules rouges et de l'hématocrite et des baisses du taux de fer sérique. Des suppléments de fer ajoutés au régime ont exercé un effet nettement protecteur contre l'anémie imputable à l'étain, tandis que cette anémie s'aggravait quand on diminuait la teneur en fer du régime. Ces observations suggèrent que certains composés du l'étain peuvent inhiber l'hématopoièse. peut-être en influant sur l'absorption intestinale du fer. Le seuil d'indifférence des sels d'étain actifs étudiés se situait à 0,1%, soit 22–23 mg d'étain/kg/jour lorsque le régime était assez riche en fer. Il peut être moins élevé quand le régime alimentaire est pauvre en fer.

Kurzzeitige Toxizitätsuntersuchungen mit einigen Zinnsalzen und -oxyden an Ratten

Zusammenfassung—An Ratten wurde Futter 4 oder 13 Wochen lang verfüttert, das 0 (Kontrolle), 0,03, 0,10, 0,30 oder 1,00% verschiedener Zinnsalz oder -oxyde enthielt. Zu den untersuchten Kriterien gehörten Mortalität, Körpergewichtsänderungen, Futterverwertung, Messungen von Blut-, Urin- und biochemischen Parametern, Organgewichte und Makro- und Mikropathologie. Keine nachteiligen Wirkungen wurden bei irgendwelchen Konzentrationen von Zinn(II)-sulfid oder -oleat oder Zinn(II)- oder Zinn(IV)-oxyd beobachtet. Starke Wachstumsverzögerung, verminderte Futterverwertung, leichte Anämie und leichte histologische Veränderungen in der Leber wurden bei 0,3% oder darüber von Zinn(II)-chlorid, -orthophosphat, -sulfat. -oxalat und -tartrat beobachtet. Die Ergebnisse zeigten einen deutlichen Unterschied der Toxizität innerhalb der Reihe der untersuchten Verbindungen. Die Zeichen von Anämie, die von bestimmten kationischen Zinnverbindungen induziert wurden, waren deutlich herabgesetzte Hämoglobinkonzentrationen, leichte Verminderungen der Erythrocytenzahl und des Hämatokritwertes und eine verminderte Konzentration von Serumeisen. Ergänzung des Futters hinsichtlich des Eisens hatte eine deutliche Schutzwirkung gegen durch Zinn induzierte Anämie, während eine Verminderung des Eisens im Futter den Zustand verschlimmerte. Diese Daten legen nahe, dass manche Zinnverbindungen die Hämatopoese hemmen können, vielleicht durch Störung der Eisenabsorption im Darm. Die wirkungsfreie Konzentration der untersuchten aktiven Zinnsalze betrug 0,1% oder 22–33 mg Zinn/kg/Tag in einem Futter, das eine grössere Menge Eisen enthielt. In Futter mit sehr geringem Eisengehalt kann die Konzentration niedriger sein.

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Teratology Studies in Mice with 2-*sec*-Butyl-4,6-dinitrophenol (Dinoseb)

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Abstract—2-*sec*-Butyl-4,6-dinitrophenol (dinoseb) was administered daily to groups of pregnant Swiss–Webster mice during organogenesis, on days 8–16, 10–12 or 14–16 of gestation, by ip or sc injection or by oral intubation. Caesarean-delivered foetuses were examined for gross, soft-tissue and skeletal anomalies. Administration of dinoseb in daily doses of 5 mg/kg ip, 10 mg/kg sc or 20 mg/kg orally on days 8–16 had no adverse effect on embryonic or foetal growth and development. Doses of 17·7 mg/kg/day given ip on days 10–12 or 14–16 were toxic to the dams (hyperthermia) and embryos (increased resorption, reduced foetal size), but teratogenic effects (e.g. imperforate anus, acaudia) resulted from treatment only on days 10–12. Dinoseb given in sc doses of 17·7 mg/kg/day on days 14–16 showed maternal toxicity and embryotoxicity but not teratogenicity, while oral doses of 20 or 32 mg/kg/day showed maternal toxicity but essentially no embryotoxicity or teratogenicity. Dinoseb was concluded to have a low potential for the induction of terata in mice.

INTRODUCTION

2-*sec*-Butyl-4,6-dinitrophenol (dinoseb) is the active constituent of Premerge® herbicide, a formulation of The Dow Chemical Company containing the ethanol and isopropanolamine salts of dinoseb and used to control many seedling weeds and grasses. The widespread use of this formulation in the USA and other countries raises questions concerning its possible perinatal toxicity to man and animals. No studies on the teratogenicity of dinoseb are known, although the injection of 2,4-dinitrophenol (DNP) into the egg of the chick embryo results in cataract induction (Feldman, Ferguson & Couch, 1959). The purpose of the present study was to determine the teratogenic potential of dinoseb administered to mice by several routes, and the results obtained indicate that the compound has a low potential for the induction of terata in mice. Comparison of dinoseb with its parent structure, DNP, indicated a unique action of the *sec*-butyl derivative, since DNP was not teratogenic in mice.

EXPERIMENTAL

Animals. Swiss–Webster mice (from Spartan Research Animals, Haslett, Mich.) were housed in groups of ten in stainless-steel cages and allowed food and water *ad lib.* A 12-hr dark–light cycle was maintained (lights on at 8 a.m.). Pregnant mice were obtained by daily pen breeding from 8 to 9 a.m. (one male/five females). Mice with vaginal plugs were identified as being at day 1 of gestation and were isolated into treatment groups.

Treatments. Dinoseb (The Dow Chemical Company, lot no. 7200206, 1966) was administered daily, ip, sc or by oral intubation, to groups of pregnant mice either throughout organogenesis (days 8–16 of gestation), during early organogenesis (days 10–12), or during

late organogenesis (days 14–16). The dinoseb dosage levels were selected as those expected to produce (1) maternal toxicity, (2) no maternal toxicity and (3) no embryotoxic or teratogenic effect. The treatment schedule is outlined in Table 1. DNP (Eastman Organic

TABLE 1. *Schedule of treatment of pregnant mice with dinoseb during organogenesis and number of deaths during treatment*

Dose level (mg/kg/day)	Mortality of dams (no. dead/no. treated) following treatment by		
	Ip injection	Sc injection	Oral intubation
	On days 10–12		
0	0/8	0/7	0/6
10	0/11	0/7	–
12·5	0/7	–	–
15·8	0/7	–	–
17·7	1/14	0/7	–
18·8	5/11	–	–
20	4/4	–	0/8
32	–	–	0/8
50	–	–	6/8
	On days 14–16		
0	0/7	0/8	–
10	–	0/8	–
12·5	0/7	–	–
17·7	4/12	1/8	–
20	–	–	0/14
32	–	–	2/11
	On days 8–16		
0	–	0/5	–
5	0/7	–	–
10	–	0/8	–
17·7	–	0/8	–
20	–	–	1/8
32	–	–	2/9

– Not included in test schedule.

Chemicals) was administered on days 10–12 to other groups of pregnant mice in ip doses of 7·7 and 13·6 mg/kg and oral doses of 25·5 and 38·3 mg/kg (equimolar to dinoseb doses of 10, 17·7, 32 and 50 mg/kg, respectively). Freshly prepared aqueous solutions of dinoseb and DNP (dissolved in 1 N-NaOH and titrated to neutral pH with 1 N-HCl) were prepared in such concentrations that 10 ml/kg body weight gave the appropriate dose for the routes of administration and gestation times mentioned above. Control animals received water.

Foetal examinations. Caesarean section was performed on day 19 of gestation and the number and position of live, dead and resorbed foetuses was noted. Foetuses were removed by severing the umbilical vessels with a cautery knife to prevent loss of foetal blood, dried on absorbent paper and weighed and the foetal crown–rump distance was measured with a vernier caliper. Individual foetuses were examined for external anomalies, and each litter was divided into two sub-groups for further examination. One was fixed in Bouin's solution

for 2 wk, after which the tissues were sectioned by hand into 2–3 mm sections and examined under a dissecting microscope for soft-tissue anomalies by the method of Wilson (1965). The other sub-group was fixed in 95% ethanol and the foetal skeletons were subsequently stained with Alizarin Red S. Stained skeletons were examined under a dissecting microscope to assess skeletal anomalies.

Statistics. Statistical analysis of measured parameters was by analysis of variance (completely random design). Treatment differences were detected by the least significant difference test (Steel & Torrie, 1960). The level of significance was chosen as $P < 0{\cdot}05$.

RESULTS

Intraperitoneal administration of dinoseb

In ip doses of 17·7–20·0 mg/kg/day, dinoseb produced signs of toxicity (hyperthermia) in the dams and some maternal deaths (Table 1). Surviving dams bore litters that were smaller in number and size than those of controls regardless of the gestational stage at the time of administration (Table 2). Doses of dinoseb that were sub-toxic to the dams (10–15·8 mg/kg/day) did not affect resorption rate or foetal size when given during early organogenesis (days 10–12) but were foetotoxic when given later (days 14–16). Dinoseb given throughout organogenesis (days 8–16) in ip doses of 5 mg/kg/day had no adverse effect on embryonic or foetal growth and development (Table 2).

Statistically significant incidences of gross, soft-tissue and skeletal anomalies were induced in the offspring of dams given dinoseb ip during early organogenesis (Table 3). It should be noted that the effects were considered to be significant relatively frequently in the group given 17·7 mg/kg/day in which only 1/14 dams died, but were rarely significant in the group given 18·8 mg/kg/day ip in which 5/11 died. No gross, soft-tissue or skeletal anomalies were detected in foetuses from dams given 5 mg/kg/day ip throughout gestation (days 8–16), nor in foetuses from dams given any dose level on gestational days 14–16. On the basis of these parameters, the 5 mg/kg/day ip dose of dinoseb was considered to have produced no effect.

Subcutaneous administration of dinoseb

Dinoseb administered sc in doses of 17·7 mg/kg/day produced overt maternal toxicity similar to that observed with ip administration. At this dosage, dinoseb significantly affected foetal survival and size, but only when administered during late organogenesis (days 14–16) or throughout organogenesis, days 8–16 (Table 4).

Administered sc, the herbicide only produced statistically significant gross or soft-tissue anomalies when given in doses of 17·7 mg/kg/day on days 14–16. This treatment resulted in an incidence of cleft palate of 10·6 $\pm$ 6·8% (mean % response/litter $\pm$ SEM). The actual significance of cleft-palate induction by dinoseb is uncertain because this anomaly did not have statistical significance in any ip-treated group or in the sc-treated group given 17·7 mg/kg/day throughout days 8–16. Skeletal examination showed an increased incidence of certain anomalies in the offspring of dams treated with 17·7 mg dinoseb/kg/day on gestational days 10–12 and 8–16 (Table 5), a finding which indicated foetotoxicity as well as maternal toxicity. Examination of foetuses from the dams given 10 mg/kg/day revealed no effect on the parameters studied (Table 5).

Table 2. *Resorption rate and foetal size in pregnant mice given dinoseb by ip injection during various stages of organogenesis*

Dose level (mg/kg/day)	No. of pregnant mice		No. of implantations†	No. of foetuses†	Resorptions† (%)	Foetal body weight† (g)	Foetal crown–rump length† (cm)
	Treated	Surviving					
			Days 10–12‡				
0	8	8	13 ± 1	13 ± 1	3·5 ± 1·3	1·357 ± 0·036	2·6 ± 0
10	11	11	13 ± 1	12 ± 1	11·3 ± 4·0	1·334 ± 0·026	2·6 ± 0
12·5	7	7	13 ± 1	11 ± 1	8·1 ± 2·4	1·250 ± 0·047	2·5 ± 0
15·8	7	7	12 ± 1	11 ± 1	8·1 ± 2·7	1·283 ± 0·028	2·5 ± 0
17·7	14	13	12 ± 0	8 ± 1*	33·9 ± 7·4*	1·060 ± 0·036*	2·5 ± 0
18·8	11	6	13 ± 1	12 ± 1	4·5 ± 2·1	1·138 ± 0·024*	2·3 ± 0*
20·0	4	0	–	–	–	–	–
			Days 14–16‡				
0	7	7	13 ± 1	12 ± 1	8·7 ± 4·5	1·355 ± 0·030	2·5 ± 0
12·5	7	7	13 ± 1	10 ± 2	36·7 ± 11·1*	1·169 ± 0·036*	2·5 ± 1
17·7	12	8	14 ± 1	4 ± 2*	71·2 ± 11·6*	1·101 ± 0·040*	2·4 ± 0*
			Days 8–16‡				
5	7	7	12 ± 1	11 ± 1	8·8 ± 3·2	1·270 ± 0·036	2·6 ± 0

†Mean response/litter ± SEM.
‡Days of gestation on which treatment was given.
Values marked with an asterisk differ significantly from those of controls: *$P < 0·05$.

Table 3. *Gross, soft-tissue and skeletal anomalies in offspring of pregnant mice given dinoseb by ip injection during early organogenesis (days 10–12)*

Anomalies	No. of litters examined....	Incidence of anomalies following treatment at dose levels (mg/kg/day) of					
		0	10·0	12·5	15·8	17·7	18·8
		8	11	7	7	13†	6
Gross							
Oligodactyly		0	0	1·3 ± 1·3	0	35·9 ± 9·8*	15·0 ± 13·1
Imperforate anus		0	0	0	2·9 ± 2·9	19·2 ± 8·7*	6·7 ± 6·7
Acaudia		0	0	0	2·9 ± 2·9	17·5 ± 7·7*	1·7 ± 1·7
Microcaudia		0	0	0	0	25·3 ± 8·0*	7·8 ± 6·5
Brachygnathia		0	0	0	0	2·5 ± 2·5	0
Amelia		0	0	0	0	16·3 ± 8·7*	0
Micromelia		0	0	0	0	5·5 ± 3·9	8·3 ± 8·3
Open eyes		0	3·6 ± 3·0	1·1 ± 1·1	8·3 ± 5·9	0	0
Soft-tissue							
Internal hydrocephalus		14·6 ± 8·4	92·0 ± 3·0*	97·1 ± 2·9*	55·9 ± 11·5*	76·2 ± 8·6*	20·5 ± 5·5
Hydronephrosis		5·4 ± 5·4	15·6 ± 4·5	23·4 ± 6·3	18·9 ± 10·2	31·6 ± 8·1*	18·5 ± 5·7
Cleft palate		1·8 ± 1·8	1·3 ± 1·3	0	0	0	0
Enlarged bladder		3·6 ± 2·4	0	0	9·4 ± 4·6	1·4 ± 1·4	8·2 ± 5·3
Adrenal agenesis		0	0	0	0	16·2 ± 9·9	10·0 ± 10·0
Skeletal							
Ribs: supernumerary		27·2 ± 12·2	13·1 ± 6·8	23·4 ± 13·6	20·7 ± 6·1	26·0 ± 8·9	24·7 ± 5·9
fused		0	0	22·1 ± 9·4	14·3 ± 14·3	54·5 ± 11·6*	37·2 ± 13·9*
absent		0	0	0	0	12·5 ± 7·7*	0
Sternebrae: fused		0	0	0	0	15·6 ± 7·8*	0
absent or not ossified		6·6 ± 4·3	11·2 ± 3·6	19·7 ± 7·2	34·0 ± 13·9	56·5 ± 9·8*	25·8 ± 13·6
Vertebrae: fused		0	0	4·4 ± 2·9	2·0 ± 2·0	76·2 ± 8·1*	37·2 ± 13·9*
not ossified		0	0	0	0	19·5 ± 8·8*	0
absent		0	0	0	7·1 ± 7·1	30·8 ± 10·5*	0
Long bones absent or not ossified		0	0	4·8 ± 3·1	9·1 ± 7·1	41·3 ± 10·7*	13·3 ± 9·9

†Only 12 litters examined for soft-tissue anomalies.
Values are the mean percentage responses/litter ± SEM and those marked with asterisks differ significantly from those of controls: $*P < 0.05$.

Table 4. *Resorption rate and foetal size in pregnant mice given dinoseb by sc injection during organogenesis*

Dose level (mg/kg/day)	No. of pregnant mice		No. of implantations†	No. of foetuses†	Resorptions† (%)	Foetal body weight† (g)	Foetal crown–rump length† (cm)
	Treated	Surviving					
			Days 10–12‡				
0	7	7	11 ± 1	11 ± 1	4·4 ± 2·2	1·339 ± 0·032	2·6 ± 0
10	7	7	14 ± 1	13 ± 1	5·2 ± 3·0	1·348 ± 0·033	2·6 ± 0
17·7	7	7	13 ± 1	12 ± 1	10·0 ± 5·4	1·224 ± 0·040	2·6 ± 0
			Days 14–16‡				
0	8	8	13 ± 0	12 ± 0	5·6 ± 2·4	1·373 ± 0·030	2·6 ± 0
10	8	8	14 ± 1	12 ± 1	14·0 ± 6·4	1·309 ± 0·041	2·6 ± 0
17·7	8	7	14 ± 1	7 ± 2*	46·4 ± 15·3*	1·209 ± 0·065*	2·5 ± 0*
			Days 8–16‡				
0	5	5	13 ± 1	12 ± 1	6·4 ± 1·7	1·304 ± 0·042	2·6 ± 0
10	8	8	14 ± 1	13 ± 0	7·2 ± 2·8	1·315 ± 0·026	2·6 ± 0
17·7	8	8	12 ± 1	10 ± 1	14·0 ± 7·4	1·083 ± 0·039*	2·4 ± 0*

†Mean response/litter ± SEM.
‡Days of gestation on which treatment was given.
Values marked with an asterisk differ significantly from those of controls: *$P < 0·05$.

Table 5. *Skeletal anomalies in offspring of pregnant mice given dinoseb by sc injection during organogenesis*

Dose level (mg/kg/day)	No. of litters examined	Incidence of skeletal anomalies (mean percentage response/litter ± SEM)						
		Ribs		Sternebrae		Vertebrae		
		Supernumerary	Fused	Absent or not ossified	Fused	Supernumerary	Fused	Absent
				Days 10–12†				
0	7	26·1 ± 10·2	0	2·4 ± 2·4	0	0	0	0
10	7	14·3 ± 11·7	4·7 ± 4·7	22·8 ± 8·4	0	0	0	0
17·7	7	39·0 ± 8·8	33·0 ± 10·7*	35·6 ± 15·0*	2·4 ± 2·4	6·0 ± 4·0	50·3 ± 9·8*	2·9 ± 2·9
				Days 14–16†				
0	8	20·5 ± 10·8	0	16·0 ± 5·2	0	0	0	0
10	8	7·2 ± 4·8	0	11·6 ± 6·0	0	1·6 ± 1·6	2·5 ± 2·5	0
17·7	5	19·4 ± 9·8	0	31·2 ± 9·5	0	0	0	0
				Days 8–16†				
0	5	28·0 ± 9·9	0	2·8 ± 2·8	0	0	0	0
10	8	44·1 ± 12·0	0	16·4 ± 5·0	0	8·0 ± 6·2	0	0
17·7	8	88·4 ± 6·6*	8·8 ± 4·4	39·5 ± 6·6*	6·6 ± 4·3	26·2 ± 11·8*	7·1 ± 5·4	0

†Days of gestation on which treatment was given.
Values marked with an asterisk differ significantly from those of controls: $*P<0.05$.

Table 6. *Resorption rate and foetal size in pregnant mice given dinoseb by oral intubation during organogenesis*

Dose level (mg/kg/day)	No. of pregnant mice		No. of implantations†	No. of foetuses†	Resorptions† (%)	Foetal body weight† (g)	Foetal crown–rump length† (cm)
	Treated	Surviving					
			Days 10–12‡				
0	6	6	10 ± 2	9 ± 2	4·7 ± 3·3	1·315 ± 0·042	2·6 ± 0
20	8	8	11 ± 1	11 ± 1	3·5 ± 1·8	1·296 ± 0·030	2·6 ± 0
32	8	8	14 ± 1	13 ± 1	4·4 ± 1·3	1·326 ± 0·026	2·6 ± 0
50	8	2	13 ± 0	12 ± 0	4·0 ± 4·0	1·268 ± 0·039	2·5 ± 0
			Days 14–16‡				
20	14	14	13 ± 0	11 ± 0	8·0 ± 2·7	1·261 ± 0·022	2·5 ± 0
32	11	9	12 ± 1	11 ± 1	7·4 ± 2·8	1·214 ± 0·033	2·5 ± 0
			Days 8–16‡				
20	8	7	14 ± 1	14 ± 1	3·1 ± 2·1	1·296 ± 0·033	2·6 ± 0
32	9	7	12 ± 1	11 ± 1	9·0 ± 2·2	1·202 ± 0·057	2·4 ± 0*

†Mean response/litter ± SEM.
‡Days of gestation on which treatment was given.
Values marked with an asterisk differ significantly from those of controls: $*P < 0{\cdot}05$.

Oral administration of dinoseb

Dinoseb given orally in the doses used in the ip and sc studies produced no overt maternal toxicity, so higher dose levels were selected. Doses of 50 mg/kg/day given on days 10–12 of gestation approximated to an LD_{75} (Table 6) for the maternal animal but had no effect on foetal survival or size. Likewise doses of 20 or 32 mg/kg/day administered orally during early or late organogenesis or throughout this whole period caused maternal toxicity but did not significantly affect foetal survival or size, except in mice given 32 mg/kg/day on days 8–16, in which the foetal crown–rump distance was reduced to 2·4 cm compared with 2·6 cm in controls.

Oral administration of dinoseb produced no statistically significant gross or soft-tissue anomalies at any time. Statistically significant incidences of skeletal anomalies were observed in some of the groups (Table 7) but only at dose levels that were lethal to some dams (Table

Table 7. *Skeletal anomalies in offspring of pregnant mice given dinoseb by oral intubation during organogenesis*

		Incidence of skeletal anomalies (mean percentage response/litter ± SEM)		
		Ribs	Sternebrae	Vertebrae
Dose level (mg/kg/day)	No. of litters examined	Supernumerary	Absent or not ossified	Supernumerary
		Days 10–12†		
0	6	19·0 ± 16·4	16·7 ± 16·7	0
20	8	28·8 ± 11·8	16·5 ± 7·1	2·5 ± 2·5
32	8	6·2 ± 4·4	3·5 ± 3·5	0
50	2	7·0 ± 7·0	7·0 ± 7·0	0
		Days 14–16†		
20	14	7·4 ± 3·8	13·2 ± 5·5	0
32	9	26·9 ± 9·0	46·8 ± 10·2*	0
		Days 8–16†		
20	7	65·0 ± 10·3*	15·9 ± 4·6	5·6 ± 2·7
32	7	92·1 ± 5·6*	21·1 ± 11·6	43·0 ± 13·7*

† Days of gestation on which treatment was given.
Values marked with an asterisk differ significantly from those of controls: * $P < 0{\cdot}05$.

1). High incidences of the same anomalies in controls in specific comparisons precludes any assessment of the actual significance of these findings.

At the lowest treatment level (20 mg dinoseb/kg/day), no pregnant mice died when treated orally on days 10–12 or days 14–16. However, 1/8 mice died (Table 6) when treatment was given throughout the entire 9-day period (days 8–16). Even so, the foetuses from dams treated at this level showed no effects of practical significance (Tables 6 & 7).

Administration of DNP

The ip or oral administration of DNP to mice during early organogenesis produced no morphological defects but high doses were embryotoxic (Table 8). The higher DNP dosages

Table 8. *Effect on resorption rate and foetal size of 2,4-dinitrophenol (DNP) administered to mice during early organogenesis (days 10–12 of gestation)*

Dose level of DNP (mg/kg/day) and route	Molar equivalents of dinoseb (mg/kg)	No. of pregnant mice treated	No. of implantations	No. of foetuses†	Resorptions† (%)	Foetal body weight† (g)	Foetal crown–rump length† (cm)
0	—	9	13 ± 1	12 ± 1	4·4 ± 2·0	1·409 ± 0·036	2·7 ± 0
7·7 (ip)	10	8	13 ± 1	12 ± 0	5·6 ± 1·8	1·383 ± 0·034	2·6 ± 0
13·6 (ip)	17·7	8	13 ± 1	11 ± 1	14·1 ± 7·1	1·307 ± 0·038*	2·6 ± 0*
25·5 (oral)	32	7	14 ± 1	13 ± 1	9·6 ± 3·0	1·351 ± 0·029	2·7 ± 0
38·3 (oral)	50	7	13 ± 1	12 ± 1	6·1 ± 2·9	1·366 ± 0·037	2·6 ± 0

†Mean response/litter ± SEM.
Values marked with an asterisk differ significantly from those of controls: *$P < 0{\cdot}05$.

also produced overt toxic signs (hyperexcitability and hyperthermia) in the dams, but were not lethal.

DISCUSSION

The embryotoxic and teratogenic effects of dinoseb in mice were dependent on the route of administration and dose. Such effects were induced when dinoseb was administered ip but not sc or orally, even though maternal toxicity was very similar by all routes. Teratogenic doses were toxic to the dams and were in the lethal range. Orally, dinoseb produced some terata, but the anomalies were different from those produced by ip injection of the herbicide. The anomalies produced by dinoseb ranged in severity from imperforate anus and acaudia (with ip administration) to non-ossification of bone (associated with all routes). Schwetz, Sparschu & Gehring (1971), observing similar delayed ossifications in the foetuses of rats treated with 2,4-D, noted the transient nature of these bone anomalies. Perhaps the skeletal anomalies induced by dinoseb are also of a transient nature.

The present study demonstrated apparent no-effect dose levels for teratogenicity and embryotoxicity for dinoseb. The no-effect level for administration throughout organogenesis (days 8–16) was 5 mg/kg/day ip, 10 mg/kg/day sc or 20 mg/kg/day orally. Therefore, dinoseb was administered throughout organogenesis in mice at doses and by routes of administration that produced no detectable teratogenic effect. As noted above, positive findings were present only at dose levels associated with maternal toxicity.

In his proposed guidelines for the design of studies intended to predict the degree of safety of a potentially toxic substance for man, Weil (1970) suggested the use of species that metabolize the material similarly to man, the use of several dose levels because effects are dose-related, reliance on high doses only to define mechanism of action and reliance on low doses to define a level below which the effect will not occur, application of statistics only to the units that are randomly distributed, and administration of the substance to test animals by the same route or routes as those by which man will be exposed. The importance of these suggested guidelines was apparent in this study. Although the metabolism of dinoseb is unknown in man and only partially known in other species, it appears that man and other mammalian species, with the exception of ruminants, may metabolize this herbicide similarly (Ernst & Bär, 1964; Fröslie & Karlog, 1970).

In the present study, dinoseb was found to have a dose-response effect by all routes of administration, producing terata only at high doses while a lower dose level could be defined below which teratogenicity did not occur. Statistics were applied only to the units that were randomly distributed (pregnant females).

The most important guideline with regard to the determination of dinoseb teratogenicity appears to be the route of administration. The compound was teratogenic by the ip route but less so by the sc route, and when given orally it was only slightly teratogenic and/or embryotoxic.

Dinoseb is used on the green forage of some crops and therefore animals and man could probably ingest the substance in food. Therefore one possible route of exposure for man or animals would be the oral route. In contrast, ip or sc administration would be an unlikely route for dinoseb exposure and therefore the relevancy of its embryotoxicity and teratogenicity by these routes is questionable. The explanation for the dependence of dinoseb embryotoxicity and teratogenicity on route of administration was not clear, since peak blood levels of the compound were similar following ip administration of 17·7 mg/kg and oral administration of 40 mg/kg (Gibson, 1972).

DNP doses equimolar to the teratogenic and embryotoxic doses of dinoseb were not teratogenic. These results suggest, but do not establish, that the active teratogenic constituent of dinoseb is not the parent structure, DNP.

Dinoseb was concluded to have a low potential for the induction of terata in mice. Effects were dependent on the route of administration and the dosage, and the greatest number of statistically significant anomalies was induced when dinoseb was administered ip. Administration by the sc or oral route had less effect on embryonic and foetal development. Doses required to induce these anomalies caused toxic effects in the dams and were in the lethal range. The no-effect dose levels of dinoseb given throughout organogenesis (days 8–16) were 5, 10 and 20 mg/kg/day by the ip, sc and oral routes, respectively.

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Etude tératologique du 2-*sec*-butyl-4,6-dinitrophénol (dinoseb) sur la souris

Résumé—Le 2-*sec*-butyl-4,6-dinitrophénol (dinoseb) a été administré quotidiennement pendant l'organogenèse à des souris Swiss-Webster gravides, en injections ip on sc ou en intubations orales pratiquées du 8e au 16e, du 10e au 12e ou du 14e au 16e jour de gestation. On a relevé les anomalies générales, celles des tissus mous et celles du squelette que présentaient les foetus extraits par césarienne. L'administration du dinoseb aux doses quotidiennes de 5 mg/kg ip, de 10 mg/kg sc ou de 20 mg/kg par voie orale du 8e au 16e jour, n'a pas eu d'effet néfaste sur la croissance et le développement des embryons et des foetus. Les doses de 17,7 mg/kg/jour injectées par voie intrapéritonéale du 10e au 12e ou du 14e au 16e jour ont eu des effets toxiques sur les souris traitées (hyperthermie) et sur les embryons (résorption accrue, foetus plus petits), mais seul le traitement appliqué du 10e au 12e jour a eu des effets tératogènes (par exemple la non-perforation de l'anus ou l'absence de queue). Le dinoseb administré en doses sous-cutanées de 17,7 mg/kg/jour du 14e au 16e jour a eu des effets toxiques sur les mères et les embryons, mais n'a pas eu d'effets tératogènes. Les doses orales de 20 et de 32 mg/kg/jour ont eu des effets toxiques sur les mères, mais pas d'effets essentiellement embryotoxiques ou tératogènes. On conclut de ces observations que le dinoseb n'a, chez la souris, qu'un faible potentiel tératogène.

Teratologische Untersuchungen an Mäusen mit 2-*sec*-Butyl-4,6-dinitrophenol (Dinoseb)

Zusammenfassung—2-*sec*-Butyl-4,6-dinitrophenol (Dinoseb) wurde täglich einer Gruppe trächtiger Swiss-Webster-Mäuse während der Organogenese, am 8.–16., 10.–12. oder 14.–16. Tag der Trächtigkeit, durch ip oder sc Injektion oder mit der Schlundsonde verabreicht. Durch Kaiserschnitt auf die Welt gebrachte Foeten wurden auf makroskopische Anomalien der weichen Gewebe und des Skeletts untersucht. Die Anwendung von Dinoseb in täglichen Dosen von 5 mg/kg ip, 10 mg/kg sc oder 20 mg/kg oral am 8.–16. Tag hatte keine nachteilige Wirkung auf das Wachstum und die Entwicklung von Embryo oder Foetus. Dosen von 17,7 mg/kg/Tag, die ip am 10.–12. oder 14.–16. Tag verabreicht wurden, waren toxisch für das Muttertier (Hyperthermie) und die Embryos (verstärkte Resorption, verminderte Foetusgrösse), aber teratogene Wirkungen (z.B. Atresia ani, Acaudie) ergaben sich nur durch Verabreichung am 10.–12. Tag. Dinoseb in sc Dosen von 17,7 mg/kg/Tag am 14.–16. Tag wirkte toxisch auf Muttertier und Embryo, jedoch nicht teratogen, während orale Dosen von 20 oder 32 mg/kg/Tag toxisch auf das Muttertier, aber praktisch weder embryotoxisch noch teratogen wirkten. Daraus wurde der Schluss gezogen, dass Dinoseb ein geringes Potential für die Induzierung von Terata bei Mäusen hat.

Fd Cosmet. Toxicol. Vol. 11, pp. 45–52. Pergamon Press 1973. Printed in Great Britain

Disposition of 2-*sec*-Butyl-4,6-dinitrophenol (Dinoseb) in Pregnant Mice

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Abstract—The disposition of 2-*sec*-butyl-4,6-dinitrophenol (dinoseb) was determined in pregnant mice after ip or oral administration. Plasma levels of dinoseb were determined colorimetrically and isotopically, and dinoseb was separated from its metabolites in tissues by methyl ethyl ketone extraction. Dinoseb was found in all maternal tissues and was shown to cross the placenta, although a barrier was clearly present since embryo levels never exceeded 2·5 % of maternal plasma levels. The rate of absorption of dinoseb was 40 times greater after ip than after oral administration. Elimination was first order, but the rate was dependent on the route, the k_e being 0·02 and 0·09 hr^{-1} after oral and ip administration, respectively. Pregnant mice excreted dinoseb in urine and faeces regardless of the route of administration, and metabolites, which were not identified, were found in maternal liver and kidney and in the embryo, but not in maternal blood. While embryonic levels of dinoseb were similar in both cases, peak levels were reached much earlier after ip than after oral administration (8 min and 12 hr respectively), and the initial levels of the herbicide and its metabolites in the embryos were higher after ip administration. These differences may be responsible for the observed teratogenicity and embryotoxicity of dinoseb following ip but not oral administration.

INTRODUCTION

2-*sec*-Butyl-4,6-dinitrophenol (dinoseb) is teratogenic and embryotoxic in mice when administered by the ip route in doses that also produce maternal toxicity. Similar teratogenic and embryotoxic effects are not produced in mice, however, when dinoseb is administered by oral intubation (Gibson, 1972 & 1973). The perinatal toxicity of dinoseb is therefore dependent both on dose and route of administration.

The present study was undertaken to determine the disposition of dinoseb in pregnant mice after ip or oral administration. The study indicated that embryos of animals given dinoseb ip contained higher initial levels of the herbicide and its metabolites than did embryos of animals given the compound orally.

EXPERIMENTAL

Animals. Swiss–Webster mice (from Spartan Research Animals, Haslett, Mich.) were housed in groups of ten in stainless-steel cages and allowed food and water *ad lib.* A 12-hr dark-light cycle was maintained (lights on at 8 a.m.). Pregnant mice were obtained by daily pen breeding from 8 to 9 a.m. Copulation was confirmed by the presence of the vaginal plug at examination. This time was called day 1 of gestation.

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Colorimetric determination of dinoseb in plasma. Groups of five adult female mice were given 17·7 mg dinoseb/kg ip or 40 mg/kg orally. Fresh aqueous solutions of dinoseb (dissolved in 1 N-NaOH and titrated to neutral pH with 1 N-HCl) were prepared in such concentrations that 10 ml/kg body weight gave the appropriate dosage. At various times (0·03–24 hr) after administration, the mice were lightly anaesthetized with ether and blood was collected by cardiac puncture into heparinized syringes. After centrifugation of the blood, plasma samples (0·4–0·5 ml) were diluted with distilled water to 5 ml and mixed. After addition of 5 ml methyl ethyl ketone (MEK) and 1 g $NaCl$–Na_2CO_3 (9:1, w/w), the tubes were shaken. The MEK phase was withdrawn and the MEK extraction was repeated. The combined MEK extracts were then read at 430 nm against dinoseb standards (0–10 μg/ml).

Disposition of [^{14}C]dinoseb in mice at day 11 of gestation. Uniformly ring-labelled [^{14}C]-dinoseb (3·04 mC/m-mole; Mallinkrodt Nuclear Corp., St. Louis) was mixed with non-radioactive dinoseb (The Dow Chemical Company, lot no. 7200206, 1966) in such proportion that, in final concentration, 10 ml/kg body weight gave the appropriate dosage. The dose levels studied were 17·7 mg/kg ip and 32 mg/kg given orally, with each animal receiving approximately 1 μC of radioactivity (25 μC/kg). At various times (1 min–48 hr) after administration of [^{14}C]dinoseb, samples of maternal blood, liver, kidney, brain, muscle and adipose tissue were collected. In addition, samples of embryo, placenta and uterus were taken. Embryos and placentae from each animal were pooled. Samples were minced with scissors, transferred to tared glass liquid-scintillation vials and weighed. Plasma samples (100 μl) were placed in separate liquid scintillation vials. All samples were solubilized in 1·0 ml Soluene 100® and 15 ml toluene counting solution (5 g PPO plus 200 mg dimethyl POPOP/litre toluene) was added. Radioactivity levels were measured with a liquid scintillation counter. Quench corrections were made using [^{14}C]toluene internal standardization. Disintegrations/min/g tissue were converted to μg dinoseb/g tissue to eliminate the effect of differences in the specific activities of injected dinoseb.

Pharmacokinetics of dinoseb in mice. Using the kinetic model of Wiegand & Sanders (1964), rate constants for dinoseb absorption (k_a) and elimination (k_e) were calculated from plasma levels of the compound. From the kinetic model, the volume of distribution was also estimated.

Excretion of [^{14}C]dinoseb by female mice. The radioactivity of urine and faeces collected for periods of 1–64 hr after oral or ip administration of [^{14}C]dinoseb was determined in groups of non-pregnant mice treated as described above. Three groups of three mice were used for each sampling time. Weighed faecal samples were homogenized in 2 vols 1 N-HCl and aliquots of the faecal homogenate were transferred to liquid scintillation vials. Faeces and urine samples were solubilized and counted as described above. Excretion was expressed as a cumulative percentage of the administered dose for each time period. In other experiments the common bile duct of female mice anaesthetized with 60 mg pentobarbitone/kg was cannulated using polyethylene tubing (Adams P.E.10). The abdominal incision was closed and dinoseb was administered ip or by stomach injection and bile was collected into tared scintillation vials. The bile was weighed, solubilized and counted for radioactivity as described above.

Separation of dinoseb from its metabolites in tissues. [^{14}C]Dinoseb was separated from its metabolites in blood, liver, kidney and embryo tissues. Tissues were collected 3 hr after ip or oral administration of the compound to pregnant mice on day 11 of gestation. The MEK extraction procedure already described was used, with the following modification. Tissues were homogenized in distilled water (1 g in 4 ml) and one 10-ml MEK extraction was used.

An aliquot of the MEK extract was counted for radioactivity as previously described. The remaining MEK was evaporated to 0·1 ml under nitrogen and spotted on cellulose thin-layer chromatography (TLC) plates. MEK extracts were chromatographed against standard [^{14}C]dinoseb using two solvent systems; *n*-butanol–ethanol–benzene–NH_4OH (2:4:2:2, by vol.) and *n*-butanol–acetic acid–H_2O (6:2:2, by vol.). Radioactivity on the TLC plates was located with a radiochromatogram scanner. Only dinoseb was extracted into MEK (recovery >99%).

Statistics. Statistical analyses of data were made by the grouped *t* test (Steel & Torrie, 1960). The level of significance was chosen as $P < 0{\cdot}05$.

RESULTS

The administration of dinoseb in an ip dose of 17·7 mg/kg to female mice produced peak blood levels, measured colorimetrically, comparable with those resulting from an oral dose of 40 mg/kg (Fig. 1). For subsequent studies an ip dose of 17·7 mg/kg and an oral dose of 32 mg/kg were chosen, since these dose levels were, respectively, teratogenic and essentially non-teratogenic when given repeatedly during gestation.

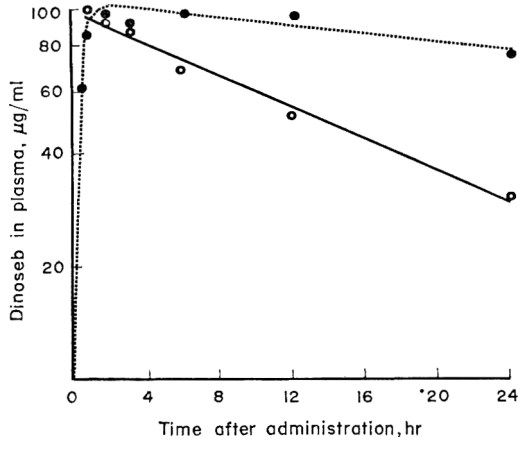

FIG. 1. Dinoseb levels in the plasma of female mice given a single oral or ip dose. Levels were determined colorimetrically after MEK extraction and each point is the mean of five determinations. The standard errors of the means are included within the points and were excluded from the graph. The absorption rate of dinoseb given ip was too rapid to be depicted on the graph.

Placental transfer and embryonic uptake of dinoseb

[^{14}C]Dinoseb crossed the placenta and reached the embryo, although a barrier to such transfer was present since embryonic levels never exceeded 2·5% of the maternal plasma levels (Fig. 2). The ip and oral administration of [^{14}C]dinoseb produced similar embryonic levels of radioactivity, but peak levels were reached much earlier after ip administration; in 8 min compared with 12 hr in the case of oral treatment.

In the maternal animal treated with [^{14}C]dinoseb, radioactivity reached all tissues, although a blood-brain barrier was noted. Brain radioactivity was of the same order of magnitude as that in the embryo. In all tissues, peak levels of radioactivity were noted earlier

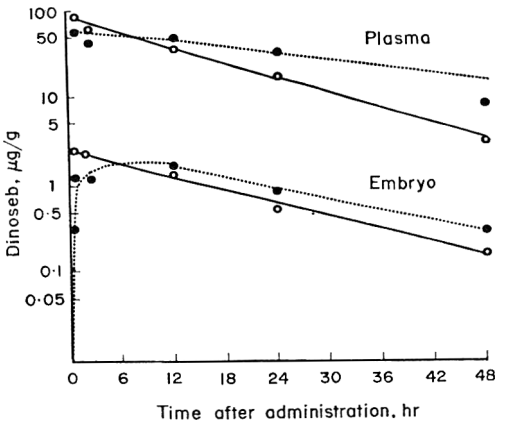

FIG. 2. Maternal plasma and embryonic levels of dinoseb after administration of ^{14}C-labelled compound orally or ip on day 11 of gestation. Each point represents the mean of at least three determinations. Absorption rates and embryo uptake following ip administration were too rapid to be clearly depicted on the graph. The embryonic levels reflect the levels of parent compound and its metabolites.

after ip than after oral administration. Radioactivity in all tissues studied was at levels lying between those in the plasma and in the embryo, except in the case of the liver, which initially had a higher level of radioactivity than plasma.

Pharmacokinetics

When administered ip, dinoseb was absorbed 40 times faster than after po administration (Table 1). Elimination of dinoseb was first order and the rate was dependent upon the route of administration. The k_e after oral administration was 0·02 hr^{-1} and after ip administration 0·09 hr^{-1}. In addition the volume of distribution of dinoseb appeared to depend on the route of administration. Following oral administration it was distributed to total body water but after ip administration only to extracellular water (Table 1).

Dinoseb excretion

[^{14}C]Dinoseb and its metabolites were excreted in the urine and faeces of mice regardless of the route of administration (Table 2), although this factor influenced the rate of excretion of radioactivity into the faeces. The difference was dependent on the biliary excretion of

Table 1. *Pharmacokinetics of [^{14}C]dinoseb in female mice following oral or ip administration*

Route of administration	Dose (mg/kg)	k_a (hr^{-1})	k_e (hr^{-1})	Volume of distribution (litres/kg)
Oral intubation	32	7 ± 4	0·02 ± 0·01	0·66 ± 0·13*
Ip injection	17·7	299 ± 205	0·09 ± 0·02	0·18 ± 0·18†

*Volume of distribution indicates distribution to total body water.
†Volume of distribution indicates distribution to extracellular water.
Values are means for groups of five mice ± SEM.

