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INFORMATION SECTION

ARTICLES OF GENERAL INTEREST*

TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS*

*These items have been contributed by the staff of the British Industrial Biological Research Association. Comments on them will be welcomed and should be sent to the Assistant Editor at BIBRA.

Fd Cosmet. Toxicol. Vol. 13, p. ii. Pergamon Press 1975. 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*:

Effects of prolonged ethylene thiourea ingestion on the thyroid of the rat. By S. L. Graham, K. J. Davis, W. H. Hansen and Carleene H. Graham.

Tissue distribution, excretion and biological effects of [^{14}C]tetrachlorodibenzo-*p*-dioxin in rats. By J. R. Allen, J. P. Van Miller and D. H. Norback.

Lack of dominant lethality in rats treated with polychlorinated biphenyls (Aroclors 1242 and 1254). By S. Green, Frances M. Sauro and L. Friedman.

Generation and inhalation toxicity of dichloroacetylene. By D. Reichert, D. Ewald and D. Henschler.

The effect of CTAB, a cationic surfactant, on the absorption rate of [^{14}C]tripalmitate from a test meal in the rat. By B. Isomaa and G. Sjöblom.

Long-term toxicity of four fluorescent whitening agents. F. L. Lyman, J. Schulze, C. R. Ganz, P. S. Stensby, M. L. Keplinger and J. C. Calandra.

Long-term safety studies of a chloroform-containing dentifrice and mouth-rinse in man. By S. De Salva, A. Volpe, G. Leigh and T. Regan.

The rabbit as a model for evaluating skin irritants: A comparison of results obtained on animals and man using repeated skin exposures. By F. N. Marzulli and H. I. Maibach.

Absence of carcinogenic activity in BD rats after oral administration of high doses of bismuth oxychloride. By R. Preussmann and S. Ivankovic. (Short Paper)

An improved semi-quantitative method for the estimation of aflatoxin M_1 in liquid milk. By D. S. P. Patterson and B. A. Roberts. (Short Paper)

Monographs on fragrance raw materials. By D. L. J. Opdyke.

The influence of milk and related dietary constituents on lead metabolism. By R. Stephens and H. A. Waldron. (Review Paper)

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Research Section

IN VIVO CHROMOSOME-DAMAGING EFFECT OF CYCLOHEXYLAMINE IN THE CHINESE HAMSTER

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(*Received 19 December 1974*)

Abstract—Experiments performed in Chinese hamsters treated orally with cyclohexylamine (CHA) of high purity given in three daily doses of 200 mg/kg/day indicated that CHA has a chromosome-damaging effect *in vivo* in this species. The purity of the CHA was checked by mass spectrometry and in view of the instability of this compound, it was found advisable to handle it at a low pH (2·2–2·4) and in a nitrogen atmosphere.

INTRODUCTION

Cyclamate has been, and in many countries still is, used as an artificial sweetener. Chromosomal aberrations have been found in humans using cyclamate for therapeutic reasons (Bauchinger, Schmid, Pieper & Zöllner, 1970). In view of the interest in the mutagenic and carcinogenic activity of cyclamate and the contradictory results in the literature, we felt that research into the *in vivo* chromosome-damaging activity of cyclohexylamine (CHA), the most important metabolite of cyclamate, was justified.

An association between carcinogenic and chromosome-damaging activity is not unlikely; many malignant tumours have an aberrant karyotype, while leukaemia can be caused by environmental factors giving rise to chromosome aberrations in man and animals. Arguments for this association were put forward recently by Rowley (1974) and van Went-de Vries & Kragten (1975). Moreover, an increased risk of leukaemia is also found in certain hereditary diseases associated with chromosome abnormalities, such as Bloom's syndrome, Fanconi's anaemia and ataxia telangiectasia.

The chromosome-breaking activity of CHA was tested by oral administration of the chemical to Chinese hamsters. Chromosome analysis was carried out on phytohaemagglutinin-stimulated lymphocytes of the peripheral blood before and after the administration.

EXPERIMENTAL

Animals. Because of their small number of chromosomes ($2n = 22$), Chinese hamsters were chosen as the test animals. The age of the animals was about 7 months and their weight varied between 30 and 40 g, males usually being some 10 g heavier than females of the same age. Although male and female animals were used in the experiments, it was not possible to use an equal number of animals of both sexes, since males were available in much greater quantities than females. There was no evidence, however, that males and females differed in their response to CHA administration in respect of chromosome abnormalities.

CHA preparation. Because CHA is a relatively unstable product, CHA sulphate is often used in cases where stability is important. The CHA sulphate intended for use in this experiment was investigated by mass spectrometry and two unknown impurities were found. Free CHA (BDH 27875) of stated purity by spectrometric analysis, from BDH Chemicals Ltd., Poole, Dorset, was therefore used instead and checked by mass spectrometry (Fig. 1) as having a purity of 99–100·5%. Since it oxidizes easily, it was necessary to handle the CHA in a nitrogen atmosphere; once a bottle had been opened, the CHA degraded considerably in 1–2 months even when stored under nitrogen. It was found necessary to work with CHA hydrochloride, as this was more stable than CHA itself, the CHA solution being diluted for this purpose with hydrochloric acid to a pH of 2·2–2·4. Because the pH of the hamster stomach is probably below 2·2, it was assumed that oral administration of the acid itself would have no effect. For this reason it was not thought necessary to use a control group of animals treated with the solvent alone, in addition to the CHA-treated group.

Calculation of dose. While the administered dose of a chemical is usually calculated on a body-weight basis, it is probably more accurate, especially in small animals to base the calculation on basal metabolism (Van Noordwijk, 1964). In man, the basal metabolism is relatively low compared with that of small animals. The dose for a Chinese hamster can be related to the comparable dose for man (Table 1) by multiplying it by the ratio of human basal metabolism to that of the hamster and using the formula of Brody (1945): log (basal metabolism) = 0·734 log (body weight) + a constant. In this way, the dose of 200 mg/kg body weight given to the hamsters in this study is shown to be equivalent to 1·43 g for a 60-kg man.

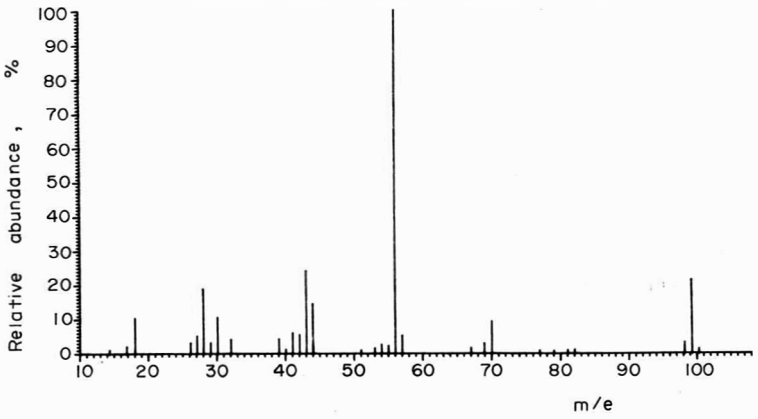

Fig. 1. Mass spectrum of the CHA used.

Bauchinger *et al.* (1970) reported a daily consumption of cyclamate varying from 2 to 5 g/day by the patients they studied. Burbank & Fraumeni (1970) gave similar doses. Complete metabolism of this cyclamate to CHA would yield 1–2·5 g CHA. The calculated dose of 1·43 g, mentioned above, thus lies within this range. It could be objected, that complete transformation of cyclamate into CHA is improbable. On the other hand, CHA was administered to the animals only three times in our experiments, while some of the individuals considered by Bauchinger *et al.* (1970) used it for years. A dose of 200 mg/kg body weight/day was therefore considered appropriate, taking into account the doses given to rats, mice and Chinese hamsters in other reported studies.

Experimental procedure. The group of Chinese hamsters used consisted of 13 males and seven females. Each animal was given a dose of 200 mg CHA/kg by oral intubation on three successive days. Blood samples for lymphocyte culture were taken from the orbital plexus before and 4 days after the CHA treatment.

Tissue culture. Lymphocytes from these samples were cultured according to a technique described by De Jong & Anders (1972). Hamsters are more resistant to colchicin than other animals (Orsini & Pansky, 1952), so vincristin (Lilly) replaced the colchicin normally used in the microtechnique applied in our laboratory (De Vries, Geleijnse, De France & Hogendoorn, 1975). The animals had to be at least 6 months old for successful cultures with many mitoses to be possible. Cultures for each animal were initiated before and 4 days after the treatment, and chromosome analyses were done in all cases.

Chromosome analysis. The slides were numbered at random before the analysis and the numbers, together with the experimental data of the animal, were handed to a person who did not participate in the experiment. The persons performing the chromosome analysis were not aware of the origin of the slides. Fifty metaphases were analysed from each culture. Selection of mitoses for analysis was carried out with a low-power objective (× 10). In every culture, the total numbers of aneuploid cells, polyploid cells and structural abnormalities of the chromosomes were noted with a high-power oil-immersion objective (Zeiss Planapo, × 100). The number of polyploid cells was related to the total number of mitoses observed in the course of selection of the 50 metaphases for analysis. The total number of breaks was expressed in terms of the minimal number of breaks necessary for the formation of the total number of structural abnormalities in 50 metaphases.

Statistical analysis. The observed frequencies (x) were assumed to follow approximately a Poisson distribution. To obtain a normal distribution, the data were transformed in accordance with the transformation of Freeman and Tukey: $y = \sqrt{x} + \sqrt{(x+1)}$ (De Jonge, 1964). A Student's t test was applied to the transformed data. Because we were not interested in a possible decrease in chromosome aberrations by CHA, only the probability of an increase in abnormalities by chance was tested. For this reason a one-tailed test was used.

T·ble 1. *Calculation for different mammals of dose levels equivalent to a human dose of 5 mg/kg body weight*

Species	Body weight (g)	f*	Equivalent dose* (mg)	Equivalent dose (mg/kg body weight)
Man	6×10^4	1	300	5
Rhesus monkey (male)	8×10^3	$2{\cdot}28 \times 10^{-1}$	68·36	8·6
Rabbit	3×10^3	$1{\cdot}09 \times 10^{-1}$	32·7	10·9
Rat	2×10^2	$1{\cdot}50 \times 10^{-2}$	4·50	22·5
Golden hamster	1×10^2	$9{\cdot}20 \times 10^{-3}$	2·76	27·6
Mouse and Chinese hamster	2×10	$2{\cdot}81 \times 10^{-3}$	0·84	42·0

*The equivalent animal dose was calculated by multiplying the human dose by a factor, f = [weight of animal/weight of man]$^{0{\cdot}734}$. For this purpose Brody's formula was used: log (basal metabolism) = 0·734 log (body weight) + a constant.

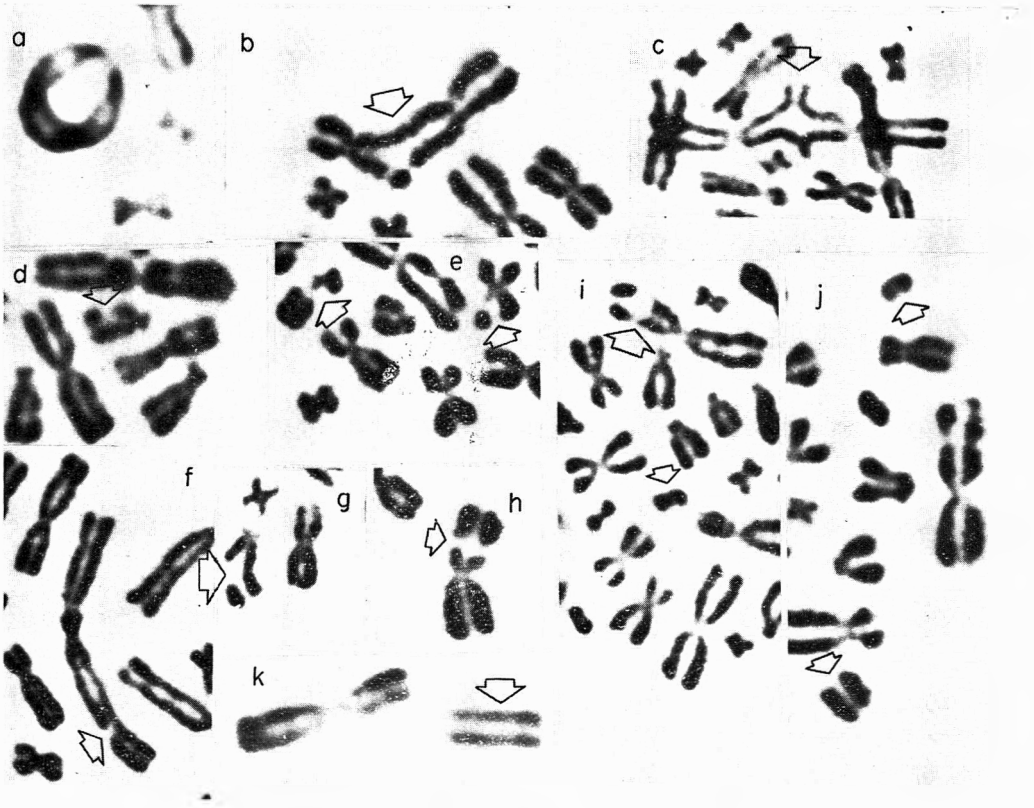

Fig. 2. Structural aberrations in the chromosomes of Chinese hamsters treated with CHA: (a) ring chromosome; (b,c) exchange figures; (d,f,g) chromatid breaks; (e) chromatid and isochromatid break; (h) isochromatid break; (i) isochromatid break and fragment; (j,k) fragments.

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RESULTS

The results of the chromosome analysis are given in Table 2. It appears from the statistical analysis that a significant increase ($P < 0.0005$) in the number of structural aberrations of the chromosomes was found after treatment of the animals with 200 mg CHA/kg body weight on three successive days (Table 2). A ring chromosome, several exchange figures and numerous fragments and breaks were registered. Examples of these abnormalities are shown in Fig. 2. The increases in the numbers of aneuploid and polyploid cells were not significant (Table 2).

DISCUSSION

As CHA is the most important metabolite of cyclamate, its possible carcinogenic and mutagenic properties have aroused interest. Price, Biava, Oser, Vogin, Steinfeld & Ley (1970) administered a daily dose of 15 mg CHA/kg body weight to rats. A tumour of the bladder was found in one (male) animal of the 17 that survived this treatment for 2 yr.

Studies *in vitro* of the chromosome-damaging activity of CHA have produced conflicting results. A significant increase in chromosome breaks was found in cultures of human and Chinese hamster fibroblasts by Bladon & Turner (1971) and Dixon (1973), in a rat-kangaroo cell line by Green, Palmer & Legator (1970) and in human lymphocyte cultures by Stoltz, Khera, Bendall & Gunner (1970). On the other hand, Brewen, Pearson, Jones & Luippold (1971) did not establish a significant increase in chromosome aberrations in human lymphocyte cultures. No mutagenic effects were found in the host-mediated assay by Brewen *et al.* (1971) or Voogd, Van der Stel & Jacobs (1973). In the dominant lethal test, no indication of mutagenic activity was noted in rats or mice by Bailey, Morgareidge, Cox, Vogin & Oser (1972), Cattanach & Pollard (1971) or Lorke & Machemer (1974). However in the same type of test, a significant increase in dominant lethal effects was demonstrated by Petersen, Legator & Figge (1972) in C57 Bl/Fe mice.

Table 2. *Numbers of chromosomal aberrations/50 metaphases in cultures of lymphocytes taken from Chinese hamsters before and after oral administration of cyclohexylamine*

Hamster no. and sex	Number found (x)* Aneuploid cells		Polyploid cells		Structural abnormalities†	
	Before	After	Before	After	Before	After
Males						
395	1	1	1	0	4	16
396	1	1	0	0	5	5
399	1	1	0	0	4	7
401	2	2	0	0	8	16
402	1	3	0	0	5	8
405	1	3	2	0	2	12
409	0	1	2	0	5	19
410	2	0	0	0	9	12
413	2	3	0	0	3	7
423	0	0	0	0	5	6
434	2	0	1	0	15	18
460	1	0	0	0	5	16
461	1	0	1	0	6	19
Females						
438	1	1	0	0	9	3
440	1	0	0	0	5	13
441	0	0	0	0	6	16
445	1	0	0	0	9	22
448	0	0	0	2	6	8
449	0	0	0	0	4	13
457	0	0	0	0	10	15
t (19 df) . . .	−1·1137		−1·4741		5·2327	
P_R	>0·75 (NS)		>0·80 (NS)		<0·005	

NS = Not statistically significant

*Transformed values (y) for the above x values found before and after treatment are (with the corresponding x value in brackets): 1 (0), 2·41 (1), 3·15 (2), 3·73 (3), 4·24 (4), 4·69 (5), 5·10 (6), 5·47 (7), 5·83 (8), 6·16 (9), 6·48 (10), 7·07 (12), 7·35 (13), 7·87 (15), 8·12 (16), 8·59 (18), 8·82 (19) and 9·48 (22). P_R indicates the probability of y being greater after treatment than before.

†Structural abnormalities are recorded as the minimal number of breaks necessary for the total number of aberrations in each culture.

Administration of CHA to rats in the drinking-water was shown to decrease significantly the number of implantations per litter, probably by pre-implantation loss (Khera & Stoltz, 1970). Sex-linked recessive lethals were not found in Drosophila by Knaap, Kramers & Sobels (1973). Cytogenetic analysis *in vivo* also produced contradictory results. No increase in the relative frequency of chromosome abnormalities in rat and mouse spermatogonia was demonstrated by Bailey *et al.* (1972) or by Cattanach & Pollard (1972). Dick, Schniepp, Sonders & Wiegand (1974) found no chromosome-damaging effect of CHA in rats or in a group of four human subjects. From the experiments of Legator, Palmer, Green & Petersen (1969) and of Turner & Hutchinson (1974), and also from our own data, however, *in vivo* chromosome-damaging activity was apparent. Legator *et al.* (1969) found a significant increase in chromosome breaks in the spermatogonia and bone-marrow cells of rats, while Turner & Hutchinson (1974) indicated an increase in chromosome abnormalities in the peripheral lymphocytes of the Chinese hamster.

A further argument centred on the fact that chromosome damage in man was found to be induced by a metabolite of pencyclidine. This compound produces a metabolite similar to CHA from cyclamate (Walker & Seig, 1973). In radiation experiments, Brewen & Preston (1974) reported that the yield of reciprocal translocations caused by irradiation in animals was related to the effective chromosome arm number. In their opinion, an extrapolation from cytogenetic data obtained with laboratory animals to man is possible. A chromosome-damaging effect found in the Chinese hamster, which has 38 chromosome arms, must be even stronger in man, with 81 chromosome arms.

As we mentioned earlier, CHA is readily oxidized, although the stability is improved at low pH. Very few of the authors quoted kept a check on the purity of the CHA; Green *et al.* (1970), Legator *et al.* (1969) and Dick *et al.* (1974) used gas chromatography, while Turner & Hutchinson (1974) effected control by infrared spectrometry. In our experiments mass spectrometry was used (Fig. 1). In all publications in which pH was mentioned, the value was between 7·0 and 7·3, which is not optimal in our experience. So far no other workers seem to have handled the CHA under nitrogen. Much of the lack of agreement among the published results may be due to the use of impure CHA.

It is striking that, in our study, a high percentage of structural abnormalities of the chromosome was found in a proportion of the control cultures. In

nearly all cases, however, these values increased after treatment. The same trend towards abnormalities in control cultures was mentioned by A. Schinzel (personal communication 1973) as occurring in fibroblast cultures of Chinese hamsters. As yet no explanation has been found for this phenomenon, and no similar effect was found in Chinese hamster bone-marrow (van Went-de Vries & Kragten, 1975) or in lymphocyte cultures of man, several Macaca species or rabbits (De Vries *et al.* 1975), but it is arguable that this makes the system more sensitive for mutagenicity screening.