Table 2. *Excretion of [^{14}C]dinoseb by female mice following oral or ip administration*

Time after administration (hr)	Treatment ..... Dose (mg/kg)	Mean cumulative excretion (% of administered radioactivity) in					
		Bile		Urine		Faeces	
		Oral 32	Ip 17·7	Oral 32	Ip 17·7	Oral 32	Ip 17·7
0·5		0·1 ± 0	0·2 ± 0·1	–	–	–	–
1		0·4 ± 0·1	0·6 ± 0·1	0·8 ± 0·2	1·4 ± 0·2	–	–
2		0·6 ± 0·3	1·4 ± 0·4	1·9 ± 0·4	3·9 ± 0·1	–	–
4		0·9 ± 0·4	3·9 ± 0·6	3·2 ± 0·4	7·0 ± 0·1	–	–
8		1·4 ± 0·6	9·6 ± 1·4*	6·8 ± 1·4	13·4 ± 1·3	0·5 ± 0	3·3 ± 0·9
16		–	–	14·4 ± 2·0	22·1 ± 2·1	4·3 ± 1·1	11·1 ± 1·1*
32		–	–	23·2 ± 3·5	26·3 ± 1·9	9·7 ± 3·7	28·7 ± 4·8*
64		–	–	26·3 ± 3·3	28·2 ± 2·5	30·4 ± 7·5	40·8 ± 6·5

Values are means for groups of three mice ±SEM. Those marked with asterisks differ significantly from the corresponding value for orally treated animals: *$P < 0{\cdot}05$.

radioactivity (Table 2). Urinary excretion was not affected by the route of administration. Between 67 and 78% of the administered dose was recovered in urine and faeces within 64 hr of administration of a single dose of dinoseb, regardless of the route of administration.

Metabolism of dinoseb in mice

Dinoseb was metabolized by pregnant mice (Table 3). The results suggest that more metabolism occurred after ip than after oral administration. In embryos, a smaller fraction

Table 3. *Separation of dinoseb from its metabolites 3 hr after oral or ip administration to pregnant mice on day 11 of gestation*

		Mean tissue levels (μg/g)† of dinoseb and metabolites			
		Total‡		Dinoseb alone§	
	Treatment.....	Oral	Ip	Oral	Ip
Tissue	Dose (mg/kg)..	32	17·7	32	17·7
Blood		31·3 ± 5·4	45·0 ± 1·4*	29·7 ± 4·6	46·6 ± 3·9
Liver		26·5 ± 4·1	32·9 ± 1·3	14·2 ± 0·6	15·6 ± 1·8
Kidney		21·8 ± 3·9	28·6 ± 4·4	13·8 ± 1·4	15·4 ± 1·4
Embryo		2·1 ± 0·2	5·1 ± 0·4*	1·8 ± 0·1	2·9 ± 0*

†Based on the molecular weight of dinoseb.
‡Measured by total radioactivity in samples.
§Measured as MEK-extractable material.
Values are means for groups of at least three animals ±SEM and those marked with an asterisk differ significantly from the corresponding value for orally treated animals: *$P < 0{\cdot}05$.

of dinoseb metabolites was found after oral than after ip treatment. However, it cannot be concluded that unchanged dinoseb is teratogenic and embryotoxic, since after ip injection dinoseb and its metabolites were found in equal amounts. Metabolites of dinoseb were not found in maternal blood. Those in the tissues were not identified.

DISCUSSION

The present experiments were undertaken to investigate the factors responsible for the teratogenic and embryotoxic properties of dinoseb, shown to be dependent on the route of administration. Gross differences in maternal absorption and/or elimination of dinoseb after administration by the two routes (ip and oral) do not explain the route-dependent responses. However, the relatively rapid rate of embryonic uptake of dinoseb and its metabolites after ip administration compared with that after oral administration may explain the teratogenic-embryotoxic effects. Indeed significant levels of dinoseb or its metabolites were noted within 1 min of ip administration (peak at 8 min), but no dinoseb was found until 30 min after oral administration (peak at 12 hr). The rate of uptake by the embryo was therefore markedly dependent on the route of administration, although peak embryonic levels of dinoseb radioactivity and the rate of dinoseb disappearance were not remarkably different. Nevertheless the rate of embryonic uptake of dinoseb could explain

the appearance of teratogenic effects after ip administration, since the amount present in the embryo over the time studied was greater after ip than after oral administration.

Plasma concentrations of dinoseb after ip and oral administration to mice were found to be similar by two methods of determination, colorimetric and isotopic (Figs 1 & 2), but the rate of elimination from plasma was dependent on the route of administration. The differences in elimination rates may be due to differences in the rate and mode of dinoseb metabolism.

The metabolism of dinoseb has not been studied in the mouse, but it has been shown to be metabolized by rabbits, rats (Ernst & Bär, 1964) and various ruminants (Fröslie & Karlog, 1970). Rabbits but not rats reduced one nitro group with the formation of the 6-amino derivative conjugated as the *O*-glucoside. A carboxylic acid was also formed by oxidation of the terminal carbon of the secondary side chain in both rats and rabbits. Dinoseb in rumen fluid, under anaerobic conditions, was converted to the 6-amino derivative, with successive reduction to the diamino compound. This reduction did not take place in heat-sterilized rumen. *In vivo*, the diamino compounds were not demonstrated in blood plasma in the cow (Fröslie & Karlog, 1970). In view of these observations, the possibility of microbial metabolism of dinoseb in the gut of the mouse must not be overlooked, since this could lead to differential metabolism and thus to differential embryotoxicity and teratogenicity by the ip and oral routes. Metabolism was confirmed in mice 3 hr after administration of dinoseb (Table 3), when liver and kidney were found to contain approximately 50% unchanged dinoseb and 50% unidentified metabolite regardless of the route of administration. In contrast, no metabolites were found in blood. Embryonic radioactivity 3 hr after oral administration of dinoseb was primarily (85%) in the form of the unchanged compound, whereas after ip treatment only 57% of the material found was in the unmetabolized form. This suggests but does not confirm, that the teratogenicity of dinoseb may be due, in part, to unidentified metabolites.

The rate of elimination of [^{14}C]dinoseb from the embryo paralleled the rate of elimination of the herbicide from plasma and other tissues (Fig. 2). This observation suggests that neither dinoseb nor its metabolites accumulates in the embryo.

It was concluded that the teratogenic and embryotoxic effects of ip-administered dinoseb were due to rapid and relatively extensive uptake of the compound or its metabolites by the embryo. The relative lack of teratogenic and embryotoxic effects after oral administration of the herbicide may be accounted for by the slow uptake of dinoseb by the embryos.

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Répartition du 2-*sec*-butyl-4,6-dinitrophénol (dinoseb) chez la souris gravide

Résumé—On a étudié, chez la souris gravide la répartition du 2-*sec*-butyl-4,6-dinitrophénol (dinoseb) administré par voie intrapéritonéale ou par voie orale. La méthode colorimétrique et celle des isotopes ont été appliquées pour déterminer les taux plasmatiques de dinoseb et ce produit a été séparé de ses métabolites tissulaires par extraction avec la méthyléthylcétone. On l'a retrouvé dans tous les tissus de la mère et constaté qu'il traverse le placenta, nonobstant l'existence d'une barrière, clairement mise en évidence par le fait que les taux plasmatiques chez les embryons ne dépassaient jamais 2,5% des taux plasmatiques relevés chez les mères. Le taux d'absorption du dinoseb administré par voie intrapéritonéale était 40 fois plus élevé que celui du produit administré par voie orale. L'élimination suivait une cinétique d'ordre 1, mais sa rapidité différait selon la voie d'administration: l'indice k_e était de 0,02 h^{-1} après l'administration orale et de 0,09 h^{-1} après l'administration intrapéritonéale. Les souris gravides ont excrété le dinoseb dans l'urine et les fèces, quelle que fût la voie d'administration. Des métabolites, qui n'ont pas été identifiés, ont été trouvés dans le foie et les reins des mères et chez l'embryon, mais pas dans le sang des mères. Les taux de dinoseb étaient similaires chez les embryons pour l'un et l'autre mode d'administration, mais des taux maximaux ont été atteints beaucoup plus tôt après l'administration intrapéritonéale qu'après l'administration orale (respectivement en 8 min et 12 h). Les taux initiaux de dinoseb et de ses métabolites étaient plus élevés, chez les embryons, après l'administration intrapéritonéale. Ces différences pourraient expliquer les effets tératogènes et embryotoxiques observés après l'administration intrapéritonéale et non après l'administration orale.

Verteilung von 2-*sec*-Butyl-4,6-dinitrophenol (Dinoseb) in trächtigen Mäusen

Zusammenfassung—Die Verteilung von 2-*sec*-Butyl-4,6-dinitrophenol (Dinoseb) wurde bei trächtigen Mäusen nach ip oder oraler Anwendung bestimmt. Die Plasmakonzentrationen von Dinoseb wurden kolorimetrisch und isotopisch bestimmt, und Dinoseb wurde von seinen Stoffwechselprodukten in den Geweben durch Extraktion mit Methyläthylketon getrennt. Dinoseb wurde in allen Geweben des Muttertiers gefunden, und es zeigte sich, dass es durch die Placenta geht, obwohl eindeutig eine Sperre vorhanden sein muss, da die Konzentrationen im Embryo niemals 2,5% der Plasmakonzentrationen des Muttertiers überschritten. Die Absorptionsgeschwindigkeit von Dinoseb war nach ip Anwendung 40mal grösser als nach oraler Verabreichung. Die Ausscheidung erfolgte nach erster Ordnung, aber die Geschwindigkeit hing vom Weg ab, wobei k_e nach oraler Anwendung 0,02 h^{-1} und nach ip Anwendung 0,09 h^{-1} betrug. Trächtige Mäuse schieden Dinoseb im Urin und in den Faeces unabhängig vom Anwendungsweg aus, und Stoffwechselprodukte, die nicht identifiziert wurden, wurden in Leber und Nieren des Muttertiers und im Embryo, aber nicht im Blut des Muttertiers gefunden. Während die Dinosebkonzentrationen im Embryo in beiden Fällen gleich waren, wurden maximale Konzentrationen viel früher nach ip als nach oraler Anwendung (8 min bzw. 12 h) erreicht, und die Anfangskonzentrationen des Herbicids und seiner Stoffwechselprodukte in den Embryonen waren nach ip Anwendung höher. Diese Unterschiede können für die beobachtete Teratogenität und Embryotoxizität von Dinoseb nach ip, aber nicht nach oraler Anwendung verantwortlich sein.

Fd Cosmet. Toxicol. Vol. 11, pp. 53–62. Pergamon Press 1973. Printed in Great Britain

Dominant Lethal Study of *p,p'*-DDT in Rats

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Abstract—*p,p'*-DDT was tested by the dominant lethal system in rats. Male rats were given DDT either ip or orally and were mated with two untreated females in each of the following 3 wk. The pregnant females were sacrificed at approximately mid-term and were scored for corpora lutea, total implants, dead implants and pre-implantation losses. A statistically significant effect was found in the proportion of females having one or more dead implants after being mated during wk 3 with males given DDT orally in a dose of 100 mg/kg. No significant effects were found in females mated with ip-treated males. It is concluded that DDT is only marginally positive with respect to the dominant lethal test.

INTRODUCTION

In recent years, several carcinogens have been shown to be mutagenic when tested in animals or when an active metabolite has been identified and evaluated in sub-mammalian species (Miller & Miller, 1971). The reported carcinogenicity of the persistent pesticide, DDT, prompted a series of investigations into the possible mutagenic activity of this compound and its metabolites (Innes, Ulland, Valerio, Petrucelli, Fishbein, Hart, Pallotta, Bates, Falk, Gart, Klein, Mitchell & Peters, 1969). Palmer, Green & Legator (1972) reported that chromosome abnormalities were produced by DDT and its metabolites in a cultured mammalian cell line, but in a collaborative study reported by Legator, Palmer & Adler (1972), no cytogenetic abnormalities were found in bone-marrow cells of rats treated with DDT. The present study extends the evaluation of this pesticide in animals, by the use of the dominant lethal test as an indicator of *in vivo* mutagenic activity.

EXPERIMENTAL

Materials and animals. The purity of the *p,p'*-DDT, supplied by the Reference Standard Section of the FDA, was at least 99·9%, as determined by gas-chromatographic comparison with a reference standard of known purity. Random-bred male and female albino rats weighing 180–200 g were supplied by Holtzman Breeders, Madison, Wisc.

Procedure. A modification of the dominant lethal test (Bateman & Epstein, 1971; Epstein & Röhrborn, 1971; Epstein & Shafner, 1968) was used. In each of the six experiments, at least ten males were given the solvent (corn oil) alone and groups of five males were given *p,p'*-DDT in the required doses or triethylenemelamine (TEM), the positive control compound. *p,p'*-DDT was given by oral intubation in a single dose of 25, 50 or 100 mg/kg in

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experiments 1–3 and ip in daily doses of 20, 40 or 80 mg/kg for five consecutive days in experiments 4–6. Corn oil was given either orally or ip in doses of 1·0 ml/kg and TEM was given only ip, as a single dose of 0·5 mg/kg. The males were mated with two virgin females immediately after intubation or after their last injection and then at weekly intervals. The females were sacrificed 13 days from the mid-week of mating. In the first oral and ip experiments, the males were sequentially mated to untreated virgin females for wk 1–3 and wk 5–7, to cover the three major divisions (post-meiotic, meiotic and pre-meiotic) of spermatogenesis. The initial studies indicated that the first 3 wk after treatment was the critical period, and in subsequent experiments only this period was considered. At autopsy the females were examined for corpora lutea, total implantations and dead implantations.

Statistical analysis of results. The data from each dosage level were compared with control data, using the *t* test with appropriate transformation of the raw data, as noted on the tables. The data for females with one or more dead implants were tested with the chi-square test by comparing each level with control values, and by Armitage's trend for linear proportions to determine whether the proportions were related to the dose.

RESULTS

Oral intubation

Table 1 shows the average numbers of corpora lutea and implantations per pregnant female at each dose level for the three oral experiments. In experiment 3, the average number of corpora lutea was significantly decreased, as compared with the control value, for wk 3 with DDT at the 50 mg/kg dose level. The average number of implantations was significantly decreased at wk 2 in experiment 1, for the group given 100 mg DDT/kg and increased at wk 2 in experiment 3 in the group given 50 mg/kg. No dose-related responses to DDT treatment were observed at any week either within a single experiment or in the combined results.

The average pre-implantation losses and numbers of dead implants per pregnant female are given in Table 2. There were no significant differences from control values at any level of DDT treatment; the apparent increase in pre-implantation loss during wk 3 of experiment 2 with a DDT dose of 50 mg/kg was due mainly to a single outlier and was not statistically significant.

Table 3 shows the proportion of females with one or more dead implants for each week and each experiment, as well as the combined data from the three experiments. In the analysis of each separate experiment, there were no significant differences between values for any level of DDT treatment and the control values. Analysis of the combined data by the chi-square test showed a statistically significant difference ($P < 0{\cdot}05$) at wk 3 between the value for DDT at 100 mg/kg and the control value. For both wk 2 and 3, there appears to be a dose-related effect in the combined data from the three experiments, but this dose-relationship is not statistically significant.

Fertility was affected by oral treatment with DDT only during wk 2 of experiment 2 at the 25 mg/kg dose level. This was not repeated in the following experiment or at higher doses.

Intraperitoneal administration

Tables 4–6 present the results of the ip experiments. None of the values for the parameters studied in females mated to DDT-treated males differed significantly from those of controls. In addition, fertility was not affected by ip administration of DDT.

Table 1. *Corpora lutea and implants in rats after treatment of males with* p,p′-*DDT by oral intubation*

Experiment no.	Mating period (wk after treatment)	Nos of corpora lutea and total implants after treatment with									
		0 (control)		25 mg DDT/kg		50 mg DDT/kg		100 mg DDT/kg		0·5 mg TEM†/kg	
		No.§	Mean‖	No.	Mean	No.	Mean	No.	Mean	No.	Mean
		Corpora lutea									
1	1	115/9	12·78					76/6	12·67	104/8	13·00
	2	212/15	14·13					129/10	12·90	115/9	12·78
	3	255/19	13·42					120/9	13·33	110/10	11·00*
2	1	224/18	12·44	104/9	11·56	116/10	11·60	105/8	13·13	92/8	11·50
	2	226/17	13·29	38/3	12·67	217/15	14·47	73/6	12·17	26/3	8·67**
	3	173/12	14·42	126/10	12·60	105/7	15·00	109/7	15·57	45/4	11·25*
3	1	161/13	12·38	113/9	12·56	94/7	13·43	112/9	12·44		
	2	198/15	13·20	79/6	13·17	138/10	13·80	112/9	12·44		
	3	221/15	14·73	123/10	12·30	113/9	12·55**	121/9	13·44		
1–3 combined	1	500/40	12·50	217/18	12·06	210/17	12·35	293/23	12·74	196/16	12·25
	2	636/47	13·53	117/9	13·00	355/25	14·20	314/25	12·56	141/12	11·75**
	3	649/46	14·11	249/20	12·45	218/16	13·63	350/25	14·00	155/14	11·07**
		Total implants									
1	1	111/9	12·33					78/6	13·00	80/8	10·00*
	2	198/15	13·20					106/10	10·60*	55/9	6·11**
	3	224/19	11·79					115/9	12·78	43/10	4·30**
2	1	164/18	9·11	62/9	6·89	77/10	7·70	90/8	11·25	53/8	6·63
	2	200/17	11·76	38/3	12·67	208/15	13·87	67/6	11·17	1/3	0·33**
	3	157/12	13·08	112/10	11·20	76/7	10·86	105/7	15·00	18/4	4·50**
3	1	158/13	12·15	111/9	12·33	89/7	12·71	100/9	11·11		
	2	179/15	11·93	74/6	12·33	131/10	13·10*	91/9	10·11		
	3	198/15	13·20	120/10	12·00	107/9	11·89	115/9	12·78		
1–3 combined	1	433/40	10·83	173/18	9·61	166/17	9·76	268/23	11·65	133/16	8·31*
	2	577/47	12·28	112/9	12·44	339/25	13·56	264/25	10·65	56/12	4·67**
	3	579/46	12·59	232/20	11·60	183/16	11·44	335/25	13·40	61/14	4·36**

†Triethylenemelamine was administered ip.
§Total no./total no. of pregnancies.
‖Average no. per pregnancy.
Values marked with asterisks differ significantly (Student's *t* test) from the control values: *$P < 0.05$; **$P < 0.01$.

Table 2. *Pre-implantation losses and dead implants in rats after treatment of males with* p,p'-*DDT by oral intubation*

Experiment no.	Mating period (wk after treatment)	Nos of pre-implantation losses and dead implants after treatment with									
		0 (control)		25 mg DDT/kg		50 mg DDT/kg		100 mg DDT/kg		0·5 mg TEM†/kg	
		No.§	Mean‖	No.	Mean	No.	Mean	No.	Mean	No.	Mean
		Pre-implantation losses									
1	1	4/9	0·44					1/6	0·17	24/8	3·00**
	2	14/15	0·93					23/10	2·30	60/9	6·67**
	3	31/19	1·63					5/9	0·56	67/10	6·70**
2	1	60/18	3·33	42/9	4·67	39/10	3·90	15/8	1·88	39/8	4·88
	2	26/17	1·53	0/3	0·00	9/15	0·60	6/6	1·00	25/3	8·33**
	3	16/12	1·33	24/10	2·40	29/7	4·14	4/6	0·57	27/4	6·75*
3	1	3/13	0·23	2/9	0·22	5/7	0·71	12/9	1·33		
	2	19/15	1·27	5/6	0·83	7/10	0·70	22/9	2·44		
	3	23/15	1·53	3/10	0·30	6/9	0·67	6/9	0·67		
1–3 combined	1	67/40	1·68	44/18	2·44	44/17	2·59	28/23	1·22	63/16	3·94**
	2	59/47	1·26	5/9	0·56	16/25	0·64	51/25	2·04	85/12	7·08**
	3	70/46	1·52	27/20	1·35	35/16	2·19	15/25	0·60	94/14	6·71**
		Dead implants									
1	1	2/9	0·22					4/6	0·67	38/8	4·75**
	2	10/15	0·67					9/10	0·90	41/9	4·55**
	3	7/19	0·37					7/9	0·78	37/10	3·70**
2	1	15/18	0·83	2/9	0·22	2/10	0·20	5/8	0·63	18/8	2·25
	2	8/17	0·47	0/3	0·00	18/15	1·20	6/6	1·00	1/3	0·33
	3	8/12	0·67	6/10	0·60	3/7	0·43	9/7	1·28	18/4	4·50
3	1	4/13	0·31	3/9	0·33	0/7	0·00	3/9	0·33		
	2	10/15	0·67	4/6	0·67	4/10	0·40	4/9	0·44		
	3	3/15	0·20	5/10	0·50	1/9	0·11	2/9	0·22		
1–3 combined	1	21/40	0·53	5/18	0·28	2/17	0·12	12/23	0·52	56/16	3·50**
	2	28/47	0·60	4/9	0·44	22/25	0·88	19/25	0·76	42/12	3·50*
	3	18/46	0·39	11/20	0·55	4/16	0·25	18/25	0·72	55/14	3·93**

†Triethylenemelamine was administered ip.
§Total no./total no. of pregnancies.
‖Average no. per pregnancy.
Values marked with asterisks differ significantly (Student's *t* test after Freeman–Tukey square-root transformation) from control values: $*P < 0.05$; $**P < 0.01$.

Table 3. *Proportion of female rats with one or more dead implants after treatment of males with* p,p′-*DDT by oral intubation*

Experiment no.	Mating period (wk after treatment)	Nos of females with dead implants after treatment with									
		0 (control)		25 mg DDT/kg		50 mg DDT/kg		100 mg DDT/kg		0·5 mg TEM†/kg	
		No.	Proportion	No.	Proportion	No.	Proportion	No.	Proportion	No.	Proportion
1	1	2/9	0·22					2/6	0·33	6/8	0·75
	2	6/15	0·40					5/10	0·50	7/9	0·78
	3	6/19	0·32					6/9	0·67	9/10	0·90*
2	1	9/18	0·50	2/9	0·22	2/10	0·20	3/8	0·38	5/8	0·63
	2	7/17	0·41	1/3	0·33	9/15	0·60	5/6	0·83	1/3	0·33
	3	6/12	0·50	5/10	0·50	3/7	0·43	6/7	0·86	4/4	1·00
3	1	3/13	0·23	2/9	0·22	0/7	0·00	3/9	0·33		
	2	5/15	0·33	3/6	0·50	3/10	0·30	4/9	0·44		
	3	2/15	0·13	4/10	0·40	1/9	0·11	2/9	0·22		
1–3 combined	1	14/40	0·35	4/18	0·22	2/17	0·12	8/23	0·35	11/16	0·69
	2	18/47	0·38	4/9	0·44	12/25	0·48	14/25	0·56	8/12	0·67
	3	14/46	0·30	9/20	0·45	4/16	0·25	14/25	0·56*	13/14	0·93*

† Triethylenemelamine was administered ip.
Values marked with an asterisk differ significantly (chi-square test) from control values: *$P < 0·05$. Armitage's trend for linear proportion was used to determine whether the proportion was dose-related.

Table 4. *Corpora lutea and implants in rats after treatment of males with* p,p′-*DDT by ip administration for 5 days*

Experiment no.	Mating period (wk after treatment)	Nos of corpora lutea and total implants after daily treatment with: 0 (control) No.†	0 (control) Mean§	20 mg DDT/kg No.	20 mg DDT/kg Mean	40 mg DDT/kg No.	40 mg DDT/kg Mean	80 mg DDT/kg No.	80 mg DDT/kg Mean	0·5 mg TEM/kg No.	0·5 mg TEM/kg Mean
				Corpora lutea							
4	1	191/14	13·64	105/8	13·13	110/8	13·75	94/7	13·43		
	2	265/19	13·95	134/10	13·40	122/9	13·56	95/7	13·57		
	3	276/20	13·80	144/10	14·40	93/6	15·50	109/7	15·57		
5	1	226/19	11·89					224/19	11·79	38/4	9·50
	2	285/22	12·95					262/21	12·48	35/4	8·75*
	3	302/24	12·58					259/22	11·77		
6	1	253/22	11·50					262/23	11·39		
	2	323/26	12·42					268/22	12·18		
	3	328/27	12·15					332/27	12·30		
4–6 combined	1	670/55	12·18					580/49	11·84		
	2	873/67	13·03					625/50	12·50		
	3	906/71	12·76					700/56	12·50		
				Total implants							
4	1	175/14	12·50	90/8	11·25	102/8	12·75	83/7	11·86		
	2	225/19	11·84	127/10	12·70	119/9	13·22	93/7	13·29		
	3	268/20	13·40	126/10	12·60	82/6	13·67	99/7	14·14		
5	1	207/19	10·89					222/19	11·68	24/4	6·00*
	2	273/22	12·41					251/21	11·95	16/4	4·00*
	3	285/24	11·88					249/22	11·32		
6	1	211/22	9·59					223/23	9·70		
	2	311/26	11·96					262/22	11·91		
	3	309/27	11·44					319/27	11·81		
4–6 combined	1	593/55	10·78					528/49	10·78		
	2	809/67	12·07					606/50	12·12		
	3	862/71	12·14					667/56	11·91		

†Total no./total no. of pregnancies.
§Average no. per pregnancy.
Values marked with an asterisk differ significantly (Student's *t* test) from control values: $*P < 0.05$.

Table 5. *Pre-implantation losses and dead implants in rats after treatment of males with* p,p'-*DDT by ip administration for 5 days*

Experiment no.	Mating period (wk after treatment)	Nos of pre-implantation losses and dead implants after daily treatment with									
		0 (control)		20 mg DDT/kg		40 mg DDT/kg		80 mg DDT/kg		0·5 mg TEM/kg	
		No.†	Mean§	No.	Mean	No.	Mean	No.	Mean	No.	Mean
		Pre-implantation losses									
4	1	16/14	1·14	15/8	1·88	8/8	1·00	11/7	1·57		
	2	40/19	2·11	7/10	0·70	3/9	0·33	2/7	0·29		
	3	8/20	0·40	18/10	1·80	11/6	1·83	10/7	1·43		
5	1	19/19	1·00					2/19	0·11	14/4	3·50*
	2	12/22	0·55					11/21	0·52	19/4	4·75**
	3	17/24	0·71					10/22	0·45		
6	1	42/22	1·91					39/23	1·70		
	2	12/26	0·46					6/22	0·27		
	3	19/27	0·70					13/27	0·48		
4–6 combined	1	77/55	1·40					52/49	1·06		
	2	64/67	0·96					19/50	0·38		
	3	44/71	0·62					33/56	0·59		
		Dead implants									
4	1	8/14	0·57	2/8	0·25	6/8	0·75	11/7	1·57		
	2	9/19	0·47	7/10	0·70	4/9	0·44	4/7	0·57		
	3	12/20	0·60	3/10	0·30	5/6	0·83	2/7	0·29		
5	1	4/19	0·21					3/19	0·16	17/4	4·25**
	2	16/22	0·73					9/21	0·43	5/4	1·25
	3	12/24	0·50					9/22	0·41		
6	1	7/22	0·32					19/23	0·83		
	2	15/26	0·58					12/22	0·55		
	3	15/27	0·56					10/27	0·37		
4–6 combined	1	19/55	0·35					33/49	0·67		
	2	40/67	0·60					25/50	0·50		
	3	39/71	0·55					21/56	0·38		

†Total no./total no. of pregnancies.
§Average no. per pregnancy.
Values marked with asterisks differ significantly (Student's *t* test after Freeman–Tukey square-root transformation) from control values: *$P < 0.05$; **$P < 0.01$.

Table 6. *Proportion of female rats with one or more dead implants after treatment of males with* p,p′-*DDT by ip administration for 5 days*

Experiment no.	Mating period (wk after treatment)	Nos of females with dead implants after treatment with									
		0 (control)		25 mg DDT/kg		40 mg DDT/kg		80 mg DDT/kg		0·5 mg TEM/kg	
		No.	Proportion	No.	Proportion	No.	Proportion	No.	Proportion	No.	Proportion
4	1	7/14	0·50	2/8	0·25	5/8	0·63	5/7	0·71		
	2	9/19	0·47	4/10	0·40	4/9	0·44	2/7	0·29		
	3	7/20	0·35	3/10	0·30	3/6	0·50	2/7	0·29		
5	1	4/19	0·21					3/19	0·16	2/4	0·50
	2	10/22	0·45					8/21	0·38	3/4	0·75
	3	8/24	0·33					7/22	0·32		
6	1	6/22	0·27					7/23	0·30		
	2	11/26	0·42					9/22	0·41		
	3	12/27	0·44					9/27	0·33		
4–6 combined	1	17/55	0·31					15/49	0·31		
	2	30/67	0·45					19/50	0·38		
	3	27/71	0·38					18/56	0·32		

The chi-square test was used to compare data with control values. Armitage's trend for linear proportions was used to determine whether the proportion was dose-related.

As in the oral-intubation studies, results obtained with the TEM-treated animals (Tables 1–6) were similar to those previously published by Bateman (1960).

DISCUSSION

The toxicity of DDT varies widely in different animal species (Woodard, Nelson & Calvery, 1944). Epstein & Shafner (1968) reported negative results for the dominant lethal test in mice given a single ip injection of DDT. In our study, a statistically significant effect was found in the proportion of females with one or more dead implantations only in animals mated during the post-meiotic stage of spermatogenesis (wk 3) following oral treatment with 100 mg DDT/kg. The results of this study lead to the conclusion that DDT causes a marginally positive dominant lethal effect. An attempt should be made to extend the *in vitro* findings to determine whether the genetic effect of this compound is due to the action of major or minor metabolites.

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Etude de la létalité dominante du *p,p'*-DDT chez le rat

Résumé—On a étudié la létalité dominante du *p,p'*-DDT en administrant le DDT par voie intrapéritonéale ou par voie orale à des rats mâles, auquel on faisait saillir à chacune des 3 semaines suivantes deux femelles non traitées. On sacrifiait ensuite les femelles gravides à peu près à mi-terme et enregistrait le nombre de corps jaunes, d'implantations totales, d'implantations mortes et de pertes avant l'implantation. On a reconnu un effet statistiquement significatif dans la proportion de femelles qui présentaient une ou plusieurs implantations mortes après avoir été accouplées le 3e semaine avec des mâles auxquels on avait administré le DDT en une dose orale de 100 mg/kg. Aucun effet significatif n'a été observé chez les femelles saillies par les mâles traités par voie intrapéritonéale. On en conclut que le DDT n'est que marginalement positif en ce qui concerne le test de létalité dominante.

Dominant letaler Versuch mit *p,p'*-DDT an Ratten

Zusammenfassung—Mit *p,p'*-DDT wurde nach dem dominant letalen System ein Versuch an Ratten durchgeführt. Männliche Ratten erhielten DDT entweder ip oder oral und wurden in jeder der folgenden 3 Wochen mit zwei unbehandelten weiblichen Tieren gepaart. Die trächtigen weiblichen Tiere wurden ungefähr in der Mitte der Trächtigkeitsperiode getötet und auf Gelbkörper, Gesamtzahl der Implantationen, abgestorbene Implantationen und Verluste befruchteter Eizellen vor der Implantation untersucht. Ein statistisch signifikanter Effekt wurde bei dem Anteil der weiblichen Tiere gefunden, der eine oder mehrere abgestorbene Implantationen nach der Paarung mit männlichen Tieren in der 3. Woche, nachdem diese DDT oral in einer Dosis von 100 mg/kg erhalten hatten, aufwies. Keine signifikanten Effekte wurden bei weiblichen Tieren gefunden, die mit ip behandelten männlichen Tieren gepaart worden waren. Daraus wird der Schluss gezogen, dass DDT nur marginal positiv hinsichtlich des dominant letalen Versuchs ist.

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Effect of the Quality of Dietary Protein on Heptachlor Toxicity*

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Abstract—The toxicity of heptachlor was studied in male weanling rats which had been fed different types of dietary protein for 10 days at two levels of protein intake. With a dietary protein level of 10%, heptachlor was less acutely toxic in rats fed an unsupplemented gluten diet than in animals pair-fed diets containing gluten plus amino acids or casein plus 0·2% DL-methionine. When the level of dietary protein was raised to 18%, heptachlor was twice as toxic to rats pair-fed casein diets as to rats fed unsupplemented gluten. The clinical syndrome of intoxication was not affected by the quality of dietary protein fed, but the acute toxicity of heptachlor varied according to the method of feeding used. Animals fed casein diets *ad lib.* were less susceptible to heptachlor intoxication than were rats fed the same diets at a restricted level of intake.

INTRODUCTION

The toxicity of pesticides can either be enhanced or reduced by variation in a number of dietary factors, including the level of dietary protein. Boyd & Tanikella (1969) found that malathion was more toxic to rats given low protein diets (3·47% protein) than to rats fed a commercial laboratory chow or a purified diet containing 26% protein as casein. The authors postulated that protein deficiency possibly depressed the detoxification of malathion by hepatic enzymes.

In contrast to the findings of Boyd & Tanikella (1969), Weatherholtz, Campbell & Webb (1969) reported that rats fed a 5%-casein diet were less susceptible to the lethal effects of heptachlor than were their pair-fed mates given 20- or 40%-casein diets. In this case, the low-protein diet impaired the conversion of heptachlor to the more toxic metabolite, heptachlor epoxide.

The present report describes the effects of feeding a low-quality protein, gluten, on the toxicity of heptachlor.

EXPERIMENTAL

Animals. Weanling male albino rats were obtained from Flow Laboratory Animals, Dublin, Virginia. After a 2-day adjustment in the laboratory, the animals were weighed and distributed randomly by weight into various treatment groups. All the animals were housed individually in stainless-steel cages in a room where lighting, temperature and humidity were controlled.

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Diets. The basal diet used in all experiments was based upon that used by Weatherholtz *et al.* (1969). It consisted of sucrose, protein, corn oil, salt mixture and vitamins. The two types of protein used, gluten and casein, and the vitamin and mineral mixtures were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. The proteins were incorporated into the basal diet at the expense of sucrose in amounts required to provide a dietary level of protein of 10 or 18%. The casein diets were supplemented with 0·2% DL-methionine. Diets containing gluten were either unsupplemented or supplemented with 0·49% L-lysine, 0·22% DL-methionine, 0·17% L-threonine and 0·14% L-valine.

Experimental design and conduct

Before the experiments were begun, all rats were adjusted to a 20% casein diet for 2 days. To evaluate the effects of the quality of dietary protein on heptachlor toxicity, two experiments were performed. In experiment A, male weanling rats, weighing 55 ± 5 g, were divided into four groups each of 48–50 animals, and were fed diets containing 10% protein. Group 1a was given an unsupplemented gluten diet, group 2a a gluten diet plus amino acids, and groups 3a and 4a a casein diet plus 0·2% DL-methionine. Both group 1a and 4a animals were fed *ad lib.* while group 2a and 3a animals were pair-fed to group 1a.

In experiment B, male weanling rats weighing 55 ± 5 g were divided into three groups each of 50 animals and were fed diets containing 18% protein. Group 1b animals were fed the unsupplemented gluten diet *ad lib.* Group 2b, pair-fed to group 1b, received a casein diet, and group 3b was fed the same diet *ad lib.* In both A and B experiments, food intakes of the rats were measured daily whereas the body weights were recorded at the beginning and end of each experiment.

After 10 days on their respective diets, the animals were injected intraperitoneally with heptachlor (from Velsicol Chemical Corp.), 8–10 animals being used at each dose level. The dosages of the pesticide were determined from previous range-finding experiments. Food was withheld for 12 hr before the administration of heptachlor. The volume of the heptachlor injection was always 0·5 ml/100 g body weight, a 20:80 mixture of polyoxyethylene sorbitan monooleate (Tween 80) and saline (0·9% NaCl) serving as the vehicle.

After the pesticide administration, the animals were observed for overt signs of toxicity. Deaths were recorded at regular intervals up to 120 hr and a computer programme was used to calculate the LD_{50} values.

RESULTS

Nutritional effects of the diets

Table 1 shows the food consumption and weight gain of animals fed the various diets and the calculated protein efficiency ratios (PER). During a 10-day period, the average food consumption of group 1a animals was much lower than that of group 4a animals. The difference in food consumption between group 1a and the pair-fed 2a and 3a is a reflection of the method of pair-feeding. On the last day the rats in 2a and 3a ate approximately 4 g more than 1a rats, prior to withdrawal of diet from all animals 12 hr before heptachlor administration. A relationship similar to that between groups 1a and 4a was seen in experiment B, in which 18% protein diets were fed, group 1b rats having lower food intakes than those in group 3b. When weight gains were related to protein intake, casein gave the highest PER while unsupplemented gluten showed the lowest PER, both being fed *ad lib.* and at dietary protein levels of 10%.

Table 1. *Body weight gains, food consumption and protein efficiency ratios (PER) of rats fed gluten and casein diets for 10 days*

Experiment	Group*	Diet	Mean weight gain (g)	Mean food consumption (g)	PER
A (10% protein)	1a	Gluten (*ad lib.*)	0·9	54·8	0·2
	2a	Gluten plus amino acids† (pair-fed to 1a)	14·3	59·0	2·4
	3a	Casein (pair-fed to 1a)	14·6	58·5	2·5
	4a	Casein (*ad lib.*)	28·3	83·6	3·4
B (18% protein)	1b	Gluten (*ad lib.*)	4·6	61·0	0·4
	2b	Casein (pair-fed to 1b)	24·1	65·0	2·0
	3b	Casein (*ad lib.*)	43·0	91·6	2·5

*No. of animals per group was 50 except for groups 1a (49) and 3a (48).
†The amino acid supplements were 0·49% L-lysine, 0·22% DL-methionine, 0·17% L-threonine and 0·14% L-valine.

Toxicity of heptachlor

The results of the LD_{50} determinations are summarized in Table 2. In experiment A using a 10% dietary protein level, the LD_{50} of heptachlor was higher in group 1a than in group 2a or 3a, but was highest when casein was fed *ad lib.* (group 4a). In experiment B, in which 18% protein diets were fed, the LD_{50} of heptachlor tended to be slightly higher in the group given the unsupplemented gluten diet than in the group fed the casein diet *ad lib.* The LD_{50} value was considerably reduced when casein was fed in restricted amounts (group 2b).

Table 2. *LD_{50} values of heptachlor (ip) for rats fed gluten and casein diets*

Experiment	Group*	Diet	LD_{50} (mg/kg)	95% confidence interval
A (10% protein)	1a	Gluten (*ad lib.*)	68·2	64·5–71·9
	2a	Gluten plus amino acids (pair-fed to 1a)	40·6	37·3–43·9
	3a	Casein (pair-fed to 1a)	41·0	37·6–44·4
	4a	Casein (*ad lib.*)	83·9	76·2–91·6
B (18% protein)	1b	Gluten (*ad lib.*)	57·2	53·2–61·1
	2b	Casein (pair-fed to 1b)	26·6	22·5–30·7
	3b	Casein (*ad lib.*)	48·1	45·3–50·9

*No. of rats per group was 50 except for groups 1a (49) and 3a (48).

The clinical signs of intoxication observed in the animals were circling, jumping, increased sensitivity to touch and sound, extension of limbs, pawing, salivation, tremors and convulsions. Additional signs were shrieking, bleeding from the nostrils and mouth, biting of the wire cage, listlessness and prostration. The effects were observed as early as 30 min after the injection of the pesticide. The onset or severity of the symptoms was not related to the

dietary treatment. Death was attributed mainly to respiratory failure following a convulsive seizure. In some cases an animal recovered from an initial convulsion but eventually died from subsequent convulsive attacks.

The interval between pesticide administration and death varied from 45 min to 96 hr. The majority of recorded deaths occurred within 4 hr of the heptachlor injection.

DISCUSSION

Wheat gluten, the protein source of the gluten diets, is an 'unbalanced protein' which is deficient in some amino acids, particularly lysine (Grau & Kamei, 1950). Thus, the depression in food intake and growth of the animals fed the unsupplemented gluten diet can be attributed to the limiting amino acids.

Despite its adverse effect on growth, unsupplemented gluten afforded some protection to rats against the lethal action of heptachlor. Heptachlor was more lethal to rats pair-fed better diets containing casein plus 0·2% DL-methionine or gluten plus amino acids than to rats fed unsupplemented gluten. The effect of protein quality on heptachlor toxicity was more marked at higher levels of dietary protein. Rats pair-fed casein diets containing 18% dietary protein were twice as susceptible to the toxic effect of heptachlor than animals fed the unsupplemented gluten diet.

The differences in heptachlor toxicity associated with variations in the quality of the dietary protein may be due to differences in the rate of activation of heptachlor *in vivo*. In a previous study, Weatherholtz *et al.* (1969) observed that protein-deficient diets impaired the production of heptachlor epoxide from heptachlor. Their results showed that the ratio of heptachlor epoxide to heptachlor in the livers of rats fed a 5% casein diet was lower than in animals pair-fed a 20% casein diet. This impairment of the conversion of heptachlor to heptachlor epoxide probably also occurs in rats fed a poor-quality protein and might account for the differential toxicity of heptachlor.

The susceptibility of rats to heptachlor toxicity also varied with the method of feeding. This variation is difficult to explain. It is possible that heptachlor epoxidation was occurring at a higher rate in the pair-fed animals, which were actually partially starved as a result of food restriction, than in the animals fed casein diets *ad lib.* Kato (1967) and Gram, Guarino, Schroeder, David, Reagan & Gillette (1970) have reported that starvation can increase the oxidation of certain compounds. An alternative explanation may lie in the difference between the pair-fed animals and those fed *ad lib.* in respect of the amount of body fat, which could trap the injected pesticide. More depot fat was present in animals fed casein diets *ad lib.* (groups 4a and 3b) than in pair-fed animals (groups 3a and 2b). Being lipophilic, heptachlor and heptachlor epoxide, once sequestered in the fat depots, may not reach the target organs to produce the acute toxic effects. The effects of a higher rate of heptachlor metabolism in well-fed animals may, possibly, be cancelled by the presence of the fat depots. This possibility may not only explain the variation in LD_{50} values between groups 3a and 4a but also between groups 1a and 4a (Table 2).

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Effet de la qualité des protéines alimentaires sur la toxicité de l'heptachlore

Résumé—On a étudié la toxicité de l'heptachlore chez de jeunes rats mâles sevrés, auxquels on avait fait consommer pendant 10 jours différents types de protéines alimentaires, incorporées à deux taux différents dans le régime. Aux régimes à 10% ce protéines alimentaires, la toxicité de l'heptachlore était moins aiguë chez les rats soumis à un régime de gluten non supplémenté que chez ceux qui étaient soumis, en rationnement apparié, à un régime supplémenté d'acides aminés ou de caséine et de 0,2% de DL-méthionine. Lorsque le taux de protéines alimentaires était porté à 18%, l'heptachlore était deux fois plus toxique pour les rats soumis en rationnement apparié aux régimes à la caséine que pour les rats qui recevaient le gluten non supplémenté. La qualité de la protéine alimentaire consommée n'influait pas sur le syndrome clinique de l'intoxication, mais la toxicité aiguë de l'heptachlore variait selon la méthode d'alimentation utilisée. Les animaux qui pouvaient consommer *ad lib.* l'aliment à la caséine étaient moins sensibles à l'intoxication par l'heptachlore que ceux qui recevaient le même aliment, mais étaient rationnés.

Einfluss der Qualität von Futterprotein auf die Toxizität von Heptachlor

Zusammenfassung—Die Toxizität von Heptachlor wurde an abgesetzten männlichen Ratten untersucht, die 10 Tage lang verschiedene Typen von Futterprotein mit zwei Konzentrationen der Proteinaufnahme erhalten hatten. Bei einer Proteinkonzentration des Futters von 10% war Heptachlor weniger akut toxisch für Ratten, die Futter nur mit Gluten als Proteinanteil erhalten hatten, als für entsprechend gefütterte Tiere, deren Futter Gluten plus Aminosäuren oder Kasein plus 0,2% DL-Methionin enthielt. Wenn die Konzentration des Futterproteins auf 18% erhöht wurde, war Heptachlor doppelt so toxisch für Ratten, die Kaseinfutter erhielten, wie für Ratten, die nur Gluten als Futterprotein erhielten. Das klinische Syndrom der Intoxikation wurde von der Qualität des verfütterten Proteins nicht beeinflusst, aber die akute Toxizität von Heptachlor unterschied sich nach der angewendeten Fütterungsmethode. Tiere, die Kaseinfutter *ad lib.* erhielten, waren weniger empfindlich gegen Heptachlorintoxikation als Ratten, welche die gleichen Futtertypen in beschränkten Mengen fressen konnten.

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The Safety Testing of Medical Plastics. I. An Assessment of Methods

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Abstract—The toxicity of a variety of plastics medical devices used in medicine and of a number of polyvinyl chloride (PVC) samples of known constitution was evaluated by tissue culture techniques, implantation tests in rabbit muscle and rat subcutaneous tissue, and examination of the systemic effects of administering extracts of the plastics to rats and mice. PVC samples containing known concentrations of an organotin compound were used as positive controls. None of the test plastics exhibited toxicity as high as that of the positive control plastics. The tissue culture technique was the most sensitive method used and toxic effects were detected with a few of the test plastics. Implantation in rabbit sacrospinalis muscle for 7 days was the most sensitive of the implant systems tested and the results showed a good correlation with tissue culture. The systemic tests on extracts failed to demonstrate toxicity, even with the positive control plastics. In view of the high sensitivity of tissue culture, positive results should not necessarily indicate rejection of a plastics material for medical use. Ir such a situation, additional experimental evidence is required.

INTRODUCTION

Plastics are widely used in medicine in place of the more traditional steel, glass and rubber. Their chief advantages lie in the ease of manufacturing a desired shape, in the greater potential for controlling physical properties by altering composition, and in making disposable sterile products economically viable. Plastics are currently used mainly in devices for obtaining access to body compartments in order to collect, administer or facilitate the passage of fluids (e.g. transfusion sets, catheters, tracheostomy tubes and syringes), for the storage of blood and solutions for injection, and for prosthetic devices (e.g. heart-valves, blood vessels and joints). The absence of ill-effects over a long period may be acceptable evidence of the safety of specific plastics for particular uses. However, with new plastics and new uses, testing is necessary to minimize risks to the patient. A number of test methods have been suggested (Autian, 1968) and several are included in a standard for plastics containers in the *United States Pharmacopeia* (1965).

Cruickshank, Hooper, Lewis & MacDougall (1960) found a correlation between the toxicity of rubbers and plastics to tissue cultures and the incidence of thrombophlebitis following iv infusions through transfusion sets assembled with the materials tested. Tissue culture techniques have since been modified for use in the routine testing of plastics (Guess, Rosenbluth, Schmidt & Autian, 1965) and the results obtained with different techniques have been published (Conning & Firth, 1969; Eubanks & Autian, 1971; Guess & Autian,

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1964; Guess & Stetson, 1968; Gullbring, Eklund & Svartz-Malmberg, 1964; Lawrence, Turner & Autian, 1969; Millet, Dony, Gerard & L'Hoest, 1962; Roggen, Bertschmann, Berchtold & Mühlemann, 1964). Correlation with the results of other tests was good, the technique appearing to have a higher sensitivity than another commonly used procedure, implantation in rabbit muscle (Guess & Stetson, 1968; Lawrence *et al.* 1969; Rosenbluth, Weddingdon, Guess & Autian, 1965).

Since the report of Cruickshank *et al.* (1960), little attempt has been made to assess the value of tissue culture and other tests by relating the results of tests on a plastic to the safety-in-use of devices made from it. In the present study, tests have been carried out on the plastics parts of various medical devices not associated with any reports of adverse clinical effects, to investigate whether the high sensitivity of the tissue culture test could result in the unnecessary rejection of materials used in making medical devices. The sensitivity of the tissue culture technique is compared with that of a number of other biological test methods. Attempts were made to achieve conditions of high sensitivity for each of the test methods used.

This work is part of a larger study on the reproducibility of the results of tissue culture tests between a number of research establishments. The results and conclusions reported in the present paper refer only to the BIBRA work. It is hoped that an account of the comparative study will be published later. These investigations were stimulated by the British Standards Institution Technical Committee SGMH/26—Toxicity Tests for Medical Rubber and Plastics, which has the ultimate objective of introducing British Standard Specifications in this field.

EXPERIMENTAL

Test materials

Test samples were taken from polyvinyl chloride (PVC) tracheostomy tubes, ethylene vinyl acetate (EVA) Guedel airways, PVC endotracheal tubes, PVC scalp-vein sets (PVC tubing with hypodermic needle, plastic skin attachment, and luer fitting and plug assembly), polypropylene heart-valve prostheses, two samples (TA and TB) of medical-grade PVC tubing and eight PVC sheets (08–15) of a formulation commonly used in blood transfusion equipment and manufactured under a variety of conditions, some of which might be expected to cause degradation in the material.

In addition to these clinically acceptable plastics, five samples (TC–TG) of PVC tubing containing combinations of three widely used plasticizers and three common stabilizer systems were prepared by Imperial Chemical Industries Ltd. The composition of these tubings, disclosed after completion of the tests, is shown in Table 1.

Pure polyethylene (1 mm thick) was used throughout as a negative control. Two plastics were employed as positive controls, PVC D (supplied by ICI Ltd.), a sheet 1 mm thick and containing 1·4% dibutyltin diacetate, and PVC G (supplied by Dr. W. L. Guess, Drug-Plastic Research and Toxicology Laboratories, University of Texas) composed of 37% di-2-ethylhexyl phthalate, 2% dibutyltin maleate and 61% Geon 101 EP. PVC D was known from previous experience to produce a severe tissue reaction on implantation in rabbit muscle and to be toxic to tissue cultures. Also studied were a PVC without tin (PVC A), which was non-toxic to tissue culture under the same experimental conditions and produced minimal tissue reaction on implantation, and two other PVC-tin formulations, PVC B and

Table 1. *Composition of experimental PVC tubings*

Component	Level (%) used in PVC samples				
	TC	TD	TE	TF	TG
Plasticizers					
Di-isooctyl phthalate	32	–	–	32	32
Dibutyl phthalate	–	32	–	–	–
Acetyl tributyl citrate	–	–	32	–	–
Stabilizers					
Calcium/zinc	2	2	2	–	–
Di-*n*-octyltin thioglycollate	–	–	–	1	–
Cadmium/barium	–	–	–	–	1·3
Stearic acid	–	–	–	0·16	0·16

The PVC and the other ingredients were the same in all compositions.

C, which contained 0·17 and 0·5% tin, respectively. PVCs A, B, C and D contained no plasticizer.

The plastics devices had been sterilized with ethylene oxide, the PVC tubings and transfusion PVC samples by autoclaving and the control plastics by irradiation. The heart valves were received unsterile and were washed in 1% Lab-brite (British Hydrological Corp. London), rinsed in tap-water and then distilled water, and autoclaved for 30 min at 121°C.

Extracts of the plastics were prepared from samples with a total surface area of 60 sq cm cut into about 12 pieces. For aqueous extracts, the samples were autoclaved for 30 min at 121°C with 10 ml double-distilled water. Sodium chloride was added to a final concentration of 0·9% just before use in the systemic toxicity tests. Other samples were incubated at 37°C with 20 ml of a physiological medium (10% rat serum in Krebs–Henseleit solution, sterilized by filtration) for 7 days or with 10 ml neat serum for 72 hr.

Implantation tests

Plastics samples for implantation were cut into 5 × 5 mm squares, any sharp edges being trimmed with scissors. Curved segments of 5 × 5 mm were cut from the walls of the medical tubings TA and TB (5·5 mm OD, 4 mm ID) and the PVC tubings TC–TG (6 mm OD, 3 mm ID), but the vein-set tubing (2 mm OD, 0·5 mm ID) was cut into 8 mm lengths.

Female New Zealand White rabbits weighing 1·9–3·2 kg were anaesthetized with pentobarbitone sodium (30 mg/kg iv) and ether. The dorsal skin surface was clipped and swabbed with 1% Cetrimide, and 1–1·5 cm skin incisions were made over the sacrospinalis muscle. The muscle fascia was split and the muscle fibres were parted using an opening action of a pair of scissors. Plastics samples were placed in the pockets so formed and the incisions in the muscle fascia and in the skin were closed with 5/0 silk. Two or four different test samples were placed 2 cm apart in each animal, together with one each of the negative and positive control samples. The animals were killed at 65 hr or 7 days, and the implantation site was excised in a block of muscle and fixed in 10% buffered formalin for 7 days. A central portion of the implantation site was trimmed for embedding in paraffin wax and 8μ sections were cut and stained with haematoxylin and eosin. Macroscopic assessment of the tissue reaction was made at autopsy and when the blocks were trimmed.

Implants in male Wistar rats (355–380 g body weight) were made under light ether

anaesthesia. Two 1·5 cm mid-line incisions were made approximately 2 cm apart in the dorsal skin and the sc tissue was parted for a distance of 2 cm on both sides of each incision. Plastics samples were placed in each of the four pockets formed and the skin incisions were closed with 5/0 silk. The rats were killed with ether at 65 hr or 7 days and 2 × 2 cm squares of skin including the implantation sites were excised, examined macroscopically, fixed in 10% buffered formalin and prepared for histological examination.

On both macroscopic and microscopic assessments the control implants were known to the observer but the test materials implanted were unidentified. The tissue sections were assessed independently by two observers. For each section, the amounts of fibroblastic proliferation, tissue necrosis and total leucocyte infiltration were each scored, with a maximum of 4 for the greatest response encountered.

Systemic toxicity tests

Acute systemic toxicity was investigated by iv infusion of plastics extracts, warmed to 38°C, into male Wistar rats weighing 170–230 g under pentobarbitone sodium anaesthesia (60 mg/kg ip). Blood pressure was measured with a pressure transducer (Bell and Howell, Type 4-327-L221) connected via saline-filled polyethylene tubing to the right carotid artery. Rate and depth of respiration were recorded using a mercury-in-rubber strain-gauge around the thorax, and the ECG was recorded from needle electrodes (standard lead II). Slow recordings (2·5 mm/min) were made continuously on a Devices Multichannel hot-wire recorder, and intermittent fast recordings (2·5 cm/sec) were taken for counting pulse rate and respiration and for observing the ECG pattern. Infusions of 2·5 ml/100 g body weight were made into the right external jugular vein at 0·5 ml/min using a Palmer syringe pump. Recordings were made before and up to 30 min after the end of infusions.

Effects on mouse behaviour were examined by injecting each plastics extract ip at a dose rate of 1 ml/25 g body weight into two male white mice (TFI strain from A. Tuck & Co. Ltd.) The mice were observed for changes in exploratory activity, body position and movement, respiratory movements, response to touch and in the startle and righting reflexes. Behaviour was observed continuously for 30 min, at 10–20 min intervals up to 3 hr, at 4 and 5 hr and then daily for 7 days. The extracts, including those of the control plastics, were presented for examination with coded labels and in random order.

Red-cell osmotic fragility tests

The effect of aqueous extracts on the osmotic fragility of human red cells was examined by the method of Parpart, Lorenz, Parpart, Gregg & Chase (1947) as modified by Gullbring *et al.* (1964). To 1·4 ml aliquots of the extracts were added 0·1 ml $NaCl/PO_4$ solution to produce concentrations covering the most sensitive part of the osmotic fragility curve (0·35–0·45%). Heparinized human blood (10 μl) was added and the samples were incubated for 40 min at 30°C. Isotonicity was restored by the addition of 1·5 ml of a complementary $NaCl/PO_4$ solution, the cells were removed by centrifugation and the optical density was measured at 540 mμ against a 1% $NaCl/PO_4$ blank. Percentage haemolysis was calculated, assuming that the blood in the 0·1% $NaCl/PO_4$ was completely haemolysed.

Tissue culture tests

Primary rat-kidney cells (BRK) were obtained by trypsin treatment of kidneys from 1–3-day-old rats and the L929 cell line was obtained from Flow Laboratories Ltd.,

Ayrshire. The BRK cells were grown in Eagle's Minimum Essential Medium with Hanks' salts and 5% foetal calf serum, and the L929 cells in Eagle's Basal Medium with Earle's salts and 10% calf serum (all supplied by Flow Laboratories Ltd.). Both penicillin and streptomycin were added, to 100 IU/ml.

The agar overlay method was similar to that described by Guess *et al.* (1965). Confluent cell monolayers in 6 cm Petri dishes were overlayed with 5 ml 1% agar (May & Baker Ltd., Dagenham, Essex) in growth medium which was buffered to pH 6·9 with HEPES instead of with bicarbonate, thus avoiding the need to provide an air/CO_2 atmosphere. One piece each of the test, positive and negative control plastics were placed on the surface of the agar. The surface area of each sample in contact with the agar was 0·5–1 sq cm. The cells were stained either with 0·01% neutral red in isotonic saline and then examined 24 hr after the addition of the plastics samples, or, as suggested by Cooper (1959), with 2-*p*-(iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., 2 ml of 1·5 mg INT/ml isotonic saline being added at 24 hr with incubation for a further 24 hr to develop the colour. Monolayers stained with INT showed greater plaque contrast than with neutral red and had the advantage that they could be stored for long periods after the addition of formalin. In a trial experiment, agar-suspension cultures of L929 cells were used instead of monolayers (Cooper, 1961). A suspension of 5×10^6 cells in 2 ml molten agar-growth medium was poured on to a nutrient base layer of 5 ml agar-growth medium. Plaques produced with this method were smaller than with the monolayer method.

Cell monolayers were also grown on cover-slips in Leighton tubes and exposed for 3 and 18 hr to growth media made up with serum extracts of the plastics. Microscopic examination for changes in cell morphology was carried out after cells had been stained with May–Grünwald–Giemsa stain.

BRK cells exposed to test media for 3 hr were also examined for changes in lysosomal-enzyme activity, as described by Grasso, Gaydon & Hendy (1973), by staining for acid phosphatase.

RESULTS

Implantation tests

Sensitivity of the implantation test in rats and rabbits. Figure 1 shows assessments of the tissue reactions produced by six samples of plastics containing graded levels of toxic agents, implanted sc in rats and im in rabbits for 65 hr or 7 days. Variation in the tissue response is illustrated in Fig. 2, which shows the tissue reactions resulting from implantation of a larger number of the least and most toxic of these plastics. Examples of the tissue reactions seen are shown in Figs 3–6.

Differences in the assessments given by the two observers were not large enough to alter the interpretation of the results. Little fibroblastic proliferation had occurred at either site by 65 hr but at 7 days a significant amount was present around all the plastics (Figs 1a,b). This proliferation tended to be greater with the more toxic materials in the rat (Figs 1b & 2b), but no such correlation was seen in the rabbit (Figs 1a & 2a).

Muscle necrosis seen at all the sites in the rabbit (Figs 1c & 2c) was at least partly a result of operative trauma, since it was seen also at sham-operated sites without plastics. Although tissue necrosis was always present with PVC G (2·0% organotin) in the rat implants, its incidence and severity were variable with PVC D (1·4% organotin) and it was rare with

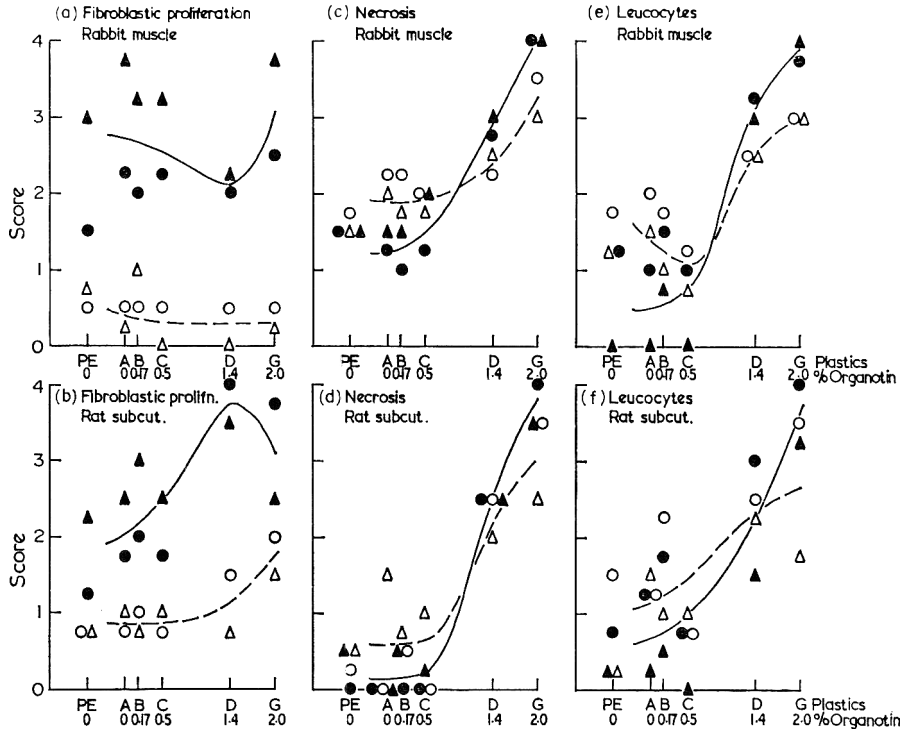

FIG. 1. Assessment of tissue reactions to implantation of PVC-tin samples in rabbits and rats for 65 hr (○- - -○, △- - -△) or 7 days (●——●, ▲——▲). Each point marks the mean score for two implants, with circles and triangles representing the same set of slides assessed independently by two observers.

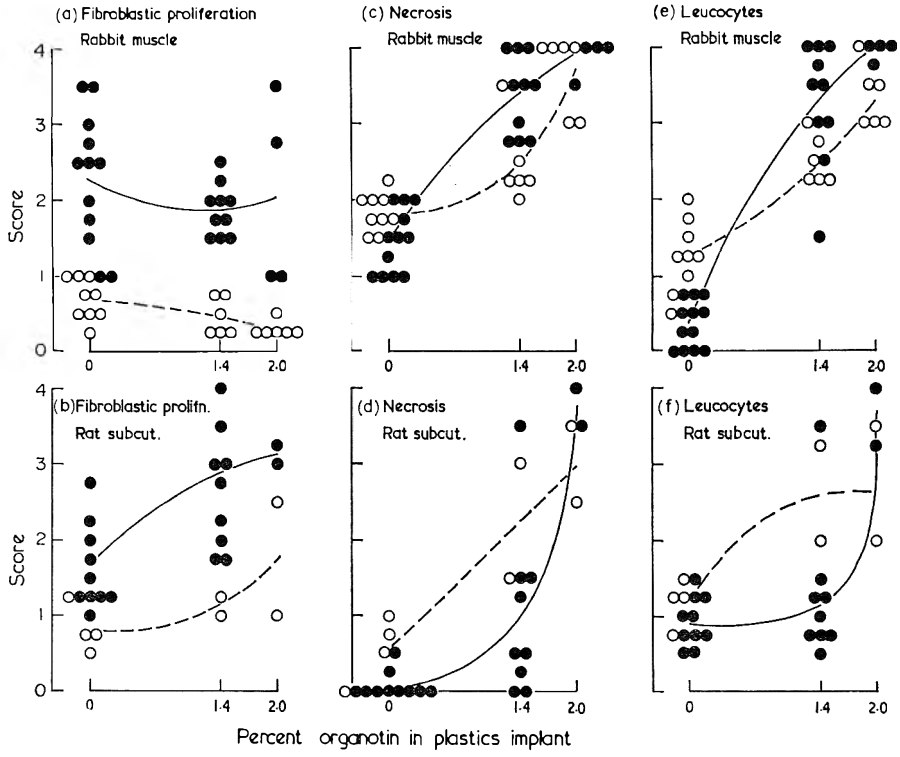

FIG. 2. Effect of tin content on tissue reactions to plastics implanted in rabbits and rats for 65 hr (○- - -○ or 7 days (●——●). Each point represents the mean score given by two observers to one implant.

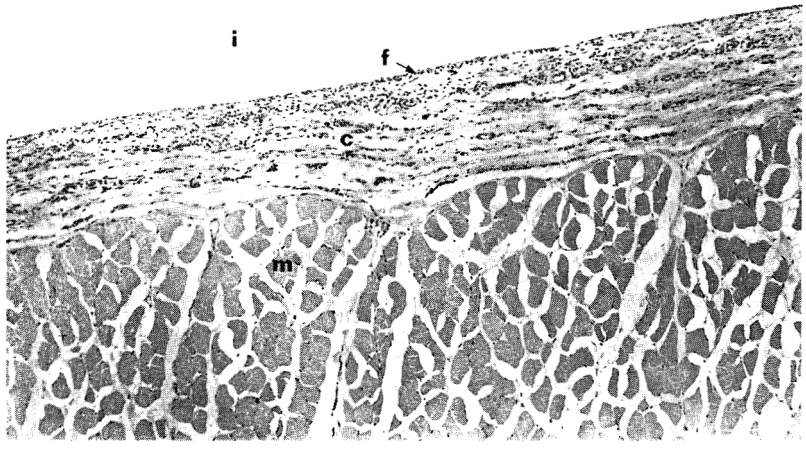

FIG. 3. Sc tissue adjacent to PE (negative control) implanted for 7 days in the rat. Normal connective tissue (c) lies between the muscle (m) and the implant site (i) except for a thin layer of fibroblasts (f) actually in contact with the plastics. Haematoxylin and eosin × 4 objective.

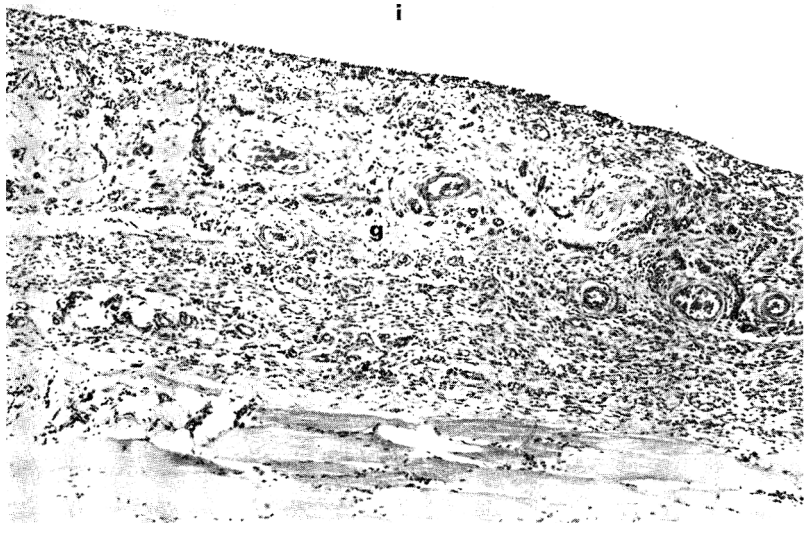

FIG. 4. Sc tissue adjacent to PVC containing 1·4% dibutyltin diacetate (positive control) implanted for 7 days in the rat. The implant site (i) is surrounded by granulation tissue (g). This, the most severe reaction found with PVC D using this preparation, was given the maximum score for fibroblastic proliferation. Haematoxylin and eosin × 4 objective.

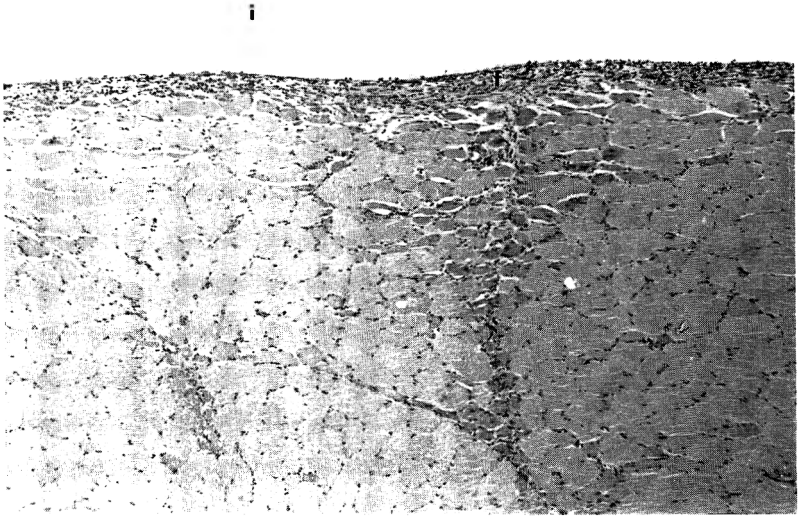

FIG. 5. Sacrospinalis muscle adjacent to PE (negative control) implanted for 7 days in the rabbit. A thin fibrous capsule (f) has formed. Haematoxylin and eosin ×4 objective.

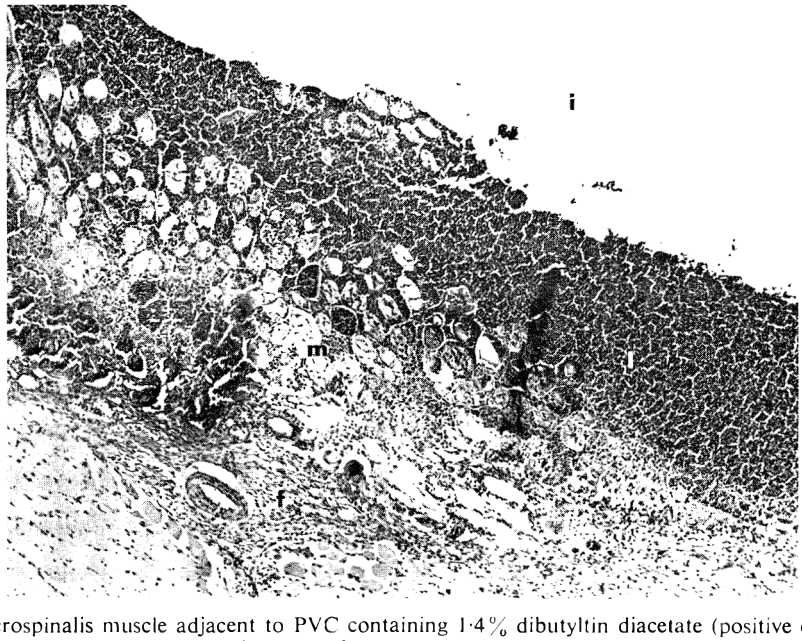

FIG. 6. Sacrospinalis muscle adjacent to PVC containing 1·4% dibutyltin diacetate (positive control) implanted for 7 days in the rabbit. A wide zone of necrotic muscle (m) with leucocyte infiltration (l) surrounds the implant and there is some fibroblastic proliferation (f). Haematoxylin and eosin ×4 objective.

implants of PVCs B and C (Figs 1d & 2d). An absence of tissue necrosis in the rat would therefore not exclude the presence of toxicity equivalent to that of PVC D.

Leucocyte infiltration had occurred by 65 hr in both the rabbit and rat (Figs 1e,f & 2e,f). With the less toxic materials it had decreased slightly by 7 days, but it had increased with the more toxic plastics. In the rabbit, leucocyte infiltration was always seen with PVC D and PVC G (Figs 1e, 2e & 6), whereas in the rat its incidence was low and the amount was variable with PVC D (Fig. 2f).

Of the systems tested, the 7-day implantation in rabbit muscle appeared to be the most sensitive to the presence of highly toxic substances in plastics. However, in all the test systems, the plastics containing 0·17 and 0·5% organotin (PVCs B and C) failed to produce

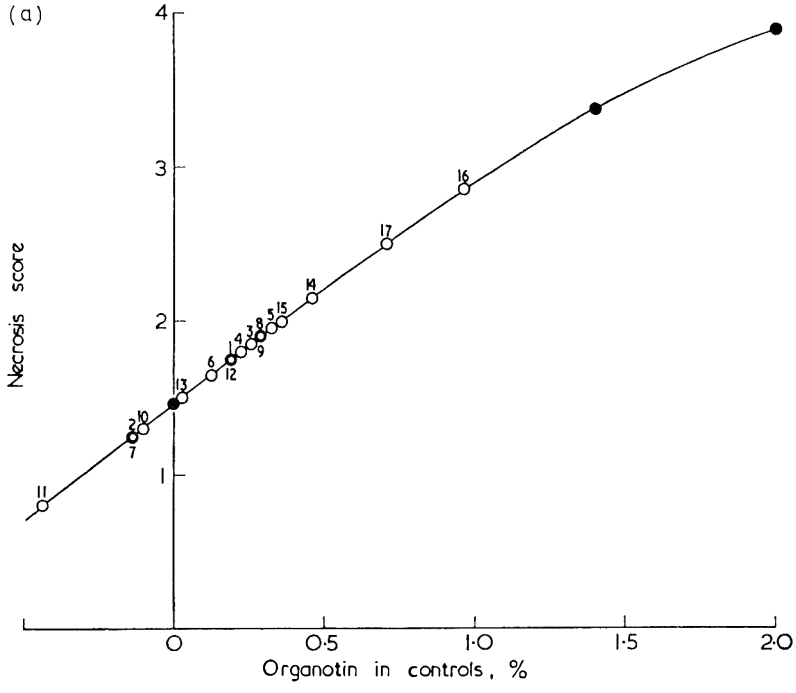

FIG. 7(a).

tissue reactions distinguishable from those with the plastics containing no organotin (PE and PVC A). The threshold concentration of organotin in PVC for the detection of toxicity with the 7-day rabbit-muscle system was between 0·5 and 1·4% and for the 7-day rat sc system between 1·4 and 2·0%.

Implantation tests on medical plastics. Three samples of each of the test plastics were implanted into the sacrospinalis muscle of rabbits for 7 days and the tissue reactions were evaluated in the same way as in the preceding experiment. The average scores for muscle necrosis and leucocyte infiltration are compared with those produced by negative and positive control plastics in Fig. 7. None of the samples taken from the medical devices produced tissue reactions that were clearly more severe than those produced by the negative

control samples. The special PVC-tubing samples, TF and TG, produced tissue reactions that appeared to be more severe than those produced by the negative control implants but less severe than those produced by PVC D. Their toxicity lay near the threshold of detectability by this method. Sample TF enhanced fibroblastic proliferation when implanted for 7 days in rat sc tissue.

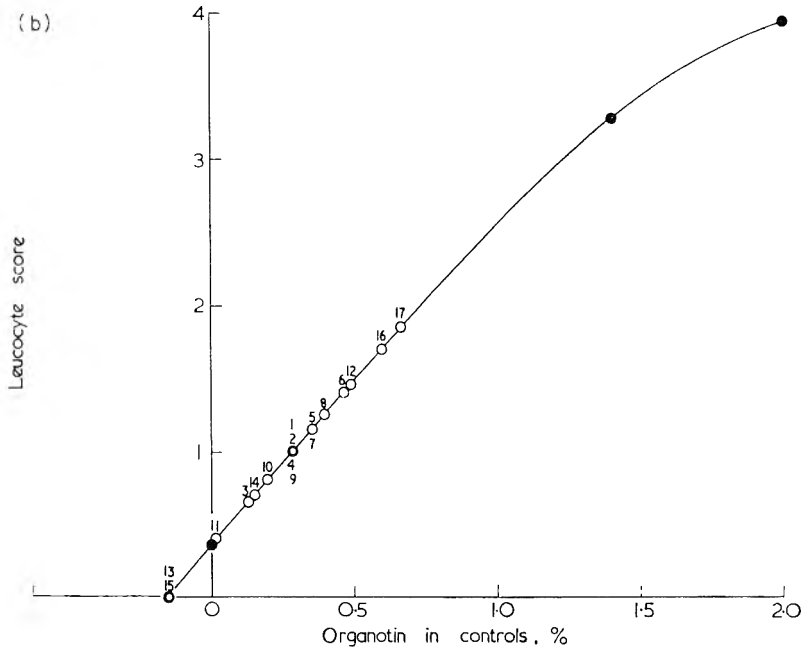

FIG. 7(b).

FIG. 7. Assessments of (a) necrosis and (b) leucocyte infiltration with implantation of medical plastics in rabbit muscle for 7 days, showing mean scores for control plastics of known toxin content taken from Fig. 2 (●) and mean scores (two assessments on each of three implants) for medical plastics (○) fitted to the control curves. Test samples were a tracheostomy tube (1), endotracheal tube (2), Guedel-airway tube (3), Guedel-airway mouthpiece insert (4), scalp vein-set tubing (5), skin flange (6) and luer assembly (7), medical-grade tubing TA (8) and TB (9), heart-valve disc (10) and ring (11), transfusion plastics (12) and PVC tubings TC (13), TD (14), TE (15), TF (16) and TG (17).

Systemic toxicity tests

The use of neat rat serum as an extraction medium was abandoned after it was found that pressor activity developed in the serum on incubation at 37°C without the addition of plastics samples. Dilution of the serum to 10% with Krebs–Henseleit solution reduced this activity, although acute changes in blood pressure (up to 15 mm Hg) and respiration rate (up to 25%) were recorded during the infusion in some experiments. Control infusions (blanks and with extracts of PVC D and PVC G) produced changes similar to those found with extracts of the test plastics. Infusion of aqueous extracts produced no significant changes in blood pressure, pulse rate, ECG and respiration in the rats.

The behaviour of mice was not affected by injection of aqueous extracts of the plastics. However, approximately half of the mice injected with 10% serum/Krebs extracts exhibited a period of slow deep breathing which started soon after injection and lasted 20–30 min. This also occurred with the samples incubated without plastics and is therefore not attributable to extracted material.

Red-cell osmotic fragility tests

The aqueous extract of PVC D produced an increase in osmotic fragility of 0·05% NaCl compared with the control autoclaved without plastics. No other extract produced such a large shift in the osmotic fragility curve. While extracts of all the transfusion plastics, PVC tubing TE, medical-grade tubing TB and tracheostomy, endotracheal and vein-set tubings had little effect, osmotic fragility was reduced by PVC tubings TA and TD and increased by PVC tubings TC, TF and TG and Guedel airway tubings. The Guedel airway tubing melted during extraction. Typical results are shown in Fig. 8.

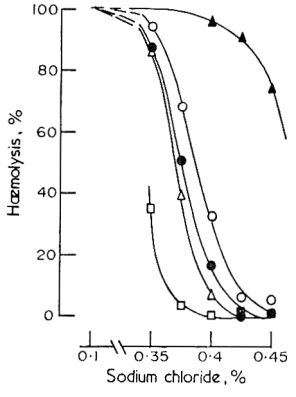

FIG. 8. Effect of plastics extracts on osmotic fragility of red cells, including those producing the largest changes in osmotic fragility: Blank control (●); PVC-tin positive control (▲); PVC tubing TF (○); medical-grade tubing A (□); medical-grade tubing B (△).

To simulate conditions of use, osmotic fragility was also determined on samples of acid/citrate/dextrose-treated blood stored for 21 days at 4°C in contact with the transfusion plastics (6 sq cm plastics/ml blood). Under these conditions, the fragility of cells exposed to the positive control plastic, PVC D, was not increased and some reduction in fragility was apparent with the transfusion plastics.

Tissue culture

With the agar overlay method, pieces of the negative control plastics produced no observable effect on the cell monolayers, whereas the positive control plastic (PVC D) produced a plaque of unstained cells of approximately 2 cm diameter. Tubings TF and TG (L929 cells) and TG (BRK cells) produced plaques which extended beyond the edges of the plastics sample but the plaques were smaller than those produced by PVC D. Slight toxicity was detected in other cases (Table 2).

The morphology of cells treated with medium containing the serum extract of the negative control plastic was similar to that of cells grown in normal medium. With the positive

Table 2. *Tissue culture tests on plastics and their extracts*

		Assessment of						
		Agar overlay technique		Changes in cell morphology				Changes in lysosomal enzymes
Test samples	Cells ..... Exposure (hr)..	L929 24	BRK 24	L929 3	L929 18	BRK 3	BRK 18	BRK 3
PVC B		+*	+	−	−	−	−	−
PVC C		+	+	+	+	+	+	+
PVC tubing: TD		+*	+*	−	−	−	−	−
TF		+	+*	−	−	−	+	+
TG		+	+	−	−	−	+	−
Guedel-airway insert		+†	−	−	−	−	−	−
Scalp vein-set tube		+†	−	−	−	−	−	−

− = Reactions similar to those with negative control plastic (PE). Similar reactions were obtained throughout with PVC A, PVC tubings TC and TE, medical tubings TA and TB, tracheostomy tube, Guedel airway, endotracheal tube and scalp vein-set luer assembly and skin fixture.
+ = Reaction similar to that with positive control plastic (PVC D). No reactions were more severe than those to PVC D.
*An area of mixed stained and unstained cells seen beneath and within 3–4 mm of edge of sample.
†Small plaques seen near one end of the plastics on only one of three plates.

control (PVC D) extract, the characteristic spindle shape of the cells was lost and the continuous cell sheet gave place to isolated rounded cells. Apart from this, rounding of cells was seen only with extracts of TF and TG in BRK cells treated for 18 hr.

BRK cells treated with the TF extract enhanced acid-phosphatase activity, but after a 3-hr recovery period in normal medium this activity was the same as in the negative and untreated controls. Extract of PVC D caused increased acid-phosphatase activity, clumping of lysosomes and the appearance of autophagic vacuoles in the cells, which failed to recover in fresh medium.

The effects of the PVC-tin samples A, B, C and D on tissue cultures are described in another publication (Grasso *et al.* 1973).

DISCUSSION

Implantation tests

Brewer & Bryant (1960) suggested that plastics for use in contact with body tissues be tested for toxicity by observation of the tissue reaction to im implantation of test strips in the rabbit. The test has since been used for this purpose (Guess & Stetson, 1968; Powell, Lawrence, Turner & Autian, 1970), and also for testing plastics not intended for use in direct contact with body tissues (Eubanks & Autian, 1971; Guess & Autian, 1964; Lawrence, Mitchell, Guess & Autian, 1963; Lawrence *et al.* 1969). The procedure assumed official status as a biological test for plastics containers in the *United States Pharmacopeia* (1965).

In view of the economic advantages of using the rat, tissue reactions to sc implantation in rats were compared with reactions to the same materials implanted in rabbit muscle. Although several authors (Calnan, 1963; LeVeen & Barberio, 1949; Usher & Wallace, 1958) claim greater sensitivity for sites other than the sc site, and Powell *et al.* (1970) stated

that the sub-dermal implant system in rats was less sensitive to a toxic or irritant response than rabbit-muscle implantation, there appears to be no published evidence supporting this. However, in the present study the muscle implantation sites showed tissue necrosis and leucocyte infiltration at lower levels of toxicity than did the sc sites (Fig. 2). It is not known whether this was due to differences in species or site sensitivities or in the ease of assessing reactions in sections of the two tissues.

Lawrence *et al.* (1963) interpreted the presence of fibrous connective tissue around plastics samples implanted in rabbit muscle as an indication of severe damage to the tissues. In a follow-up publication (Lawrence *et al.* 1969), a value for the intensity of fibro-endothelial proliferation was included in the calculation of a "toxic index" for implanted materials. Others (Little & Parkhouse, 1962) implied that production of a fibroblastic reaction was an undesirable property of polymers intended for insertion in the body. Roydhouse (1968), on the other hand, assessed the irritant qualities of polymerizing materials by their ability to prevent or delay fibrous encapsulation in the rat sc tissue. In the rabbit-muscle implants of the present study, there was no clear relationship between the amount of fibroblastic proliferation and the expected toxicity of the material. The tissue reactions produced by some of the most toxic plastics were given low scores for fibroblastic proliferation (Fig. 2a). Although a correlation was seen between toxicity and fibroblastic proliferation in rat sc tissue at 7 days, the appreciable overlap in response between samples containing and those not containing organotin would make it difficult to identify toxic materials on this basis. Fibroblastic proliferation is a normal reaction of the tissues to injury and therefore an expected consequence of operative trauma. Our results indicate that the extent to which this response is modified by the release of irritant substances from an implant does not bear a simple relationship to the level of toxicity. It is suggested that the amount of fibroblastic proliferation encountered after 7 days implantation in rabbit sacrospinalis muscle is not a reliable index of toxicity.

All the rabbit sections examined showed some muscle necrosis, attributed in part to operative trauma. Necrotic muscle was also seen in 'hollows' in irregularly shaped implants (e.g. in the concavity of plastics-tubing segments) where it may have resulted from ischaemia. With the toxic plastics, a discrete zone of necrotic muscle surrounded the implant. The locality of the muscle necrosis therefore gave some indication of its origin. The coincidence of a zone of necrotic muscle and an accumulation of polymorphonuclear cells and cell debris was characteristic of reactions to those plastics expected to have toxic potential (PVCs D and G) and was also seen with the test plastics that gave clear positive results in the tissue culture tests. Lawrence *et al.* (1969) also reported good correlation between necrosis in rabbit muscle and tissue culture.