One of the variables that requires consideration is the haematopoiesis that occurs after the first blood puncture. The lymphocyte cultures necessitate removal of a rather large volume of blood from the hamsters (0·7 ml is equal to about one-third of the total blood volume), and after this the animals need 3–4 wk to recover haematologically. Experiments on the possible influence of haematopoiesis on the number of chromosome aberrations did not indicate any such effect. The complete results of this study are presented in a subsequent paper (van Went-de Vries, Kragten & van den Bosch, 1975).

It may be concluded from this experiment that CHA caused a significant increase in structural chromosome aberrations in Chinese hamsters *in vivo*. It was clearly essential to monitor the purity of the CHA preparation and it was found advisable to work at low pH and in a nitrogen atmosphere.

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REFERENCES

Bailey, D. E., Morgareidge, K., Cox, G. E., Vogin, E. E. & Oser, B. L. (1972). Chronic toxicity, teratology, and mutagenicity studies with cyclohexylamine in rats. *Toxic. appl. Pharmac.* **22**, 330.

Bauchinger, M., Schmid, E., Pieper, M. u. Zöllner, N. (1970). Zytogenetische Wirkung von Cyclamat in menschlichen peripheren Lymphozyten in vivo. *Dt. med. Wschr.* **95**, 2220.

Bladon, M. T. & Turner, J. H. (1971). Independent and joint effects of caffeine and cyclohexylamine upon (HDCS) WI-38. *Mamm. Chrom. Newsl.* **12**, 5.

Brewen, J. G., Pearson, F. G., Jones, K. P. & Luippold, H. E. (1971). Cytogenetic effects of cyclohexylamine and *N*-hydroxy-cyclohexylamine on human leukocytes and Chinese hamster bone marrow. *Nature New Biology* **230**, 15.

Brewen, J. G. & Preston, R. J. (1974). Cytogenetic effects of environmental mutagens in mammalian cells and the extrapolation to man. *Mutation Res.* **26**, 297.

Brody, S. (1945). *Bioenergenetics and Growth. With Special Reference to the Efficiency Complex in Domestic Animals.* Hafner Publishing Co. Inc., Rheinhold, New York.

Burbank, F. & Fraumeni, J. F., Jr. (1970). Synthetic sweetener consumption and bladder cancer trends in the United States. *Nature, Lond.* **227**, 296.

Cattanach, B. M. & Pollard, C. E. (1971). Mutagenicity tests with cyclohexylamine in the mouse. *Mutation Res.* **12**, 472.

De Jong, B. & Anders, G. J. P. A. (1972). Lymphocyte cultures from Chinese hamster (*Cricetulus griseus*). *Experientia* **28**, 86.

De Jonge, H. (1964). Introduction to Medical Statistics. Essay of the Dutch Institute for Preventive Medicine XLI. part II, p. 688.

De Vries, Greta F., Geleijnse, Maria E. M., De France, H. F. & Hogendoorn, Anne M. (1975). Lymphocyte cultures of *Macaca mulatta* and *Macaca fascicularis*. *Lab. Anim. Sci.* **25**, 33.

Dick, C. E., Schniepp, Marilyn L., Sonders, R. C. & Wiegand, R. G. (1974). Cyclamate and cyclohexylamine: lack of effect on the chromosomes of man and rats *in vivo*. *Mutation Res.* **26**, 199.

Dixon, C. H. (1973). *In vitro* effects of sodium and calcium cyclamates, cyclohexylamine and sucrose on growth rate and chromosomes of Chinese hamster fibroblasts. *Diss. Abstr.* 59-33 B.

Green, S., Palmer, K. A. & Legator, M. S. (1970). *In vitro* cytogenetic investigation of calcium cyclamate, cyclohexylamine and triflupromazine. *Fd Cosmet. Toxicol.* **8**, 617.

Khera, K. S. & Stoltz, D. R. (1970). Effects of cyclohexylamine on rat fertility. *Experientia* **26**, 761.

Knaap, Ada G. A. C., Kramers, P. G. N. & Sobels, F. H. (1973). Lack of mutagenicity of the cyclamate metabolites in Drosophila. *Mutation Res.* **21**, 341.

Legator, M. S., Palmer, K. A., Green, S. & Petersen, K. W. (1969). Cytogenetic studies in rats of cyclohexylamine, a metabolite of cyclamate. *Science, N.Y.* **165**, 1139.

Lorke, D. & Machemer, L. (1974). Investigation of cyclohexylamine sulfate for dominant lethal effects in the mouse. *Toxicology* **2**, 231.

Orsini, Margaret W. & Pansky, B. (1952). The natural resistance of the golden hamster to colchicine. *Science, N.Y.* **115**, 88.

Petersen, K. W., Legator, M. S. & Figge, F. H. J. (1972). Dominant-lethal effects of cyclohexylamine in C57 Bl/Fe mice. *Mutation Res.* **14**, 126.

Price, J. M., Biava, C. G., Oser, B. L., Vogin, E. E., Steinfeld, J. & Ley, H. L. (1970). Bladder tumors in rats fed cyclohexylamine or high doses of a mixture of cyclamate and saccharin. *Science, N.Y.* **167**, 1131.

Rowley, Janet D. (1974). Do human tumors show a chromosome pattern specific for each ethologic agent? *J. natn. Cancer Inst.* **52**, 315.

Stoltz, D. R., Khera, K. S., Bendall, R. & Gunner, S. W. (1970). Cytogenetic studies with cyclamate and related compounds. *Science, N.Y.* **167**, 1501.

Turner, J. H. & Hutchinson, D. L. (1974). Cyclohexylamine mutagenicity: An *in vivo* evaluation utilizing fetal lambs. *Mutation Res.* **26**, 407.

Van Noordwijk, J. (1964). Communication between the experimental animal and the pharmacologist. *Stat. Neerl.* **18**, 403.

van Went-de Vries, G. F. & Kragten, M. C. T. (1975). Saccharin: Lack of chromosome-damaging activity in Chinese hamsters *in vivo*. *Fd Cosmet. Toxicol.* **13**, 177.

van Went-de Vries, Greta F., Kragten, Maria C. T. & van den Bosch, Renée A. (1975). Lack of effect of blood sampling induced haematopoiesis on *in vivo* chromosome damage by cyclohexylamine in Chinese hamsters. *Fd Cosmet. Toxicol.* **13**, 419.

Voogd, C. E., Van der Stel, J. J. & Jacobs, J. J. J. A. A. (1973). Investigation of Mutagenic Activity of Sodium Cyclamate, Sodium Saccharinate and Cyclohexylamine. National Institute of Public Health Report 15/73 Chemo.

Walker, F. A. & Seig, Jill A. (1973). Phencyclidine and environmental teratogen. *Mutation Res.* **21**, 348.

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LACK OF EFFECT OF BLOOD SAMPLING-INDUCED HAEMATOPOIESIS ON *IN VIVO* CHROMOSOME DAMAGE BY CYCLOHEXYLAMINE IN CHINESE HAMSTERS

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(*Received 19 December 1974*)

Abstract—Chromosomes of Chinese hamster lymphocytes were analysed before and after oral administration of cyclohexylamine and before and after reactive haematopoiesis. The reactive haematopoiesis was induced by the taking of the relatively large sample of blood necessary for lymphocyte cultures. No increase in chromosome aberrations was found in the cultures prepared after the production of new blood cells. Cyclohexylamine, however, induced a significant increase in structural chromosome abnormalities.

INTRODUCTION

Cyclohexylamine (CHA) has been shown to damage Chinese hamster chromosomes *in vivo* (De Vries, Freudenthal, Hogendoorn & Kragten, 1973; van Went-de Vries, Freudenthal, Hogendoorn, Kragten & Gramberg, 1975). In these experiments, many structural chromosome abnormalities were found in a proportion of the control cultures. In connexion with this phenomenon, it was necessary to investigate the possibility that haematopoiesis following the first blood puncture might have been involved in the increase in chromosome aberrations mentioned above. It is impossible to avoid this haematopoiesis, because even the minimal amount of blood (0·7 ml) necessary for the lymphocyte cultures accounts for about one-third of the total blood volume in these small animals. Haematological recovery requires 3–4 wk. We therefore decided to study the effect of reactive haematopoiesis without CHA. Chromosome analysis was performed on phytohaemagglutinin-stimulated lymphocytes of peripheral blood. The hamsters studied formed two groups, one untreated and analysed before and after the haematopoiesis, the other treated with CHA and analysed before and after the oral administration of this compound.

EXPERIMENTAL

Animals. Two groups of 12 Chinese hamsters, aged about 7 months and weighing 30–40 g, were chosen as test animals. One group received an oral dose of 200 mg CHA/kg body weight on three successive days, while the other one was left untreated in order to test the effect of reactive haematopoiesis.

CHA preparation. CHA (from BDH Chemicals Ltd., Poole, Dorset), with a purity of 99–100%, was used as in the earlier experiments (van Went-de Vries *et al.* 1975). The CHA was diluted with hydrochloric acid to a pH of 2·2–2·4. Handling was carried out under nitrogen, because the CHA was readily oxidized.

Tissue culture. A technique described by De Jong & Anders (1972) was used for the culture of lymphocytes from peripheral blood. Blood was obtained by puncturing the orbital plexus. Cultures from each animal were prepared before and after CHA treatment, or before and after the haematopoiesis. Chromosome analysis was performed on all samples.

Chromosome analysis. Before the chromosome analysis the slides were numbered at random, and persons participating in the analysis had no information about the origin of the slides. From each culture, 50 metaphases were analysed. Mitoses suitable for analysis were selected with a low-power objective (× 10). The total numbers of aneuploid cells, polyploid cells and structural aberrations of the chromosomes were counted in every culture with a high-power oil-immersion objective (Zeiss Planapo, × 100). The number of polyploid cells was related to the total number of mitoses observed in the course of selection of the 50 metaphases for analysis. The total number of breaks was expressed in terms of the minimal number of breaks necessary for the formation of the total number of structural abnormalities in 50 metaphases (van Went-de Vries & Kragten, 1975).