These observations indicate that if a scoring system for evaluating tissue response is to be used, the values should reflect a careful comparison of test with positive and negative control reactions. Summation of the scores given to each feature of the reaction is not justified unless the characteristics evaluated can be shown to correlate with toxicity. For the detection of tissue irritancy by organotin compounds in PVC, 7-day implantation in the sacrospinalis muscle of the rabbit was more sensitive than 65-hr implantation in the rabbit and either 65-hr or 7-day implantation in rat sc tissue.

Tests on extracts

Serum-containing extraction media were included in some of the tests in view of evidence that the serum content may promote the appearance of biologically active substances

during contact with plastics (Bowery & Lewis, 1968; Gullbring *et al.* 1964; Jaeger & Rubin, 1970; Nevanlinna, Penttinen, Saxen & Bremer, 1964). When undiluted serum was used, pressor activity developed during the incubation period at 37°C whether or not plastics samples were added. This was not due to angiotensin, since similar activity developed in serum obtained from nephrectomized (renin-free) donors. Sterility checks failed to demonstrate contamination with micro-organisms. Some biological activity was still recorded on administration of a physiological extraction medium containing only 10% rat serum. No extracts of the test or control plastics produced physiological changes differing clearly from those seen with the blank extraction media, although it cannot be excluded that small differences in response were masked by the activity that developed in the serum-containing media.

Guess & Autian (1964) demonstrated a depressor effect in the rabbit with iv infusion of a concentrated alcohol extract of a plastics feeding-tube. Using more physiological extraction conditions, Conning & Firth (1969) found no clinical, haematological or histological abnormality in dogs infused with large volumes of an extract of PVC containing 5% dibutyltin diacetate. The failure to detect toxicity with extracts of the positive control plastics supports the opinion of Conning & Firth (1969) that toxic concentrations in tissue fluids may not be achieved in normal animals with small volumes at acceptable rates of infusion. No systemic effects followed administration of extracts of the medical plastics. This may have been due to inefficiency of the extraction procedure, insensitivity of the animal preparation, or both. Nevertheless, attempts to increase the efficiency of extraction by the use of rigorous extraction conditions are not easily justified if these conditions differ substantially from the extremes of normal use.

That aqueous extraction effectively extracted toxic material from PVC-tin is evident from the large increase in red cell fragility that took place. Smaller increases in fragility occurred with two test plastics that were also toxic in tissue culture (TF and TG) and with one that was not (TC). The decrease in fragility with other test plastics may have been due to osmotic protection or to a direct effect on the red-cell membrane, but in any case indicates that something had been extracted by autoclaving. However, the finding that fragility was unaltered in cells stored in contact with PVC-tin under conditions simulating use suggests that the test on aqueous extracts was not relevant to clinical use. Wall, Buckley & Doan (1953) found poor correlation between *in vitro* and *in vivo* tests on stored blood, and Strumia, Colwell & Dugan (1959), while recommending *in vitro* testing as a screening method, considered that *in vivo* studies should be carried out before acceptance of a product for clinical use.

Tissue culture tests

Although modification of the agar technique by suspension of cells in agar (Cooper, 1961) produced smaller areas of unstained cells than the monolayer method, it provided a means of increasing the life of cultures without sacrificing the proximity of cells and plastics, by increasing the volume of nutrient base layer. This may be an advantage when longer contact between cells and plastics is necessary to confirm a result.

The results of the tissue culture tests showed good correlation with the rabbit implantation tests. Plastics that produced a significant tissue reaction were also toxic in tissue culture. No plastics produced a significant tissue reaction on implantation but no reaction in tissue culture. The results are in agreement with other work (Lawrence *et al.* 1969; Rosenbluth *et al.* 1965). Two of the PVC samples (TF and TG) whose composition was disclosed after

completion of the tests gave positive reactions both in tissue culture and on implantation. The toxicity was probably related to the stabilizers used (Table 1).

A positive result was found more frequently with the established cell line (L929) than with the primary cultures of rat kidney cells. Guess *et al.* (1965) found four plastics out of 100 which gave positive results with L929 cells but had no apparent effect on chick embryo cells. Recent work further supports the idea that malignant cells in culture are at least as sensitive to toxic agents as are non-malignant cells. On addition of a range of biologically active environmental chemicals to the media, growth inhibition did not differ significantly in HeLa cell and normal human skin fibroblast cultures (Litterst & Lichtenstein, 1971). There was a tendency for the non-malignant cells to be less sensitive. For routine testing, malignant cell lines have several advantages over primary cultures, particularly convenience of preparation and cheapness.

The finding that most of the plastics samples taken from medical devices had no detectable effect on tissue cultures, demonstrates that these plastics can be manufactured in a way that allows mammalian cells to exist in their proximity without damage. We would suggest that, provided its reproducibility between laboratories is confirmed, a tissue culture test could form part of a specification for plastics used in those classes of medical device in which it can be demonstrated that freedom from toxicity in tissue culture is attainable.

A small number of samples produced, in tissue culture, effects that indicated toxicity of a low order. With two plastics, a small plaque appeared at the edge of one of the three samples tested, suggesting the presence of local concentrations of toxic agent. Other plastics produced small plaques in which only a proportion of the cells remained unstained. This type of response would be expected under conditions of borderline toxicity where the culture comprises a cell population of non-uniform susceptibility. Since the devices showing such responses were without known ill-effects in clinical use, toxicity of this order may have no practical significance, although it should be noted that the parts of the devices showing a toxic response do not make direct contact with body tissues in normal use.

In view of the high sensitivity of the tissue culture test, a plastics material giving a positive result should not be rejected on these grounds alone, except perhaps where the device is implanted for long periods in the body at a sensitive site (as with ophthalmic implants). Unless alternative materials are available, plastics giving positive results in tissue culture should be given further consideration, particular attention being paid to experimental evidence relating to the intended use of the material.

Since the tissue culture test bears little relation to conditions of usage of most plastics medical devices, it cannot be used as a general criterion for rejecting plastics until it can be shown that a positive result is likely to have clinical significance. It is suggested that to validate the technique fully, the predictive value of the test should be investigated using a variety of devices that have been rejected for use in medicine following clinical evidence of toxicity.

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Etude de l'innocuité de plastiques à usage médical. I.Examen de méthodes de test

Résumé—Afin d'évaluer la toxicité d'un certain nombre d'articles de plastique à usage médical et d'échantillons de chlorures de polyvinyle (PVC) de compositions connues, on a procédé à des cultures de tissus, à des tests d'implantation dans les muscles du lapin et dans les tissus sous-cutanés du rat; ainsi qu'à l'analyse des effets systémiques de l'administration d'extraits de ces plastiques à des rats et à des souris. Des échantillons de PVC à teneurs connues d'un composé d'étain organique faisaient office de témoins positifs. Aucun des plastiques testés n'a fait preuve d'une toxicité aussi grande que celle de ces témoins. La culture de tissus s'est affirmée la plus sensible des méthodes essayées et a révélé des effets toxiques chez quelques-uns des plastiques testés. Parmi les méthodes d'implantation étudiées, la plus sensible était celle qui consistait à implanter un échantillon dans le muscle sacrovertébral du lapin et à l'y maintenir pendant 7 jours; ses résultats concordaient bien avec ceux de la culture de tissus. Aucune toxicité n'a pu être établie par les tests systémiques à l'aide d'extraits, même pas pour les plastiques utilisés comme témoins positifs. La méthode des cultures de tissus est si sensible, que ses résultats, quand ils sont positifs, ne signifient pas nécessairement que le plastique en cause soit à rejeter pour les usages médicaux. Des preuves expérimentales supplémentaires sont nécessaires dans ce cas.

Die Sicherheitsprüfung von medizinisch verwendeten Kunststoffen. I.Vergleich der Methoden

Zusammenfassung—Die Toxizität verschiedener medizinischer Geräte aus Kunststoff und einer Anzahl von Polyvinylchlorid(PVC)-Proben bekannter Zusammensetzung wurde mit Gewebekulturmethoden, Einpflanzungen in den Kaninchenmuskel und das subcutane Gewebe der Ratte und Untersuchung der systemischen Wirkungen der Anwendung von Extrakten der Kunststoffe auf Ratten und Mäuse geprüft. PVC-Proben, die bekannte Konzentrationen einer organischen Zinnverbindung enthielten, wurden als positive Kontrollen verwendet. Keiner der geprüften Kunststoffe zeigte eine so hohe Toxizität wie die der als positive Kontrollen dienenden Kunststoffe. Die Gewebekulturmethode war die empfindlichste der angewendeten Methoden, und bei einigen der untersuchten Kunststoffe wurden toxische Wirkungen festgestellt. Die Einpflanzung in den Sacrospinalmuskel des Kaninchens für die Dauer von 7 Tagen war die empfindlichste der angewendeten Einpflanzungsmethoden, und die Ergebnisse zeigten eine gute Korrelation mit der Gewebekultur. Die systemischen Prüfungen mit den Extrakten ergaben keine nachweisliche Toxizität, auch nicht bei den als positive Kontrollen dienenden Kunststoffen. In Anbetracht der hohen Empfindlichkeit von Gewebekulturen sollten positive Ergebnisse ein Kunststoffmaterial für medizinischen Gebrauch nicht unbedingt ausschliessen. In so einem Fall werden zusätzliche Experimentalbefunde benötigt.

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An Evaluation of the Criterion "Organ Weight" Under Conditions of Growth Retardation

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Abstract—In order to study the effects of a reduced gain in body weight on organ weight/body weight ratios, a "non-toxic" growth retardation was induced by *ad lib.* feeding of rats on diets containing high levels of several crude fibre materials (raw potato starch, sawdust, oat-hull feed and cellulose) for 4 or 13 wk. The feeding of cellulose at levels of 45–70% turned out to be a suitable method for the induction of growth retardations ranging from 11 to 58%.

Growth retardation had a marked effect on the relative weight of the brain (increased) and the testes (decreased after 4 wk, increased after 13 wk). The relative weights of other organs were only slightly, if at all, affected by retarded growth. It appears, therefore, that in cases of growth reduction, an increase in the relative weights of the liver or the kidneys must be considered to be an effect of the compound.

As brain weight is known to be mainly a function of actual age and to alter little with changing body weights, whereas the weights of most of the other organs are more liable to change with body weight, the use of organ weight/brain weight ratios may lead to erroneous conclusions in cases of animals in which growth has been retarded.

INTRODUCTION

The organ weight parameter has traditionally been used as a criterion in toxicological experiments (Peters & Boyd, 1966; Pfeiffer, 1968). It is generally recognized that organ weights are among the most sensitive biological indicators for organ damage, although it is often difficult to ascertain whether a difference in organ weight between treated and untreated animals is a reflection of a toxic effect of the test compound or of a physiological response by the experimental animal (Pfeiffer, 1968).

Organ weights are usually expressed either by their absolute values or by the ratio of organ weight to body weight (relative organ weight). The latter value is frequently used in an attempt to diminish the variation in absolute organ weights resulting from the unavoidable differences in individual body weights. Occasionally, the use of organ weight/body weight ratios has been criticized, *inter alia* because it disregards completely the possibility that the weights of some organs are more a function of age than of body weight (Dikstein, Kaplanski, Koch, Locker, Sulman & Guterman, 1967; Pfeiffer, 1968).

In toxicity studies showing reduced body-weight gain (growth retardation) in treated groups, the absolute weight of an organ cannot be interpreted unless it is compared in some manner to body weight. It should be realized, however, that an effect on organ weight/body weight ratios may be caused by the growth retardation itself rather than by the test compound. Constants for adjusting organ weight/body weight ratios for the possible effect

of reduced body-weight gain have been estimated in rats for several organs (Robbins, Small & Anderson, 1965).

In order to allow a better understanding of the significance of changes in organ weight under conditions of growth retardation in toxicity studies, observations were made on rats which gained weight at different rates. Restricted feeding is a common method for inducing growth depression in rats. This method entails periodic fasting, whereas in toxicity studies organ weight/body weight ratios are normally obtained under conditions of *ad lib.* feeding. In order to avoid the possibility of a direct effect of fasting on organ weights, we conducted *ad lib.* feeding studies in which growth depression was brought about by incorporating into the diets high levels of largely indigestible materials.

EXPERIMENTAL

Materials. Raw potato starch, sawdust from deal, oat-hull feed (80% indigestible for rats) and pure cellulose were examined as sources of roughage in preliminary experiments. The final experiments were carried out with pure cellulose (Solka Floc, BW 100, Brown Company, New York). The basal diet consisted of the Institute's stock diet enriched with casein (5%), complete B vitamin mixture (0·2%), vitamin-ADE preparation (0·4%) and trace minerals in NaCl (0·5%).

Animals. Weanling Wistar-derived rats from the Institute's colony were housed in groups of five in screen-bottomed cages, in a room maintained at 24–26°C. Diets and tap water were constantly available.

Experimental design and conduct

Preliminary experiments. Four or five groups of rats, each consisting of ten males and ten females, were given the basal diet containing 25–55% raw potato starch, sawdust, oat-hull feed or cellulose for 4 wk. Body weights and food intake were recorded weekly. Determinations of faecal dry-weights were used to monitor the recoveries, and therefore the digestibility, of the test components of the diets.

Final experiments. Groups of ten male and ten female rats were given 45–70% cellulose in the basal diet for 4 and 13 wk. Body weights were recorded weekly and food intake was recorded during the first 4 wk and during wk 10 and 11. At the end of the experiments the animals were killed by decapitation and the heart, kidneys, liver, spleen, lungs, brain, gonads, thymus, pituitary, thyroid and adrenals of each rat were weighed.

RESULTS

Preliminary observations

Some results of the preliminary experiments are shown in Table 1. Considerable growth depression occurred on diets containing sawdust or oat-hull feed, whereas the growth rate was only slightly retarded when potato starch or cellulose was fed at the same dietary level of 40%.

The diets containing sawdust and oat-hull feed were not always consumed as offered, because the rats turned out to be able to select certain components of the diet for consumption or rejection as a result of the relatively large particle size of these crude fibrous materials. Such selection results in a gradual increase in the roughage content of the food in the feeders and may even lead to starvation if fresh portions of the diet are not made available

Table 1. *Mean values of body weight, food consumption and food conversion of rats fed various sources of roughage in the diet for 4 wk*

Crude fibre added to basal diet	Level of addition (%)	Body weight (g) at wk					Food consumption (g/rat/day) in wk		Food conversion during wk 1–4	
		0	1	2	3	4	1 + 2	3 + 4	Total diet/ growth	Basal diet/ growth
Males										
None	–	63	96	129	165	201	10·5	15·2	2·6	2·6
Potato starch	40	63	89	112*	143**	172*	11·4	16·9	3·6	2·8
Sawdust	40	63	77**	88***	87***	130***	9·4	16·2	5·3	3·2
Oat-hull feed	40	63	82*	100**	122***	149***	10·4	16·4	4·4	3·0
Cellulose	40	64	90	116*	147	175*	14·6	22·9	4·7	2·8
None	–	55	85	114	140	174	10·3	13·8	2·7	2·7
Cellulose	25	55	85	108	132	163	12·1	17·9	3·7	2·8
	35	55	87	112	134	164	13·9	21·0	4·3	2·7
	45	55	80	104	122**	148**	14·8	22·9	5·4	2·9
	55	55	80	99*	107***	120***	16·2	22·4	7·8	3·5
Females										
None	–	58	82	102	121	137	8·9	11·6	3·6	3·6
Potato starch	40	58	77	95	113	131	10·1	13·0	4·4	3·6
Sawdust	40	58	67**	76***	74***	105***	8·5	14·3	6·7	4·1
Oat-hull feed	40	59	73*	92	113	133	10·2	15·9	4·9	3·3
Cellulose	40	58	79	97	113	132	12·2	17·9	5·8	3·5
None	–	46	72	94	111	124	9·3	11·6	3·6	3·6
Cellulose	25	46	70	92	107	120	10·6	14·9	4·6	3·4
	35	46	68	88	103	119	11·6	18·8	5·6	3·6
	45	46	68	87	100	115	13·0	21·5	6·7	3·6
	55	46	61**	78***	87***	99***	13·5	23·5	9·2	4·0

Values are the means for groups of ten rats. Those marked with asterisks differ significantly (Wilcoxon test) from controls: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

in time. This may be the cause of the irregularities in the body-weight figures and the increase in food conversion (last column, Table 1) recorded for rats on the diet containing sawdust. The marked growth depression in animals given sawdust or oat-hull feed is, therefore, not necessarily caused by some toxic factor but may be due to large particle size and consequent selection of the diets. As a result of these complications, sawdust and oat-hull feed were considered unsuitable as sources of dietary roughage for rats.

From determinations of the dry matter excreted with the faeces per unit of food consumed, it appeared that the sawdust and cellulose ingested were completely recovered in the faeces, whereas with oat-hull feed and raw potato starch the recoveries amounted to 80 and 50% respectively. As a result of its relatively high digestibility and its well-known capacity for inducing diarrhoea and caecal enlargement (Fischer, 1957) potato starch was considered less suitable for the purpose of our studies than cellulose.

Increasing dietary levels of cellulose resulted in decreasing average body weights, but levels as high as 55% had to be fed in order to obtain a statistically significant growth depression in both sexes (Table 1). The food intake figures increased with increasing levels of cellulose. The additional amounts of food consumed on the high cellulose diets increased in the course of the experimental period up to approximately twice the normal intake. This high capacity for adaptation, which was most pronounced in females, was also observed in other tentative observations with increasing dietary levels of powdered polyethylene.

Food conversion (the amount of food consumed/g body-weight gain) was increased considerably by the addition of the crude fibre from various sources. If calculated on the basis of the amount of basal diet consumed, the food conversion figures showed much less marked differences between groups. At the highest dietary level of cellulose, these conversion figures were slightly increased in both sexes, possibly as a result of the heavily loaded intestinal transport function.

On the basis of the above results, cellulose was chosen as the source of crude fibre in this study of the effects of a non-toxic growth depression on organ weight/body weight ratios under conditions of *ad lib.* feeding.

Final experiments

The reduction in body-weight gain (Table 2) increased with increasing dietary levels of cellulose, and was more pronounced in males than in females, especially during the first 4 wk. During the first 4 wk of the experiments, the food conversions calculated on the basis of the amount of basal diet were only slightly increased up to cellulose levels of 60%, but were clearly unfavourably affected at the 70% cellulose level. On the contrary, during wk 10–11 of the experimental period the less heavy animals of the 70% cellulose group showed distinctly lower food conversion figures.

The absolute weights of all organs generally decreased with decreasing body weights (Table 2). However, the extent of this decrease varied markedly from one organ to another, as is demonstrated by the slight decrease in the weight of the testes after 13 wk and in brain weight both after 4 and 13 wk, and by the considerable decrease in the weights of the gonads and liver of males after 4 wk.

The organ weight/body weight ratios are shown in Table 3. The relative brain weights increased with decreasing body weights. The relative weight of the testes decreased with increasing growth retardation after the feeding of the diets for a period of 4 wk, but the opposite effect occurred after feeding for 13 wk. Growth depression had no effect on the relative weights of heart and pituitary. The relative weights of the kidneys and liver and, in

Table 2. *Reduction in body-weight gain, food conversion and absolute organ weights of rats fed different levels of cellulose in the diet for 4 or 13 wk*

Sex and dietary level (%)	Initial body weight (g)	Terminal body weight (g)	Reduction in body-weight gain (g)	Food conversion† in wk		Absolute organ weight (g)									
				1–4	10–11	Heart	Kidneys	Liver	Spleen	Lung	Brain	Gonads	Thymus	Pituitary‡	Thyroid‡	Adrenals‡
4 wk																
Males																
0	55	174	0	2·7		0·704	1·39	7·75	0·470	1·056	1·59	1·96	0·441	6·8	12·4	27·9
45	55	148**	22	2·9		0·587**	1·05**	5·87**	0·343*	0·843*	1·56	1·47*	0·379	5·1**	11·0	25·2
55	55	120***	45	3·5		0·470***	0·86***	4·59***	0·262***	0·706**	1·53*	0·98***	0·213***	4·7**	9·2**	22·1**
Females																
0	47	134	0	3·3		0·549	1·15	5·71	0·329	0·845	1·50	0·046	0·336	7·7	10·7	32·2
45	47	124*	11	3·1		0·556	0·95***	5·23	0·291	0·714***	1·47	0·049	0·336	6·0***	11·6	32·2
55	47	121***	15	2·8		0·464*	0·90***	4·89***	0·264**	0·690***	1·44*	0·039	0·347	6·2***	11·9*	30·0
13 wk																
Males																
0	79	329	0	2·8	21·3	1·064	2·26	10·92	0·579	1·47	1·77	2·71	0·323	7·0	16·9	41·4
50	79	276***	21	3·2	25·4	0·963	1·87**	9·64*	0·468*	1·32	1·70	2·75	0·253*	6·6	13·2	31·6**
60	80	243***	35	3·4	16·1	0·766***	1·60***	7·76***	0·386***	1·20	1·65**	2·57	0·255*	5·2	14·4	31·3***
70	80	186***	58	4·9	12·4	0·622***	1·27***	6·12***	0·282***	0·89***	1·58***	2·40	0·178***	4·3**	9·7***	27·8***
Females																
0	72	194	0	4·2	25·1	0·724	1·31	6·08	0·367	1·12	1·60	0·053	0·237	8·5	12·6	44·6
50	72	181	11	4·2	23·4	0·632	1·11**	5·83	0·341	1·01	1·57	0·046	0·215	7·1	11·1	36·2
60	77	166***	23	4·4	25·5	0·589**	1·12**	5·50	0·331	1·00*	1·57	0·043*	0·201	7·3	10·9	38·3
70	72	137***	47	5·8	19·0	0·523***	0·97***	4·71***	0·253***	0·91***	1·50**	0·030***	0·173*	5·2***	9·5	32·1**

†Calculated on the basis of the amount of basal diet consumed by the rats in the various groups.
‡Values of these organs 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: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$; $***P < 0{\cdot}001$.

Table 3. *Relative organ weights of rats fed different levels of cellulose in the diet for 4 or 13 wk*

Sex and dietary cellulose level (%)	Terminal body weight (g)	Reduction in body-weight gain (%)	Organ weight/body weight ratio (g/100 g body weight)										
			Heart	Kidneys	Liver	Spleen	Lung	Brain	Gonads	Thymus	Pituitary†	Thyroid†	Adrenals†
4 wk													
Males													
0	174	0	0·404	0·79	4·44	0·269	0·61	0·92	1·12	0·253	3·8	7·2	16·1
45	148**	22	0·397	0·71**	3·93***	0·231*	0·57	1·06*	0·97	0·260	3·4	7·5	17·1
55	120***	45	0·391	0·72**	3·80***	0·216**	0·58	1·28***	0·80*	0·177***	3·9	7·6	18·4*
Female													
0	134	0	0·410	0·86	4·26	0·245	0·63	1·12	0·035	0·252	5·8	8·0	24·0
45	124*	11	0·450	0·77**	4·24	0·235	0·58*	1·19*	0·040	0·272	4·9*	9·3	26·0
55	121***	15	0·385	0·75**	4·06	0·219*	0·57*	1·20*	0·032	0·288	5·1	9·9*	24·8
13 wk													
Males													
0	329	0	0·323	0·69	3·32	0·175	0·45	0·54	0·82	0·099	2·1	5·2	12·8
50	276***	21	0·349	0·68	3·50	0·169	0·47	0·62***	1·00***	0·092	2·4	4·8	11·4
60	243***	35	0·315	0·66	3·18	0·159	0·49	0·68***	1·06***	0·106	2·1	6·0	12·8
70	186***	58	0·334	0·68	3·28	0·152*	0·48	0·85***	1·30***	0·096	2·3	5·2	14·9
Females													
0	194	0	0·374	0·68	3·15	0·191	0·58	0·83	0·027	0·123	4·4	6·6	23·2
50	181	11	0·350	0·62	3·23	0·189	0·56	0·88	0·026	0·119	3·9	6·2	20·2
60	166***	23	0·354	0·67	3·30	0·199	0·60	0·95**	0·026	0·121	4·4	6·6	23·2
70	137***	47	0·381	0·72	3·48	0·186	0·67*	1·10***	0·022*	0·127	3·8	7·0	23·6

†Values of these organs are expressed in mg/100 g body weight.
Values are means of groups of ten rats. Those marked with asterisks differ significantly (Student's *t* test) from the control values: *$P<0·05$; **$P<0·01$; ***$P<0·001$.

males, of the thymus were slightly decreased after the 4-wk treatment, but were not influenced by treatment for 13 wk. There were indications of a slight increase in the relative weights of the thyroid and adrenals only after 4 wk. The relative ovary weight was not affected after 4 wk, but was slightly decreased after 13 wk only in the group showing 47% reduction in body-weight gain. The spleen-to-body weight ratio was slightly diminished. The relative lung weight was slightly decreased after 4 wk and slightly increased after 13 wk, the effects being more marked in females than in males.

Table 4 presents an attempt to summarize in practical terms the data listed in Table 3 and described above. This table shows that after treatment for both 4 and 13 wk the relative weights of only two of the 12 organs, namely the brain and testes were considerably affected

Table 4. *Summary of the effects of reduced body-weight gain on relative organ weights of rats fed cellulose at dietary levels of 45–70% for 4 or 13 wk*

Organ	Effects* of retarded growth on organ-weight/body weight ratios after wk	
	4	13
Heart	0	0
Kidneys	−	0
Liver	−	0
Spleen	−	−
Lung	−	+
Brain	++	++
Testes	−−	++
Ovaries	0	−
Thymus	−	0
Pituitary	0	0
Thyroid	+	0
Adrenals	+	0

*0 = not affected; − = slightly decreased; −− = considerably decreased; + = slightly increased; ++ = considerably increased.

by the reduction in growth. After 4 wk the relative weights of all other organs were either unaffected or were only slightly changed. The relative weights of as many as seven organs were not influenced by the reduction in body-weight gain after 13 wk, and only slight changes were noticeable for the spleen, ovaries and lungs.

DISCUSSION

The results of the present study show clearly that a "non-toxic" growth retardation can readily be induced in rats by *ad lib.* feeding of a diet containing high levels of cellulose. The growth retardation was accompanied by a distinct increase in relative brain weight. This finding, which is in agreement with previous reports (e.g. Peters & Boyd, 1966; Winick & Noble, 1966), suggests that in toxicity tests in which growth depression is observed, an increased brain weight/body weight ratio has to be considered primarily an effect of a reduction in body-weight gain.

The decrease in the relative weight of the testes observed after a feeding period of 4 wk in groups displaying growth retardation had changed into a striking increase after 13 wk,

which indicates a retardation of testicular development during the first few weeks after weaning and an enhancement in development thereafter. These different effects of growth retardation on the relative weights of the testes at different ages, which is possibly related to sexual maturation, should be borne in mind by the toxicologist when interpreting results of subacute toxicity studies.

Apart from the brain and the testes, the relative weights of the organs examined were only slightly, if at all, affected by reduced growth. Therefore, marked changes in the relative weights of these other organs under conditions of growth depression must be considered to be compound related.

The relative weights of liver and kidneys, which are very sensitive criteria in toxicological experiments (de Groot & Til, 1965; Smyth, Weil, Adams & Hollingsworth, 1952; Weil & McCollister, 1963), were either not affected or were slightly decreased by reduced growth. Therefore, an increase in the relative weight of these organs at dose levels associated with growth retardation cannot be attributed to the lower body weights and has to be considered as a probable effect of the compound, an effect possibly predictive of injury. This is not in agreement with the opinion of other authors (Gaunt, Farmer, Grasso & Gangolli, 1967), who consider such increased weights of liver and kidneys a reflection of reduced body-weight gain, if the increase is no longer apparent when these organ weights are expressed relative to brain weight. However, brain weight is mainly a function of actual age and hardly changes with fluctuations in body weight, whereas the weights of most of the other organs, with the possible exception of the ovaries and testes (Dikstein *et al.* 1967), are much more liable to change when the body weight alters. In order to exclude as far as possible the effects of differences in body weight, the organ weights should, therefore, be expressed per unit of body weight, rather than per unit weight of an organ that is largely independent of body weight. It is our opinion that the use of organ weight/brain weight ratios may lead to erroneous conclusions from experiments in which growth is retarded, and in general may be considered undesirable and illogical, as has recently been illustrated by Weil (1970).

From a quantitative point of view, it is of little importance whether a dose level responsible for toxic growth retardation also affects other criteria adversely, because the growth depression itself suffices to establish that level as a toxic-effect level. Consequently, if a change in the relative weight of an organ occurs in an animal showing growth retardation, a rough knowledge (such as that given in Table 4) about the nature and extent of the effect of growth depression on the relative weight of that organ will be sufficient for the experienced toxicologist to judge as to how far this change in organ weight has to be considered an effect of reduced growth or an effect of the compound. We feel that in such a situation more accurate analysis of organ weight data, such as by application of correction factors as suggested by Robbins *et al.* (1965), is of little practical significance.

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Evaluation du critère "poids des organes" dans des conditions de croissance retardée

Résumé—Afin d'étudier les effets d'une réduction du gain de poids total sur le rapport entre les poids des organes et le poids total, on a provoqué chez des rats un ralentissement "non toxique" de leur croissance en soumettant ces animaux *ad lib.* pendant 4 et pendant 13 semaines à un régime riche en différentes fibres brutes (amidon de pommes de terre cru, sciure de bois, aliment aux glumes d'avoine et cellulose). Un régime à 45–70% de cellulose s'est révélé efficace pour retarder la croissance de 11–58%.

Le retardement de la croissance a eu un effet marqué sur le poids relatif du cerveau qui augmentait) et des testicules (qui avait diminué après 4 semaines, mais augmenté après 13 semaines). Les poids relatifs des autres organes n'ont guère, voire pas du tout été affectés par le retard de croissance. Il semble donc qu'en cas de croissance réduite une augmentation des poids relatifs du foie ou des reins doit être considérée comme un effet du produit testé.

On sait que le poids du cerveau est principalement fonction de l'âge réel et que des modifications du poids du corps n'ont que peu d'effet sur lui, alors que le poids de la plupart des autres organes subit plus l'effet des modifications du poids total du corps. On risque donc d'arriver à des conclusions erronées quand on se base sur les rapports entre le poids des organes et le poids du cerveau dans le cas d'animaux dont la croissance a été retardée.

Eine Bewertung des Kriteriums "Organgewicht" unter den Bedingungen der Wachstumsverzögerung

Zusammenfassung—Um die Einflüsse einer verminderten Zunahme des Körpergewichts auf Verhältnisse des Organgewichts zum Körpergewicht zu untersuchen, wurde eine "nicht-toxische" Wachstumsverzögerung durch die Fütterung *ad lib.* von Ratten eingeführt, deren Futter hohe Anteile verschiedener Rohfasermaterialien (rohe Kartoffelstärke, Sägemehl, Haferschrot und Cellulose) für die Dauer von 4 oder 13 Wochen enthielten. Die Verfütterung von Cellulose in Konzentrationen von 45–70% erwies sich als geeignete Methode für die Induktion von Wachstumsverzögerungen im Bereich von 11 bis 58%.

Wachstumsverzögerung hatte einen deutlichen Einfluss auf das relative Gewicht des Gehirns (erhöht) und der Hoden (nach 4 Wochen vermindert, nach 13 Wochen erhöht). Das relative Gewicht der anderen Organe war, wenn überhaupt, vom verzögerten Wachstum nur leicht beeinflusst. Es scheint daher, dass in Fällen von Wachstumsverminderung eine Zunahme des relativen Gewichts der Leber oder der Nieren als eine Wirkung der Verbindung angesehen werden muss.

Da das Gehirngewicht bekanntlich hauptsächlich eine Funktion des erreichten Alters ist und sich mit Änderungen des Körpergewichts wenig ändert, während das Gewicht der meisten anderen Organe sich meist mit dem Körpergewicht ändert, kann die Verwendung von Verhältnissen des Organgewichts/Gehirngewicht zu irrigen Schlüssen in den Fällen von Tieren führen, deren Wachstum verzögert ist.

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MONOGRAPHS

Monographs on Fragrance Raw Materials

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INTRODUCTION

The Research Institute for Fragrance Materials, Inc. (RIFM) was established in 1966 by the principal suppliers of raw materials to the fragrance industry. At present, RIFM is supported by 38 of these companies, representing nearly 97% of the industry in the United States and Europe. The sole purpose of RIFM is to assure the safety of perfumery raw materials.

The Board of Directors is composed of the chief executive officers of the member companies, elected at an annual meeting. In order to ensure an independent scientific status for the Institute, it is structured so that the only link between the administrative branches and the scientific arms is the President, who performs a dual role as scientist and administrator. The President has available to him the advice of a Scientific Advisory Committee composed of perfumers, research scientists and analytical chemists drawn from the fragrance industry. Judgements in matters pertaining to the evaluation of safety are made completely independently by a Panel of Experts, who are toxicologists, pharmacologists or dermatologists drawn from the academic world and have no connexion whatever with the fragrance industry.

The monographs on fragrance materials have been prepared by the Institute as part of its programme to evaluate the safety of the raw materials used in perfumery and cosmetics. They will appear, in alphabetical order, as a regular feature of *Food and Cosmetics Toxicology*.

MATERIALS AND METHODS

The raw materials are selected on the basis of the following criteria: (1) they must be representative of the material in actual use by the industry; (2) they must conform to the specifications and standards, if any, of the Essential Oil Association of the USA (EOA); (3) they must be supplied to the RIFM without indication of the supplier, with name and an identification number only; (4) they must be accompanied by gas-chromatographic, ultraviolet or infra-red curves to "thumb-print" the materials.

The specifications and standards in the monographs will be those of the EOA, where available. The levels of usage to be reported are the result of an industry-wide survey.

When each raw material arrives at the Institute, a retain sample is taken and the rest is sent out to various commercial laboratories for testing. A sample in petrolatum is prepared by the Institute for repeated insult patch testing (Shelanski & Shelanski, *Proc. scient. Sect. Toilet Goods Ass.* 1953, **19,** 46) or maximization testing (Kligman, *J. invest Derm.* 1966, **47,** 393) on human skin using, where feasible, a tenfold exaggeration of the maximum use level to which human skin could be exposed.

Acute oral and acute dermal LD_{50}s are determined as a general measure of toxicity and, wherever pertinent, a test for phototoxicity to human skin is included. The results of these preliminary data are reviewed by the Panel of Experts, who decide whether additional work is indicated. The monographs appearing in this and subsequent issues are the result of this combined effort.

While every attempt has been made to ensure that these monographs will be complete, some of the materials have extensive toxicological documentation in the literature. In such instances, the intention has been to limit the data to those pertinent to the use of the materials as fragrance items (as distinct from possible use as flavourings) and to their use on the skin.

Acknowledgements—Special appreciation is due to all the members of the Board of Directors, without whose financial support and wisdom there would be no Institute, to all the members of the Presidential Scientific Advisory Committee, who validate the levels of usage and select the materials to be tested, to the President of the EOA and the Chairman of that Association's Scientific Committee, who supply the materials, and to the very special Expert Panel, without whose experience and judgemental evaluation the work would be useless.

ACETATE C-9

Synonyms: 1-Nonyl acetate; pelargonyl acetate.
Structure: $CH_3 \cdot [CH_2]_8 \cdot OCOCH_3$
Description and physical properties: Food Chemicals Codex (1966).
Occurrence: Apparently it has not been reported to occur in nature.
Preparation: By acetylation of alcohol C-9.
Uses: In public use since the 1900s. Use in fragrances in the USA amounts to less than 5000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	–
Maximum	0·09	0·015	0·02	0·20

Analytical data: Gas chromatogram, RIFM no. 71–5; infra-red curve, RIFM no. 71–5.

Status

Acetate C-9 was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) listed acetate C-9 (nonyl acetate), giving an ADI of 1 mg/kg. The *Food Chemicals Codex* (1966) has a monograph on acetate C-9.

Biological data

Acute toxicity. The acute oral LD_{50} value (RIFM sample no. 71–5) was reported as $> 5{\cdot}0$ g/kg in the rat (Levenstein, 1972). The acute dermal LD_{50} for sample no. 71–5 was reported to be $> 5{\cdot}0$ g/kg (Levenstein, 1972).

Human testing. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at 2% concentration in petrolatum and no case of sensitization was reported (Kligman, 1972).

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ACETOPHENONE

Synonym: Methyl phenyl ketone.
Structure: $C_6H_5 \cdot CO \cdot CH_3$
Description and physical properties: EOA Spec. no. 37.
Occurrence: In at least 15 essential oils, including green tea, labdanum, orris and castoreum (Gildemeister & Hoffman, 1960).
Preparation: Obtained as a by-product in the manufacture of phenol by the hydroperoxidation of cumene, it may also be prepared by the Friedel–Crafts method or by oxidation of ethyl benzene with air (Bedoukian, 1967).
Uses: In public use since the beginning of this century. Use in fragrances in the USA amounts to less than 10,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·002	0·005	0·09
Maximum	0·15	0·024	0·03	0·20

Status

Acetophenone was considered GRAS by FEMA (1965) and is permitted by the FDA in foods (21 CFR 121.1164). The Council of Europe (1970) listed acetophenone (1-phenyl ethanone) giving an ADI of 1 mg/kg. Browning (1965) has provided an extensive monograph on acetophenone.

Biological data

Acute toxicity. The acute oral LD_{50} in rats was 3·2 g/kg (Jenner, Hagan, Taylor, Cook & Fitzhugh, 1964). The LD_{50} by dermal application was >20 ml/kg in the guinea-pig (Rowe & Wolf, 1963).

Chronic toxicity. In an FDA feeding study, 10,000 ppm fed to rats in the diet for 17 wk produced no effects (Hagan, Hansen, Fitzhugh, Jenner, Jones, Taylor, Long, Nelson & Brouwer, 1967).

Human testing. A maximization test (Kligman, 1966) on acetophenone at 2% concentration produced no reaction (Kligman, 1971).

Inhalation toxicity. Apparently no TLV has been established, but Rowe & Wolf (1963) reported no deaths in rats exposed for 8 hr to an atmosphere saturated with acetophenone. [Flash point, 82°C; vapor pressure (% in saturated air), 0·45%; evaporation rate (ether = 1), 0·06.]

Metabolism. At one time, acetophenone was used as a hypnotic. Its conversion to benzoic acid and methylphenylcarbinol in dogs and rabbits was observed by a number of early workers. Small amounts (up to 2%) are also excreted as mandelic acid. In the rabbit about half the dose is excreted as methylphenylcarbinyl glucuronide and about 20% as hippuric acid. It is probable that the ketone is first asymmetrically reduced to the carbinol, which is the precursor of benzoic and mandelic acids (Williams, 1959).

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ALCOHOL C-8

Synonyms: 1-Octanol; *n*-octyl alcohol.
Structure: $CH_3 \cdot [CH_2]_6 \cdot CH_2OH$.
Description and physical properties. EOA Spec. no. 166.
Occurrence: Found in several citrus oils and at least ten other natural sources (Gildemeister & Hoffman, 1960).
Preparation: By sodium reduction or high-pressure catalytic hydrogenation of the esters of the naturally occuring caprylic acid, or by oligomerization of ethylene using aluminium alkyl technology.
Uses: In public use since the 1900s. Use in fragrances in the USA amounts to less than 10,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·003	0·005	–
Maximum	0·09	0·014	0·03	0·20

Analytical data: Gas chromatogram, RIFM sample no. 71–7; infra-red curve, RIFM sample no. 71–7.

Status

Alcohol C-8 was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) listed alcohol C-8 (octyl alcohol), giving an ADI of 1 mg/kg. The *Food Chemicals Codex* (1966) has a monograph on alcohol C-8 (octanol).

Biological data

Acute toxicity. The acute oral LD_{50} in the rat was $>3{\cdot}2$ g/kg, while in the guinea-pig, the LD_{50} by dermal application was $>0{\cdot}5$ g/animal and skin irritation was slight (Treon, 1963). The acute oral LD_{50} for alcohol C-8 (RIFM sample no. 71–7) was found to exceed 5·0 g/kg in the rat and the acute dermal LD_{50} in rabbits exceeded 5·0 g/kg (Levenstein, 1972).

Human testing. A maximization test (Kligman, 1966) was carried out on 25 volunteers. The material was tested at 2% concentration in petrolatum and no evidence of sensitization was reported (Kligman, 1972).

Metabolism. "The primary aliphatic alcohols undergo two general reactions *in vivo*, namely oxidation to carboxylic acids and direct conjugation with glucuronic acid. The first reaction proceeds with the intermediate formation of an aldehyde, and the carboxylic acid from this may be either oxidized completely to carbon dioxide or excreted as such or combined with glucuronic acid as an ester glucuronide. The extent to which an alcohol undergoes the second reaction, i.e. direct conjugation to an ether glucuronide, appears to depend upon the speed of the first reaction, for alcohols which are rapidly oxidized form very little ether glucuronide unless given in high doses" (Williams, 1959).

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ALCOHOL C-9

Synonyms: 1-Nonanol; pelargonic alcohol.
Structure: $CH_3 \cdot [CH_2]_7 \cdot CH_2OH$.
Description and physical properties: Food Chemicals Codex (1966).
Occurrence: Found in a number of citrus oils and about ten other oils (Gildemeister & Hoffman, 1960).
Preparation: Usually by sodium or high-pressure catalytic reduction of esters of pelargonic acid (Bedoukian, 1967).
Uses: In public use since the 1900s. Use in fragrances in the USA amounts to less than 10,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·003	0·005	0·09
Maximum	0·09	0·014	0·03	0·25

Analytical data: Gas chromatogram, RIFM no. 71–8; infra-red curve, RIFM no. 71–8.

Status

Alcohol C-9 has been granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) listed alcohol C-9 (nonyl alcohol), giving an ADI of 1 mg/kg. The *Food Chemicals Codex* (1966) has a monograph on alcohol C-9 and an extensive monograph on nonanol has been compiled by Browning (1965).

Biological data

Acute toxicity. Treon (1963) reported the single-dose oral LD_{50}s in mice and rats as 6·4–12·8 and 3·56 g/kg, respectively, and the 24-hr dermal LD_{50} in the rabbit as 5·66 ml/kg. There was no skin irritation in the rabbit. Gerarde & Ahlstrom (1966) have reported on the aspiration hazard of a series of alcohols, including alcohol C-9.

Human testing. Alcohol C-9 does not appear to produce skin irritation (Peterson & Hall, 1946). A maximization test (Kligman, 1966) was carried out on 25 volunteers using a 2% concentration in petrolatum and no case of sensitization was reported (Kligman, 1972).

Metabolism. See alcohol C-8.

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ALCOHOL C-10

Synonyms: 1-Decanol; *n*-decyl alcohol.

Structure: $CH_3 \cdot [CH_2]_8 \cdot CH_2OH$.

Description and physical properties: EOA Spec. no. 167.

Occurrence: Reported to occur in sweet orange and a few other essential oils (Gildemeister & Hoffman, 1960).

Preparation: By sodium reduction or high-pressure catalytic hydrogenation of the esters of naturally occurring capric acid, or by oligomerization of ethylene using aluminium alkyl technology.

Uses: In public use since the 1900s. Use amounts to approximately 20,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·003	0·005	0·09
Maximum	0·09	0·02	0·03	0·25

Analytical data: Gas chromatogram, RIFM no. 71-9; infra-red curve, RIFM no. 71-9.

Status

Alcohol C-10 was classified GRAS by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) included alcohol C-10 (decyl alcohol) in the list of admissible artificial flavouring substances at a level of 5 ppm.

Biological data

Acute toxicity. The acute oral LD_{50} has been reported as 12·8 g/kg in the rat (Bär & Griepentrog, 1967), and as 6·4–12·8 g/kg in the mouse and 9·80 g/kg in the rat (Treon, 1963). The dermal LD_{50} value in the rabbit was 3·56 g/kg and skin irritation in the rabbit after 24 hr was severe with the mixed isomers of decyl alcohol (Treon, 1963).

Human testing. A maximization test (Kligman, 1966) was carried out on 25 volunteers using a 3% concentration in petrolatum. No subject became sensitized to alcohol C-10 (Kligman, 1972).

Metabolism. See alcohol C-8.

References

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ALCOHOL C-11

Synonyms: 10-Undecen-1-ol; undecylenic alcohol.
Structure: $CH_2 \cdot CH[CH_2]_8 \cdot CH_2OH$.
Description and physical properties: Givaudan Index (1966).
Occurrence: Found in the leaves of *Litsea odorifera* Val. (Gildemeister & Hoffman, 1960).
Preparation: Usually by sodium reduction of the esters of undecylenic acid, which is obtained by the cracking of castor oil (Bedoukian, 1967).
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 10,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	–	–	0·005	0·06
Maximum	–	–	0·03	0·10

Analytical data: Gas chromatogram, RIFM no. 71–10; infra-red curve, RIFM no. 71–10.

Status

Alcohol C-11 is permitted by the FDA for use in foods (21 CFR 121.1164).

Biological data

Acute toxicity. Both the oral LD_{50} value in rats and the dermal LD_{50} value in rabbits exceeded 5 g/kg (Levenstein, 1972).

Human testing. A maximization test (Kligman, 1966) was carried out on 25 volunteers, using a 1% concentration in petrolatum and no evidence of sensitization was reported (Kligman, 1972).

Metabolism. See alcohol C-8.

References

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ALCOHOL C-12

Synonyms: 1-Dodecanol; lauric alcohol.

Structure: $CH_3 \cdot [CH_2]_{10} \cdot CH_2OH$.

Description and physical properties: EOA Spec. no. 184.

Occurrence: In lime oil and several other essential oils (Gildemeister & Hoffman, 1960).

Preparation: By sodium reduction or high-pressure catalytic hydrogenation of esters of naturally occurring lauric acid, or by oligomerization of ethylene using aluminium alkyl technology.

Uses: In public use since the 1900s. Use in fragrances in the USA amounts to approximately 20,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·002	0·005	0·09
Maximum	0·09	0·018	0·02	0·25

Analytical data: Gas chromatogram, RIFM no. 71–11; infra-red curve, RIFM no. 71–11.

Status

Alcohol C-12 (lauryl alcohol) was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) listed alcohol C-12 (lauryl alcohol), giving an ADI of 1 mg/kg. There is a monograph on alcohol C-12 in the *Food Chemicals Codex* (1966).

Biological data

Acute toxicity. The acute oral LD_{50} in rats was reported by Treon (1963) to exceed 36·0 ml/kg and the LD_{50} by skin absorption in the guinea-pig to exceed 10 ml/kg. Seven rats and seven rabbits, which survived a dose of either 24 or 36 ml technical lauryl alcohol/kg body weight, demonstrated no significant gross or microscopic change (Treon, 1963). Bär & Griepentrog (1967) reported the oral LD_{50} in rats as 12·8 g/kg.

Human testing. Alcohol C-12 is said to have "low toxicity" (*Merck Index*, 1968). A maximization test (Kligman, 1966) was carried out on 25 volunteers using a 4% concentration in petrolatum, and no case of sensitization was reported (Kligman, 1972).

Metabolism. See alcohol C-8.

References

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ALDEHYDE C-6

Synonyms: 1-Hexanal; hexaldehyde.

Structure: $CH_3 \cdot [CH_2]_4 \cdot CHO$.

Description and physical properties: A colourless mobile liquid with a powerful fatty-green odour (Arctander, 1969).

Occurrence: Reported to occur in about a dozen essential oils (Gildemeister & Hoffman, 1960).

Preparation: By oxidation of *n*-hexanol.

Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 1000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·005	0·001	0·0015	0·018
Maximum	0·03	0·005	0·01	0·05

Analytical data: Gas chromatogram, RIFM no. 71-12; infra-red curve, RIFM no. 71-12.

Status

Aldehyde C-6 was classified GRAS by FEMA (1965), and is approved by the FDA for food use (21 CFR 121.1164).

Biological data

Acute toxicity. The oral LD_{50} in rats is given as 5 g/kg in the *Merck Index* (1968) and as 4·9 g/kg by Fassett (1963), who reported the material to be slightly irritating to the skin and eye of the rabbit. Smyth, Carpenter, Weil, Pozzani, Striegel & Nycum (1969) reported an acute oral LD_{50} of 9·51 g/kg for the mixed isomers. The dermal LD_{50} in rabbits is given as > 10 ml/kg (Smyth *et al.* 1969).

Human testing. A maximization test (Kligman, 1966) was carried out on 25 volunteers using a 1% concentration in petrolatum and no case of sensitization was reported (Kligman, 1972).

References

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ALDEHYDE C-8

Synonyms: 1-Octanal; octyl aldehyde.
Structure: $CH_3 \cdot [CH_2]_6 \cdot CHO$.
Description and physical properties: EOA Spec. no. 106.
Preparation: By catalytic oxidation of octyl alcohol.
Occurrence: In about 20 essential oils, including a number of citrus oils (Gildemeister & Hoffman, 1960).
Uses: In public use since the 1900s. Use in fragrances in the USA amounts to less than 10,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·0025	0·018
Maximum	0·06	0·009	0·01	0·05

Analytical data: Gas chromatogram, RIFM no. 71–13: infra-red curve, RIFM no. 71–13.

Status

Aldehyde C-8 was given GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) listed aldehyde C-8 (octanal), giving an ADI of 1 mg/kg. The *Food Chemicals Codex* (1966) includes a monograph on aldehyde C-8, and the Joint FAO/WHO Expert Committee on Food Additives (1967) has published a monograph and specifications for octanal.

Biological data

Acute toxicity. The oral LD_{50} in rats was found to be 5·63 ml/kg, the dermal LD_{50} in rabbits was 6·35 ml/kg (4·70–8·59 ml/kg), inhalation of concentrated vapour by rats for 8 hr produced no deaths, and skin and eye irritation was reported as very mild (Smyth, Carpenter Weil, Pozzani & Striegel, 1962). Penetration through intact mouse skin has been reported (Meyer, Meyer & Kerk, 1959).

Human testing. A standard repeated insult patch test using a 0·25% concentration in alcohol did not sensitize any of 40 subjects (Majors, 1972).

Metabolism. Aldehydes C-8, C-10, C-12 and C-14 (myristic), the lower unsubstituted aliphatic aldehydes, are readily oxidized in the animal body to the corresponding fatty acids, which normally undergo oxidation and are eventially oxidized to carbon dioxide and water (Williams, 1959).

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ALDEHYDE C-9

Synonyms: 1-Nonanal; pelargonaldehyde.

Structure: $CH_3 \cdot [CH_2]_7 \cdot CHO$.

Description and physical properties: EOA Spec. no. 91.

Occurrence: Found to occur in at least 20 essential oils, including rose oils, citrus oils and several species of pine oil (Gildemeister & Hoffman, 1960).

Preparation: By catalytic oxidation of alcohol C-9.

Uses: In public use since the 1900s. Use in fragrances in the USA amounts to less than 10,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·002	0·0025	0·018
Maximum	0·06	0·008	0·01	0·05

Analytical data: Gas chromatogram, RIFM no. 71–14; infra-red curve, RIFM no. 71–14.

Status

Aldehyde C-9 was given GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1970) listed aldehyde C-9 (nonanal), giving it an ADI of 1 mg/kg. The *Food Chemicals Codex* (1966) includes a monograph on aldehyde C-9 (nonanal) and the Joint FAO/WHO Expert Committee on Food Additives (1967) has also published a monograph on this compound.

Biological data

Acute toxicity. Both the acute oral LD_{50} in albino rats and the acute dermal LD_{50} in albino rabbits exceeded 5·0 g/kg (Shelanski, 1971).

Human testing. A maximization test (Kligman, 1966) was carried out on 25 volunteers using a 1% concentration and no case of sensitization was reported (Kligman, 1971).

Metabolism. See aldehyde C-8.

References

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Review Section

REVIEWS OF RECENT PUBLICATIONS

Food Additives and Contaminants Committee Report on the Review of the Preservatives in Food Regulations 1962. Ministry of Agriculture, Fisheries and Food. HMSO, London, 1972. pp. 89. £0.57.

The Food Additives and Contaminants Committee (FACC) has recently reviewed the Preservatives in Food Regulations 1962. Its report, published on 7 July, re-examines the list of preservatives at present permitted in the UK and considers representations that have been made requesting changes. Opinions of the Pharmacology Sub-Committee (PSC) and of the Antibiotics Panel of the Committee on Medical Aspects of Food Policy regarding the safety-in-use of the additives are included.

Technological need has been a major consideration for the Committee throughout the review and it is stressed that evidence of this need as well as adequate toxicological data are required before the use of any additive can be recommended. In fact, no new compounds have been proposed for use as preservatives.

The definition of 'preservative' and the present system of control have been reconsidered by the FACC, and the definition has been amended to underline the restriction of the term 'preservative' to compounds that protect food against deterioration due to micro-organisms. Deterioration due to oxidation is controlled by the Antioxidants in Food Regulations 1966, and is thus outside the scope of this review, but recommendations are made to clarify the position with regard both to compounds used to protect food during storage (e.g. pesticides) and to carry-over preservatives (e.g. formaldehyde).

Substances considered exempt from the definition of 'preservative' include those controlled by other Statutory Instruments (such as colourings, emulsifiers and solvents), bleaching and improving agents, lecithin, sodium chloride, sugars, tocopherol, carbon dioxide and nitrogen gases. Nicotinic acid and its amide are not recommended for control other than under the restrictions at present imposed by the Meat (Treatment) Regulations 1964. Smoke solutions, which are normally used as flavourings but which also have a preservative effect, are to be considered in a separate review. Meanwhile, where wood is used to produce smoke it should not be impregnated, painted or otherwise treated with any material. Foods in which preservatives are not recommended include margarine, low-fat foods, fish preserves and cold processed packaged fish and meat.

The use of antibiotics as preservatives has been considered and the report recommends that the present provision for tetracyclines in raw fish should be deleted. No change is recommended in the present uses of nisin, but the continued use of nystatin on banana skins is not approved because the compound is not effective. An alternative, thiabendazole, is recommended for use on both bananas and citrus fruit at a maximum level of 6 ppm (on

the weight of the whole fruit), provided this level is subject to review when further toxicological data become available. The Committee was not convinced of the necessity for pimaricin as an additional mould inhibitor for use on the rind of hard cheeses, even though this use has been accepted within limits by the Joint FAO/WHO Expert Committee on Food Additives (*Tech. Rep. Ser. Wld Hlth Org.* 1969, **430**).

Several representations for preservatives not currently permitted have been rejected, mainly because of insufficient evidence of technological need. These include the use of octyl gallate and *n*-heptyl *p*-hydroxybenzoate in beer, formic acid in soft drinks, hexamethylenetetramine in fish preserves, nordihydroguaiaretic acid as a general preservative, fumaric acid combined with sodium benzoate in fish and benzoyl peroxide as a bleaching and preserving agent in blue cheeses. Diethyl pyrocarbonate, for which a definite technological need was recognized, was not recommended for inclusion in the revised permitted list until the PSC had re-evaluated some new toxicological findings.

The preservatives most commonly used under the 1962 Regulations are nitrates and nitrites, sulphur dioxide and benzoic acid, all of which act as bactericides. Sorbic acid is less widely used and acts mainly as a fungistat. Extensions for the use of these currently permitted preservatives were considered by the FACC. For sorbic acid, the recommended extra uses (in sweetened nut pastes, dried prunes, wine and mead) would be unlikely to lead to a significant increase in the present daily intake, and the foods concerned are not subjected to processing that might lead to chemical conversion of the fungistat. No further extensions are likely until the results of further work on parasorbic acid, currently in progress at BIBRA, have been assessed by the PSC.

Extended uses of sulphur dioxide, sulphurous acid and its salts include its use in wine or cider vinegar, concentrated grape juice for home-made wines, dehydrated Brussels sprouts, horseradish sauce, French mustard, garlic powder, fruit yogurt (including carry-overs from sulphited fruit pulp), wine and beer finings, grapes, canned cauliflower and dried hops. Representations for the use of sulphur dioxide in desiccated coconut, butchers' minced meat and vegetables in brine have been rejected.

Extended uses of benzoic acid include its use in fruit yogurt, low-acid sauces, diabetic and low-carbohydrate jams, tomato pulp and purées, glacé and crystallized fruit and beer. The use of benzoic acid in wines and powdered rennet is not recommended.

The use of methyl and propyl *p*-hydroxybenzoates and their sodium salts is permitted by the 1962 Regulations. The FACC has now recommended that the ethyl ester and the potassium salts of all three esters should be permitted in addition. Moreover, their use is to be considered interchangeable with benzoic acid. A specific use (250 ppm of sodium methyl *p*-hydroxybenzoate in prepacked cooked beetroot) is also recommended.

Provision was not recommended for the presence of diphenyl in citrus drinks, for copper carbonate on pears from tissue wrappings, for the new use of *o*-phenylphenol on fresh tomatoes, strawberries, bananas or grapes, or for ethylene oxide as a sterilant for egg albumen and desiccated coconut. In most cases the technological need was again considered insufficient. The need for ethylene oxide as a sterilant for spices, however, was considered to be well established.

The question of nitrate and nitrite preservation of meats has been reviewed by the FACC and the PSC, particular attention being paid to the possible formation of hepatotoxic *N*-nitroso compounds, which may also be liver carcinogens. An extensive research programme is recommended to determine the value of nitrites in the prevention of *Clostridium botulinum* toxin, the interaction of amines and nitrites in food and the gut, and the long-

term dose-related effects of small quantities of nitrosamines in the diets of experimental animals. Meanwhile, the FACC considers that the use of nitrates and nitrites in new applications should not be recommended. The present levels of 500 ppm nitrate and 200 ppm nitrite permitted in meat curing, as laid down by the recent amendment to the 1962 Regulations (*Cited in F.C.T.* 1971, **9,** 879) should be retained for a further 2 years, but manufacturers should explore the possibility of reducing the amounts of these substances in meat products.

In the PSC report, each preservative has been classified into one of three groups, namely group A (additives that the available evidence suggests are acceptable for food use), group B (additives that are provisionally acceptable but must be reviewed within a specified time on the basis of further information) or group E (additives for which the available evidence is inadequate for assessment of safety-in-use). Diethyl pyrocarbonate and *o*-phenylphenol esters of acetic, isobutyric and lauric acids were classified in group E. Additives classified in group B were diphenyl, ethylene oxide, heptyl *p*-hydroxybenzoate, *o*-phenylphenol and its sodium salt, sodium and potassium nitrates and nitrites, sorbic acid and its sodium, potassium and calcium salts and thiabendazole. Group A additives were benzoic acid and its sodium and potassium salts, copper carbonate, methyl, ethyl and propyl *p*-hydroxybenzoates, propionic acid and its sodium and calcium salts, and sulphur dioxide and sulphurous acid and its sodium, potassium and calcium salts. Much of the additional work required on preservatives is likely to form the basis of further discussion within the BIBRA Panel III Working Party.

It is interesting to note that specifications have been proposed for preservatives in food, based for the most part on EEC specifications, which require limits of 3 ppm As, 10 ppm Pb and 50 ppm Zn and Cu in combination.

Survey of Lead in Food. Working Party on the Monitoring of Foodstuffs for Heavy Metals: Second Report. Ministry of Agriculture, Fisheries and Food. HMSO, London, 1972. pp. 31. £0.21.

When the Working Party on the Monitoring of Foodstuffs for Heavy Metals published its survey of mercury in food last year (*Cited in F.C.T.* **10,** 399), it was announced that further reports would deal with lead and cadmium. The first of these is now available.

The lead content of the staple foodstuffs analysed—cereals, meat, fish, vegetables, fruit, fats and milk—proved to be generally low, with mean levels ranging from 0·03 ppm in milk to 0·24 ppm in green vegetables. On the basis of these figures, the lead content of the average daily diet was estimated as 0·13 ppm, equivalent to about 200 μg in the 1·5 kg of food consumed daily by the average person in the UK. A 1967 estimate suggested that a further 20 μg could be assumed to be ingested daily from beverages. These values were, if anything, lower than estimates made as early as 1949, suggesting that total dietary levels had not increased significantly during the intervening years.

In most foodstuffs, the lead levels detected were well below the limits prescribed in the Lead in Food Regulations 1961, although the small number of canned fruit juices examined contained on average 0·66 ppm (range $<$0·01–1·9 ppm), compared with the permitted level of 0·5 ppm. A few samples of canned corned beef and canned sardines also contained excessive amounts of lead. Mean levels of 0·24 ppm (range 0·01–1·1 ppm) were detected in canned baby food, for which no limit other than the general one of 2 ppm is prescribed in

the current Regulations. In fish, lead was generally below the limit of detection, even in samples from the Bristol Channel and Cardigan Bay, which are subject, respectively, to industrial discharges and to run-off from metalliferous rocks and old mine-workings. Levels in shellfish from these two areas were higher than in shellfish generally, reaching 18 ppm (5–6 times the normal level) in some winkle samples. However, the mean level in UK shellfish as a whole, weighted according to landings from different areas, was only 1·0 ppm. Proximity to metal smelting or refining plants or to busy roads increased the lead content of crops, but this was mainly because of surface deposition, which was largely removed during preparation and cooking. Milk from cows grazing in such areas did not appear to be significantly affected. Work is being continued to assess lead uptake in crops growing in the vicinity of refining plants and on land treated with sewage sludge, for which no data are yet available.

The health implications of these findings were assessed by the Pharmacology Sub-Committee (PSC). Dietary levels were generally not regarded as excessive, but it was recommended that the average level of 0·24 ppm in canned baby food should be reduced, at least to the 0·13 ppm level existing in the adult diet and preferably below this. In view of the fact that average shellfish consumption is only 1·5 g/day in this country, the lead levels in this commodity were not thought to present a hazard to the majority of the population, but further studies of individuals with a high dietary intake of lead-rich shellfish were recommended, to determine whether their body burden of lead was excessive.

The Food Additives and Contaminants Committee (FACC) also regarded canned baby foods as a major problem and advised an amendment of the Lead in Food Regulations to impose a limit of 0·5 ppm on the lead content of foods specially prepared for infants and young children. It was further recommended that every effort should continue to be made to reduce further contamination of the environment with lead. There was thought to be no need to continue monitoring for lead on the present scale; in most cases, samples taken as part of the Total Diet Study would be sufficient, but special attention should be given to foods particularly liable to lead contamination.

The FACC's recommendation on baby food has been endorsed by the Minister of Agriculture, Fisheries and Food, as has the PSC's recommendation for a study of high-risk shellfish consumers.

The report makes a useful contribution to the present debate on lead. A similarly detailed report on cadmium, due later this year, will be eagerly awaited.

Mercury Pollution Control 1971. By H. R. Jones. Pollution Control Review no. 1. Noyes Data Corporation, Park Ridge, New Jersey, 1971. pp. vii + 251. $35.

The discovery in March 1970 that fish from two Canadian lakes contained high levels of mercury led in less than a year to the withdrawal of over a million cans of tuna fish and practically every brand of frozen swordfish from the American market. Chapter 1 of the above-named publication describes this story chronologically up to February 1971, adding several pages of references on the occurrence of mercury in fish and the aquatic environment for good measure. Another 23-page chapter deals with the toxicology of mercury, beginning with a discussion of specific episodes of human mercury poisoning. Strangely, this omits mention of either the New Mexican family poisoned by pork from pigs fed mercury-dressed seed or the occurrence of Minamata disease, although references to the

latter are listed later. The chapter also covers mercury's metabolism and its ability to inhibit enzymes, the acute and chronic toxicity of both the inorganic and organic forms in man and the effects on animals and plants. Each of these sub-sections is well documented, with extensive lists of references.

Further chapters deal with air pollution by mercury, stressing the hazard of evaporation from residual quantities of the spilled material, and with environmental standards. The latter section includes a discussion on the validity of present threshold limit values for mercury in working atmospheres and concludes that different tolerance levels may be necessary for the alkyl and the aryl mercury compounds, in view of the greater toxicity of the former. Mention is also made of attempts to establish tolerances for mercury in food and water, and the lack of data on which these could reliably be based.

Additional chapters cover mercury production, properties, uses and detection, the clean-up of spilled mercury and its removal from gases, liquids and other materials. It is somewhat disturbing to find that mercury still finds a variety of applications in pharmaceutical and cosmetic products, including diuretics, antiseptics, skin preparations and preservatives. While some of these uses are expected to decline over the next few years, cosmetic uses are likely to increase. Dental use is also expected to increase, despite the potential hazard to dental assistants.

The book has been published in paperback form, "to close the time gap" between "manuscript" and "completed book", and although this object is to be applauded it makes the price appear somewhat high. However, the extensive bibliographies on specific topics (including references from patent as well as journal literature) and the emphasis on measures that can minimize further environmental contamination should be of value to all those concerned with mercury technology and its side-effects.

Fd Cosmet. Toxicol. Vol. 11, pp. 123–130. Pergamon Press 1973. Printed in Great Britain

BOOK REVIEWS

Food Science. By G. G. Birch, A. G. Cameron and M. Spencer. Pergamon Press, Oxford, 1972. pp. viii + 189. £2.50.

This informative little book gives an up-to-date account of the preparation and preservation of foodstuffs. It outlines the many factors involved in giving food maximum palatability while maintaining its nutritive value. The book is made all the more readable by the use of a partly historical approach, in which the present methods of food processing are illustrated in the light of the original discovery and the refinements that have occurred over the years. The author also stresses the need for public awareness of the quality of our food, and cites numerous cases showing that there is much room for improvement in this respect.

After a briefing on the components of food and drink, there is a chapter devoted to the need for food and the way in which it is assimilated in the body. The bulk of the work, however, is devoted to the processing and preservation of food, although the most important chapters from BIBRA's point of view are the last two, which deal with the subject of food additives and with future speculation regarding increased efficiency of food production. In the first of these, both intentional and unintentional additives are discussed, and their benefits and potential hazards are debated. The obvious conclusion is drawn that modern man cannot do without small quantities of these chemicals, albeit within certain prescribed limits. The title of this chapter "Eating Chemicals" is misleading, since it seems to imply—and the text does not clarify the point with authority—that there are different meanings for the word 'chemical' when it refers to a food additive and when it refers to substances present in the basic food. In the final chapter, new ways of processing food are documented, potential foods of the future are considered, and the conclusion is drawn that a much greater efficiency of production could be achieved if man were to assume a more responsible attitude to the problem of food shortage.

As stated in the preface, the book has been written mainly for the layman. Its simple, lucid style and general lack of scientific jargon should provide easy and interesting reading for anyone looking for an introduction to the increasingly important subject of food science.

A History of Make-up. By Maggie Angeloglou. Studio Vista, London, 1970. pp. 144. £3.60.

If the fair Elaine was indeed a character in the original Celtic Arthurian Court, Maggie Angeloglou would undoubtedly contend that her toilet owed much to the Romans, who in their turn had learnt from the Egyptians and Greeks. However, it appears that to dye their hair red or blonde the Romans themselves used a soap that originated in Gaul. If so, it is tempting to surmise that Elaine, with or without Roman help, could have contrived to choose the colour of her hair, even if she had decided that woad was somewhat outmoded.

It is more than likely, however, that Elaine was a figment of the fertile mediaeval imagination, in which case she would have had a whole range of cosmetics to command, many of them brought back by the Crusaders from the Eastern Mediterranean. Nevertheless, aristocratic mediaeval ladies played it cool and made themselves as pale as possible. Hair was made pale, skin that had been exposed to the sun was hastily whitened with a flour powder, and eyebrows were plucked, so that the whole face was smooth, white and as egg-like as possible. More adventurous ladies certainly painted, however, and possibly gained in suitors what they lost in respectability. Somehow one feels that Elaine was one of the pale sort, only succeeding in arousing Sir Lancelot's attention after her death.

Fortunately, the author of this book does not confine herself to cosmetics in the narrowest sense but covers the whole field of personal adornment through the ages, from tattooing to wigs. There are many interesting sidelights. For example, everyone knows the date 1066, some even know what happened then, but surely very few know that Harold was recognized among the many dead because of his distinctive tattoos, allegedly including "Edith" inscribed over his heart.

Cosmetics manufacturers, sleepless in the small hours from worrying about the unknown sensitizers, irritants and toxins that may be lurking in their products, should take comfort from the fact that none of their clients are likely to suffer the fate of Maria Gunning who died in 1760, a "victim to Cosmetics". An acknowledged beauty, she insisted on painting her face with white lead, in spite of (or because of?) her husband's objections. Her reign was brief. Within 6 years of her triumphant introduction to London society and her marriage to the Earl of Coventry, she had died of lead poisoning.

Men have often outdone women in their use of paints and personal adornments of various kinds. Maggie Angeloglou ends her book with a prediction that the next major development in cosmetics will "almost certainly be intended for men". She recommends that men should not attempt the more colourful range of reds but "various shades of brown blended with a paler brown lipstick", well-shaped eyebrows "crayoned in with dark grey pencil" and pale nail lacquer "complemented by sunburnt hands". However, those men, and presumably women, who really want to break away from the tedious routine of greys, browns and muted pinks, can always use "elaborate decoration above and below the eye" and mock tattoo designs appliquéd on both face and body. At least we have advanced a bit. Poor Harold was stuck with "Edith" tattooed above his heart forever; now he could wash off his "tattoo" every night (in a perfumed bubble bath) and start again with the name of any lady he fancied over his heart and a brand new tattoo appropriate to the latest attraction.

In short, this is a book that no-one interested in either the use or the manufacture of cosmetics should be without.

Occupational and Environmental Cancers of the Urinary System. By W. C. Hueper. Yale University Press, London, 1969. pp. xix + 465. £9.

Cancer of the urinary bladder resulting from occupational or environmental exposure to carcinogens has stimulated the interest of epidemiologists and experimentalists for at least two decades. Epidemiological studies revealed that workers in the aniline-dye industry had an exceptionally high incidence of bladder cancer and this was traced to contact with a

number of aromatic amines, including fuchsin, benzidine, 4-aminodiphenyl and β-naphthylamine. On the environmental front, bladder cancer has been associated with infection by *Schistosoma hematobium*. The eggs of this blood parasite lodge in the bladder mucosa causing, in addition to carcinoma, a variety of pathological lesions.

Over the years, numerous studies on the production of bladder tumours in experimental animals have been undertaken in attempts to gain some idea of the mechanisms responsible for bladder carcinogenesis in man. As in most areas of experimental carcinogenesis, much of the evidence obtained has been conflicting or difficult to interpret. One notable exception, however, was the finding that the dog developed carcinoma of the bladder with all the compounds suspected of being carcinogenic to man.

The author of the book cited above traces the history of this important chapter in cancer research. The epidemiological studies are recorded in detail and supported by a large number of references. The experimental work is given equal coverage. One feels, however, that whereas a commendably objective approach is maintained throughout the chapters relating to epidemiology, this is not altogether the case in the discussion of experimental results. As the author rightly points out, bladder cancer in man and in the dog is due to some chemical interaction between a metabolite of the carcinogen and the transitional epithelium of the urinary tract. It is questionable, however, whether such interaction can account for bladder carcinomas in rodents under all circumstances. One accepts that the induction of bladder cancer by substances given orally or parenterally is very likely to be a manifestation of chemical interaction, but the induction of tumours by compounds incorporated into pellets and implanted in the bladder is another matter. Pellets of paraffin wax, glass and cholesterol containing no added carcinogen have been shown to induce carcinoma of the bladder in rodents. Such findings are clearly of major importance in the evaluation of the carcinogenic effects of chemicals implanted in the bladder, but while the author is obviously aware of these pitfalls, they are not given the prominence they deserve.

The author also slips from strict objectivity on another point. The chemical relationship between some food colourings and certain other azo compounds that have induced bladder tumours led him to consider the possibility that some of the food colourings might be carcinogenic to man and to suggest that too little is being done to eliminate possible carcinogenic dyes from foods. There is no concrete evidence to substantiate this. Most of the tumours induced by these dyes under experimental conditions do not constitute valid evidence of carcinogenicity.

Elsewhere in the book, there is an interesting discussion on the possible role of endogenous carcinogens, particularly tryptophan metabolites, in causing cancers of the urinary tract. Legal and public health measures for controlling urinary cancer hazards are also considered. The book as a whole is lucid as well as informative, and the long list of references at the end will be welcomed by many a harassed research worker delving into the confused depths of urinary tract carcinoma.

Hazards in the Chemical Laboratory. Edited by G. D. Muir. Royal Institute of Chemistry, London, 1971. pp. xi + 266. £3 (paperback £2).

The old maxim that accidents only happen to other people must surely be the reason for the apathy and indifference shown by most research workers to the hazards confronting them in the laboratory. Anyone who has worked in any kind of laboratory cannot fail to notice the contradictions between the normal laboratory practices they have seen and

followed and the procedures recommended in the present volume, the Royal Institute of Chemistry's sequel to its "Laboratory Handbook of Toxic Agents", first published in 1960.

The new volume discusses not only toxic hazards but also fire prevention, radiation protection and first-aid procedures. Perhaps the most revealing chapter is that on "planning for safety", because it is here that the inadequacies of the present systems are most apparent. Sensible planning can, for instance, provide less dangerous alternatives to some of the commoner toxic or flammable compounds (e.g. benzene and acetone) and can certainly prevent the construction of laboratories without adequate ventilation, access or fire-fighting equipment. While cases such as that in which students were discovered pipetting by mouth the potent carcinogen, benzidine, will always attract the greatest publicity, it is the maintenance of sensible day-to-day laboratory hygiene and safety precautions that can have the greatest overall benefit to laboratory staff.

The greater part of this book is given over to summaries of the hazardous properties of over 430 laboratory chemicals. The description of the chemical, physical and biological properties of each compound is linked to the hazards that they are therefore likely to offer and to the best ways in which such hazards can be lessened and if necessary dealt with.

This is a reference book deserving a place in every laboratory. One hopes that education will remain a good cure for apathy.

Chromatography of Environmental Hazards. Vol. 1. Carcinogens, Mutagens and Teratogens. By L. Fishbein. Elsevier Publishing Company, Amsterdam, 1972. pp. vii + 499. $42.25.

Considerable attention has been paid in recent years to the identification and determination of environmental chemicals likely to present a hazard to health. The analyst clearly has a big part to play in this work, and chromatography is probably the most important technique at his disposal. It is very gratifying, therefore, to find a book that contains such a wealth of information on this subject.

This volume, the first of a series of three, is concerned with carcinogens, mutagens and teratogens, and contains chapters dealing with alkylating agents, pesticides, drugs, food and feed additives and contaminants and miscellaneous toxicants. The other two volumes will deal with air, water, industrial pollutants, metals, pesticide residues and drugs. Any sub-division into categories is likely to lead to difficulties when a chemical is eligible for inclusion under two or more headings, and inevitably certain substances one might expect to find described in this volume are held back for a later one.

In general, each chemical is given a fairly full background treatment, the information on methods of preparation, uses, natural and 'accidental' occurrence, toxicity and metabolism being sufficient to enable the chromatographer to start with the comforting feeling that the problem has been adequately defined for his purpose—a feeling enhanced by a liberal supply of structural formulae. The analytical sections deal primarily with thin-layer, paper and gas–liquid chromatography, and here the author covers the ground very thoroughly and in a reasonable amount of detail, with the aid of numerous tables and diagrams of chromatograms. The copious references at the end of each chapter add considerably to the value of the book. An analyst requiring greater detail will not be satisfied with anything less than the original papers. The index of nine pages is, however, disappointingly brief.

The volume is well printed and produced, and will be a welcome addition to the bookshelves of those working in this field.

Organ Culture. Edited by J. A. Thomas. Academic Press, New York, 1970. pp. xiii + 512. $29.50.

This book is the first English translation of a classic work on organ culture—"Cultures Organotypiques" by Etienne Wolff and his colleagues—published in France in 1964. The chapters comprising this work were originally presented as a series of lectures forming part of a course in cell biology. The authors have, however, taken this opportunity to update the work to 1969 by including after each chapter an addendum outlining some of the more important work that has been carried out since the original material was assembled.

The book has been expertly translated and its readability is further enhanced by the good layout of the chapters and the abundance of photographs and diagrams. A summary at the end of each chapter provides useful clarification of the main points made. Each chapter and addendum ends with a comprehensive bibliography.

The first chapter, by Professor Wolff himself, is in the form of a general introduction to the principles of organ culture and is followed by nine further contributions showing how these principles have been applied in various areas of research. The main point to emerge from the first three of these is that, generally speaking, embryonic tissues in culture follow the same pattern of differentiation as they would *in vivo*. Strangely enough, such differentiation takes place quite well in synthetic media. The addition of sex hormones to culture media can alter the development of the gonads. For example, in some amphibia the addition of male sex hormones to cultures of female embryonic gonadal tissue produces a male type of gland.

The rest of the book considers the action of hormones and inhibitors on organogenesis *in vitro*, the factors governing the re-association of dissociated cells and the processes of induction of growth and differentiation. A chapter devoted to organ culture in the invertebrates, a branch of the technique still in its infancy, is one of the few parts of the book where the culture of adult organs is considered. The final contribution, devoted to organ chimeras and the organ culture of malignant tumours, will be of particular interest to workers in cancer research.

On the whole this is a very readable book and should prove of value to many biologists, not only those working in the fields of developmental biology and tissue culture.

Control Mechanism of Growth and Differentiation. Symposia of the Society for Experimental Biology. No. XXV. Edited by D. D. Davies and M. Balls. Cambridge University Press, 1971. pp. vii + 498. £8.

This volume is a collection of the papers presented to the 25th Symposium of the Society for Experimental Biology, jointly organized by the Society and the British Society for Developmental Biology. The 26 papers range over subject matter as varied as wall-pattern formation in angiosperm microsporogenesis and the form of the mouse blastocyst. Such diversity reflects the complex and extensive subject matter pertinent to the work of the developmental biologist.

Early contributions deal with the relatively simple systems of development in the prokaryotes and lower eukaryotes. The first communication reviews the response of *Escherichia coli* and other micro-organisms to starvation, a reaction that is basically concerned with the synthesis of new enzymes to metabolize alternative nutrients. The occurrence of long-lived messenger RNA is also a common feature of these primitive organisms.

Several papers consider molecular aspects of development. One discusses the role electron microscopy has played in attempts to visualize directly some complex biological structures assembled from macromolecular components, such as collagen fibrils, virus particles and enzymes. Another examines the structure and function of chromatin (interphase chromosomes).

A series of papers follows on the formation and control of pattern in the differentiation and development of more complex organisms, from plants to mammals. These encompass fields of investigation as diverse as the precision positioning of organs on the growing points of plants and the inductive interactions involved in the development of the vertebrate metanephric kidney. The book closes with a group of papers concerned with theoretical aspects of control mechanisms of growth and differentiation.

The contributions are all concerned with very specialized topics, but the ease with which readers not actively engaged in similar work are likely to be able to assimilate the information presented varies considerably from paper to paper. Too many papers suffer from an excessive use of jargon without adequate explanations of the terminology used, and much-needed summaries of detailed papers are often lacking. As a general criticism, titles to the papers are frequently long and unnecessarily obscure.

On the whole, the book needed more than the addition of an author and subject index if it aspired to be more than the sum of its papers. The absence of a general introduction by the editors is a particularly obvious omission in this connexion. Nevertheless, the general presentation of the book, the illustrations and the references are good.

It seems that the zenith of imaginative ideas and philosophies in the science of embryology propounded by Waddington, Needham and Brachet some 40 years ago has been followed by a period of searching and wandering in a jungle of complexity, while basic mechanisms of organization, regulation and differentiation have remained unexplained. This symposium emphasizes our lack of knowledge, poses more questions and does not suggest many short cuts to the answers. However, the definition of problems is the first stage of research, and for this at least the volume can be recommended to scientists working or interested in the fields of developmental biology.

The Blood Supply of Bone. An Approach to Bone Biology. By M. Brookes. Butterworths, London, 1971. pp. ix + 338. £6.50.

Detailed texts on the anatomy and physiology of bone are few, and even fewer contain references to the mass of relevant research conducted in the last decade. The author of this book has paid particular attention to the vascular supply of bone and gives up-to-date consideration to its role in bone modelling in health and disease.

The early chapters are concerned primarily with the function of the circulation in the nutrition of osseous tissue and cartilage, emphasis being placed on the disposition of the important arteries in the major long bones of common laboratory animals and man, as demonstrated by angiographic and Indian ink-injection techniques. The development of the blood supply to bone is considered with regard to the different regions of a typical long bone, such as the femur. A separate chapter describes other bones in similar detail, including the vault of the skull, the vertebrae and small long bones.

The vasculature of marrow cavities would seem to be a very complex system, as discussed

with reference to thorotrast and other marker techniques. However, with the use of excellent diagrams and photographs, comprehensive coverage is given to this controversial subject.

Later chapters are diverse in content, dealing among other matters with faults in bone morphology and physiology resulting from dysgenesis or malfunction of the vascular supply. The vascular control of osteogenesis is particularly well covered, from both morphological and biochemical standpoints.

In all, *The Blood Supply of Bone* appears to be an admirable work of reference for the researcher in the teratology of physiology laboratory. The ample supply of clear diagrams supplemented by photographs of high quality illustrates more than satisfactorily a carefully worded text. References to animal models as well as to human material are also helpful. The author has given adequate consideration to the various aspects of his subject and has skilfully supported his views by recent observations.

Advances in Clinical Chemistry. Vol. 12. Edited by O. Bodansky and C. P. Stewart. Academic Press, New York, 1969. pp. xvi + 462. $19.50.

This book is part of an excellent serial publication established in 1958 with the clear aim of presenting both reliable diagnostic and prognostic procedures and current views on the biochemical mechanisms underlying disease. The volume under review contains six sections contributed by a total of nine authors, many of whom are eminent authorities in their fields.

The biochemical changes that occur after injury are reviewed with consummate clarity by D. P. Cuthbertson and W. J. Tilstone. The influence of various factors, such as the nutritional state of the subject and the environmental temperature, on the "ebb" and "flow" of metabolic activity and the endocrine response to injury are fully discussed.

The following section, by I. E. Bush, is a critical review of methods for the determination of steroids in plasma and urine. An excellent introduction to the chemistry and metabolism of steroids and their clinical significance precedes the main part of this contribution, which deals with methodology. The special problems associated with steroid studies in early infancy are stressed by F. L. Mitchell and C. H. L. Shackleton, who point out that results obtained in neonates by means of techniques designed for work with adults must be interpreted with caution. Special techniques are available for studies in infants and these are particularly useful for investigating suspected defects in steroid synthesis, but it is clear that steroid metabolism in early life is a field in which many questions still remain unanswered.

The section by H. V. Street on the use of gas–liquid chromatography in clinical chemistry covers the main areas of application of this technique. Any review of this rapidly expanding field clearly has built-in disadvantages, particularly with regard to obsolescence of methods. In spite of this limitation, the author provides a concise and clear introduction to a complex subject.

The clinical chemistry of bromsulphthalein and other dyes employed in liver-function tests forms the subject of a contribution by Paula Jablonski and J. A. Owen. The pharmacokinetics of dyes and other related cholephilic compounds are discussed in relation to normal and pathological conditions.

In the final section, R. D. Leeper reviews recent advances in the biochemistry of thyroid regulation. The rapid advances being made in the understanding of the physiology and biochemistry of the thyroid have been assiduously collected and clearly collated.

This volume maintains the high standard established by its predecessors and, with the numerous references listed at the end of each section, should prove of considerable value to many workers in the field of clinical chemistry.

BOOKS RECEIVED FOR REVIEW

Peptide Transport in Bacteria and Mammalian Gut. A Ciba Foundation Symposium. Elsevier Excerpta Medica, North-Holland, 1972. pp. viii + 161. £2.40.

Progress in Experimental Tumor Research. Vol. 16. Pathology of the Syrian Hamster. Edited by F. Homburger. S. Karger, Basel, 1972. pp. xvi + 637. £20.70.

Autoradiography for Biologists. Edited by P. B. Gahan. Academic Press, London, 1972. pp. xi + 124. £2.

Inhaled Particles, III. Proceedings of an International Symposium organized by the British Occupational Hygiene Society in London, 14–23 September, 1970. Vols I and II. Unwin Brothers Limited, Surrey, 1971. pp. xxiii + 1090. £15.00.

Chemical Mutagens. Principles and Methods for Their Detection. Vols 1 and 2. Edited by A. Hollaender. Plenum Press, New York, 1971. pp. cxxx + 610. £8.20 per volume.

International Encyclopedia of Pharmacology and Therapeutics. Section 71, Vols I and II. Pharmacology and Toxicology of Naturally Occurring Toxins. Edited by H. Rašková. Pergamon Press, Oxford, 1971. pp. xxix + 368 and xxix + 299. £7.50 per volume.

Aging in Cell and Tissue Cultures. Edited by Emma Holečková and V. J. Cristofalo. Plenum Press, New York, 1970. pp. xx + 163. £5.85.

Biochemical Responses to Environmental Stress. Edited by I. A. Bernstein. Plenum Press, New York, 1971. pp. xii + 153. £3.75.