Statistical analysis. It was assumed that the observed frequencies (x) before and after treatment or haematopoiesis followed approximately a Poisson distribution. In order to obtain a normal distribution, the data were transformed to the corresponding y values in accordance with the transformation of Freeman and Tukey: $y = \sqrt{x} + \sqrt{(x+1)}$ (De Jonge, 1964). A Student's t test for differences was applied to the transformed data. We were not interested in a decrease in chromosome aberrations by CHA or haematopoiesis, so only the probability of an increase in abnormalities by chance was tested. Therefore a one-tailed test was used.

RESULTS

The results of the experiments are given in Table 1. The analyses before and after haematopoiesis do not show a significant increase in any of the chromosome abnormalities. The lower half of Table 1 confirms the chromosome-damaging activity of CHA, the

Table 1. *Numbers of chromosomal aberrations/50 metaphases in cultures of lymphocytes taken from Chinese hamsters either before and after haematopoiesis (control group) or before and after oral administration of cyclohexylamine*

Hamster no.	Number found (x)* Aneuploid cells		Polyploid cells		Structural abnormalities†	
	Before	After	Before	After	Before	After
Control group						
493	1	0	0	0	15	5
494	0	0	1	6	18	20
497	0	0	0	1	15	9
509	2	0	0	1	5	5
510	0	0	1	6	7	9
511	1	0	3	5	6	6
570	0	2	0	0	19	5
571	0	0	1	0	12	21
572	0	0	0	0	13	19
574	0	0	0	1	17	6
575	1	0	0	0	21	13
576	0	5	1	0	19	2
t (11 df)...	−0·09634		1·64569		−1·78283	
P_R...	>0·75 (NS)		<0·10 and >0·05 (NS)		>0·90 (NS)	
Cyclohexylamine-treated group						
519	1	1	5	3	2	11
521	0	1	3	1	12	16
522	0	0	1	0	11	11
547	0	0	1	1	9	17
551	0	0	1	1	4	14
552	0	1	1	0	14	22
556	0	0	0	2	11	6
557	3	1	0	0	7	12
560	0	0	0	1	8	16
561	0	1	2	0	9	25
562	0	0	1	1	9	17
564	0	0	0	0	13	10
t (11 df)...	1·05297		−0·86352		3·23499	
P_R...	<0·25 and >0·10 (NS)		>0·75 (NS)		<0·005 and >0·001	

NS = Not statistically significant

*Transformed values (y) for the x values found before and after haematopoiesis or treatment are (with the corresponding x value in brackets): 1 (0), 2·41 (1), 3·15 (2), 3·73 (3), 4·24 (4), 4·69 (5), 5·10 (6), 5·47 (7), 5·83 (8), 6·16 (9), 6·48 (10), 6·78 (11), 7·07 (12), 7·35 (13), 7·61 (14), 7·87 (15), 8·12 (16), 8·37 (17), 8·59 (18), 8·82 (19), 9·05 (20), 9·27 (21), 9·48 (22), 10·09 (25). P_R indicates the probability of y being greater after treatment than before.

†Structural abnormalities are recorded as the minimal number of breaks necessary for the total number of aberrations in each culture.

number of structural chromosome aberrations showing a significant increase after treatment ($0{\cdot}005 > P_R > 0{\cdot}001$). The same effect was found in earlier experiments (van Went-de Vries *et al.* 1975). The numbers of aneuploid and polyploid cells were not increased significantly.

DISCUSSION

Chromosome-breaking activity of CHA *in vitro* has been established by several authors. This effect was found in human and Chinese hamster fibroblasts, rat-kangaroo cells and human lymphocytes (Bladon & Turner, 1971; Dixon, 1973; Green, Palmer & Legator, 1970; Stoltz, Khera, Bendall & Gunner, 1970). No increase in chromosome abnormalities was demonstrated, however, in human lymphocyte cultures by Brewen, Pearson, Jones & Luippold (1971). An *in vivo* chromosome-damaging effect was found by Legator, Palmer, Green & Petersen (1969), by Turner & Hutchinson (1974) and also in our laboratory (van Went-de Vries *et al.* 1975).

No such effect could be demonstrated *in vivo* by Bailey, Morgareidge, Cox, Vogin & Oser (1972), Cattanach & Pollard (1971) or Dick, Schniepp, Sonders & Wiegand (1974). As mentioned before (van Went-de Vries *et al.* 1975), many of the controversial results in the literature may possibly be attributable to working with impure CHA.

In our experiments, the factor of haematopoiesis after blood puncture remained to be checked as a possible cause of structural chromosome abnormalities. In the group of untreated animals no significant increase in structural or other chromosome aberrations was found, while in the CHA-treated Chinese hamsters the same significant increase in chromosome abnormalities was established as was found in earlier experiments (van Went-de Vries *et al.* 1975). In view of these results, haematopoiesis may be ruled out as a possible cause for chromosome aberrations.

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REFERENCES

Bailey, D. E., Morgareidge, K., Cox, G. E., Vogin, E. E. & Oser, B. L. (1972). Chronic toxicity, teratology, and mutagenicity studies with cyclohexylamine in rats. *Toxic. appl. Pharmac.* **22**, 330.

Bladon, M. T. & Turner, J. H. (1971). Independent and joint effects of caffeine and cyclohexylamine upon (HDCS) WI-38. *Mamm. Chrom. Newsl.* **12**, 5.

Brewen, J. G., Pearson, F. G., Jones, K. P. & Luippold, H. E. (1971). Cytogenetic effects of cyclohexylamine and *N*-hydroxy-cyclohexylamine on human leukocytes and Chinese hamster bone marrow. *Nature New Biology* **230**, 15.

Cattanach, B. M. & Pollard, C. E. (1971). Mutagenicity tests with cyclohexylamine in the mouse. *Mutation Res.* **12**, 472.

De Jong, B. & Anders, G. J. P. A. (1972). Lymphocyte cultures from Chinese hamster (*Cricetulus griseus*). *Experientia* **28**, 86.

De Jonge, H. (1964). Introduction to Medical Statistics. Essay of the Dutch Institute for Preventive Medicine. XLI. Part II, p. 688.

De Vries, G. F., Freudenthal, J., Hogendoorn, A. M. & Kragten, M. C. T. (1973). Experiments on the chromosome damaging activity of cyclohexylamine in Chinese hamsters *in vivo*. National Institute of Public Health Report 162/73 Farm.

Dick, Claire E., Schniepp, Marilyn L., Sonders, R. C. & Wiegand, R. G. (1974). Cyclamate and cyclohexylamine: lack of effect on the chromosomes of man and rats *in vivo*. *Mutation Res.* **26**, 199.

Dixon, C. H. (1973). *In vitro* effects of sodium and calcium cyclamates, cyclohexylamine and sucrose on growth rate and chromosomes of Chinese hamster fibroblasts. *Diss. Abstr.* 59-33 B.

Green, S., Palmer, K. A. & Legator, M. S. (1970). *In vitro* cytogenetic investigation of calcium cyclamate, cyclohexylamine and trifluprom azine. *Fd Cosmet. Toxicol.* **8**, 617.

Legator, M. S., Palmer, K. A., Green, S. & Petersen, K. W. (1969). Cytogenetic studies in rats of cyclohexylamine, a metabolite of cyclamate. *Science, N.Y.* **165**, 1139.

Stoltz, D. R., Khera, K. S., Bendall, R. & Gunner, S. W. (1970). Cytogenetic studies with cyclamate and related compounds. *Science, N.Y.* **167**, 1501.

Turner, J. H. & Hutchinson, D. L. (1974). Cyclohexylamine mutagenicity: An *in vivo* evaluation utilizing fetal lambs. *Mutation Res.* **26**, 407.

Van Went-de Vries, G. F. & Kragten, M. C. T. (1975). Saccharin: Lack of chromosome-damaging activity in Chinese hamsters *in vivo*. *Fd Cosmet. Toxicol.* **13**, 177.

van Went-de Vries, Greta F., Freudenthal, J., Hogendoorn, Anne M., Kragten, Maria C. T. & Gramberg, L. G. (1975). *In vivo* chromosome-damaging effect of cyclohexylamine in the Chinese hamster. *Fd Costmet. Toxicol.* **13**, 415.

Fd Cosmet. Toxicol. Vol. 13. pp. 423–425. Pergamon Press 1975. Printed in Great Britain

FAILURE TO INDUCE TUMOURS IN GUINEA-PIGS AFTER CONCURRENT ADMINISTRATION OF NITRITE AND DIETHYLAMINE

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Abstract—Simultaneous administration of diethylamine hydrochloride (2 or 4 g/litre) and sodium nitrite (0·4 or 0·8 g/litre) in the drinking-water to guinea-pigs for a period of up to 30 months failed to induce any tumours in the experimental animals. However, 18 of 20 guinea-pigs given diethylnitrosamine (15 mg/litre) in the drinking-water developed liver tumours within 1 yr. None of the controls or animals receiving only the amine (4 g/litre) or nitrite (0·8 g/litre) developed liver tumours. The data suggest that the amount of *in vivo* synthesis of diethylnitrosamine from the ingested amine and nitrite occurring in the stomachs of the animals was insufficient to induce cancer in the animals within the experimental period. The strong basicity of diethylamine was probably responsible for the low *in vivo* yields of diethylnitrosamine.

INTRODUCTION

Druckrey, Steinhoff, Beuthner, Schneider & Klärner (1963) first suggested that secondary amines and nitrite may react with each other in the acidic environment of the human stomach and produce *N*-nitrosamines, many of which are potent carcinogens (Druckrey, Preussmann, Ivankovic & Schmähl, 1967; Magee & Barnes, 1967). However, these workers (Druckrey *et al.* 1963) failed to induce tumours in rats fed high doses of diethylamine and sodium nitrite for their entire lifespan over two generations. This negative finding was attributed to the low acidity (pH > 4) of the rat stomach. Later studies (Alam, Saporoschetz & Epstein, 1971; Mirvish, 1970; Sander, 1967; Sen, Smith & Schwinghamer, 1969) provided conclusive evidence for both the *in vitro* and *in vivo* formation of nitrosamines from secondary amines and nitrite in the stomach of experimental animals.