Particle Size Analysis in Industrial Hygiene. By L. Silverman, C. E. Billings and M. W. First. Academic Press, New York, 1971. pp. xiv + 317. £7.95.

Carbon–Fluorine Compounds. Chemistry, Biochemistry & Biological Activities. A Ciba Foundation Symposium. Edited by Katherine Elliott and Joan Birch. Associated Scientific Publishers, Amsterdam, 1972. pp. viii + 417. £6.26.

Fd Cosmet. Toxicol. Vol. 11, pp. 131–144. Pergamon Press 1973. Printed in Great Britain

Information Section

ARTICLES OF GENERAL INTEREST

IF ALL THE SEA WERE PCB . . .

The polychlorinated biphenyls (PCBs) have now well and truly lined up with DDT and mercury in the ranks of materials recognized as important environmental contaminants. The qualifications for membership of this group are widespread occurrence, relatively high toxicity and, most importantly, non-biodegradability. The PCBs score well in all these categories, a fact borne out by the large number of review articles written on the subject of PCB pollution during the past year or so (Edwards, *Chemy Ind.* 1971, p. 1340; Peakall & Lincer, *BioScience* 1970, **20,** 958; Hammond, *Science, N.Y.* 1972, **175,** 155; Tinker, *New Scient. & Sci. J.* 1971, **50,** 16).

In simple terms, the problem seems to be that the PCBs, used *inter alia* as plasticizers and solvents in paints, sealants, adhesives and printing inks, are escaping into the environment, probably from sewage combined with industrial effluent, and are slowly accumulating in rivers and sea areas. The structure of the biological food-chains then ensures that these PCBs are widely distributed throughout the animal kingdom, including man. This much is fact (*Cited in F.C.T.* 1971, **9,** 568). The exact toxicological significance of PCB residues is not fully understood, however, although any marked accumulation of even a metabolically inert compound must be considered cause for concern. This concern was somewhat alleviated last year by the decision of the Monsanto group, sole producers of PCBs in the USA and the UK, to restrict PCB sales to those destined for use in enclosed applications, such as capacitors and transformers, providing a minimal possibility of leakage into the environment (*ibid* 1971, **9,** 571). As PCBs have been known to cause poisoning in man (Kuratsune *et al. HSMHA Hlth Reps* 1971, **86,** 1083) and as they are clearly accumulating in the environment from some of the less controllable sources, this decision represents an important advance in reducing the potential hazard proffered by these compounds.

Levels of PCB residues

Aquatic animals and the birds feeding on them are the obvious material to use as a starting point for a survey of PCB contamination. Furthermore, as the PCBs, especially the more highly chlorinated ones, have an extremely low solubility in water, one would expect them to be principally associated with the sediment on the bottom of seas and to enter into food chains via bottom-living organisms.

Practical verification of this theory has come from Escambia Bay, Florida, where PCB residues of up to 120 and 1·5 ppm have been found in shrimps and crabs respectively (Nimmo *et al. Nature, Lond.* 1971, **231,** 50). The PCBs, which were mainly located in the sediments of the bay, originated from an up-stream chemical plant. Samples taken from

places up-stream from the plant were unpolluted. Experimental work, involving exposure of shrimps and crabs to polluted sediment in the laboratory for 30 days, confirmed that both species were capable of absorbing PCBs from their environment to such an extent that the final residue level in the animals after 30 days often exceeded that in the sediment itself. A similar conclusion was reached by Wildish & Zitko (*Mar. Biol.* 1971, **9**, 213), using the small shrimp, *Gammarus oceanicus*. In this case, exposure was for shorter periods measured in hours rather than days. The uptake of PCBs was found to depend on both the external PCB concentration and the total exposure period, but after 4 hours there was a reduction in the rate of uptake. In another study (Sanders & Chandler, *Bull. env. contam. & Toxicol.* (*U.S.*) 1972, **7**, 257), eight invertebrate species, including shrimps, crayfish and several types of immature aquatic insect, were exposed to PCB at concentrations below 3 ppb*, a level similar to that found in the waters of Escambia Bay. After exposure for 4 days, total body concentrations ranged from 0·2 ppm in crayfish to 16 ppm in the glass shrimp and 52 ppm in daphnia. In scud (*Gammarus pseudolimnaeus*) exposed to 1·6 ppm PCB, the concentration in the organism reached equilibrium in 14 days and was then 27,500 times higher than the concentration in the water.

As one might expect, the levels found in fish are somewhat less, most commonly in the 0–1 ppm range. In a Canadian survey of fish taken from lakes, rivers and the sea and reported by Zitko (*ibid* 1971, **6**, 464), values of 0·3–0·4 ppm were most common but levels up to 1·01 ppm were recorded for some samples. Much higher residue levels were seen in fish kept experimentally in water containing known concentrations of PCBs (Hansen *et al. ibid* 1971, **6**, 113). For example, a concentration of 5 ppb resulted in levels of over 100 ppm in both pinfish and spot; furthermore this concentration was fatal to some 50–60% of the fish. A level of 1 ppb PCB was tolerated, however, even though residue levels of around 30 ppm were recorded after 30 days and the livers of these animals contained up to 200 ppm PCB. Interestingly, after 84 days in non-polluted water, the PCB levels of the whole fish fell by some 73%.

Similar figures have been reported in a review of PCB contamination by Zitko & Choi (Fisheries Research Board of Canada Technical Report no. 272, 1971). Once again the levels in fish were generally less than 1 ppm, although occasionally figures as high as 10–20 ppm were recorded from waters near highly industrial areas. In aquatic birds, and especially their eggs, higher levels were found, figures of 1–10 ppm being not uncommon. Fish-eating birds seem to have particularly high PCB levels, occasionally in excess of 100 ppm. Other animals have also been shown to accumulate PCBs. The blubber of grey seals, for instance, commonly contains 10–50 ppm PCB, and one sample was found to have a level as high as 1800 ppm. Of greater importance to man, however, is the finding of up to 3 ppm PCBs in some commercial fish oils and of 0·18, 0·21 and 0·47 ppm in margarines manufactured, respectively, from 100% vegetable oils and 60 and 80% marine oils.

In view of the known environmental contamination, it is not surprising that PCBs are to be found in man. Two samples of adipose tissue analysed by Biros *et al.* (*Bull. env. contam. & Toxicol.* (*U.S.*) 1970, **5**, 317) yielded 200 and 600 ppm PCB and further work has detected PCBs at a concentration of 0·103 μg/ml (or 3·5 ppm based on the milk fat) in human milk. In the latter case, fatty tissue was also examined and found to contain 5·7 ppm PCB (Acker & Schulte, *Naturwissenschaften* 1970, **57**, 497). However, a survey of 79 lactating women, 32 from villages in New Guinea and 47 from cities in Texas, failed to

* b = 10^9 throughout this article.

confirm these findings (Dyment *et al. Bull. env. contam. & Toxicol.* (*U.S.*) 1971, **6,** 532). While residues of chlorinated hydrocarbon pesticides were present, there was no evidence of PCBs in the milk of any of these women.

Toxicity of PCBs

The results of Hansen *et al.* (*loc. cit.*) suggest that the threshold of toxicity of the PCBs to fish lies around 1 ppb. The pink shrimp, *Penaeus duorarum*, seems equally sensitive (Nimmo *et al. Mar. Biol.* 1971, **11,** 191). The adults were killed within 17–53 days by Aroclor 1254 concentrations of from 2·4–4·3 ppb, but the juvenile shrimps were found to be less resistant and failed to tolerate even 1 ppb. There were indications that a similar increase in susceptibility to PCBs occurred during moulting. Accumulation (up to 510 ppm) occurred in the hepatopancreas of the animals but subsequently the material was transferred to other tissues and eventually lost. There was very little indication of how or why the animals died, but *in vitro* inhibition of fish ATPase by PCBs was recently reported (Yap *et al. Nature, Lond.* 1971, **233,** 61; Desaiah *et al. Biochem. Pharmac.* 1972, **21,** 857) and the possibility that this may also occur *in vivo* perhaps deserves further study.

The signs of PCB poisoning are more apparent in birds. For example, chicks fed a diet containing known quantities of PCB have presented with a characteristic group of reactions. Levels of 100–150 ppm PCB (Aroclor 1248) in the diet for up to 5 wk have been shown to depress weight gain, cause oedema, breathlessness, hydropericardium and internal haemorrhages, depress secondary sexual characteristics and increase liver weight (Rehfeld *et al. Poult. Sci.* 1971, **50,** 1090). At 50 ppm these effects were less marked and at 10 ppm they were absent. Month-old cockerels fed a diet containing 500 ppm Aroclor 1254 for up to 12 weeks showed a significant decrease in the size of combs and testicles, a reduction in body weight, an increase in liver weight and an accumulation of PCB principally in adipose tissue, kidney, liver, brain, muscle and blood (Platonow & Funnell, *Can. J. comp. Med.* 1972, **36,** 89). Tissue levels increased linearly with time. Behavioural effects have been seen in caged robins receiving as little as 5 μg PCB (Clophen A50) daily, however. After 20 or so days of treatment, the experimental birds were significantly more active than the controls and residue levels in breast muscle (0·1–0·4 ppm) were four times higher (Ulfstrand *et al. Nature, Lond.* 1971, **231,** 467).

Other effects of PCBs in birds include an increase in susceptibility to viral hepatitis seen in ducks challenged with the virus 5 days after the termination of a 10-day course of feeding with diets containing 25–100 ppm Aroclor 1254 (Friend & Trainer, *Science, N.Y.* 1970, **170,** 1314). In pigeons and quails fed wheat dressed with 250–500 ppm Aroclor 1242 or 1254 for 20–28 days, residue levels have been found varying from over 100 ppm in fat to less than 10 ppm in heart and brain (Bailey & Bunyan, *Nature, Lond.* 1972, **236,** 34). The most important result of this work, however, was the finding that PCBs with a relatively low degree of chlorination are fairly rapidly metabolized and are not as persistent as the more highly chlorinated compounds. The anomaly therefore arises that the less widely used compounds, the highly chlorinated PCBs, are more common in the environment than their technologically more popular relations.

The metabolism of an orally administered PCB with a fairly high mean chlorine content (Aroclor 1254) has been studied in the rat by Grant *et al.* (*Bull. env. contam. & Toxicol.* (*U.S.*) 1971, **6,** 102). The PCB used consisted of six components recognizable by gas chromatography. In line with the results of Bailey & Bunyon (*loc. cit.*), tissue residues of the less chlorinated components were significantly lower than the residues of the more chlorinated

ones. In addition, total residue figures were increased by treatment of the rats with carbon tetrachloride, a result which, when considered alongside the increase in liver weight observed in this and several other studies, suggests that the liver may be the site of PCB metabolism.

An increase in the activity of the microsomal processing enzymes of the liver has been observed in PCB-treated birds (Bailey & Bunyon, *loc. cit.*), rabbits (Villeneuve *et al. Bull. env. contam. & Toxicol. (U.S.)* 1971, **6,** 120) and rats (Bickers *et al. Res. Commun. chem. Path. Pharmac.* 1972, **3,** 505). The more highly chlorinated biphenyls were the most potent inducers, producing a statistically significant and long-lasting effect in both species. Reduction of pentobarbitone sleeping time in rats by a number of PCBs has been shown to increase with the degree of chlorination of the compound and the extent of its storage in fatty tissues (Villeneuve *et al. Bull. env. contam. & Toxicol. (U.S.)* 1972, **7,** 264).

In the rabbit study (Villeneuve *et al.* 1971, *loc. cit.*) pregnant animals were used, so that additional data were generated showing that 10 mg PCB/kg/day was without effect on this mammalian reproductive system. However, daily doses of 12·5 mg Aroclor 1254/kg or more given to rabbits on days 1–28 of gestation led to an increase in abortions, maternal deaths and stillbirths, although the dead foetuses showed no consistent skeletal abnormalities. The rat appeared to be less sensitive, since doses up to 100 mg/kg/day caused no foetal deaths or malformations (Villeneuve *et al. Envir. Physiol.* 1971, **1,** 67). Placental transfer of the PCBs was verified by human experience during the PCB poisoning incident in Japan (Kuratsune *et al. loc. cit.*). Pregnant women suffering from the poisoning gave birth to generally small babies with a characteristic brown discoloration of the skin, especially around the groin, dark-coloured nails and discharging eyes. The condition disappeared within a few months.

An increase in the metabolic activity of the liver is associated with an increase in smooth endoplasmic reticulum (SER). Proliferation of SER and hyaline degeneration have been observed in the livers of PCB-treated mice and monkeys (Nishizumi, *Archs envir. Hlth* 1970, **21,** 620). Associated with these effects were disturbances in lipid metabolism and a reduction in liver-glycogen stores.

Similar results have been reported in rabbits following the percutaneous absorption of several PCB preparations (Vos & Beems, *Toxic. appl. Pharmac.* 1971, **19,** 617). Besides liver hypertrophy, damage to the kidney tubules, lymphopenia and atropy of the thymus were noted. The quantities of PCBs involved, 118 mg applied five times weekly for 38 days, also had a marked effect on the skin. The lesions induced were typified by hyperplasia and hyperkeratosis of the epidermal and follicular epithelia. However, as some PCB preparations produced more severe symptoms than others, the possibility that these effects may have been caused by toxic impurities such as benzofurans must be borne in mind.

The sources and the hazard to man

Monsanto's recent refusal to sell PCBs for "open" applications and for use in food-processing plants will clearly, in the long term, reduce the potential hazard of these compounds. At present, however, PCBs are present in human food. The levels involved are low—a recent US estimate put the average daily intake of a person on a high-consumption diet at only 0·1 μg/kg/day (*Food Chemical News* 1972, **13** (51), 36); nevertheless the biological characteristics of the PCBs warrant the continued monitoring of man's exposure to them.

Fish remains man's prime source of PCBs (*ibid* 1971, **13** (40), 34). Indeed, it is difficult to see how this situation can change in the foreseeable future because, once polluted, the oceans must take a considerable time to recover. Other sources of PCBs can more easily

be reduced and eventually eradicated. The finding of PCB residues in poultry and eggs (*ibid* 1971, **13** (23), 3) should gradually diminish as PCB-containing meal is removed from the market and the use of biphenyls in food plants is discontinued. Similarly, careful control over the use of PCBs as silo sealants (Skrentny *et al. Bull. env. contam. & Toxicol. (U.S.)* 1971, **6,** 409) should reduce the possibility of residues in milk. PCBs have been found in the milk of cows fed contaminated silage (*Food Chemical News* 1971, **13** (9), 22), and Saschenbrecker (*Vet. Rec.* 1972, **90,** 100) has shown that milk is by far the most important route of excretion of ingested PCB in the lactating cow. After cows have been removed from any PCB contamination, the rate of reduction in the residue level in milk has been found to be similar to that of DDT in the same circumstances (Fries *et al. Bull. env. contam. & Toxicol. (U.S.)* 1972, **7,** 252).

The other important source of dietary PCBs has been food-packaging materials manufactured from recycled papers containing carbonless copy paper (Masuda *et al. Nature, Lond.* 1972, **237,** 41) and printing inks (*Food Chemical News* 1971, **13** (28), 3). Nevertheless, while PCB levels in packages have occasionally been very high, food itself seems to be only rarely contaminated to any significant extent (*ibid* 1972, **13** (51), 36). In the UK, manufacturers of carbonless copy paper have stressed that alternative solvents are now used (Lister & Bennett, *Nature, Lond.* 1972, **237,** 414).

In the USA, the FDA has set a tentative acceptable daily intake for the PCBs of 150–300 μg (*Food Chemical News* 1971, **13** (26), 12). The actual daily intake, even before the recent tolerance levels set for various foodstuffs in America (*ibid* 1972, **13** (52), 38), was well below this. As PCB residue levels are now likely to fall, it seems that man at least should not suffer unduly from this particular environmental hazard. The effect of PCBs on oceans, lakes and rivers and their flora and fauna may be another question.

LITHIUM: USEFUL DRUG OR HAZARDOUS TERATOGEN?

Lithium (Li), or strictly speaking the Li^+ ion, has only recently aroused the interest of toxicologists. The discovery that it is an effective treatment for manic-depressive psychiatric disorders has resulted in the exposure of many individuals to relatively high serum levels of Li, and this has led to a corresponding expansion in the literature on the likely side-effects of such treatment.

Serum levels and acute toxicity

The dose of Li given in psychiatric treatment is critical. It must be such that the serum- or plasma-Li level is on the one hand above 0·6 m-equiv./litre, the lowest therapeutically effective level, and yet, on the other, below the toxic threshold of 1·6 m-equiv./litre (Schou, *Acta psychiat. neurol. scand.* 1969, **45** (Suppl. 207), 49). Plasma monitoring is one way in which the necessary control may be maintained. Fry & Marks (*Lancet* 1971, **i,** 886) have successfully used this technique in a group of 100 patients on Li therapy but, somewhat disappointingly, they found that only 29 of them had plasma concentrations within the optimum range of 0·8–1·2 m-equiv./litre. Of the rest, 18 were probably receiving insufficient

Li, while eight had toxic levels of the ion in their blood. Some others, as is common in this type of therapy, had stopped taking their tablets altogether.

Given that patients can be persuaded to adhere to the dosage regime prescribed for them, however, it is the retention and excretion of Li in the kidney that is the major cause of variation in blood levels. The two important factors influencing these levels are the sodium intake and the fluid intake (Platman & Fieve, *Archs gen. Psychiat.* 1969, **20,** 285; Thomsen & Schou, *Am. J. Physiol.* 1968, **215,** 823). An increase in either has been shown to result in a marked rise in Li excretion. It is possible to use renal Li clearance as a measure of the quantity of Li in the system, but the considerable variation between individuals must be taken into account. The normal range in adults is from 10 to 30 ml/minute, which corresponds to a daily maintenance dose of Li carbonate between 600 and 1800 mg (Schou *loc. cit.*).

The characteristic signs of acute Li toxicity are drowsiness, coarse tremor or muscle twitching, slurred speech, loss of appetite, vomiting and diarrhoea (Schou *loc. cit.*). Quite commonly, these effects are caused not by Li overdosing but by transient fluctuations in sodium or fluid intake or a degree of associated kidney damage. For instance, in one recent case (Acki & Ruedy, *Can. med. Ass. J.* 1971, **105,** 847), Li intoxication was probably brought on by a policy of dietary salt restriction and diuresis and by the dehydration resulting from the patient's diarrhoea and the recent birth of her child (Lapierre, *ibid* 1972, **106,** 112; Brown, *ibid* 1972, **106,** 112). Similarly, an inadequate fluid intake was probably a contributing factor in two further cases described by Allgén (*Acta psychiat. scand.* 1969, **45** (Suppl. 207), 98), and in one case chronic nephritis was an additional complication. On the other hand, overdosing does occur; as much as 13 g Li carbonate was administered over a 5-day period to one patient described recently by Castaing *et al.* (*Eur. J. Toxicol.* 1971, **4,** 412). The symptoms produced were characteristic of Li poisoning.

Effects on thyroid

Thyroid depression and the development of goitre are two of the more specific side-effects of Li treatment. A survey of 330 patients who had been receiving Li for periods varying from 5 months to 2 years (Schou *et al. Br. med. J.* 1968, **3,** 710) revealed 12 cases of goitre. Several other isolated cases of both goitre and hypothyroidism have since been described (Lazarus & Bennie, *Acta endocr., Copenh.* 1972, **70,** 266; Myers, *Lancet* 1972, **i,** 1287). These effects could be caused by a variety of mechanisms, but all of them rest on concentration of Li in the thyroid and a reduced plasma-iodide level in these patients. Concentration of Li could lead to competition with serum iodide and therefore to an iodide deficiency within the thyroid. Alternatively, Li could interfere at one of several stages in the biosynthesis of thyroxine or cause excessive response to the thyroid-stimulating hormone (TSH) released by the pituitary. At present, opinion is divided as to which of these theories is the most probable (Shopsin, *Dis. nerv. Syst.* 1970, **31,** 237). Attention has been drawn to the interesting possibility that the effects of Li on the thyroid may be aggravated by the high level of iodine in the xanthine derivatives used to colour certain Li tablets (Shopsin *et al. Compreh. Psychiat.* 1969, **10,** 214).

Whatever the mechanism, the condition does not seem to be unduly serious. Withholding Li leads to a disappearance of the goitres (Schou *et al. loc. cit.*) as does the commencement of thyroxine treatment (Myers *loc. cit.*). The latter method can be used without discontinuing Li therapy and therefore represents the most practical of the solutions to the problem.

Other side-effects of Li

Excessive weight gain is another side-effect of Li therapy that is receiving increased attention. Plenge *et al.* (*Lancet* 1969, **ii,** 1012) have shown that intraperitoneally administered Li chloride (500–1200 μmoles) can result in an almost complete disappearance of liver glycogen within 30 minutes in the rat. Furthermore, the effect lasts for over 24 hours and is accompanied initially by a corresponding increase in blood glucose. A stimulation of glucagon secretion as the explanation for these effects was indicated by further studies.

Three other conditions probably related to oral Li treatment have also been described. Tseng (*Archs Path.* 1971, **92,** 444) reported a case of interstitial myocarditis in a female patient. Her serum-Li levels during treatment were not excessively high, but while no Li was detectable in the brain at autopsy, a very high level was found in the heart muscle. This author therefore recommended routine ECG tracings to detect toxic levels of Li in the myocardium. In three other patients, polyuria and polydipsia developed after they had been on Li therapy for some time (Ramsey *et al. J. Am. med. Ass.* 1972, **219,** 1446). Plasma-Li levels were within the acceptable therapeutic range. These cases, and others reported by Angrist *et al.* (*Compreh. Psychiat.* 1970, **11,** 141) are particularly interesting because, as was mentioned earlier, kidney damage is not normally a symptom of Li intoxication but more often a reason for its appearance. A recent discussion of this phenomenon (*British Medical Journal* 1972, **2,** 726) suggests that Li may interfere with the action of vasopressin on the nephron, but adds a timely reminder that the resulting nephrogenic diabetes insipidus cannot be treated by salt depletion and diuretics, as is customary for other forms of this condition. Finally, the occurrence of cutaneous lesions similar to those described earlier (*Cited in F.C.T.* 1968, **6,** 540) has been reported in two further patients (Kusumi, *Dis. nerv. Syst.* 1971, **32,** 853).

The toxic effects described above should be assessed in the light not only of the therapeutic usefulness of Li but also of the overall incidence of the effects themselves. The four cases of classic Li intoxication reported by Allgén (*loc. cit.*), for instance, occurred in a total of more than 1000 patients over a period of 11 years. Over 20,000 serum analyses had been carried out. Clearly, in the proper hands, Li therapy does not carry a high risk of toxic side-effects. Of interest in this connexion is a recent report (Zisook, *J. Am. med. Ass.* 1972, **219,** 755) of a patient with long-standing ulcerative colitis who was treated with oral Li. Far from being exacerbated by this treatment, the patient's diarrhoea actually subsided after the first few days and his ulcerative colitis receded and has remained quiescent for 16 months.

Teratogenicity of Li

Following a report that Li carbonate given to pregnant mice in daily doses of 300 mg/kg or more increased the incidence of cleft palate in the young, the literature on Li teratogenicity has expanded considerably.

The original brief report of this finding (*Cited in F.C.T.* 1970, **8,** 232) has been supplemented by further communications from the same laboratories (Szabo, *Nature, Lond.* 1970, **225,** 73; Szabo *et al. Toxic. appl. Pharmac.* 1970, **17,** 274). The dosage regime in these studies was designed so that the plasma-Li levels were comparable with those found in patients receiving Li treatment (a 400 mg/kg/day regime giving a range of plasma levels of 1·01–1·70 m-equiv./litre). A study similar to that originally reported, but using larger numbers of animals, yielded comparable results, the incidence of cleft palate being 16%

with doses of 465 mg/kg/day on days 6–15 of gestation, 0·4% with 200 mg/kg and 0% in controls. There was also evidence of other dose-related embryotoxic effects, such as a high incidence of resorptions and a decrease in mean litter size and foetal survival at the high dose level. This subsequent work has thus supported the earlier indication of a very low spontaneous incidence of cleft palate in this particular strain of mouse (randomly bred HaM/ICR) and indicates a dose of 200 mg/kg/day as a possible threshold for teratogenicity in this strain.

The lesions produced were restricted to the back palate and were not associated with cleft lip. Li appears, therefore, to be a selective teratogen, a conclusion partially verified by further experimental work in Sprague–Dawley rats (Wright *et al. Lancet* 1970, **ii,** 876; *idem*, *Teratology* 1971, **4,** 151). Four groups of three pregnant rats were given an intraperitoneal injection of 50 mg Li chloride on day 1, 4, 7 or 9 of gestation, followed thereafter by a daily dose of 20 mg. These were judged to be the maximum sublethal doses. The foetuses were removed and examined on day 17. Cleft palate occurred in 45 (39%) of the 115 foetuses examined, but was not the only teratological effect observed. Eye and external-ear defects were seen in 63 and 45% of the foetuses, respectively, and the incidence of resorptions was also higher than that in either the untreated controls or in a further group given sodium chloride instead of Li chloride. The incidence of eye, ear and palate defects was 3, 0 and 3%, respectively, in the sodium chloride-treated group and there were no malformations in the untreated controls.

Tests on three non-mammalian species, the chick, frog and toad, were carried out by De Bernardi *et al.* (*Experientia* 1969, **25,** 211). In each case, incubation in 0·01 M-Li chloride resulted in the development of malformed embryos. The effects were thought to be caused by an inhibition of ribosome synthesis and consequently of incorporation of amino acid into protein.

Nevertheless, not all investigations have demonstrated teratological effects in laboratory animals. Johansen & Ulrich (*Acta psychiat. neurol. scand.* 1969, **45** (Suppl. 207), 91), for instance, found no malformations in young born to pregnant Wistar rats given 1 or 3 m-equiv. Li/kg/day in the diet until day 20 of gestation. Histological examinations were not carried out, but there was no evidence of a reduction in litter size or in foetal, uterine or placental weights. Li did cross the placenta, however, with the result that the serum concentration in the foetuses was up to one half of that found in their mothers.

A more recent report (Gralla & McIlhenny, *Toxic. appl. Pharmac.* 1972, **21,** 428) was also negative as regards embryotoxic effects, in this case in Charles River rats, albino rabbits and rhesus monkeys. In the rat study, four groups of 20 pregnant animals were given 0, 0·675, 2·025 and 4·05 m-equiv. Li carbonate/kg/day from day 5 to day 15 of gestation. At all three dosage levels, corresponding to plasma levels of up to 1·4 m-equiv. Li/litre, the young born appeared normal and no teratological properties could be attributed to the Li treatment. Similar results were obtained in three groups of ten rabbits given 0, 0·675 or 1·08 m-equiv. Li carbonate/kg/day from day 5 to day 18 of pregnancy and in six rhesus monkeys similarly treated with 0·67 m-equiv./kg/day from day 14 to day 35. Species variation and the differences in experimental method are two obvious explanations which could possibly account for the different results obtained in this study and in those of Szabo *et al.* (*loc. cit.*) and Wright *et al.* (*loc. cit.*). It should also be remembered that, while realistic, the doses of Li given in this study were slightly lower than those used earlier. Li teratogenicity in experimental animals appears to be a dose-related phenomenon.

The data on the incidence of malformations in children born to mothers receiving Li

therapy are equally conflicting. One of the first cases of a severely deformed child being born under these circumstances has been described by Aoki & Ruedy (*loc. cit.*). The mother had been on Li carbonate throughout her pregnancy and at term her serum-Li level was 1·06 m-equiv./litre. While her child was grossly deformed, it is impossible to attribute this with any certainty to the Li therapy. On the other hand, it seems fairly clear that characteristic signs of Li intoxication can be seen in the newborn children of women receiving Li treatment (Wilbanks *et al. J. Am. med. Ass.* 1970, **213,** 865; Woody *et al. Pediatrics, Springfield* 1971, **47,** 94). Li is capable of crossing the placental barrier and can therefore be present in toxic quantities in the blood of newborn infants. Such effects were not seen, however, in the young born to female rats maintained on a serum Li level of 1·4 m-equiv./litre during both pregnancy and weaning (Gralla & McIlhenny, *loc. cit.*).

There has been considerable discussion as to the biological significance of the decreased mitotic index seen in blood-lymphocyte cultures of manic-depressives on Li therapy (Genest & Villeneuve, *Lancet* 1971, **i,** 1132; *idem, ibid* 1971, **ii,** 1325). It is possible that this could be the genetic mechanism behind the teratological properties of Li, but the methodology of the experiments indicating these effects has been criticized. Furthermore, *in vitro* work seems to show that Li is unlikely to decrease the mitotic rate at therapeutic dose rates (Timson & Price, *ibid* 1971, **ii,** 93; *idem, ibid* 1972, **i,** 449). The earlier report of a normal child born to a woman who received Li throughout her pregnancy (*Cited in F.C.T.* 1971, **9,** 161) supports this conclusion. Equally reassuring is a further similar report (Silverman *et al. Am. J. Obstet. Gynec.* 1971, **109,** 934). Li, at a concentration of 1·1 m-equiv./litre was found in the plasma of the newborn baby, but was totally eliminated within 9 days. There was no evidence of Li toxicity.

As with the toxic side-effects of Li therapy, the incidence of malformations must be assessed with reference to the number of normal pregnancies seen under these circumstances, and in relation to the incidence of malformations in the general population. To this end, a register of "lithium babies" born to mothers treated with Li during pregnancy has been established (Schou & Amdisen, *Lancet* 1970, **i,** 1391; *idem, ibid* 1971, **i,** 1132). When the latest figures were published the register showed three cases of malformation out of a total of 60 infants, an incidence of the same order as that found in the general population. So far, therefore, there is no indication that Li has a teratogenic effect at the dosage levels that are therapeutically effective in the treatment of manic-depressives.

ALUMINIUM TAKES THE RAP

On the whole, aluminium (Al) and its salts are considered to be relatively inert from a toxicological standpoint, a view supported by demonstrations that, in man, absorbed Al is readily excreted by the intact kidney (Berlyne *et al. Lancet* 1970, **ii,** 494). The toxic effects of Al that have been recorded are for the most part attributable to phosphate depletion resulting from the reaction of Al and phosphates in the alimentary tract. Prolonged administration of Al chloride to mice has been found to retard growth in the second and third generations, without affecting the size of the litters (*Cited in F.C.T.* 1968, **6,** 102), while a high dietary intake of Al sulphate led in rats to body retention of Al, particularly in the

adrenals, with a negative phosphorus balance and adverse alterations in the phosphorylation reactions in the tissues. Oral administration of Al hydroxide is used extensively in the treatment of patients with chronic renal failure, to counteract the high phosphate levels that tend to accumulate in the serum in this condition. It has generally been assumed that most of this Al is excreted in the faeces as insoluble Al phosphates. It appears, however, that this is not necessarily the case, and Al absorption, allied to the impairment of kidney function, may lead to a rise in Al levels in the plasma (Berlyne *et al. loc. cit.*).

In an attempt to throw further light on this problem, studies have been carried out by Berlyne *et al.* (*Lancet* 1972, **i,** 564) in nephrectomized rats. Groups of rats subjected to a 5/6 nephrectomy by total removal of one kidney and removal of two-thirds of the other were given 1 or 2% Al chloride or sulphate solution as drinking water or oral doses of Al hydroxide equivalent to 150 mg Al/kg/day. Others were given sc or ip injections of Al hydroxide equivalent to 90 mg Al/kg/day. Liver homogenates from the animals were studied for oxygen consumption, and tissue concentrations of Al were measured by spectrophotometry. All nephrectomized animals given the 1% solutions died within 8 days, and those given the 2% solutions died within 3 days. They were lethargic, failed to thrive, developed periorbital bleeding associated with ulceration of the conjunctival epithelium, and showed a reduction in spontaneous movement. Al hydroxide given ip caused a high mortality among nephrectomized and control animals within 5 days. Sc injections did not kill the animals but induced periorbital bleeding in the nephrectomized group. In normal animals, 2% Al sulphate caused periorbital bleeding in three of five rats but no deaths. Patchy pneumonia with desquamation of the bronchial epithelium appeared in animals dying with periorbital bleeding. Animals given Al hydroxide ip showed perihepatic Al granulomas and fibrinous peritonitis, but the heart, spleen, pancreas and skeletal muscle appeared normal.

In control rats, the highest Al concentrations were in bone. In those given Al hydroxide ip, the highest Al concentration was in the liver, with high concentrations also in muscle, brain, bone and heart. Animals in all the test groups showed high serum levels of Al, with a maximum of 39·4 mg Al/litre in the nephrectomized animals on oral 2% Al sulphate. Oxygen consumption by the liver fell by 25% in Al-treated animals.

Much criticism has been levelled against these findings and against the suggestion of Berlyne *et al.* (1972, *loc. cit.*) that the widespread therapeutic use of Al salts in patients with impaired renal function should be suspended pending further study of the problem. Verberckmoes (*Lancet* 1972, **i,** 750) remarked that Berlyne *et al.* (1972, *loc. cit.*) found no correlation between the Al concentrations in serum and tissues and the observed toxicity, and suggested that the high mortality among rats given ip injections of Al hydroxide may have been due to local inflammation. On the evidence of the rat experiments, the oral administration of Al hydroxide, the usual clinical practice, appears to be relatively safe. In patients undergoing dialysis, daily oral doses of 15–40 mg Al/kg in the form of the hydroxide have been found to be effective in lowering the predialysis Ca × P product without producing any evidence of intoxication.

Thurston *et al.* (*Lancet* 1972, **i,** 881) have concluded from experiments in rats that Al retention during chronic renal failure is not toxic provided hypophosphataemia is avoided. Rats were fed a diet containing 0·32% Al hydroxide or a similar diet containing also 1% disodium hydrogen phosphate. The animals consumed 6–10 mg Al daily, equivalent to a daily intake of 60 ml Al hydroxide suspension by an adult man. This intake caused significant impairment of growth in the rats at 3 and 4 weeks. However, animals taking the

combined Al and phosphate diet showed a normal rate of growth. Uraemic rats suffered some growth impairment on the Al hydroxide diet, but had the same growth curves as untreated uraemic rats. Rachitic changes appeared in the bones of normal rats fed Al alone. No eye lesions such as those described by Berlyne *et al.* (1972, *loc. cit.*) appeared, though other untreated animals in a poor state of health from the same colony showed haemorrhagic eye lesions, which were attributed to infection.

Replying to their critics, Berlyne *et al.* (*Lancet* 1972, **i,** 1070) have pointed out that in their large colony of rats, periorbital bleeding has only been seen in animals that had received Al. They agree that the appearance of toxic effects requires higher plasma levels of Al than can be produced by orally administered Al hydroxide and maintain that periorbital bleeding is the most reliable sign of Al intoxication in rats whose plasma-Al levels approach those found in patients with advanced renal failure receiving 6–8 g Al hydroxide daily. Subsequent correspondence (Thurston & Swales, *ibid* 1972, **i,** 1241; Sherrard, *ibid* 1972, **i,** 1241) stresses that the Al compounds and routes of administration that combined to produce toxic signs in rats, namely the chloride and sulphate given orally and parenterally injected Al hydroxide, are seldom, if ever, used clinically. The relevance of these studies to the use of Al hydroxide in the therapy of patients with chronic renal failure is thus highly questionable.

These studies on the oral administration of soluble Al salts and also on the parenteral administration of Al have, however, inevitably prompted further thoughts on the use of Al containers for food and other materials (Shaw, *Br. med. J.* 1972, **2,** 169). This correspondent points out that in tests carried out by the Department of Health and Social Security, the water present in Al containers that had been subjected to sterilization was found to contain Al at a level of 500 μg/litre. Since these containers were of a type recommended for the long-term low-temperature storage of blood, Shaw (*loc. cit.*) asserts that further studies on blood stored in this way are necessary to prevent the direct intravenous administration of possibly harmful levels of Al compounds.

It seems unlikely that Al migration of this order would occur under most of the conditions encountered in the cooking and storage of food, but assuming that some Al does find its way into our diet from such sources, there seems little likelihood that the hazard is any greater than has hitherto been assumed. There is still no evidence to contradict the assumption that the greater part of ingested Al is excreted by man in the faeces. Given an adequate diet, the small amounts of Al involved would be unlikely to promote any deficiency of absorbed phosphate, and any Al that may escape this eliminating net can be assumed to be rapidly excreted by normally functioning kidneys.

TOXICOLOGY'S NEED FOR NEW ANALYTICAL METHODS

At the present time, one of the major problems facing toxicologists is the fact that many of the methods available for analysis of hazardous substances lack either specificity or sensitivity. The purpose of this article is to highlight the problems that arise in establishing chemical identity and measuring small amounts of toxic substances, to indicate some areas in toxicology where improvement may be made, and to suggest some basic approaches that might produce fruitful results.

These topics may be suitably introduced by reference to the comments of a speaker at a symposium held in Boston in April by the American Chemical Society (*Food Chemical News* 1972, **14** (4), 44). Dr. P. Issenberg of the Massachusetts Institute of Technology is reported as having emphasized the need for new methodological approaches, and particularly for new techniques for the detection of various nitrosamines and their derivatives in a wide variety of foods. He continued by criticizing the lack of specific analytical methods and the low instrumental sensitivity that characterized much earlier work in this field, and made a plea for the wider application of mass spectrometry to the problem of nitrosamine detection.

The use of the mass spectrometer coupled to the gas–liquid chromatograph (GLC–MS) is indeed one of the most versatile analytical procedures currently available for the detection of organic compounds, and is widely used for specific determinations of as little as 10^{-15} g (femtogram) quantities of various chemicals. However, the use of this powerful analytical tool is not general in toxicology, except in the measurement of pesticides. For example, in a recent review of the use of mass spectrometry, Millard (*Adv. Drug Res.* 1971, **6,** 157) made virtually no mention of previous uses in connexion with toxicology. Indeed it is sad to note that at a recent international symposium on GLC–MS held in Italy, only two of the 31 contributions were directly concerned with toxicological problems (International Symposium on Gas Chromatography–Mass Spectrometry, Isle of Elba, 17–19 May 1972).

Fields in which GLC–MS is currently generating important data include the study of hallucinogenic drugs, including the measurement of tissue concentrations of the cannabinoids derived from marihuana, and the vast fields of catecholamine research and adenine nucleotide physiology.

From the toxicological viewpoint the field of pesticide determinations shows the most sophisticated methodology. Here the use of the latest techniques has been forced upon the analyst because of the need to detect extremely small amounts of residues. In reviewing the procedures used in pesticide research, Gunther (*Ann. N.Y. Acad. Sci.* 1969, **160,** 72) referred to the "new methodology", citing GLC–MS and infra-red, ultraviolet and emission spectroscopy. Only the widespread use of sensitive methods could permit the low tolerance limits that have been established for many organochlorine pesticides in Europe and the USA.

Similar remarks on methodology have been made more recently, and in a wider context, by Egan (*Fd Cosmet. Toxicol.* 1971, **9,** 81), who referred to polychlorinated biphenyls, trace metals and nitrosamines and pointed out the fact that all analytical methods are not of equal value. This raises a most important point—that the use of a single analytical procedure is seldom, if ever, adequate to establish chemical identity. This has been the case particularly in those situations, such as in pesticide research, where the quantities to be detected are so small that analytical instruments are operating at the limits of their sensitivity.

In view of the fact that world awareness of toxicological hazards is increasing, there is a widespread need for precise detection and estimation of a multitude of toxic chemicals. In this connexion, there is an important modification to GLC–MS that has not, as yet, been applied to toxicological problems, although its use is increasing in other fields. This involves the use of the mass spectrometer as a universal gas-chromatographic detector. GLC–MS sensitivity can thus be increased up to 10^7-fold compared with other quantitative procedures (Koslow *et al. Science, N.Y.* 1972, **176,** 177) and the specificity of qualitative identification can be assured by monitoring three or more mass numbers corresponding to fragments

of the original compound. This procedure, devised by Hammer *et al.* (*Analyt. Biochem.* 1968, **25,** 532), was termed mass fragmentography and was first used to identify chlorpromazine and its metabolites in blood. Mass fragmentography has subsequently been used to measure cannabinoids, catecholamines and indolylalkylamines in tissues (Costa *et al.* and Koslow *et al.*, presented at the International Symposium on Gas Chromatography–Mass Spectrometry, Isle of Elba, May 1972) and various other drugs (Hammar *et al. Adv. Pharmacol.* 1969, **7,** 53; Koslow *et al. Science, N.Y.* 1972, **176,** 177).

There are many toxicological problems to which GLC–MS and mass fragmentography could usefully be applied, including the detection of additives leaching from plastics into the atmosphere in a confined space (e.g. phthalates from PVC car seats) and from packaging materials into foods. Another vitally important toxicological problem for which GLC–MS is already used but to which mass fragmentography could also usefully be applied is the detection of nitrosamines. Sen (*Fd Cosmet. Toxicol.* 1972, **10,** 219) has shown that 10–80 ppb dimethylnitrosamine ($b = 10^9$) can be detected in various meat products by GLC alone or GLC–MS. The use of mass fragmentography would be appropriate here because column retention times and mass numbers are known for some nitrosamines, and therefore much smaller quantities could be detected. Also it is gratifying to note that GLC–MS is now being applied to aflatoxin determinations (Rao & Anders, *Fedn Proc. Fedn Am. Socs exp. Biol.* 1972, **31,** 540). This is a welcome advance upon the currently used biological and chromatographic methods, which have recently come under considerable criticism (*Cited in F.C.T.* 1971, **9,** 576).

What other analytical techniques could feasibly be utilized in the field of toxicology? One extremely sensitive procedure, with a detection limit of 10^{-15} g, is the electron probe microanalyser. Electron scanning has revealed deposits of lead in the renal tissues of poisoned rats (Carroll *et al. Nature, Lond.* 1970, **227,** 1056). Clearly, therefore, the use of electron probe microanalysis could be extended to assist studies on the tissue distribution of metals such as cadmium, zinc and copper. Perhaps in the future, this procedure and human biopsy techniques may facilitate routine estimations for the presence of undesirable pollutants in the tissues.

As indicated already, drug abuse has prompted much sophisticated analytical activity, in particular for the measurement of very small quantities of cannabinols, which are the active principles of marihuana. Here it has proved possible to detect small quantities of fluorescent-labelled cannabinol derivatives following their conversion to dimethylaminonaphthalene 5-sulphonates (Forrest *et al. Res. Commun. chem. Path. Pharmac.* 1971, **2,** 787). The fluorescent-labelled derivatives have then been further identified by such techniques as GLC–MS and spectrophotofluorimetry. The fluorescent-labelling method could presumably be used in toxicology with compounds of appropriate structure.