A high incidence of tumours was observed when animals were fed nitrite in conjunction with a weakly basic secondary amine such as *N*-methylbenzylamine, morpholine or piperazine, but negative results were obtained when a mixture of a strongly basic amine, such as diethylamine, dimethylamine or piperidine, and nitrite were administered to the animals (Greenblatt, Mirvish & So, 1971; Newberne & Shank, 1973; Sander, Schweinsberg & Menz, 1968).

In our earlier studies (Sen *et al.* 1969), we observed that the nitrosation of diethylamine can occur both *in vitro*, in gastric juices from various laboratory animals and man, and *in vivo* in the stomach of the rabbit and cat. More diethylnitrosamine (DEN) was formed in the gastric juices of man and the rabbit (pH 1–2) than in the rat gastric juice (pH 4–5). Since the contents of the guinea-pig stomach is as acidic as that of the human stomach, it was considered that the guinea-pig would be a suitable species for long-term feeding studies. DEN has been shown to produce specific liver tumours in guinea-pigs in a reproducible manner (Arcos, Argus & Mathison, 1969) and the incidence of spontaneous liver tumours in guinea-pigs is very low.

In many of the earlier studies mentioned above, the secondary amines and nitrite were mixed in the diet and fed to the animals. It has been observed in this laboratory that such premixing of nitrite and amines in the feed can lead to the formation of high levels of nitrosamines within a normal storage period. In this experiment, therefore, the chemicals were given in the drinking-water. By proper adjustment of the pH of the water, it was possible to keep the concentration of preformed DEN to a negligible level. Moreover, simultaneous drinking of the two chemicals in the water assured optimum conditions for the formation of DEN in the stomach of the experimental animals.

EXPERIMENTAL

Male guinea-pigs of the English short-hair variety, 21–28 days old, were distributed among six groups, each of 20 animals, so that the average weight in each group was comparable. The animals were housed five in a cage and were given Purina guinea-pig chow *ad lib.* They also received once daily (except at weekends) a moderate serving of fresh lettuce. Diethylamine hydrochloride (DEA) and sodium nitrite solutions, either alone or in combination, were mixed in the drinking-water in appropriate proportions and the pH of the mixtures was adjusted to 7·5 by the addition of sodium bicarbonate. The concentrations of the compounds in the drinking-water given to the six groups of guinea-pigs were (in g/litre): no added amine or nitrite (control group), 0·015 DEN (positive control group), 4·0 DEA (DEA control group), 0·8 sodium nitrite (nitrite control group), 4·0 DEA plus 0·8 sodium nitrite (high-mix group) and 2·0 DEA plus 0·4 sodium nitrite (low-mix group). The solutions containing both DEA and nitrite were made fresh daily, except at weekends when they were kept under refrigeration to minimize the formation of DEN. The concentration of DEN in the residual solutions (those left over after feeding) was occasionally monitored and was found to be in the range of 0·01–0·04 ppm.

The possibility of loss of DEA from the unbuffered solution (pH 7·5) was also checked, but no losses of the amine were detected after storage of the solution in a normal feeding bottle for 48 hr at room temperature. The amounts of the various solutions consumed were recorded each day and the body weights of individual guinea-pigs were taken at weekly intervals.

Moribund animals were killed and the organs were examined and preserved for histology. Animals found dead were also autopsied and when autolysis was not too advanced the tissues were saved. At the end of 2·5 yr, the surviving 22 animals were killed after they had been on test between 885 and 911 days (differences being due to the staggered start). Four animals still living after 18 months on the highest dose of DEA and nitrite were withdrawn from the test solution and were given plain water for the rest of the experimental period. This was done in order to prolong their lives and ensure adequate time for tumour development. The guinea-pigs in the low-mix group, however, were kept on the test solution throughout the duration of the experiment.

Histological examinations were carried out on the salivary and thyroid glands, lymph nodes, lungs, heart, liver, spleen, adrenals and kidneys and on all grossly detectable tumours.

RESULTS

Of the 20 animals given DEN (positive control group), 18 developed liver tumours. The tumours comprised 17 hepatocellular carcinomas and one cholangiocarcinoma. One animal from this group died of pneumonia at 9 months before tumours had time to develop; the first tumour was found in the group after 10 months. By month 13, ten of the animals had tumours and 16 months the tumour incidence had reached 90%. No liver tumours developed in animals in the other groups; deaths were due mainly to pneumonia or to streptococcal infection of the submaxillary lymph glands.

Figure 1 shows the average weight of the animals during the 2·5 yr of the experiment. DEA, either alone or in combination with nitrite, produced a marked decrease in the average body weights of the animals. The surviving guinea-pigs in the high-mix group recovered their body weight somewhat after they were taken off the test solution, and their weights approximated to those of the controls at the termination of the study. The weights of guinea-pigs on nitrite alone were not markedly different from those of the controls.

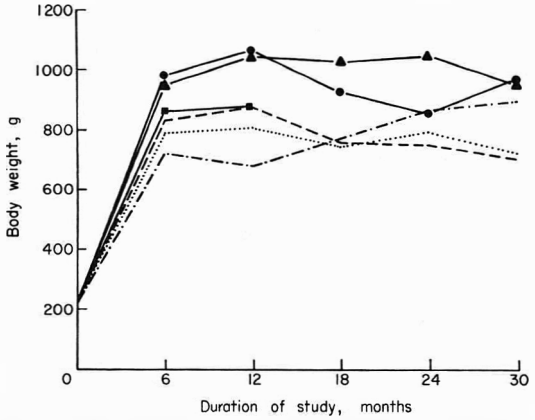

Fig. 1. Mean growth curves for guinea-pigs given drinking-water containing no added amine or nitrite (control; ▲——▲), 0·015 g DEN/litre (positive control; ■——■), 4·0 g DEA/litre (DEA control; ·····), 0·8 g sodium nitrite/litre (nitrite control; ●——●), 4·0 g DEA plus 0·8 g sodium nitrite/litre (high mix; ·—·—·) and 2·0 g DEA plus 0·4 g sodium nitrite/litre (low mix; – – – –).

Table 1. *Number of guinea-pigs surviving at 6-monthly intervals in groups given DEA and/or nitrite or DEN in the drinking-water*

Group	No. of animals surviving at month					
	0*	6	12	18	24	30
Control	20	20	17	14	10	5
Positive (DEN) control	20	20	12	0		
DEA control	20	18	14	11	5	4
Nitrite control	20	20	19	16	12	6
High-mix	20	20	15	4†	3	2
Low-mix	20	20	19	15	9	5

DEA = Diethylamine hydrochloride
DEN = Diethylnitrosamine
*Start of experiment.
†These four animals were given untreated water after 18 months.

In the high-mix group, 15 animals had died by month 18. Chronic pyelonephritis was a major contributory cause of death in these animals. This condition, normally seen in older control animals (aged 18–24 months), was aggravated by the mixture of DEA and sodium nitrite. Table 1 shows the number of animals surviving in each group at 6-monthly intervals. Although the mixtures of DEA and nitrite given to the high- and low-mix groups had concentrations in the ratio of 2:1, the daily consumptions per guinea-pig in the two groups (Table 2) were not quite in the same ratio. Presumably because of the unpleasant taste and smell of DEA, the guinea-pigs in the high-mix group drank less than those in the low-mix group. During the test period, the animals in both groups consumed, on average, 210–250 mg DEA and 40–50 mg sodium nitrite daily (Table 2).

DISCUSSION

The data obtained in this study support the earlier negative results of Druckrey *et al.* (1963) and Sander *et al.* (1968) who fed DEA and nitrite to rats.

Although many of the guinea-pigs in this study died early, a total of 34 in the high- and low-mix groups consumed the mixed chemicals for more than 1 yr and 12 survived for more than 2 yr. Although the two chemicals were fed together and the pH of the guinea-pig stomach is acidic enough for the nitrosation reaction, the amount of DEN formed *in vivo* was apparently insufficient to induce tumours during the experimental period.

The rate of nitrosation of various secondary amines depends on the basicity of the amines; the weaker the base, the faster is the rate of the nitrosation reaction (Greenblatt *et al.* 1971; Sander *et al.* 1968). The negative results of this study can probably be attributed to the fact that diethylamine is a strong base (pK_a 11·0) and, therefore, its rate of nitrosation even under ideal conditions is very slow. The low yields

Table 2. *Calculated daily and cumulative intakes of DEA, sodium nitrite and DEN by guinea-pigs given one or more of the compounds in the drinking-water*

Test compound	Intake parameter	Intake* by guinea-pigs in group designated: Positive (DEN) control	DEA control	Nitrite control	High-mix	Low-mix
DEA	Average/day	—	290	—	250	210
	Range/day	—	150–420	—	170–350	160–270
	Max cumulative†	—	391	—	194‡	240 (112‡)
Sodium nitrite	Average/day	—	—	100	50	40
	Range/day	—	—	80–120	40–70	30–50
	Max cumulative†	—	—	108	38	48 (22‡)
DEN	Average/day	1·1	—	—	—	—
	Range/day	0·7–1·3	—	—	—	—
	Max cumulative†	0·695	—	—	—	—

DEA = Diethylamine hydrochloride DEN = Diethylnitrosamine

*Values for the daily average and daily range of intakes are expressed in mg/guinea-pig, and the maximum cumulative intakes in g/guinea-pig.

†These values are not true maxima, since the animals were not caged separately.

‡Amounts consumed up to month 18; other figures refer to amounts consumed up to the termination of the experiment.

of DEN obtained in our earlier studies (Sen *et al.* 1969) also support this conclusion.

It can be concluded from the above-mentioned results and others reported in the literature that the ingestion of low levels of strongly basic secondary amines, such as diethylamine or dimethylamine, and of nitrite is unlikely to lead to the formation of significant levels of nitrosamines in the stomach. However, in view of the strongly carcinogenic nature of the nitrosamines, it would be desirable to keep the levels of the precursors of these carcinogens in foods to a minimum, thereby reducing the hazard that could arise from the *in vivo* formation of nitrosamines from the ingested nitrite and secondary amines.

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REFERENCES

Alam, B. S., Saporoschetz, I. B. & Epstein, S. S. (1971). Formation of N-nitrosopiperidine from piperidine and sodium nitrite in the stomach and the isolated intestinal loop of the rat. *Nature, Lond.* **232,** 116.

Arcos, J. C., Argus, Mary F. & Mathison, Joyce B. (1969). Hepatic carcinogenesis threshold and biphasic mitochondrial swelling response in the guinea-pig during diethylnitrosamine administration. *Experientia* **25,** 296.

Druckrey, H., Preussmann, R., Ivankovic, S. u. Schmähl, D. (1967). Organotrope carcinogene Wirkungen bei 65 verschiedenen N-Nitroso-Verbindungen an BD-Ratten. *Z. Krebsforsch.* **69,** 103.

Druckrey, H., Steinhoff, D., Beuthner, H., Schneider, H. & Klärner, P. (1963). Prüfung von Nitrit auf chronisch toxische Wirkung an Ratten. *Arzneimittel-Forsch.* **13,** 320.

Greenblatt, M., Mirvish, S. & So, B. T. (1971). Nitrosamine studies: Induction of lung adenomas by concurrent administration of sodium nitrate and secondary amines in Swiss mice. *J. natn. Cancer Inst.* **46,** 1029.

Magee, P. N. & Barnes, J. M. (1967). Carcinogenic nitroso compounds. *Adv. Cancer Res.* **10,** 163.

Mirvish, S. S. (1970). Kinetics of dimethylamine nitrosation in relation to nitrosamine carcinogenesis. *J. natn. Cancer Inst.* **44,** 633.

Newberne, P. M. & Shank, R. C. (1973). Induction of liver and lung tumours in rats by the simultaneous administration of sodium nitrite and morpholine. *Fd Cosmet. Toxicol.* **11,** 819.

Sander, J. (1967). Kann Nitrit in der menschlichen Nahrung Ursache einer Krebsentstehung durch Nitrosaminbildung sein? *Arch. Hyg. Bakt.* **151,** 22.

Sander, J., Schweinsberg, F. u. Menz, H.-P. (1968). Untersuchungen über die Entstehung cancerogener Nitrosamine im Magen. *Hoppe-Seyler's Z. physiol. Chem.* **349,** 1691.

Sen, N. P., Smith Dorothy C. & Schwinghamer, L. (1969). Formation of *N*-nitrosamines from secondary amines and nitrite in human and animal gastric juice. *Fd. Cosmet. Toxicol.* **7,** 301.

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ZUR FRAGE EINER KOMBINIERTEN WIRKUNG EINES CHEMISCHEN MUTAGENS UND STRAHLEN-STERILISIERTER NAHRUNG IM MUTAGENITÄTS- UND REPRODUKTIONSTEST BEI DER MAUS

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Zusammenfassung—Unter Verwendung von 2000 Mäusen (NMRI/Han) wurde geprüft, ob die mutagene Wirkung von Cyclophosphamid (Endoxan) durch Verabreichung strahlensterilisierten Futters (Dosis, 4,5 Mrad) verstärkt wird. In einem Dominant-Letal-Test waren die männlichen Tiere zu Versuchsbeginn mit 100 mg Endoxan/kg vorbehandelt. Die späten Spermatiden reagierten am empfindlichsten auf die Wirkung des Endoxans. Signifikante Unterschiede zwischen der Kontrollgruppe (Endoxan und unbestrahltes Futter) und der Versuchsgruppe (Endoxan und strahlensterilisiertes Futter) waren nicht zu ermitteln. Im Dominant-Letal-Test hat somit strahlensterilisierte Nahrung keine co-mutagene Wirkung zu Endoxan. In einem 7-monatigen Reproduktionstest, als Dauerpaarungsversuch ohne Laktationsphasen durchgeführt, wurden die weiblichen Tiere periodisch alle 2 Wochen mit 20 mg Endoxan/kg behandelt. Die Verringerung der Wurfgrössen mit zunehmendem Alter und Wurfzahl der Muttertiere war unter der Wirkung des Chemomutagens deutlich stärker als ohne dieses. Die Wirkung des Endoxans kam auch in einer erhöhten Zahl von Aborten und frühzeitiger Sterilität zum Ausdruck. Der Einfluss des Verzehrs von strahlensterilisierter Nahrung war dagegen in den untersuchten Parametern zu keinem Zeitpunkt des Versuches erkennbar.

Abstract—The possible intensification of the mutagenic effect of cyclophosphamide (Endoxan) by the feeding of a radiation-sterilized diet (dose, 4·5 Mrad) was studied in 2000 NMRI/Han mice. In a dominant lethal test, males were pretreated with 100 mg Endoxan/kg body weight. The greatest sensitivity towards Endoxan was observed during the late-spermatid stage. No significant differences were detected between the control group (Endoxan plus non-irradiated diet) and the experimental group (Endoxan plus radiation-sterilized diet). In this test, radiation-sterilized feed showed no co-mutagenic effect when combined with Endoxan treatment. In a reproduction study of 7 months duration (continuous mating without lactation periods), the females were treated every 2 wk with 20 mg Endoxan/kg body weight. The decline in litter size with increasing number of litters (i.e. with advancing age of the females) was more pronounced after treatment with the chemical mutagen than in the untreated group. Increases in the frequency of abortions and in premature sterility resulted from Endoxan treatment. During the entire observation period, no effects from the intake of radiation-sterilized food were detected.

EINFÜHRUNG

In einer früheren Untersuchung (Renner, Grünewald u. Ehrenberg-Kieckebusch, 1973) konnte keine mutagene Wirkung eines strahlensterilisierten Futters auf Mäuse und Ratten beobachtet werden. Seither sind uns ähnliche Untersuchungen von Bronnikova u. Okuneva (1972 u. 1973) bekannt geworden, die ebenfalls zu negativen Ergebnissen führten. Die im folgenden beschriebene Arbeit galt der Frage, ob durch den Verzehr strahlensterilisierten Futters die Wirkung eines Chemomutagens verstärkt wird.

Als Chemomutagen wurde ein bekannter Mitosehemmer, das Cyclophosphamid (Endoxan®), gewählt, dessen mutagene Eigenschaften von mehreren Autoren beschrieben wurden (Angel, 1964; Arrighi, Hsu u. Bergsagel, 1962; Brittinger, 1966; Röhrborn, 1970a). Folgende zwei voneinander unabhängige Versuche wurden durchgeführt:

In Versuch 1 wurde mittels des Dominant-Letal-Testes geprüft, ob in den verschiedenen Stadien des Spermiogenesezyklus die durch Endoxanbehandlung der männlichen Tiere induzierten dominanten Letalmutationen durch Fütterung mit strahlensterilisierter Nahrung erhöht werden (Endoxanbehandlung der männlichen Tiere).

In Versuch 2 wurde in einem Dauerpaarungsversuch geprüft, ob die Reproduktionskapazität der weiblichen Versuchstiere sich verringert, wenn diese unter periodischer Endoxanbehandlung zusätzlich mit strahlensterilisiertem Futter ernährt werden (Endoxanbehandlung der weiblichen Tiere).

EXPERIMENTELLER TEIL

Tiermaterial und Haltung. In beiden Versuchen wurde der Mäuse-Auszuchtstamm NMRI/Han, SPF

®Oder Cytoxan; Warenzeichen der Asta Werke, Brackwede.

(Zentralinstitut für Versuchstierzucht, Hannover) verwendet. Beide Versuche wurden mit 10 Wochen alten Mäusen gestartet. Alle Tiere wurden einzeln in Makrolonkäfigen Typ 2 gehalten. Die Versuchstierräume waren vollklimatisiert: Lufttemperatur 21 ± 1°C, relative Luftfeuchte 60% ± 5, Luftwechsel/h 15-fach mit Luftfilterung und 12-stündigen Hell-Dunkel-Rhythmus.

Diät. Den Tieren wurde Altromin H in pelletierter Form *ad lib.* gefüttert. Für die Tiergruppen, die strahlensterilisierte Nahrung erhielten, wurde Altromin in jeweils wöchentlichen Abständen mittels des institutseigenen Elektronen-Linearbeschleunigers mit 10 MeV Elektronen und einer Dosis von 4,5 ± 0,8 Mrad in offenen Paletten bestrahlt. Allen Tiergruppen wurde wöchentlich eine Vitaminsupplementierung über das Trinkwasser verabreicht (Multibionta, E. Merck AG, Darmstadt).

Versuch 1

Dieser Versuch wurde aus personellen und räumlichen Gründen in zwei hintereinander ablaufenden Serien (a und b) durchgeführt. Nur so konnte der Versuch mit der notwendigerweise hohen Zahl von 400 männlichen und 1200 weiblichen Mäusen durchgeführt werden. Bei Versuchsbeginn wurden allen männlichen Tieren 100 mg Endoxan/kg Körpergewicht subkutan injiziert. Einen Tag danach wurde die erste Gruppe gepaart und in wöchentlichen Abständen jeweils eine weitere Gruppe (Paarungsverhältnis 1:3). Da die Endoxanwirkung in der Woche 7 weitgehend abgeklungen war, wurde auf eine zunächst mitgeführte Gruppe für die Woche 8 verzichtet, was auch beim Vergleich mit einer unbehandelten 'Standardgruppe' ($n = 118$) aus vorangegangenen Untersuchungen (Renner *et al.* 1973) gerechtfertigt schien. Die männlichen und weiblichen Mäusen erhielten entweder unbestrahlte Nahrung (Kontrollgruppe, K) oder strahlensterilisierte Nahrung (Versuchsgruppe, V).