Another technique which may have some application to toxicology is electron paramagnetic resonance or electron spin resonance (ESR). ESR signals derived from free radicals have been detected in nerves injured by pinching (Commoner *et al. Science, N.Y.* 1969, **165,** 703) and in tumour tissue from animals and man (Emanuel *et al. Nature, Lond.* 1969, **222,** 165; Wallace *et al. Physics Med. Biol.* 1970, **15,** 198). ESR signals are normally absent from tissues and biologically active substances because of the lack of paramagnetic resonance sites, but the technique of spin labelling with a nitroxide introduced by Stone *et al.* (*Proc. natn. Acad. Sci. U.S.A.* 1965, **54,** 1010) enables a paramagnetically resonant structure to be inserted into many molecules.

Thus there seem to be at least five analytical methods that could be increasingly exploited

by toxicologists. The first two are GLC–MS with mass fragmentography and electron microprobe analysis, and the last three involve chemicals with various types of labelled molecules. The labelling may be carried out with radioactive or natural isotopes, with fluorescent molecules or by electron-spin labelling procedures. Some of these procedures cannot be used in toxicology without further development, but there is no reason why radiochemical techniques should not be used more extensively in routine toxicology than they are at present. A wealth of basic information not readily acquired by other methods can be obtained by the use of substances labelled with tritium or carbon-14. This technique may be particularly useful in the provision of extraction data, as, for example, in studies of the migration of plastics additives from packaging materials or of waxes from the linings of glass containers into foods. At present, such studies are severely hampered by a lack of sensitive analytical methods, which necessitates the use of food-simulating solvents for extraction tests although their relevance to actual conditions of use is problematic. The use of radio-labelled compounds of high specific activity would increase the possibility of determining actual levels of migration into food products. In general terms, there are many situations where it is vital to know the distribution of potentially hazardous chemicals, and the use of radiochemical techniques may provide data on the tissue distribution of compounds following acute and long-term dosage, the migration of additives from packaging materials into foods or food-simulants, intestinal-absorption rates, the persistence of chemicals on unbroken skin, percutaneous absorption rates and so on.

In conclusion, it must be stressed that neither the more widespread adoption of new methodology nor the development of the types of experiment indicated above would solve all toxicological problems. Indeed, adoption of the ideas put forward here could in a sense have the opposite effect, a point which we hope to pursue in a subsequent issue. Moreover, it is not our intention to suggest that such work can be performed cheaply and rapidly with inexpensive equipment. However, if toxicological research is to move forward at anything like the rate required to cope with the expected increases in toxic hazards resulting from all forms of environmental pollution, then steps must be taken now to update our methodology, even at considerable cost.

Fd Cosmet. Toxicol. Vol. 11, pp. 145–170. Pergamon Press 1973. Printed in Great Britain

TOXICOLOGY: ABSTRACTS AND COMMENTS

FLAVOURINGS, SOLVENTS AND SWEETENERS

2448. Acetoin continued

Gabriel, M. A., Jabara, H. & Al-Khalidi, U.A.S. (1971). Metabolism of acetoin in mammalian liver slices and extracts: Interconversion with butane-2,3-diol and biacetyl. *Biochem. J.* **124,** 793.

Acetoin (acetylmethylcarbinol) is a constituent of flavouring essences and has been shown to be metabolized by enzyme systems of mammalian liver to butane-2,3-diol. A no-untoward-effect level of 330 mg acetoin/kg/day has been established in a short-term study in rats (Gaunt *et al. Food Cosmet. Toxicol.* 1972, **10,** 131). Obviously, further knowledge of the metabolism of acetoin is desirable, and this emerges from the study cited above.

When rat-liver mince was incubated with acetoin labelled with carbon-14 in positions 2 and 3, about 70% of the acetoin disappeared from the incubation mixture but only some 0·5% of the radioactivity appeared in the carbon dioxide produced. The cumulative metabolite carrying the major proportion of the radioactivity was studied by column chromatography in the presence of bisulphite and chromatography on borate-impregnated paper and was identified as butane-2,3-diol, present in both *erythro* and *threo* forms.

Acetoin reductase was found to differ chromatographically from biacetyl reductase, which was also obtained from rat-liver mince. Acetoin dehydrogenase and butane-2,3-diol dehydrogenase catalysed the interconversion of biacetyl, acetoin and butane-2,3-diol in rat liver. Both required NAD^+ or $NADP^+$ as a cofactor, and both differed from alcohol dehydrogenase. EDTA inhibited butane-2,3-diol dehydrogenase activity, but the activity could be restored to a varying degree by Co, Cu, Zn and other divalent metal ions. The equilibrium of both reactions favoured the reduced compound, and the reaction catalysed by biacetyl reductase was virtually irreversible. Kinetic studies suggest that the enzymes discussed here have very little activity under normal conditions, although the possibility that they are primarily specific for other substrates has not been ruled out.

2449. Saccharin seeks the foetus

Pitkin, R. M., Reynolds, W. Ann, Filer, L. J., Jr. & Kling, T. G. (1971). Placental transmission and fetal distribution of saccharin. *Am. J. Obstet. Gynec.* **111,** 280.

The many studies on saccharin in rodents and small mammals have yielded no evidence of interference with reproductive performance or foetal development (*Cited in F.C.T.* 1972, **10,** 241). Direct studies, such as the one cited above, on the placental transfer of saccharin in primates are necessary, however, if convincing evidence of the safety-in-use of this sweetener is to be accumulated.

Five anaesthetized rhesus monkeys in the last trimester of pregnancy were given an infusion of [^{14}C]saccharin into the antecubital vein at a rate of 4 μg/kg/min for a period of 60 min. Samples of maternal and foetal blood and amniotic fluid were taken via indwelling catheters every 15–20 min during the infusion and after it. At intervals of 17, 31, 114, 196 and 280 min after the termination of the infusion, one foetus and placenta were delivered by hysterotomy for examination.

Radioactivity in the maternal blood increased during the infusion but rapidly dispersed afterwards, being low at 2 hr and negligible at 3 hr from the end of the infusion. Activity in the foetal blood was about 30% of that in the maternal blood during infusion, but declined very slowly afterwards. Activity was widely distributed in the foetal tissues and declined very slowly, if at all, while that in the amniotic fluid accumulated very gradually. Autoradiographs showed random dispersion of the label throughout the foetal tissue parenchyma, usually within the cell cytoplasm. Little radioactivity was detectable in central nervous tissue. Appreciable amounts appeared in the kidney glomeruli and tubule walls but there was little in the lumina of the kidney tubules and ducts. Occasional cells in the bladder and kidney showed an uptake of activity indicative of phagocytic activity. The placental villi showed some radioactivity but the absence of radioactivity from the intervillous spaces in foetuses delivered some 3 hr or more after the end of the infusion correlated well with the negligible level of activity in maternal blood by this time.

Thus, despite a lack of evidence of any adverse effect of saccharin on reproduction, its slow rate of clearance from foetal tissue suggests that repeated maternal ingestion of saccharin might lead to some accumulation in the developing foetus.

PRESERVATIVES

2450. How much benzoic acid can a cat take?

Bedford, P. G. C. & Clarke, E. G. C. (1972). Experimental benzoic acid poisoning in the cat. *Vet. Rec.* **90,** 53.

Cats are particularly susceptible to the effects of benzoic acid (BA) and have experienced hyperexcitability, convulsions and death after eating meat containing BA at a level of 2·4% (*Cited in F.C.T.* 1972, **10,** 588). The present study was designed to determine the maximum dose of BA that can be tolerated by this species.

When cats were offered 120 g meat containing 1% BA, one animal consuming only 0·45 g BA/kg developed no apparent symptoms, but three others ingesting 0·63–0·89 g/kg exhibited profuse salivation, hyperaesthesia, aggression and convulsions, terminating in two cases in death. The severity of the syndrome was proportional to the rate of feeding and one cat, which ate all the treated meat in 15 min, died within 48 hr. In these cats, liver and kidney damage and pulmonary congestion were evident at autopsy, but the central nervous system was histologically normal. Similar effects were seen in cats fed 0·5% BA, in daily amounts equivalent to 0·30–0·42 g/kg, for 3–4 days, although in these cases convulsions and aggression were absent. The cumulative effect was, however, sufficient to cause death in two of the four animals. Lower doses of BA, in the range 0·13–0·16 g/kg for 23 days or 0·20 g/kg for 15 days, had no effect on behaviour or liver or kidney function.

The development of adverse effects in the cat thus seems to depend on a single dose of more than 0·45 g/kg or repeated daily doses above about 0·2 g/kg.

In view of the ease with which BA overdosage can occur and the alleged inefficiency of this preservative at pH 5–6, it is suggested that the continued use of BA in cat meat should be seriously questioned.

2451. The effects of high-speed benzyl alcohol

Baker, N. & Huebotter, R. J. (1971). Immobilizing and hyperglycemic effects of benzyl alcohol, a common preservative. *Life Sci.* **10** (Part I), 1193.

Benzyl alcohol (I), when used at a level of 0·9% as a preservative in commercial isotonic saline and other solutions for parenteral administration, has been shown to be free from hazard on slow intravenous infusion (*Cited in F.C.T.* 1971, **9**, 908), but the study cited above describes certain untoward effects produced by large doses given intraperitoneally (ip).

Mice given an ip injection of a 0·9 or 4% solution of I in saline, in amounts equivalent to 900 or 700 mg I/kg, respectively, were totally immobilized within 2 min and remained unresponsive for about 30 min. I also induced marked hyperglycaemia in mice fasted for 1 or 6 hr, but it had no effect on liver glycogen levels. Tracer studies using radioactively-labelled glucose suggested that the hyperglycaemic effect may have resulted from increased gluconeogenesis, although diminished peripheral uptake of glucose was not ruled out. The authors warn that investigators using commercial saline should be aware of the possible effect that the presence of this preservative may have on their experimental results, and suggest that further studies on the action of I on the nervous system would prove of interest.

2452. Paraben sensitivity

Braun, W. (1971). Die allergologische Bedeutung der p-Hydroxybenzoesäureester als Konservierungsmittel in kosmetischen und dermatologischen Externa. *Hautarzt* **22**, 531.

Esters of *p*-hydroxybenzoic acid (parabens) used as preservatives in cosmetics and pharmaceutical preparations have already been implicated in cases of dermatitis and sensitization.

The paper cited above describes the results of tests carried out in 1968–1970 on a total of 2497 subjects believed to be suffering from some contact allergy. A mixture of the methyl, ethyl and propyl esters (1% of each in vaseline) was used, and some of the patients who reacted positively were also tested with the individual esters (1% in vaseline). The patients were also tested with seven other organic and inorganic compounds of known allergenic potential.

A 24-hr exposure to the paraben mixture was followed by examination after 24 and 48 hr and, if necessary, at 72 hr and thereafter. Paraben sensitization was detected in 59 subjects. Of these 59, 26 also gave positive reactions to at least one of the *p*-aminobenzene derivatives, *p*-(aminomethyl)benzenesulphonamide, *p*-phenylenediamine and ethyl aminobenzoate, and 31 reacted to Peruvian balsam. Tests with the individual methyl, ethyl and propyl parabens carried out in 19 of the paraben-positive subjects were positive in 10, 3 and 11 subjects, respectively. In the light of these results, the author comments on the apparent importance of *p*-substitution in the development of sensitization.

Of 913 cases of contact eczema examined in 1970, 48% were traced either to the paraben

esters or to one of the seven other known allergens tested. The 2·9% incidence of paraben sensitization compared, for example, with a figure of 10·4% for *p*-phenylenediamine, 6·2% for nickel sulphate, 7·9% for Peruvian balsam and 8·3% for potassium dichromate. An incidence of 2·9% was also found the previous year for paraben-sensitivity among 720 patients tested. This incidence compares closely with values quoted by other authors and its significance must be considered in the light of the known value of these parabens as preservatives in cosmetics and pharmaceuticals.

MISCELLANEOUS DIRECT ADDITIVES

2453. 1,3-Butanediol gives food for thought

Mehlman, M. A., Tobin, R. B., Hahn, H. K. J., Kleager, L. & Tate, R. L. (1971). Metabolic fate of 1,3-butanediol in the rat: Liver tissue slices metabolism. *J. Nutr.* **101,** 1711.

Tate, R. L., Mehlman, M. A. & Tobin, R. B. (1971). Metabolic fate of 1,3-butanediol in the rat: Conversion to β-hydroxybutyrate. *J. Nutr.* **101,** 1719.

Interest has centred on the potential use of butane-1,3-diol (BD) as a synthetic food substance, and investigations have been made into the usefulness of BD to rats as a source of dietary energy (*Cited in F.C.T.* 1967, **5,** 727). Rats fed for 4 wk with 20% BD in isocaloric substitution for carbohydrate grew more slowly than controls, because of reduced food intake not of reduced utilization, and the epididymal fat pad was reduced in size. These rats were less able than controls to survive extreme cold, probably because of their relative lack of adipose tissue.

The first paper cited above reports the effect of feeding rats on a diet containing 30% fat together with 25% BD as a substitute for carbohydrate for 3 and 7 wk. Animals fed BD failed to gain weight normally and food consumption was reduced, as was the weight of the epididymal fat pad in relation to body weight. Concentrations of acetoacetate and β-hydroxybutyrate in the blood rose, the level of total ketone bodies being approximately trebled. At wk 7, rats showed a significant reduction in the blood level of pyruvate. *In vitro* studies on liver slices indicated a great decrease in pyruvate and a significantly increased lactate/pyruvate ratio when BD was added to glucose in the substrate. With BD and glucose as substrates there was a marked increase in total ketone formation and with BD alone, ketone bodies were also formed. These results indicate that BD is converted to ketone bodies prior to its oxidation in the tricarboxylic acid cycle.

The second paper cited above illustrates in more detail the metabolic fate of BD in the rat. Rat-liver homogenates were found to oxidize BD and ethanol at pH 10 in the presence of NAD^+. Liver slices and homogenates from rats previously treated with pyrazole or *n*-butyraldoxime to inhibit alcohol dehydrogenase failed to produce ketone bodies from BD. These findings indicate that BD is catabolized to β-hydroxybutyrate in the rat liver, the initial oxidation to aldol being catalysed by alcohol dehydrogenase and taking place in the cytosol. Once formed, β-hydroxybutyrate follows the known metabolic pathway.

[Perhaps the conclusion to be drawn from these studies is that, regarded as a possible foodstuff to take place of carbohydrates, 1,3-butanediol closely resembles ethanol in its metabolism. In contrast to the finding, mentioned elsewhere in this issue (p. 145), that

butane-2,3-diol dehydrogenase differed from alcohol dehydrogenase, the butane-1,3-diol dehydrogenase activity of rat-liver supernatant was shown to be identical with that of alcohol dehydrogenase.]

AGRICULTURAL CHEMICALS

2454. Dermal toxicity of paraquat

McElligott, T. F. (1972). The dermal toxicity of paraquat: Differences due to techniques of application. *Toxic. appl. Pharmac.* **21,** 361.

The dermal toxicity of paraquat has been shown to be low in studies in which precautions were taken to prevent the experimental animals licking the skin (*Cited in F.C.T.* 1967, **5,** 243). The present study extends this earlier work and illuminates other factors affecting toxicity by this route.

When single doses in the range of 70–560 mg paraquat/kg were applied to rabbit skin for 24 hr and then removed with warm water, there was marked skin damage from doses of 250 mg/kg or more. Animals that were allowed to lick themselves during the fortnight following application of any of the doses showed pronounced salivation, tongue ulceration (glossitis) and anorexia. The approximate LD_{50} value in such animals was 236 mg/kg, but when grooming was strictly prevented throughout the observation period, a dose of 480 mg/kg was not lethal. The 20-day LD_{50} values followed a similar pattern, falling to 7–14 mg/kg/day when grooming was allowed after the skin washing compared with at least 24 mg/kg/day when grooming was prevented. Glossitis in some of the latter animals suggested that small amounts of paraquat were still being ingested in dust and flaked skin.

Considerably less than 0·1% of a dose of [^{14}C]paraquat applied was found to remain after routine skin washing, so that ingestion of only very low levels clearly had a major influence on apparent dermal toxicity. When licking was not prevented, a single 24-hr application of 0·48 mg paraquat, dried in warm air and covered with a light gauze dressing, resulted in severe glossitis without any evidence of systemic toxicity or mortality. When the site of application was covered with an occlusive polythene dressing, a dose of 240 mg/kg was fatal within 24 hr, causing kidney damage but not glossitis. The skin appeared sodden but intact, a finding that illustrated the importance of tissue hydration in percutaneous absorption and suggested that skin damage was not an essential prerequisite. The 20-day LD_{50} value obtained by this method was as low as 4·5 mg/kg/day, although in this case local as well as systemic toxic effects were evident.

2455. Herbicides better for birds than bacteria

Whitehead, C. C. & Pettigrew, Rosemary J. (1972). The subacute toxicity of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid to chicks. *Toxic. appl. Pharmac.* **21,** 348.

Hussain, S., Ehrenberg, L., Löfroth, G. & Gejvall, T. (1972). Mutagenic effects of TCDD on bacterial systems. *AMBIO* **1,** 32.

The herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), has a low chronic toxicity in rats and dogs (*Cited in F.C.T.* 1972, **10,** 721), and it now seems that the chick, too, is able

to tolerate fairly large doses of 2,4-D and of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) given in the form of their butoxyethyl esters.

In 4-wk-old chicks, single doses of 2,4-D and 2,4,5-T in the range 250–900 mg/kg caused a temporary reduction in food consumption and a temporary loss of weight and there were some deaths at the highest dosage level. The weight loss was made up after 3–4 days and normal growth was resumed. There was no reduction in the growth of newly-hatched chicks maintained for 3 wk on diets containing up to 1000 ppm 2,4-D or 100 ppm 2,4,5-T, but higher levels reduced food consumption and growth rate; at levels of 5000–7500 ppm, 2,4,5-T was lethal and 2,4-D, while it caused no deaths, caused histological changes in the kidney, spleen and other organs. However, the birds were able to tolerate a level of 5000 ppm of either compound for 1 wk and resume normal growth thereafter. This level did not affect plasma magnesium or calcium levels. The chicks quickly learnt to distinguish between contaminated and uncontaminated food and ate very little of the former when given the choice.

The teratogenic effects of the 2,4,5-T contaminant, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin ('dioxin'), are well known (*ibid* 1972, **10,** 722) and it has been suggested that dioxin's effects on liver enzymes resemble those of certain carcinogens (*ibid* 1972, **9,** 909), although the effects considered have also been demonstrated in non-carcinogenic compounds. It is perhaps not surprising, therefore, to find that dioxin has also been associated with mutagenic properties (second paper cited above). *Escherichia coli* Sd-4 showed a high rate of reversion to streptomycin independency in a dioxin concentration of about 2 μg/ml, at a survival rate which excluded a preferential selection of spontaneously occurring mutants. Of two *Salmonella typhimurium* strains deficient in UV excision repair, one (strain TA 1532) also showed a high mutation frequency at dioxin concentrations causing a survival of less than 50%. Thirdly, dioxin at 0·5 μg/ml partially reversed the inhibition of phage release from *E. coli* caused by DMSO. The effects resembled those of acridine, and suggested that, like this compound, dioxin acted by intercalation with DNA.

2456. Sodium chlorate toxicity in dogs

Sheahan, B. J., Pugh, D. M. & Winstanley, E. W. (1971). Experimental sodium chlorate poisoning in dogs. *Res. vet. Sci.* **12,** 387.

Heywood, R., Sortwell, R. J., Kelly, P. J. & Street, A. E. (1972). Toxicity of sodium chlorate to the dog. *Vet. Rec.* **90,** 416.

Haemolysis and renal failure are the chief signs of acute poisoning with sodium chlorate (I) in man, a dose equivalent to perhaps 0·4 g/kg having been nearly fatal (*Cited in F.C.T.* 1970, **8,** 595).

Sheahan *et al.* (first paper cited above), concerned because the unexplained deaths of dogs in County Dublin was possibly associated with I-sprayed road verges, gave four dogs oral capsules providing a dose of 0·5–2·0 g I/kg. Vomiting, anaemia, leucocytosis and haemoglobinuria occurred in all dogs and the dog given 2 g/kg died with methaemoglobinaemia, haemolysis, haematuria, kidney damage and congestion of liver and spleen. A dose of 1 g/kg also caused severe methaemoglobinaemia and death in a dog with pre-existing nephritis. There were no histopathological findings in the other two dogs given 1 or 0·5 g/kg respectively or in a fifth dog given 0·5 g/kg intravenously, although this last animal developed methaemoglobinaemia. In all cases, I could be detected in blood and

urine. The findings suggested that canine fatalities from I-sprayed herbage were unlikely, although an adverse effect of repeated doses on kidney function could not be ruled out.

These results differ somewhat from those of Heywood *et al.* (second paper cited above). In this study, dogs given a dose of 1 or 2 g I/kg in a capsule rapidly vomited and showed no further effects apart from slight methaemoglobinaemia in one animal. Solutions containing 8–9% (w/v) I (the recommended working strength) were not accepted, but death resulted from ingestion of a 2% solution in an amount equivalent to a dose of 3·3 g I/kg. Daily doses of 200–326 mg/kg given for 5 days as a 6% solution caused anaemia, with an associated increase in reticulocytes, white blood cells and plasma-urea levels. Pathological findings were limited to changes indicative of haemolysis, such as prominent Kupffer cells containing brown pigment, and haematopoietic activity in the spleen. One of these dogs died after receiving a daily dose of 308 mg/kg for 4 days, and this was the only one in which methaemoglobin values were significantly elevated. Changes observed at repeated dose levels at or below 275 mg/kg were generally mild and variable in nature.

THE CHEMICAL ENVIRONMENT

2457. Carbon disulphide—psychologically and biochemically

Hänninen, Helena (1971). Psychological picture of manifest and latent carbon disulphide poisoning. *Br. J. ind. Med.* **28,** 374.

Tarkowski, S. & Sobczak, Hanna (1971). Oxidation and phosphorylation processes in brain mitochondria of rats exposed to carbon disulphide. *J. Neurochem.* **18,** 177.

Goto, S., Hotta, R. & Sugimoto, K. (1971). Studies on chronic disulfide poisoning. Pathogenesis of retinal microaneurysm due to carbon disulfide, with special reference to a subclinical defect of carbohydrate metabolism. *Int. Arch. Arbeitsmed.* **28,** 115.

The signs of intoxication resulting from exposure of workers to carbon disulphide (CS_2) seem to depend on the degree and duration of contact. Thus, the first report cited above, relating to the behavioural effects of CS_2, shows that impairment of speed, dexterity, vigilance and intelligence in workers exposed to this compound was significantly greater in those with obvious pathological manifestations of intoxication. The exposed workers showing no pathological effects tended, in contrast, to show signs of depression, slight motor disturbances and intellectual impairment. It is suggested that these effects may be more common than has been thought and that psychological test profiles may be valuable aids in the diagnosis of CS_2 poisoning.

The second study cited, on the effects of CS_2 on brain biochemistry, examined the changes occurring in the energy powerhouse of the cell—the mitochondrion. Exposure of rats to CS_2 for a single 18-hr exposure tended to diminish the P:O ratio in brain mitochondria, as a result of decreased esterification of phosphate. A partial uncoupling of oxidative phosphorylation was also indicated by a considerable drop in respiratory control indices, due to enhanced oxidation of substrates on the respiratory chain by mitochondria in the 'resting state', the 'state 4' of Chance & Williams (*Adv. Enzymol.* 1956, **17,** 65). The anticipated decrease in the ATP–P_i (inorganic orthophosphate) exchange rate further supported

an uncoupling mechanism. Long-term exposure (for 5 hr/day on 6 days/wk for 10 months) to CS_2 gave rise to less distinct changes, though some interference with respiratory control, and hence some uncoupling, did occur. It was concluded that, besides an uncoupling effect, CS_2 poisoning could have an inhibitory effect on energy transfer in brain mitochondria. There was no evidence of any cumulative effect and the degree of tissue saturation by CS_2 seemed to be the important factor in intoxication.

The third paper cited deals with biochemical studies designed to investigate any link, in addition to the known pathological similarities, between CS_2 intoxication and diabetes mellitus. To this end, changes in carbohydrate metabolism were monitored in workers exposed to CS_2. Results of glucose tolerance tests with prior administration of prednisolone (prednisolone GTT) on CS_2 workers showed significantly higher blood-sugar levels after 1 and 2 hr than those reached in control workers. The increases observed were directly proportional to the duration of CS_2 exposure. Abnormal prednisolone GTT results and retinal microaneurysm were approximately five times more frequent in CS_2 workers than in controls, and their incidence increased with length of CS_2 exposure. Nearly 25% of CS_2 workers with microaneurysm also exhibited abnormal prednisolone GTT results, but there was no relation between these parameters in control subjects. It was concluded that CS_2 inhalation had a mild diabetogenic action and that the retinal microaneurysm associated with CS_2 exposure was induced by the same biochemical changes that caused the vascular alterations seen in diabetes mellitus.

2458. A closer look at Minamata disease

Klein, R., Herman, S. P., Brubaker, P. E., Lucier, G. W. & Krigman, M. R. (1972). A model of acute methyl mercury intoxication in rats. *Archs Path.* **93,** 408.

Methylmercury (MeHg) poisoning from contaminated fish was responsible for the outbreak of the so-called Minamata disease in Japan (*Cited in F.C.T.* 1971, **9,** 140). Observations in man and animals revealed that the main effects were located in the central nervous system. In order to study the pathogenesis of this lesion, the authors of the paper cited here gave an aqueous solution of MeHg hydroxide to a group of 28 rats by daily subcutaneous injection of doses of 10 mg/kg body weight. Groups of six rats were killed 24 hr after the second, fifth and seventh doses and the remaining ten rats were killled 8 days after the seventh dose (i.e. on day 15). Groups of control rats were killed at the same intervals. Some of the controls killed on day 15 were placed on a restricted diet from day 4 to reproduce the weight loss seen in treated rats.

No adverse effects were seen in any rats up to day 4. Loss of weight was observed from day 5 onwards and neurological signs, consisting of decreased activity and an "awkward and broad-based" gait of the hind limbs, appeared on days 9–11. Later (by day 14), the hind limbs were flaccid and atrophic and when the rats were held up by the tail the hind limbs folded across one another. Food consumption was within expected limits during the treatment period, despite the observed weight loss, but at the end of the experiment, the rats kept for 8 days after the seventh injection appeared very weak and consumed less food than the control rats.

The highest concentration of Hg (about 230 μg/g tissue) was found in the kidneys of the treated rats and was attained by day 6 of treatment. Liver concentrations reached a peak on day 8 (170 μg Hg/g tissue). The brain concentration of Hg was much lower than these levels, with a gradual increase from about 10 μg/g at day 3 to 40 μg/g at day 15.

Histologically, the nervous system and kidneys showed some changes by days 6 and 3, respectively, and in both organs advanced signs of damage were apparent by day 15. The sciatic nerve sheaths were swollen and extensive loss of myelin and damage to the nerve fibre had occurred. Groups of nerve cells were lost from the lower portions of the spinal cord, the cerebellum and some of the brain-stem nuclei. The kidney showed extensive necrosis of the proximal tubules. Blood urea nitrogen was raised in both the Hg-treated rats (58%) and the starved rats (44% above the control level).

These findings indicate that MeHg produces degenerative changes both in the peripheral nerves and in the central nervous system below the mid-brain. The white matter of the cerebral cortex and the mid-brain nuclei showed no changes.

2459. Feeding studies with unusual trace metals

Schroeder, H. A. & Mitchener, Marian (1971). Scandium, chromium (VI), gallium, yttrium, rhodium, palladium, indium in mice: Effects on growth and life span. *J. Nutr.* **101,** 1431.

This paper assesses the innate toxicity of small doses of seven abnormal trace contaminants from the results of studies in mice involving the administration of the metal ions in the drinking water from weaning until death. The parameters used for evaluation were principally those used by the same group in previous studies (*Cited in F.C.T.* 1965, **3,** 536; *ibid* 1968, **6,** 423; *ibid* 1971, **9,** 155), namely body weight, mortality and survival rates, and the incidence of benign and malignant tumours.

Groups of weanling mice (54 of each sex) were given a diet carefully controlled in respect of the content of essential and other trace minerals and drinking water similarly controlled and containing scandium (Sc), hexavalent chromium (Cr), gallium (Ga), yttrium (Y), rhodium (Rh), palladium (Pd) or indium (In) added, respectively, as Sc oxide in HCl, potassium chromate, Ga metal dissolved in HCl/HNO_3, Y nitrate and Rh, palladous and In chlorides, in amounts to give metal concentrations of 5 ppm. Animals were weighed at monthly intervals for 6 months and less frequently thereafter. All the elements caused slight growth suppression during the first 6 months, and in the cases of Ga, Sc, Y and In this effect continued throughout the test. Pd suppressed growth in males but not females, while In was more effective in females.

Longevity in females was decreased by ingestion of Ga but both Pd and Y increased the mean life-span, the former in males only and the latter in both sexes. Dead animals were weighed and autopsied and sections of the heart, lung, liver, kidneys and spleen were examined microscopically. Malignant tumours defined on the basis of microscopic changes involving mitotic figures, invasion, metastases and cell differentiation were found in both test and control animals, the most common being of the lymphoma–leukaemia types. Statistical analysis of tumour type and frequency suggested that Rh, Pd and perhaps Y might be slightly carcinogenic under these test conditions. Results with hexavalent chromium also suggested the possibility of slight carcinogenic activity.

2460. Towards defeating platinosis

Levene, G. M. (1971). Platinum sensitivity. *Br. J. Derm.* **85,** 590.

This paper reviews the problems and assesses progress in the understanding and treatment of platinum (Pt) sensitivity (platinosis). This condition occurs to a significant degree among workers exposed to the complex salts of Pt (ammonium, sodium or potassium tetra-

or hexachloroplatinates) used in refining or analysis. The respiratory reactions resemble a mixture of hay fever and asthma, and they may be accompanied by skin irritations and eruptions. Although these signs sometimes arise within minutes of exposure, individuals have been known to work with Pt salts for months or years before developing untoward reactions.

From his survey of case reports and related toxicological studies, this commentator proposes that the mechanism of histamine liberation by Pt salts, derived earlier (*Cited in F.C.T.* 1971, **9,** 300) from studies on the effects of intravenous injection of Pt salts into guinea-pigs and on isolated guinea-pig ileum, is not applicable to man. The clinical syndrome of platinosis in man, briefly outlined above, appears to be largely the result of immediate hypersensitivity to Pt salts. Further support for this is provided by the observations that scratch tests with highly diluted Pt salts are positive only from the time when the workers begin to show symptoms (Roberts, *Archs ind. Hyg.* 1951, **4,** 549), and that passive transfer tests using the patient's serum have been positive with both the Pt salt alone and in conjunction with human serum albumin (Freedman & Krupey, *J. Allergy* 1968, **42,** 233). In accord with this hypersensitivity hypothesis is a report of successful hyposensitization, analogous to that practised for hay fever treatment, effected by a course of intradermal injections with increasing amounts of ammonium hexachloroplatinate (Levene & Calnan, *Clin. Allergy* 1971, **1,** 75). Not surprisingly, in view of the difference in the degree of Pt oxidation, this patient remained sensitive to tetrachloroplatinates.

The author attempts to elucidate the mechanism of platinosis in man and its amelioration by hyposensitization, proposing that reaginic (IgE) antibody mediates the original symptoms, and that injections of the Pt salt stimulate production of IgG (blocking) antibody, which combines preferentially with the antigen to prevent further anaphylactic reactions. Although the presence of IgG antibody was not directly demonstrable, indirect support for this theory arose from the observation that intradermal injection of a Pt salt incubated with post-hyposensitization serum gave a much smaller reaction than a salt incubated with pre-hyposensitization serum.

Provision of a mechanism for contact dermatitis arising from Pt salts is more difficult. Patch tests have provided no convincing evidence that allergic contact sensitivity occurs, and in any case it seems clear that these salts cause urticaria immediately on contact, while allergic contact sensitivity is a delayed hypersensitivity reaction.

Recent reports that Pt salts may be of value in cancer chemotherapy suggest that the problem of allergic reactions to Pt may eventually move beyond the industrial field, where the problem can presumably be alleviated to some extent by the careful control of handling and ventilation.

2461. Acrylonitrile–sulphydryl interaction

Hashimoto, K. & Kanai, R. (1972). Effect of acrylonitrile on sulphydryls and pyruvate metabolism in tissues. *Biochem. Pharmac.* **21,** 635.

Acrylonitrile is toxic to nerve tissue, one of its effects being to prevent the passage of impulses along the sciatic nerve. It may react with the active hydrogen atoms of sulphydryl compounds, and this reaction, demonstrated *in vitro*, has previously been suggested as a basis for its detoxification. The originators of this suggestion have now investigated *in vivo* the reaction of this nitrile with tissue sulphydryls and its effect on pyruvate metabolism.

In control animals, the concentrations of both total and non-protein sulphydryls were higher in the liver than in the blood and brain. Substantial decreases in effective tissue concentrations were observed 1 hr after injection of a toxic dose of acrylonitrile into rabbits or guinea-pigs. By this time, the treated animals showed severe signs of toxicity. The falls were greater in the liver and brain, although early decreases in blood sulphydryls followed by a rapid recovery were also recorded. Accumulation of pyruvate, and to a lesser extent of lactate, was evident in the tissues 1 hr after treatment, and in the case of pyruvate this was particularly marked in the brain. Since such accumulation was not remedied by simultaneous administration of sodium thiosulphate, which would have counteracted the effects of inorganic cyanide liberated from the acrylonitrile, the effect was thought to be due to reactivity with the specific sulphydryl compounds involved in pyruvate oxidation.

2462. Another case of acrylamide poisoning

Cavigneaux, A. et Cabasson, G. B. (1972). Intoxication par l'acrylamide. *Archs Mal. prof. Méd. trav.* **33,** 115.

The neurotoxic effects seen in acrylamide poisoning (*Cited in F.C.T.* 1972, **10,** 270) are normally accompanied in man by contact dermatitis (*ibid* 1968, **6,** 105). The case described in the present paper was unusual in that there were no skin lesions, which frequently serve as a warning of over-exposure to acrylamide. It involved a workman employed in waterproofing operations, pumping into fissures a resin prepared on the spot from acrylamide, its *N*-methyl and *N,N*-dimethyl derivatives, ammonium persulphate and diethyl or dimethyl aminopropionitrile, sometimes together with potassium ferrocyanide. He had been supplied with protective clothing and gloves, but it is not clear whether these were always worn. After 18 months he reported paraesthesia of the hands, and after a further 6 months in the same employment he had difficulty in walking and was markedly debilitated. His condition improved little during the ensuing 3 months in hospital, where he complained of numbness and cold of the extremities (which were covered in sweat) and pain in the leg muscles. At this point his reflexes were still absent, although his hand muscles were recovering some of their former strength. After a further 2 months, he appeared to be well on the road to recovery, and failed to present himself for further examination.

[*N*-Methylacrylamide is also neurotoxic, although far less potent than acrylamide (*Cited in F.C.T.* 1970, **8,** 707), while 2-aminopropionitrile has been implicated in the neurological condition known as lathyrism (*ibid* 1970, **8,** 447). It is thus possible that one or more of the other resin components may have contributed to the neurotoxicity reported here.]

2463. The fate of chloromethane

Redford-Ellis, M. & Gowenlock, A. H. (1971). Studies on the reaction of chloromethane with preparations of liver, brain and kidney. *Acta pharmac. tox.* **30,** 49.

Redford-Ellis, M. & Gowenlock, A. H. (1971). Studies on the reaction of chloromethane with human blood. *Acta pharmac. tox.* **30,** 36.

The major toxic effects of inhaled chloromethane appear after a delay of some hours and are associated with the brain, liver and kidneys, although a reaction also occurs with human blood. Work now reported indicates that reaction of this compound with reduced

glutathione (GSH) may be a vital factor in its toxic action. The first paper cited above discusses the biochemical reaction of chloromethane with rat-tissue homogenates.

Renal and hepatic uptakes of unlabelled chloromethane were similar to the values for $[^{14}C]CH_3Cl$ uptake, the latter being only a measure of bound methyl groups. In the brain, however, uptake of radioactivity was much lower than the uptake of unlabelled chloromethane. Labelling experiments showed that *S*-methylglutathione (GSMe) and *S*-methylcysteine (S-MeCys) were formed in the liver by independent reactions, since the former compound was not hydrolysed when incubated with liver homogenate. Experiments with heat-denatured homogenates indicated that both reactions were enzymic. Since both brain and kidney homogenates can hydrolyse GSMe, formation of S-MeCys may not occur directly in these tissues, and the reactions may not be enzyme-catalysed. Only renal protein took up radioactivity into methionine as well as cysteine, a demonstration of the very specific conditions required for the reaction between chloromethane and thiol groups.

Formation of volatile products in the kidneys was suggested by the rapid losses of bound radioactivity often observed, but this was probably not due to breakdown of S-MeCys or GSMe. Loss of GSH, which assists in the catalytic breakdown of methylglyoxal to lactic acid, and accumulation of GSMe, which inhibits this breakdown, may to some extent account for the cerebrotoxic effects of chloromethane, which are similar to the signs of methylglyoxal intoxication in cats (Sjollema & Seekles, *Biochem. Z.* 1926, **176**, 431).

The second paper, from the same laboratory, deals with the reaction of chloromethane with human blood plasma and red cells. The amount of $[^{14}C]CH_3Cl$ bound to plasma following incubation was only a small fraction of the amount of unlabelled compound that became bound in a similar experiment, suggesting that the formation of volatile products is considerable. When plasma containing bound chloromethane was hydrolysed the main produce was S-MeCys. Radioactivity studies showed that $[^{14}C]CH_3Cl$ became bound to thiol groups of plasma albumin, but this reaction was not thought to be an important factor in the onset of toxic effects.

About 40% of the chloromethane taken up by the red blood cells was bound as GSMe, which appeared to be a stable compound. The reaction with GSH was probably mediated by an erythrocyte-specific enzyme, as indicated by the reduced reactivity after heating. It is speculated that a form of haemolytic anaemia found in subjects exposed to chloromethane may be due to decreased GSH levels, which may result in the breakdown of the cell membrane.

2464. Keeping an eye on fluorescein

Stein, M. R. & Parker, C. W. (1971). Reactions following intravenous fluorescein. *Am. J. Ophthal.* **72**, 861.

Intravenous fluorescein is widely used in the diagnosis of eye lesions and may also be injected intravenously for determining the circulation time in man. Toxic side-effects are normally limited to nausea and vomiting, but occasionally more severe reactions are seen and these are suspected of being of an allergenic nature. The present paper describes sixteen patients showing such conditions, including nine previously unpublished cases from the authors' own experience, and also discusses the more common urticarial reactions, which are almost certainly allergenic.

In all, evidence of 55 cases of untoward reactions to intravenous fluorescein have been collected. The majority of these (67%) were of an allergenic type and were characterized

only by urticaria. About 29% of the reactions were more severe, however, with most of the patients presenting with shock-like symptoms. In one case, myocardial infarction developed and the patient died. Of the 16 severe reactions, seven were thought to involve allergy to fluorescein. No evidence of circulating antibodies to fluorescein could be found in the cases tested, however, and the exact mechanism whereby either of these types of reaction occurs remains unclear despite considerable speculation. The value of the paper lies more in the recognition of the fact that such reactions do occur and in the authors' estimation of their frequency. The mean overall incidence of untoward side-effects from intravenous fluorescein is reported to be 0·6%, high enough for ophthalmologists and others to be concerned and to be prepared for emergency action, it is thought, but not so high as to suggest that the safety of the technique needs to be fundamentally re-examined.

2465. Neuropathy from hexane

Herskowitz, A., Ishii, N. & Schaumburg, H. (1971). *n*-Hexane neuropathy. A syndrome occurring as a result of industrial exposure. *New Engl. J. Med.* **285,** 82.

We have previously noted the relatively low acute oral toxicity of hexane in the rat in a study covering numerous organic solvents (*Cited in F.C.T.* 1972, **10,** 730) and the inability of this particular solvent to promote tumour induction when applied to mouse skin previously painted with a sub-carcinogenic dose of 7,12-dimethylbenz[*a*]anthracene (*ibid* 1967, **5,** 840). Recent reports suggest, however, that as a contaminant of factory atmospheres, *n*-hexane may be less innocuous than has been thought. Cases of polyneuropathy recently occurred among Japanese sandal-makers repeatedly exposed to atmospheric levels of hexane in excess of 500 ppm and sometimes reaching 2500 ppm (Yamamura, *Folia psychiat. neurol. jap.* 1969, **23,** 45) although, elsewhere, central nervous system depression has been the usual response to concentrations above the threshold limit value (TLV) of 500 ppm recommended by the American Conference of Governmental Industrial Hygienists. Now, however, comes a report of three cases of neuropathy caused by hexane exposure in the United States.

Three Puerto Rican women had worked as cabinet finishers in a Bronx furniture factory for 2–4 months when they developed abdominal cramps, weakness of the distal extremities and sensory loss. Achilles reflexes were absent, nerve conduction rates were significantly reduced and there were fibrillation potentials in the small muscles of hands and feet. Biopsies revealed signs of muscle denervation and changes in nerve axons and motor end plates identical to those seen in neuropathies due to acrylamide and tri-*o*-cresyl phosphate. The women had been working near an open drum of *n*-hexane in a small room, air samples from which contained on average 650 ppm hexane with occasional peaks at 1300 ppm. Their jobs entailed dipping rags into the solvent, so direct skin contact was involved as well as inhalation. The condition of the women improved slowly after they left this employment, and architectural modification of the factory eliminated the hazard to other workers. However, in view of the effects observed, a re-evaluation of the TLV for *n*-hexane is considered advisable.

2466. Hydroxylamine and the human chromosome

Brøgger, A. (1971). Chromosome damage in human cells induced by hydroxylamine. *Hereditas* **69,** 19.

It has been established that the mutagenic effect of hydroxylamine (HA) is associated

with structural alterations in DNA, and we have previously mentioned one tentative mechanism for such damage, whereby the cytosine residues in the nucleic acid undergo preferential attack by the mutagen (*Cited in F.C.T.* 1967, **5,** 107). A variety of breaks and spurious reunions become visible in the chromosome chain. A paper has now been published reporting details of the morphological effects of HA upon human leucocyte chromosomes *in vitro*.

Chromosomal damage induced in whole blood cultures bore a direct, though not strictly linear, relation to HA dose and length of treatment. Gaps and constrictions were far more widespread than breaks and exchanges in the genetic material. No clear relation could be established between distribution of aberrations and chromosome length, but the damage appeared to be in some way selective. Thus, if administered during the G2 phase, HA tended to produce aberrations at the same locus, suggesting an effect on normal folding into the metaphase conformation.