Die Männchen wurden jeweils bis zu 7 Tage in den Paarungskäfigen belassen. Weibchen mit Vaginalpfropf wurden von den Männchen getrennt. In den Fällen, in denen in einem Käfig nach Ablauf des Tages 3 noch kein Weibchen mit Pfropf festgestellt werden konnte, wurde das Männchen gegen ein anderes ausgetauscht, dessen Befruchtungsfähigkeit sich bereits erwiesen hatte. Dadurch war es möglich, männliche Sterilität aus dem Versuch auszuschalten.

Die trächtigen Weibchen wurden zwischen dem 15. und 17. Tage der Gravidität laparatomiert, wobei nach Herauspräparieren von Uteri und Ovarien die Daten für den Dominant-Letal-Test gewonnen wurden. Zur vollständigen Erfassung der frühen Postimplantationsverluste (Deciduomata) wurden die Uteri nach einer auf Wislocki u. Dempsey (1945) zurückgehenden Methode angefärbt.

Versuch 2

Im Versuch 2 wurden den weiblichen Tieren periodisch in 14-tägigen Abständen 20 mg Endoxan/kg Körpergewicht (in 0.2%iger Lösung) subkutan injiziert. Ab dem Tage nach der ersten Injektion wurden die Weibchen mit unbehandelten Männchen in permanent monogamer Paarung gehalten. Folgende Gruppen (jede 50 Paare) wurden gebildet:

Standardgruppe A: physiologische NaCl-Lösung (Weibchen) und unbestrahlte Nahrung (alle Tiere).

Standardgruppe B: physiologische NaCl-Lösung und strahlensterilisierte Nahrung.

Kontrollgruppe C: periodische Endoxanbehandlung und unbestrahlte Nahrung.

Versuchsgruppe D: periodische Endoxanbehandlung und strahlensterilisierte Nahrung.

Durch die bei diesem Versuch mitgeführten Gruppen A und B sollte geprüft werden, ob sich eine Auswirkung der strahlensterilisierten Diät (ohne Endoxan) auf die permanente Reproduktionsbelastung feststellen lässt.

Parallel zu den Endoxanbehandlungen der C- und D-Weibchen wurde den A- und B-Weibchen physiologische Kochsalzlösung injiziert. Bei Versuchsbeginn wurde die Zahl der Paare je Gruppe auf 55 erhöht, um nach Erscheinen der ersten beiden Würfe bis dahin nicht trächtig gewordene Weibchen—gegebenenfalls wahllos andere Weibchen—zu eliminieren und damit alle vier Gruppen auf 50 fertile Paare zu bringen.

Unmittelbar nach einer erfolgten Geburt wurden die Jungen entfernt, noch bevor bei den Muttertieren eine Laktation in Gang gekommen war. Dadurch konnte noch am gleichen oder folgenden Tag eine erneute Konzeption eintreten. Registriert wurden bei jedem Weibchen die Geburtstermine und die Zahl der Jungen. Der Versuch lief über 212 Tage. Nach 191 Tagen wurden alle Männchen entfernt.

ERGEBNISSE UND DISKUSSION

Versuch 1

In Tabelle 1 sind die Ergebnisse von Versuch 1 getrennt für Serie a und Serie b wiedergegeben. Die Endoxanwirkung ist in den ersten beiden Wochen nach der Behandlung der Männchen deutlich an der Höhe der toten Implantate bzw. der lebenden Feten erkennbar (Tabelle 1). Die Zahl der präimplantativen Eiverluste reflektiert die Wirkung des Chemomutagens nicht eindeutig. Bei dem Vergleich der Daten für die Kontrollgruppe mit der oben erwähnten 'Standardgruppe' ergibt sich für die Variable 'lebende Feten' ein statistisch hochsignifikanter Unterschied, d.h. das Endoxan hat die erwartete und beabsichtigte Wirkung ausgeübt. In Übereinstimmung mit den Untersuchungen von Röhrborn (1970a) und Brittinger (1966) wurde die stärkste induzierte Wirkung gefunden, wenn die männlichen Keimzellen im Stadium der späten Spermatiden von der Endoxanbehandlung getroffen waren. In Serie a wurde zunächst noch eine Kontrollgruppe für die Woche 8 mitgeführt, die aber keine erkennbaren Nachwirkungen der Endoxanbehandlung mehr zeigt. Es ist auffallend, dass die Mittelwerte/Weibchen dieser Gruppe ausser bei Präimplantationsverlusten in allen Parametern besser liegen als die der 'Standardgruppe 1973' aus dem früheren Versuch.

Kernfrage dieses Versuches ist, ob signifikante Unterschiede zwischen den Versuchs- und den Kontrollgruppen auftreten. Ein Verdacht ergibt sich aus der Feststellung, dass die Zahl der toten Implantate bei Serie a in der Woche 2 und bei Serie b in der Woche 3–6 in der Versuchsgruppe höher liegt als in der Kontrollgruppe.

Bei der Verwendung einfacher Formeln, die häufig bei der Interpretation von Resultaten aus dominanten

Tabelle 1. *Dominant-Letal-Test über die Dauer der Spermiogenese nach Vorbehandlung männlicher Mäuse mit Endoxan (Versuch 1)*

	Zahl/befruchtete Weibchen											
	n		Gelbkörper		Implantate		Präimplantations-verluste		Tote Implantate		Lebende Feten	
Woche*	K	V	K	V	K	V	K	V	K	V	K	V
						Serie a						
1	94	93	15.1	15.1	11.3	11.1	3.8	4.0	3.2	2.8	8.1	8.3
2	28	28	15.4	14.7	11.0	9.6	4.4	5.1	2.9	3.3	8.1	6.3
3	28	25	17.0	16.1	13.0	12.2	4.0	3.9	2.7	2.7	10.3	9.5
4	25	28	17.9	16.3	12.6	12.1	5.3	4.2	2.4	1.8	10.2	10.3
5	27	23	16.2	17.6	11.8	11.6	4.4	6.0	2.1	1.7	9.7	9.9
6	28	27	15.8	16.5	11.4	10.9	4.4	5.6	1.5	1.6	9.9	9.3
7	28	29	16.8	15.5	12.9	12.0	3.9	3.5	1.9	1.3	11.0	10.7
8	18		17.6		13.0		4.6		1.4		11.6	
(SG)			(16.1)		(12.2)		(3.9)		(1.5)		(10.7)	
						Serie b						
1	93	93	15.2	15.3	11.2	11.0	4.0	4.3	3.1	2.8	8.1	8.2
2	32	33	15.2	14.7	11.2	9.5	4.0	5.2	3.3	3.0	7.8	6.5
3	34	31	15.4	14.6	11.6	11.6	3.8	3.0	1.7	2.1	9.9	9.5
4	32	33	15.4	15.9	12.4	12.3	3.0	3.6	1.8	2.2	10.6	10.1
5	30	30	15.7	15.5	11.9	11.8	3.8	3.7	1.5	1.8	10.4	10.0
6	29	30	15.7	15.8	12.0	11.9	3.7	3.9	1.7	2.1	10.3	9.8
7	30	32	16.2	15.4	12.3	11.4	3.9	4.0	1.8	1.5	10.5	9.8

n = Zahl der befruchteten Weibchen K = Kontrolle (100 mg Endoxan/kg + unbestrahlte Nahrung)
V = Versuch (100 mg Endoxan/kg + strahlensterilisierte Nahrung) SG = 'Standardgruppe 1973'
*Verpaarungszeit nach der Behandlung: Die entsprechenden behandelten Stadien waren: Woche 1, reife Spermien; 2, späte Spermatiden; 3, mittlere und frühe Spermatiden; 4 und 5, Spermatozyten; 6–8, Spermatogonien.

Letalstudien angewandt werden (Röhrborn, 1970b), gelangt man im Bereich des suspekten Spermiogeneseabschnittes zu dem in Tabelle 2 gezeigten Bild. Formeln, die mit der Zahl der corpora lutea arbeiten, wurden als ungeeignet erachtet, weil sie einen weiteren Unsicherheitsfaktor beinhalten. Bei den prozentualen Postimplantationsverlusten liegen die Werte der Versuchsgruppe teilweise über, teilweise unter den Werten der Kontrollgruppe. Der deutliche Unterschied in der Woche 2, Serie a, tritt in der Wiederholungsserie (2b) nicht auf. Ein ähnliches Bild zeigt auch die Berechnung der 'induzierten Letalität': Der in der Woche 2a erhöht erscheinende Wert für diese Berechnung tritt bei der Wiederholung (2b) nicht auf.

Um zu klären, ob signifikante Unterschiede zwischen Versuchs- und Kontrollgruppen bestehen,

Tabelle 2. *Rechnerische Prüfung (Versuch 1)*

Woche	Postimplantations-verluste (%)* Kontrolle	Versuch	Induzierte Letalität nach Implantation†
1a	28.0	25.0	−4.1
1b	27.6	25.4	−3.0
2a	26.2	34.2	10.8
2b	29.9	31.4	2.2
3a	21.2	21.7	0.7
3b	14.6	18.0	3.9
4a	18.8	14.7	−5.9
4b	14.4	17.7	3.8

*(Tote Implantate/Implantate) × 100. Standardgruppe 13,3.

†$\left(1 - \frac{\text{lebende Feten/Implantate: Versuch}}{\text{lebende Feten/Implantate: Kontrolle}}\right) \times 100.$

müssen statistische Prüfverfahren herangezogen werden. Hierfür kommen Tests für zwei unabhängige Stichproben in Frage, insbesondere der χ^2-Test und der *U*-Test (Rangzahlentest nach Wilcoxon, Mann und Whitney). Bei den statistischen Tests wurde nicht mit den Mittelwerten der Tabelle 1, sondern mit den absoluten Zahlen gerechnet.