The results of this work and a similar study on HeLa S3 cells suggest that HA does not induce unscheduled DNA synthesis, its main action probably being to interact with proteins involved in chromosomal rearrangements.

2467. Checking up on MOCA

Linch, A. L., O'Connor, G. B., Barnes, J.R., Killian, A. S., Jr. &Neeld, W. E., Jr. (1971). Methylene-bis-ortho-chloroaniline (MOCA): Evaluation of hazards and exposure control. *Am. ind. Hyg. Ass. J.* **32,** 802.

MOCA (4,4′-methylene-bis-(2-chloroaniline); 3,3′-dichloro-4,4′-diaminodiphenylmethane) has gained in importance over the past 10 yr as a curing agent for isocyanate polymers and epoxy resins. However, it has been reported to produce kidney irritation in man and the dog (Mastromatteo, *J. occup. Med.* 1965, **7,** 502) and, as an aromatic amine, it is inevitably suspect as a potential carcinogen. Concern has therefore been expressed for the safety of workers handling it. A high incidence of liver, lung and mammary tumours has been reported in rats fed a protein-deficient diet containing MOCA (Steinhoff & Grundman, *Naturwissenschaften* 1969, **56,** 215; Stula *et al. Toxic appl. Pharmac.* 1971, **19,** 380), but the latter authors found no evidence of tumour formation after oral administration of MOCA to dogs for 4 yr (unpublished data, 1971). The evidence presented by Linch *et al.* (cited above) gives further grounds for hope that exposure to MOCA presents no serious hazard to man.

The data presented stem from a health survey of workers exposed to MOCA for varying periods during 17 yr of process development and production. A statistically equivalent control group and other factory personnel were also included in the study. The workers within each group were classified as to general health, frequency and duration of absenteeism, type of illness and degree of exposure to the chemical. Proof of exposure to MOCA was obtained by urine analysis, which showed up interesting personal variations in absorption, metabolism and clearance rates. Analysis for MOCA was complicated by interference from other aromatic amines, but gas chromatography proved a reliable procedure capable of detecting 40 ppb MOCA in urine ($b = 10^9$) or 10 $\mu g/m^3$ in air. Air concentrations seldom rose above this level, however. For this reason, inhalation was not a significant route of absorption and biological rather than air monitoring was the more useful measure of exposure.

No malignant tumours were diagnosed in any of the 31 workers who had been actively engaged in MOCA production for more than 15 yr, nor in the 31 controls. There were only two cases of malignancy (with tumours of the larynx and large intestine) in the 178 former MOCA workers, who had experienced some degree of exposure for varying periods of time during the first 6 yr of production, and both of these had been diagnosed before the men began working with MOCA. The incidence of cancer deaths in the total plant population over a 15-yr period was 0·115% and was thus below the national average of 0·139%. This difference may be a reflection of the relatively low age of the men involved in this study.

The incidence of other disabilities was closely comparable in the exposed and control groups, with respiratory syndromes (42 and 48%, respectively) and gastro-intestinal problems (18 and 19%) heading the list and cardiovascular disease (2% in each group) and haematological disturbances (0·5 and 0%) coming a long way down. No dermatological cases were found in either group. Thus in 209 workers currently or formerly exposed to MOCA for up to 16 yr, there was no evidence of unusual absenteeism, unexpected serious or chronic disease, abnormalities of urinary cytology or an excessive incidence of malignant tumours or deaths.

Direct absorption of MOCA through the skin occurred to a sufficient degree in the early days of production to raise urinary excretion levels to 25 mg/litre. At this level, MOCA had no observable haematological effects, in contrast to *p*-chloroaniline, which produces some degree of methaemoglobinaemia at levels of 10–20 mg/litre. This high level of MOCA absorption was later effectively reduced by improved ventilation and cleaning, the use of protective clothing, the institution of mandatory showers after each shift and other similar measures.

2468. Nitrofuran metabolism

Akao, M., Kuroda, K. & Miyaki, K. (1971). Metabolic degradation of nitrofurans by rat liver homogenate. *Biochem. Pharmac.* **20,** 3091.

Exploitation of the antibiotic properties of nitrofurans has been restricted by the reported toxic and carcinogenic effects of some of these compounds (*Cited in F.C.T.* 1967, **5,** 426; *ibid* 1970, **8,** 715; *Food Chemical News* 1971, **13** (20), 31). The present *in vitro* work was carried out to study the cellular site and rate of metabolism of nitrofurans in general.

After separate incubation of seven nitrofurans with the 8500 **g** supernatant, microsomal suspension or 105,000 **g** supernatant of rat-liver homogenate, using added $NADPH_2$ as a hydrogen donor, metabolic changes were monitored by following optical absorbance and loss of nitro groups. For most compounds, the slow changes that occurred in these two properties under aerobic conditions appeared unrelated. Under anaerobic conditions, the loss of nitro groups and the decrease in absorbance both occurred rapidly and to a comparable degree. Much of the enzyme activity responsible for the major changes in absorbance was associated with the microsomal fraction, though some was also found in the 105,000 **g** supernatant.

It was concluded that the metabolic changes were probably reductive, since metabolism was accelerated under anaerobic conditions. Nitrofuran degradation, at least *in vitro*, may therefore be associated with the same cytoplasmic structures and the same enzymes that reduce nitrophenyl compounds and azobenzenes, particularly since the presence of a nitrofuran tends to inhibit the metabolism of these other compounds.

2469. Another alkylation mechanism?

Lawley, P. D., Orr, D. J. & Shah, S. A. (1971/72). Reaction of alkylating mutagens and carcinogens with nucleic acids: N-3 of guanine as a site of alkylation by *N*-methyl-*N*-nitrosourea and dimethyl sulphate. *Chemico-Biol. Interactions* **4,** 431.

Alkylating mutagens and carcinogens have been shown to act at various sites on purine residues (*Cited in F.C.T.* 1970, **8,** 79; Lawley, *Fd Cosmet. Toxicol.* 1968, **6,** 573). The present report demonstrates some *in vitro* alkylation at the N-3 atom of guanine with both S_N1 and S_N2 reagents, and suggests a possible *in vivo* mechanism for the miscoding induced by this base.

Alkylating agents of the S_N1 and S_N2 types, typified by *N*-methyl-*N*-nitrosourea and dimethyl sulphate, respectively, were incubated with DNA and the denatured product was hydrolysed to the individual base residues. These were separated initially on a Dowex 50 column and methylated derivatives of the purine bases were identified by studying the UV absorption spectra of the elements. Alkylating agents labelled with [^{14}C]methyl were used as a more sensitive method of detecting 3-methyl-guanine, which was also formed as a result of methylation of both μ2 RNA and poly(G) by di[^{14}C]methyl sulphate.

A tautomeric form of 3-methylguanine nucleosides was postulated, with an imino group at the C-2 position, which would be conducive to mispairing of 3-methylguanine with thymine. Mutagenesis caused by an S_N1 reagent, for example in the bacteriophage T2, has been explained in terms of alkylation of the 0–6 atom of guanine; in the light of this report, miscoding induced by 3-methylguanine could be the key to a mechanism whereby S_N2 alkylating agents initiate mutagenic activity.

2470. Keep chlorinated hydrocarbons cool

Glass, W. I., Harris, E. A. & Whitlock, R. M. L. (1971). Phosgene poisoning: Case report. *N.Z. med. J.* **74,** 386.

We reported once before a case of phosgene poisoning due to the action of heat on a chlorinated hydrocarbon (methylene chloride) in a paint-stripper (*Cited in F.C.T.* 1964, **2,** 427). Another case, due to the incautious use of trichloroethylene (TCE), has now been reported. The solvent was being used to de-grease metal studs before they were welded. The studs, held in a wire basket, were immersed in an open bucket of solvent situated on the work bench close to the area where carbon dioxide arc welding (which produces temperatures of 3500–4000°C) was being carried out. The studs were welded when still damp, and by the end of the morning shift the affected welder's gloves were soaked with TCE. By this time, he was experiencing chest congestion, respiratory difficulties and general malaise. Although he had apparently recovered by the end of the day, during which further exposure was avoided, some of the effects returned when he woke the next morning. Physiological tests then showed a reduction in vital capacity and forced expiratory volume indicating airways obstruction, an increase in alveolar deadspace and venous admixture, arterial hypoxia and impaired carbon monoxide transfer.

Interpretation of these effects was complicated by the patient's history of chronic bronchitis, but they did not correspond to anything he had experienced before, and this pattern of initial onset, disappearance and recurrence seems characteristic of phosgene poisoning. Further support for this diagnosis is provided by reference to similar cases involving exposure to other heated hydrocarbons, such as carbon tetrachloride from a fire-extinguisher

(Seidelin, *Thorax* 1961, **16,** 91) and, again, methylene chloride from paint-strippers used in heated, ill-ventilated rooms (Gerritsen & Buschmann, *Br. J. ind. Med.* 1960, **17,** 187). It has been proposed that the signs of phosgene poisoning result from its slow hydrolysis, after inhalation, to hydrochloric acid and carbon dioxide (Everett & Overholt, *J. Am. med. Ass.* 1968, **205,** 243).

The danger of using chlorinated aliphatic hydrocarbons at elevated temperatures is stressed and a temperature of 400°C is cited as sufficient for the formation of phosgene.

NATURAL PRODUCTS

2471. Bitter almond poisoning

Pack, W. K., Raudonat, H. W. u. Schmidt, K. (1972). Über eine tödliche Blausäurevergiftung nach dem Genuss bitterer Mandeln (Prunus Amygdalus). *Z. Rechtsmedizin* **70,** 53.

A fatal case of cyanide poisoning following the ingestion of bitter almonds, which contain the cyanogenetic glycoside amygdalin, was reported once before (*Cited in F.C.T.* 1965, **3,** 358) and a comment on the surprisingly large number of such cases was made at that time. Since then a similar, but non-fatal, event has resulted from the consumption of apricot kernels (Gunders *et al. J. Israel Med. Ass.* 1969, **76,** 536). The present short paper is worth attention, not only as a reminder of the danger of eating this culinary commodity in its uncooked state, but also because the results of the post-mortem examination of the victim provide the sceptic with convincing evidence that bitter almonds can indeed be the source of a lethal dose of cyanide.

The victim, a 40-yr-old man, was found dead by his bed, his mouth full of reddish foam. When analysis of blood and urine had demonstrated the absence of alcohol, the stomach contents were examined and the presence therein of about 150 ml of a semi-fluid white material smelling strongly of bitter almonds gave a clue to the source of the trouble. Analysis of various body tissues and fluids confirmed the presence of cyanide, and the blood level (312 μg/100 ml, calculated as the anion) was in accordance with values found in other cases of lethal cyanide poisoning. Measurable amounts were also detected in the stomach contents (4380 μg/100 ml), kidneys (69·3 μg/100 g), liver (7·4 μg/100 g), brain (24·7 μg/100 g), muscle (122·9 μg/100 g) and urine (11 μg/100 ml).

These findings, together with microscopic evidence of bitter almond husks in the stomach and the presence therein of benzaldehyde and benzoic acid (which, together with hydrocyanic acid and glucose, are breakdown products of amygdalin) left little doubt as to the cause of death and the source of the cyanide.

2472. Pyrrolizidine alkaloids and the competitive spirit

Jago, Marjorie V. (1971). Factors affecting the chronic hepatotoxicity of pyrrolizidine alkaloids. *J. Path.* **105,** 1.

Numerous theories about the mechanism of the hepatotoxic action of pyrrolizidine alkaloids have been discussed (*Cited in F.C.T.* 1970, **8,** 607; *ibid* 1971, **9,** 307), and the latest idea is that chronic liver damage is mediated by toxic pyrrole metabolites (*ibid* 1971, **9,**

895). The mechanism of destruction still remains obscure, however, and the present work was designed to throw light on a confused situation by tracing the development of the hepatic lesion against a background of the liver cell changes attributable to age and sex and by monitoring the primary cause of these changes, namely fluctuations in the activity of the liver microsomal-enzyme system.

In the rat, sensitivity to lasiocarpine or heliotrine administered repeatedly in small doses increased with age in both sexes. Females were more sensitive than males to lasiocarpine but the situation was reversed in the case of heliotrine. Pretreatment with phenobarbitone provided some protection against the long-term and acute hepatotoxic effects of lasiocarpine, but enhanced the toxicity of heliotrine. *In vitro* microsomal degradation of lasiocarpine was far greater than that of heliotrine, irrespective of the sex of the animals used, although breakdown was always more rapid with microsomes from male rats and from those pretreated with phenobarbitone. The direct relation between accelerated metabolism and an increase in toxicity, such as is found with heliotrine following phenobarbitone treatment, was taken as evidence in favour of the theory that alkaloid toxicity depends on the formation of an active metabolite, but the inverse relation found in the case of lasiocarpine only served to increase the general confusion regarding the hepatotoxic mechanism of these compounds. It seems likely that the overall toxicity of compounds of this type depends in the long run on the relative rates of a series of competing reactions, which produce the active metabolite, inactivate this active compound and produce less active compounds directly from the original alkaloid.

2473. A feast of fat carobs

Tamir, M., Nachtomi, E. & Alumot, E. (1972). Urinary phenolic metabolites of rats fed carobs (*Ceratonia siliqua*) and carob fractions. *Int. J. Biochem.* **3,** 123.

Rats fed tannins isolated from ripe and, to a lesser extent, from green carobs have shown significant depression of growth, a finding attributed by some to the formation of an insoluble protein–tannin complex in the gut (*Cited in F.C.T.* 1971, **3,** 456). Later work indicated that tannic acid might reduce absorption by a direct effect on the gut epithelium rather than by protein-complex formation (*ibid* 1972, **10,** 733). Further light is thrown on the adverse effect of carobs in the feed by the metabolic studies cited above.

Rats were fed green or ripe carobs or a preparation of carob tannins, either in the diet or, after starvation for 16 hr, by stomach tube. In the latter case, a dose of 100 mg was given in 2 ml olive oil, and control rats were given 2 ml of the vehicle. Urinary metabolites were separated and identified by thin-layer and paper chromatography. Gallic acid and 4-*O*-methylgallic acid, evidently derived from free gallic acid in green carob pods or from ester-linked gallates in the tannin fraction were identified as the major urinary metabolites. Catechin derivatives were absent from the urine extracts, indicating that the rat cannot metabolize the catechin moiety of carob tannins.

2474. Denouement of the blackfat story

Miller, G. J., Ashcroft, M. T., Beadnell, H. M. S. G., Wagner, J. C. & Pepys, J. (1971). The lipoid pneumonia or blackfat tobacco smokers in Guyana. *Q. J. Med.* **40,** 457.

British Medical Journal (1972). Blackfat tobacco smoker's lung. *ibid* **1,** 393.

A diffuse pulmonary fibrosis found among East Indians in Guyana has been shown to

occur only in those who have smoked blackfat tobacco, a preparation of whole tobacco leaf treated with light mineral oil and petrolatum imported from the United States. The connexion between blackfat-tobacco smoking and this lung condition in Guyana was first pointed out by Miller *et al.* (*Lancet* 1968, **ii,** 259). These authors suggested that inhalation of dust or fungal spores from the leaves might be responsible for the chronic allergic or irritant alveolitis observed, the tobacco being particularly prone to fungal growth in the hot and humid climate of Guyana. There was also the possibility that pyrolysis products of the tobacco might be the cause of the trouble.

The paper cited above reports a study of 56 patients in Guyana who had developed diffuse pulmonary fibrosis after some years of blackfat-tobacco smoking. A survey of a predominantly Indian community indicated that 20·4% of those older than 55 yr were blackfat smokers and that 19·6% of the smokers, but none of the non-smokers, showed radiological evidence of pulmonary fibrosis, which was responsible for severe airways obstruction. Immunological tests produced no evidence to support the idea that a hypersensitivity reaction was involved. A limited number of autopsy specimens from the lung demonstrated a diffuse interstitial fibrosis and vasculitis. Large deposits of fat surrounded by black amorphous material were observed in the small cystic spaces in the interstitial tissue, resembling those seen in lipoid pneumonia. The widespread distribution of oil throughout the lungs was consistent with inhalation of the material. The pathological condition may therefore properly be described as lipoid pneumonia following the inhalation of vaporized mineral oil from the tobacco. It seems that blackfat may be smoked for many years, perhaps 20 or more, before the smoker becomes aware of breathlessness, although X-rays can detect the condition earlier. The disease may also appear many years later in patients who have smoked blackfat tobacco only in their youth.

The authors of the first paper cited remark that the lung hazards of tobacco additives used for flavouring or moistening have been accorded little attention. An editorial comment in the second document cited draws attention to the possibility of additives in cigarettes causing pulmonary disease with temporal relationships similar to those between smoking and lung cancer, but points out that, in Britain, tobacco manufacturers customarily add nothing but water to their product.

2475. And now bracken and the pig

Evans, W. C., Widdop, B. & Harding, J. D. J. (1972). Experimental poisoning by bracken rhizomes in pigs. *Vet. Rec.* **90,** 471.

We have recently discussed possible problems of carcinogenicity and mutagenicity in connexion with bracken (*Pteridium aquilinum*) and have reported on the demonstration of the transport of one or more active principles in the milk of cows and mice fed on this fern (*Cited in F.C.T.* 1972, **10,** 603 & 882). Another, relatively long-established, problem associated with bracken is that it causes thiamine deficiency in monogastric animals (*ibid* 1966, **4,** 635). By a curious enigma, however, the incidence of bladder tumours in bracken-fed rats is greatly increased by the concurrent administration of thiamine (*ibid* 1971, **9,** 920).

Evans *et al.* (cited above) have now produced the characteristic signs of thiamine deficiency in pigs with dietary levels of 25–33% dried and ground rhizome of bracken. Blood transketolase activity fell after 7–8 days and after 20 days a rise in blood pyruvate levels was recorded. The pigs became listless, their appetites were impaired and they died after 55–71 days, terminal signs being sudden recumbency and dyspnoea and a further steep

drop in transketolase activity. At autopsy, the hearts were mottled and enlarged, with atrophied muscle fibres (many containing basophilic granules), areas of hyperaemia, and endomysial cells both larger and more numerous than is usual. Other organs, in particular the lungs, showed congestion and oedema in areas commonly affected in this way by acute heart failure.

The dietary levels used in this study were very high, because thiaminase is to some extent deactivated during the drying process, and it was hoped that these levels would provide a thiaminase intake more relevant to natural conditions, in which pigs might ingest relatively small quantities of fresh rhizomes containing a very high degree of thiaminase activity. In another study, diets containing 20% dried bracken fronds given to pigs for up to 78 days produced no adverse effects.

The major point of this study was its demonstration that the measurement of transketolase activity in the blood could provide a useful indication of marginal thiamine deficiency. The *in vitro* addition of thiamine pyrophosphate to the enzyme system readily restored the transketolase activity to normal levels, but with blood samples from the control pig virtually no change ensued.

2476. Oesophageal cancer still a mystery

Cook, Paula & Collis, C. H. (1972). Cancer of the oesophagus and alcoholic drinks in East Africa. *Lancet* **i,** 1014.

The authors cited above and their colleagues have been carrying out a series of studies in which they have sought a possible relationship between the varying incidence of oesophageal cancer in different parts of Africa and the varying local customs with regard to the consumption of alcoholic drinks. Recently (*Cited in F.C.T.* 1972, **10,** 883), we reported on a study in which this group failed to establish any correlation between oesophageal cancer incidence and levels of nitrosamines in the local spirit drinks. In connexion with this study, however, it was mentioned that the occurrence of this type of tumour appeared to be generally high in areas where the local beer was fermented from maize rather than from bananas, millet or sorghum.

The letter cited above replies to an earlier letter in which Diller (*Lancet* 1972, **i,** 742) suggested that if maize beer were indeed associated with oesophageal tumours in Africa, aflatoxin might be the responsible carcinogen. The reply agrees that aflatoxin can survive fermentation and that its presence in beer could be a hazard, particularly if the worst-contaminated grains were rejected for food use and used instead for beer-making. However, it also points out that in order to account for the preponderance of this condition in males in any given area, it would be necessary to postulate a mycotoxin present in maize beer but not in other maize products and not in foods prepared from other grains. Moreover, although aflatoxin is a potent liver carcinogen, it has never been shown to produce oesophageal tumours in animals. This makes it unlikely, though admittedly not impossible, that it would have this effect in man. Epidemiological studies have revealed no similarity between the geographical distribution of oesophageal cancer and that of primary hepatoma in Africa. The latter is far more widespread and, while it is the second most common tumour in most areas where oesophageal cancer is rife, it is also common in many areas where oesophageal cancer is rare or almost unknown. There is also little correlation between the known distribution of aflatoxins in Africa and the occurrence of tumours of the oesophagus.

It looks as if we shall have to look beyond the arch-enemy, aflatoxin, for the culprit in this case.

2477. Troubled spirits

Boyland, E. & Down, W. H. (1971). The diethyl esters of *cis*- and *trans*-epoxysuccinic acid, and other possible carcinogens in spirits. *Eur. J. Cancer* **7,** 495.

This probe into the aetiology of cancer of the oesophagus arose from the observation that the incidence of this disease was higher among drinkers of some spirits than among imbibers of undistilled drinks (see previous abstract). It was reasoned that because *trans*-2,3-epoxysuccinic acid (*trans*-EPSA) was an intermediate in the biosynthesis of tartaric acid by *Aspergillus fumigatus*, and because tartaric acid had been detected in wine sediments, it was possible that *cis*- and *trans*-EPSA or their ethyl esters might be present in alcoholic drinks. It was further considered that, like certain other epoxy compounds (*Cited in F.C.T.* 1969, **7,** 192), these compounds might possess carcinogenic properties. An initial experiment demonstrated the stability of the two EPSA ethyl esters in spirits, little more than 10% being lost from a medium approximating to a potable spirit (35% aqueous ethanol at pH 5) in 18 days.

The acute toxicity of each diethyl ester was then examined, doses of 5–625 mg/kg being given intraperitoneally to mice. In each case, an initial weight loss was followed by rapid recovery after the first day. The accordance of this recovery with an epoxide detoxication mechanism proposed earlier (Boyland & Williams, *Biochem. J.* 1965, **94,** 190) was demonstrated by incubating ethanol solutions of each ester with glutathione (GSH) with and without rat-liver supernatant as a source of GSH-*S*-epoxidetransferase. In the presence of the supernatant, 95% conjugation with GSH occurred within 1 hr, compared with only 35% in the absence of the enzyme.

Proceeding with their carcinogenicity studies, the authors examined the alkylating properties of each ester, properties known to be significant factors in the activity of simple epoxides. Both compounds alkylated γ-(4-nitrobenzyl)pyridine, the *cis* isomer at twice the rate of the *trans* isomer. Negative results with the diethyl esters of oxaloacetic, maleic, fumaric and succinic acids demonstrated that alkylation by the epoxy esters was due to the epoxy group itself rather than to the terminal $-CH_2.CO_2C_2H_5$ ester group. Following this result, gas–liquid chromatography was used in attempts to identify the diethyl esters in "African Gin". Although these were not successful, the possibility of the presence of the esters was not excluded, because traces of diethyl *trans*-EPSA ester were later identified in residues after mesotartaric acid had been refluxed in absolute ethanol for 72 hr.

Further attempts to detect the esters in potable spirits are therefore planned, as are carcinogenicity tests on the compounds themselves. The authors also suggest several known carcinogens whose occurrence in spirits is theoretically possible, namely γ-butyrolactone, nitrosamines and certain methyl esters potentially carcinogenic by virtue of their alkylating properties.

[The presence of nitrosamines in whisky distilled from fermented African maize has already been demonstrated (*Cited in F.C.T.* 1971, **9,** 612) but their presence in British whisky could not be detected.]

2478. A tutu trip—by tractor

Chilvers, C. D. (1972). Tutu poisoning in an elderly Maori lacy. *N.Z. med. J.* **75,** 85.

Numerous cases of poisoning have resulted from ingestion of tutin, a substance related

to the picrotoxins and present in the celebrated tutu tree (*Coriaria arborea*). Tutin, which has also been detected in samples of toxic honey (*Cited in F.C.T.* 1969, **7**, 399), is believed to be present in all parts of the tutu plant except the soft black fruit. The gravity of this matter becomes apparent when it is realized that tutu poisoning can claim such diverse victims as two Indian circus elephants with diarrhoea and an elderly Maori lady, the subject of the present report.

The lady in question was found prostrate on the sofa following ingestion of a glass of a fluid prepared by boiling four tutu leaves in water. Though she appeared well shortly after her drink, when she was milking her cows, visual effects were prominent 6 hr (and another glass of the potion) later, when this 69-yr-old had trouble in controlling her tractor. Other effects included mental confusion, general weakness, lack of co-ordination and an apparent yellow coloration of the skin, but after appropriate hospital treatment, the patient made an uneventful recovery.

Despite its obvious hazards when taken orally, tutu is claimed to have its uses (for bathing rheumatic joints, healing fractures and treating boils, for example) and its reputedly effective treatment of gonorrhoea could be a claim to fame!

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

2479. Chlorhexidine as an alternative bactericide

Wahlberg, J. E. & Wennersten, G. (1971). Hypersensitivity and photosensitivity to chlorhexidine. *Dermatologica* **143**, 376.

Since the recently recommended restrictions on the use of hexachlorophene in cosmetics and toilet preparations, particularly those intended for use on babies, chlorhexidine (CH) has been considered as a possible alternative. CH solutions have been used in hospitals as an obstetric and pre-operative skin bactericide, but only occasional reports of allergic skin reactions to CH-containing products have been published and it appears that in none of these cases had patch tests been performed to prove that CH was the offending ingredient. Recently, however, a case was reported of a 31-yr-old businessman who developed dermatitis after a knee-joint operation in which the skin had been disinfected with 0·5% alcoholic CH.

Skin testing of a standard series of substances, using occlusive patches held in place for 48 hr and a photopatch technique, showed that the patient reacted to CH solutions at concentrations as low as 0·125% without light exposure. Twenty-five eczema patients used as controls produced no reactions to a 0·5% solution of CH under similar conditions. Irradiation of the original patient's patch-test sites after 24 hr, with an Osram xenon lamp to give a delayed erythemal or sub-erythemal dose or with a Wood's light, evoked adverse reactions to CH solutions at concentrations as low as 0·0156%. Again a control group of seven eczema patients showed no reaction.

The authors note that six nickel-allergic patients showed no differences in the threshold of sensitivity when irradiated and non-irradiated patches were compared. The concentrations of nickel sulphate used ranged from 1·25 to 0·0195%. They conclude that when an allergen can produce both photoallergic and allergic contact hypersensitivity, the photoallergic reaction appears to be of higher intensity.

[Although the likely incidence of sensitization to chlorhexidine in the general population is not known, this isolated case does not appear to warrant further studies. However, an eye should be kept on the increasing use of this compound and its safety-in-use.]

2480. Reaction to a QAC

Shmunes, E. & Levy, E. J. (1972). Quaternary ammonium compound contact dermatitis from a deodorant. *Archs Derm.* **105,** 91.

The increasing utilization of quaternary ammonium compounds for their antibacterial and fibre-conditioning properties has meant that the rare cases of allergic contact dermatitis resulting from their use cannot be ignored. The present report concerns a patient with a history of eczematous dermatitis who developed an allergic reaction localized in the axillae.

Patch tests on his back elicited a positive reaction to his roll-on deodorant but not to any of the 26 reagents in the diagnostic patch-test tray used for initial screening. However, testing with a 1:1000 concentration of benzalkonium chloride produced a strong reaction. Since the deodorant contained cetalkonium chloride, a quaternary ammonium compound of comparable structure to the one used for patch testing, the dermatitis was ascribed to a sensitization reaction to this component of the deodorant.

2481. Keeping track of photosensitization

Herman, P. S. & Sams, W. M., Jr. (1971). Cellular reactions in contact photoallergy. *Int. Archs Allergy appl. Immun.* **41,** 551.

We referred recently to a possible *in vitro* test for screening out cosmetics ingredients that may cause photosensitization in man (*Cited in F.C.T.* 1972, **10,** 886). The cellular reactions that occur in photoallergy have now been considered, with particular reference to the antibacterial compound, tetrachlorosalicylanilide (TCSA).

Skin taken from the sites of strongly positive photopatch tests in man and guinea-pigs has been shown histologically to contain dermal infiltrates of mononuclear cells. Histochemical techniques showed that very few of these cells contained acid phosphatase or indoxyl esterase. There are several possible explanations for the finding: either the majority of the cells were lymphocytes or the cells were monocytes whose enzyme activity had been suppressed as a result of the immune reaction that had occurred or whose enzyme content had been for the most part discharged.

In vitro work showed that peritoneal mononuclear cells from TCSA-sensitized guinea-pigs contained large amounts of cytoplasmic acid phosphatase and indoxyl esterase when they were grown in a control culture medium. When the cells were grown in a medium containing the specific hapten-protein antigen (TCSA-guineapig albumin), however, no enzyme activity was detectable. Although the authors of this paper did not identify lysosomal enzymes in the culture medium, they suggest that hydrolytic enzymes could be released from dermal macrophages and cause the epidermal damage seen in cutaneous delayed hypersensitivity. This is in agreement with the model for delayed hypersensitivity proposed by Waksman (*Hosp. Pract.* 1968, **3,** 22), with circulating sensitized lymphocytes resulting in the release of a macrophage migration inhibiting factor. This in turn causes monocytes to attach to the adjacent capillary epithelium and release lysosomal hydrolases.

[Unfortunately the release of histiocyte enzymes with subsequent epidermal damage has not been proved. Furthermore, the enzyme systems investigated in this study may not be the most relevant to skin damage.]

CANCER RESEARCH

2482. The role of structure in carcinogenicity

Wogan, G. N., Edwards, G. S. & Newberne, P. M. (1971). Structure-activity relationships in toxicity and carcinogenicity of aflatoxins and analogs. *Cancer Res.* **31,** 1936.

Edwards, G. S., Wogan, G. N., Sporn, M. B. & Pong, R. S. (1971). Structure-activity relationships in DNA binding and nuclear effects of aflatoxin and analogs. *Cancer Res.* **31,** 1943.

As with other biologically active compounds, relationships can be demonstrated between the chemical structure and the activity of carcinogens. Such a relationship was investigated in a number of aflatoxins and three analogous synthetic compounds, 5,7-dimethoxycyclopentene[*c*]coumarin (compound 2), 5,7-dimethoxycyclopentenone[3,2-*c*]-coumarin (compound 8) and 5,7-dimethoxycyclopentenone[2,3-*c*]coumarin (compound 11). These three synthetic compounds contained the fused coumarin-cyclopentene structures of the aflatoxin B molecule, but not the dihydro- or tetrahydrofurofuran rings found in the aflatoxin molecules.

Aflatoxin B_1 induced hepatocellular carcinomas in all 7, 18 and 17 rats given orally intubated doses totalling 0·5, 1·0 or 1·5 mg and made up of 20 or 40 equal doses given over 4 or 8 wk. Aflatoxin G_1 induced this type of tumour within 68 wk in three of five rats given a total dose of 1·4 mg in 14 100-μg doses over 2·5 wk and within 64 wk in 18/18 rats given a total dose of 2·0 mg orally in 40 doses over 8 wk. Aflatoxin B_2 produced no malignant liver tumours when given in a total dose of 0·5 or 1·0 mg orally. Given intraperitoneally, 150 mg B_2 (in 40 doses over 8 wk) produced hepatocellular carcinomas in three of nine rats within 59 wk. Doses of B_1 totalling 1·3 mg given over 8 wk by the same route induced tumours in all nine treated rats within 46 wk.

Differences in carcinogenic potency between aflatoxins B_1 and B_2 were also observed when the compounds were tested by the subcutaneous route. Local sarcomas were produced within 58 wk in all nine rats given a total of 0·4 mg B_1/rat over 20 wk. A total dose of 12 mg B_2 administered under similar conditions induced no tumours in 78–86 wk.

Compounds 2, 8 and 11, containing the substituted coumarin portion of the B_1 configuration, were non-carcinogenic to rats over a period of at least 87 wk when given orally in total doses of 156 mg over a period of 52 wk.

The toxicity of these compounds and of tetrahydrodeoxyaflatoxin B_1 to the duckling and rat was also investigated. The LD_{50}s of B_1 and G_1 in the duckling were 0·73 and 1·18 mg/kg, respectively, and in the rat, 1·16 and 1·5–2·0 mg/kg, respectively. Aflatoxins B_2 and G_2 were less potent to the duckling (LD_{50}s 1·76 and 2·83 mg/kg), and these two compounds, as well as the three synthetic compounds, caused no mortality in doses up to 200 mg/kg. Tetrahydrodeoxyaflatoxin B_1 was without effects in doses up to 128 mg/kg.

The results demonstrate a striking parallel between the toxic and carcinogenic potentials

of this range of compounds. The furofuran moiety of the aflatoxin structure is essential for both these biological effects, while the presence of a double bond in the terminal furan ring is an important determinant of potency. Also important in this respect are the substituents on the lactone portion of the molecule.

Biochemical and morphological changes in the hepatocyte, reflecting effects on RNA, are related to the toxic and carcinogenic potential in the second paper cited. Thus aflatoxins B_1 and G_1 inhibited RNA polymerase activity and decreased the RNA content in rat hepatocyte nuclei, B_1 being three times more potent in these respects than G_1. B_2 had neither effect at doses of 200 mg/kg. Ultrastructurally, B_1 and G_1 produced a rapid and marked segregation of the fibrillar and granular portions of the hepatocyte nucleolus, while B_2 induced only minimal segregation of these components. The tetrahydrodeoxy derivative, the three synthetic analogues and coumarin produced no observable ultrastructural alterations.

The binding of B_1, B_2, tetrahydrodeoxy B_1 and compound 8 to calf-thymus DNA did not accurately reflect their *in vivo* potency. All four compounds possess the same value for their theoretical saturation binding concentration (1 mole ligand/25 moles nucleotide), yet the non-carcinogenic and non-toxic compound 8 had the highest affinity for DNA, followed in decreasing order by B_1, B_2 and the tetrahydrodeoxy B_1. However a comparative study of the binding of aflatoxins B_1 and B_2 to rat liver *in vivo* using ^{14}C-labelled compounds revealed that twice as much radioactivity was present 3 hr after the intraperitoneal injection of a single dose of the potent carcinogen B_1 than was the case with the weakly active B_2.

[The authors are to be congratulated on an excellent investigation into the mechanisms of chemical carcinogenesis. Although not all *in vitro* tests showed correlation with the *in vivo* activities, the correlation of RNA-polymerase inhibition with the carcinogenic activity of B_1 and G_1 and the demonstration of prolonged binding of the carcinogenic B_2 with the liver are sufficiently striking to merit further investigation with similar groups of closely related compounds, only some of which are carcinogenic.]

TERATOGENESIS

2483. Cell death and teratogenesis

Scott, W. J., Ritter, E. J. & Wilson, J. G. (1971). DNA synthesis inhibition and cell death associated with hydroxyurea teratogenesis in rat embryos. *Devl Biol.* **26,** 306.

Inhibition of DNA synthesis by cytosine arabinoside has been tentatively implicated in the teratogenic effects of this compound (Ritter *et al. Teratology* 1971, **4,** 7). In the present study in Wistar rats, another inhibitor of DNA synthesis, hydroxyurea, was administered on day 12 of pregnancy, to see whether any positive relationship between inhibition of DNA synthesis and the incidence of malformation could be demonstrated in this case.

There was no clear association between the dose-related increase in teratogenesis and the observed drug-induced depression of DNA synthesis, and so no definite causal relationship between cell death and malformations could be formulated. The fact that hydroxyurea was present at elevated levels in the embryo for longer periods than in the maternal plasma was indicative of some restriction of transport to maternal blood, possibly because

of some form of binding of the drug within the embryo. Comparison of embryonic concentrations of hydroxyurea with the DNA-synthesis rate supported the hypothesis of direct drug action on the embryonic cells. Embryonic inhibition of DNA synthesis by hydroxyurea was somewhat similar to that shown by cytosine arabinoside in previous studies. Effects of the two drugs differed, however, in that only hydroxyurea gave rise to some cell synchronization, as evidenced by the observation that, over a range of doses, hydroxyurea administration caused DNA synthesis to increase rapidly to a peak of activity after the initial depression, and then to decline rapidly again before returning more slowly to a normal level.

Hydroxyurea showed certain types of malformation not exhibited by cytosine arabinoside, including a differing pattern of missing forelimb digits. Timing of drug administration in relation to pregnancy seemed to be more important in such variation than the chemical structure of the drug, a finding that implicated cell death as a key factor in determining the type of malformation induced. The true role of cell death still remains obscure, however, as shown by the observation that although extensive toxicity in the limb bud was followed by malformations of the limb, embryotoxicity of the neural tube did not lead to malformations of the central nervous system.

Fd Cosmet. Toxicol. Vol. 11, p. 171. Pergamon Press 1973. Printed in Great Britain

ANNOUNCEMENT

POSTGRADUATE COURSE IN TOXICOLOGY

A new postgraduate (M.Sc.) course providing training in the fundamentals of toxicology, with particular reference to the toxicological evaluation of medicines, food additives and environmental chemicals, will commence in October 1973 at the University of Surrey. The course will be organized by the Department of Biochemistry in conjunction with other departments of the University, the British Industrial Biological Research Association, Carshalton, the MRC Toxicology Unit, Carshalton, and other major industrial toxicology establishments. Both full-time and part-time students may be accepted. Preference will be given to those with a background in biochemistry or chemistry and/or previous experience in toxicology.

Further information may be obtained from Dr. J. W. Bridges, Department of Biochemistry, University of Surrey, Guildford, Surrey, England.

Fd Cosmet. Toxicol. Vol. 11, p. 173. 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:*

Teratology studies on food colourings. Part II. Embryotoxicity of R salt and metabolites of amaranth (FD & C Red No. 2) in rats. By T. F. X. Collins and J. McLaughlin.

Evolution, au cours du temps, de quelques effets du Bordeaux S et du Jaune de Beurre sur l'organisme du rat. Par R. Albrecht, Ph. Manchon, C. Keto-Pinto et R. Lowy.

Metabolism and elimination of sulphite by rats, mice and monkeys. By W. B. Gibson and F. M. Strong.

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. By B. M. Ulland, J. H. Weisburger, R. S. Yamamoto and Elizabeth K. Weisburger.

Effect of the antioxidant butylated hydroxytoluene (BHT) on the metabolism of the carcinogens *N*-2-fluorenylacetamide and *N*-hydroxy-*N*-2-fluorenylacetamide. By P. H. Grantham, J. H. Weisburger and Elizabeth K. Weisburger.

Short-term peroral toxicity of undegraded carrageenan in pigs. By E. Poulsen.

Acute and subacute oral toxicity of AHR-2438B, a purified sodium lignosulphonate, in rats. D. K. Luscombe and P. J. Nicholls.

Assessment of the teratogenic activity of dithiocarbamate fungicides. By T. Petrova-Vergieva and L. Ivanova-Tchemishanska.

Effects of methylmercuric chloride on the progeny of mice and rats treated before or during gestation. By K. S. Khera and S. A. Tabacova.

The safety testing of medical plastics. II. An assessment of lysosomal changes as an index of toxicity in cell cultures. By P. Grasso, Julia Gaydon and R. Hendy.

Electrocardiography and blood chemistry in the detection of myocardial lesions in dogs. By B. E. Osborne and N. J. Dent.

The renal toxicity of folic acid in mice. By C. E. Searle and J. A. Blair. (Short Paper)

A histochemical study on some enzyme changes in the kidney, liver and brain after chronic mercury intoxication in the rat. By L. W. Chang, R. A. Ware and Patricia A. Desnoyers. (Short Paper)

Metabolism as a factor in determining the toxic action of the aflatoxins in different animal species. By D. S. P. Patterson. (Review Paper)

กำหนด

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

General. Authors from the United Kingdom should send *Original Papers and Reviews* to the Assistant Editor. All other papers and reviews should be sent to the appropriate Regional Editor. All 'Letters to the Editor' should be sent to the Editor and must be signed before they can be considered for publication.

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.

Forms of Papers Submitted for Publication. Papers should be headed with the title of the paper, the surnames and initials of the authors (female authors may use one given name) and the names and addresses of the institutions where the work was done. A shortened version of the title not exceeding 45 letters and spaces, to serve as a running title, should be supplied.

In general the text should be subdivided as follows: (a) Summary: brief and self-contained, not exceeding 3% of length of paper (b) Introductory paragraphs (c) Experimental (d) Results, presented as concisely as possible (e) Discussion (if any) and general conclusions (f) Acknowledgements and (g) References. Each subdivision should commence on a separate sheet. Manuscripts should be typewritten on *one side of the paper* and *double spaced*. At least *two copies* should be supplied (one original and one, or preferably two, carbon copies). Papers will be accepted in English, French and German.

References. These should be listed on a separate page, in alphabetical order and arranged as follows: author's name and initials, year, title of the journal (abbreviated according to the rules adopted in the *World List of Scientific Periodicals 1963*. 4th Ed., Butterworth & Co. (Publishers) Ltd. London), volume, first page number:

e.g. Hickman, J. R., McLean, D. L. A. & Ley, F. J. (1964). Rat feeding studies on wheat treated with gamma-radiation. I. Reproduction. *Fd Cosmet. Toxicol.* **2**, 15.

References to books should include the author's name followed by initials, year, title of book, edition, appropriate page number, publisher and place of publication:

e.g. Dow, E. & Moruzzi, G. (1958). *The Physiology and Pathology of the Cerebellum*. 1st Ed., p. 422. The University of Minnesota Press, Minneapolis.

The names of all the authors of papers to be cited should be given when reference is first made in the text. In cases where there are more than two authors subsequent citations should give the first-named author followed by the words *et al.*:

e.g. (McLaughlin, Bidstrup & Konstam, 1963); (McLaughlin *et al.* 1963).

Where more than one paper by the same author(s) has appeared in any one year, the references should be distinguished in the text and the bibliography by the letters, a, b etc. following the citation of the year:

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Footnotes. These as distinct from literature references should be avoided as far as possible. Where they are essential, reference is made by the symbols * † ‡ § ‖ ¶ in that order.

Illustrations and Diagrams. These should be kept to a *minimum* and they should be numbered and marked on the back with the author's name. Legends accompanying illustrations should be typewritten on *separate* sheets. Diagrams and graphs must be drawn in Indian ink on good quality paper or tracing linen. The following standard symbols should be used on line drawings since they are easily available to the printers:

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