Der χ^2-Test bei Implantaten (tote Implantate + lebende Feten) deckt einen deutlichen Unterschied nur in der Woche 2, Serie a, auf. Die im χ^2-Test Woche 2a auftretende Signifikanz ($P < 0{,}05$) hat sich in der Wiederholungsserie (2b) nicht bestätigt. Wichtig ist, dass der wesentlich schärfer prüfende *U*-Test bei 'toten Implantaten' und bei 'lebenden Feten' weder in der fraglichen Woche 2 noch in einem anderen Abschnitt der Spermiogenese einen Unterschied aufdeckt. Insgesamt betrachtet lassen sich folglich eindeutige Unterschiede zwischen Kontroll- und Versuchsgruppe nicht finden. Daher wird das Versuchsergebnis dahingehend interpretiert, dass eine comutagene Wirkung von strahlensterilisierter Nahrung zu Endoxan im Dominant-Letal-Test unter den beschriebenen Versuchsbedingungen nicht nachweisbar ist.

Tabelle 3. *Dauerpaarungsversuch unter periodischer Behandlung weiblicher Mäuse mit Endoxan (Versuch 2)*

Gruppe	Reproduktionsleistung*	Wurfzahl*†	Wurfgrösse*†
A	83.5	8.1	10.3
B	82.3	8.2	10.0
C	52.5	7.5	7.0
D	54.9	7.4	7.4

*Geborene Junge/Weibchen.
†Ohne Aborte.

Tabelle 4. *Dauerpaarungsversuch: Wurfgrösse vom 1.–10. Wurf (mit Aborten)*

Nummer des Wurfs	Wurfgrösse/Weibchen bei Gruppe			
	A	B	C	D
1	11,6 ± 2,7 (50)	11,8 ± 2,9 (50)	5.1 ± 5,3 (50)	4,8 ± 5,3 (50)
2	11,0 ± 4.1 (49)	11,1 ± 4.1 (50)	8,7 ± 4,3 (50)	8.7 ± 4.0 (50)
3	12,4 ± 4.0 (49)	11,2 ± 4,6 (50)	9,4 ± 4,3 (50)	8,7 ± 4,9 (50)
4	11,8 ± 4.6 (47)	12,2 ± 3,6 (50)	7,2 ± 5,2 (50)	8,1 ± 4.4 (50)
5	11,0 ± 4.0 (45)	10,1 ± 4,8 (49)	6,4 ± 4.7 (50)	8,6 ± 4.8 (47)
6	9.8 ± 4.4 (45)	9,7 ± 3,9 (47)	6,2 ± 4.2 (47)	6,2 ± 4.7 (46)
7	8.7 ± 4,5 (42)	8,7 ± 3,8 (44)	6,4 ± 4.7 (38)	8.1 ± 4.0 (36)
8	9,1 ± 3.7 (39)	6,9 ± 4.4 (38)	6,2 ± 4,2 (28)	5,6 ± 4.4 (26)
9	7,5 ± 4.4 (28)	6,6 ± 4,1 (24)	7,2 ± 4.7 (9)	7,2 ± 5,2 (13)
10	5,1 ± 2,8 (9)	5,6 ± 3,1 (8)	5,5 ± 3,5 (2)	7,0 (1)

In Parenthesen sind die Zahlen der Weibchen, die den jeweiligen Wurf gebracht haben.

Versuch 2

Für den Versuch 2 ist das Ergebnis in Tabelle 3 an Hand der drei Parameter, Reproduktionsleistung, Wurfzahl und Wurfgrösse zusammengefasst. Die Wirkung des Endoxans (Vergleich der Gruppen A und B mit den Gruppen C und D) ist deutlich zu erkennen. Dagegen zeigt sich keine offensichtliche Wirkung der Bestrahlung (Vergleich A mit B und C mit D). Während der 7-monatigen Versuchsdauer sind aus der Gruppe A fünf, der Gruppe B eine, der Gruppe C eine und der Gruppe D drei weibliche Tiere durch Tod ausgeschieden. Die mit zunehmender Wurfzahl abnehmende Wurfgrösse wird aus Tabelle 4 deutlich. Die Reproduktionskapazität war mit dem 10. Wurf in den Gruppen C und D praktisch erschöpft, in den Gruppen A und B war sie zu diesem Zeitpunkt noch bei einem kleinen Teil der eingesetzten Weibchen mit stark reduzierten Wurfgrössen erhalten.

Die Dauerbelastung der weiblichen Versuchstiere durch Reproduktion und Endoxan kam ausser in den sich verringernden Wurfgrössen noch in häufigen Aborten oder in frühzeitiger Sterilität zum Ausdruck (12 und 13 Aborte bei 391 und 397 Würfen in den Gruppen A und B, aber 58 und 54 Aborte bei 316 und 315 Würfen in den Gruppen C und D). Die Verabreichung der bestrahlten Diät hatte dagegen keinen Einfluss auf die Häufigkeit von Aborten und Würfen.

Tabelle 5. *Dauerpaarungsversuch: Anzahl der Jungen/Gruppe je 20-Tage-Intervall*

Wurfintervall	Gruppe			
	A	B	C	D
6.–25.3.	42	61	49	48
–14.4.	573	539	210	175
– 5.5.	526	619	430	413
–25.5.	614	536	336	392
–14.6.	511	549	322	327
– 4.7.	438	437	292	373
–24.7.	400	409	218	209
–13.8.	381	345	239	288
– 2.9.	300	261	184	154
–22.9.	257	244	176	202
–12.10.	133	116	171	162
Summe...	4175	4116	2627	2743
davon:				
tot geboren...	84	61	665	699
lebend geboren...	4091	4055	1962	2044

Die erwartete induktive Wirkung des Endoxans hat zu signifikanten Unterschieden zwischen den Gruppen A und C bzw. B und D geführt: $P < 0{,}001$ (χ^2-Test). Ein analoger Vergleich zwischen den Gruppen A und B lässt dagegen keinen Unterschied aufgrund der Strahlensterilisierung des Futters erkennen, ebensowenig unterscheiden sich die Gruppen C und D.

Aus dem gleichmässigen Abfall der Wurfgrössen in den Endoxan-behandelten Gruppen C und D (Tabelle 4) kann ebenfalls gefolgert werden, dass die zusätzliche Futterbehandlung (Bestrahlung) bei der Gruppe D zu keinem sichtbaren Unterschied gegenüber C geführt hat. Diese Aussage ist aber zu pauschal, da sie nur auf den Mittelwerten (mit hoher Standardabweichung) beruht. Daher wurde der gesamte Versuchszeitraum in gleiche Trächtigkeitsintervalle (Tabelle 5) zu je 20 Tagen eingeteilt und die Zahl der in den einzelnen Zeiträumen geborenen Jungen registriert. Auch diese Art der Darstellung ergibt nur zufällige Unterschiede beim Vergleich A mit B und C mit D. Es kann gefolgert werden, dass die Reproduktionsleistung von unter periodischer Endoxan-Behandlung stehenden weiblichen Mäusen durch Verfütterung von strahlensterilisierter Diät nicht beeinflusst wird.

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LITERATUR

Angel, H. (1964). Über die Wirkung des Triäthyleniminobenzochinon-1,4 (Trenimon) und des N,N-Bis-(β-chloräthyl)-N,O-propylenphosphorsäureesterdiamid (Endoxan) auf den Schwangerschaftsverlauf, die Embryonalentwicklung, die Fertilität und die Mutagenität an dem Rattenstamm BDI. Inauguraldissertation. Universität des Saarlandes, Homburg/Saar.

Arrighi, F. E., Hsu, T. C. & Bergsagel, D. E. (1962). Chromosome damage in murine and human cells following cytoxan therapy. *Tex. Rep. Biol. Med.* **20**, 545.

Brittinger, D. (1966). Die mutagene Wirkung von Endoxan bei der Maus. *Humangenetik* **3**, 156.

Bronnikova, I. A. & Okuneva, L. A. (1972). Hygienic evaluation of food rations with predominance of vegetable

products subjected to gamma irradiation. *Vop. Pitan.* **31**(4), 74.

Bronnikova, I. A. & Okuneva, L. A. (1973). On the study of mutagenic and cytotoxic properties of irradiated food products. *Vop. Pitan.* **32**(4), 46.

Renner, H. W., Grünewald, Th. u. Ehrenberg-Kieckebusch, W. (1973). Mutagenitätsprüfung bestrahlter Lebensmittel mit dem "dominant lethal test". *Humangenetik* **18**, 155.

Röhrborn, G. (1970a). The activity of alkylating agents. I. Sensitive mutable stages in spermatogenesis and oogenesis. In *Chemical Mutagenesis in Mammals and Man.* Edited by F. Vogel and G. Röhrborn. p. 294. Springer Verlag, Berlin.

Röhrborn, G. (1970b). The dominant lethals: Method and cytogenetic examination of early cleavage stages. In *Chemical Mutagenesis in Mammals and Man.* Edited by F. Vogel and G. Röhrborn. p. 148. Springer Verlag, Berlin.

Wislocki, G. B. & Dempsey, E. W. (1945). Histochemical reactions of the endometrium in pregnancy. *Am. J. Anat.* **77**, 365.

Fd Cosmet. Toxicol. Vol. 13, pp. 433–436. Pergamon Press 1975. Printed in Great Britain

DOMINANT LETHAL MUTATIONS IN MALE MICE FED γ-IRRADIATED DIET

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Abstract—Three groups of Swiss male mice were fed a stock ration or an unirradiated or irradiated (2·5 Mrad) test diet for 8 wk. After the feeding period, the males were mated with groups of untreated female mice for 4 consecutive weeks. The females were autopsied at mid-term pregnancy for evaluation of dominant lethal mutations. Numbers of dead implantations, including deciduomas and dead embryos, showed no significant differences among the different groups, thus producing no evidence of any induced post-implantation lethality in mice fed on irradiated diet. Similarly, there was no indication of pre-implantation lethality, since implantation rates remained comparable among different groups. Consumption of irradiated diet did not affect the fertility of mice. Total pre- and post-implantation loss, as indicated by the numbers of live implantations remained comparable among all the groups of mice.

INTRODUCTION

The possible existence of a genetic risk in the consumption of irradiated foods has been pointed out in recent years on the basis of the reported induction of cytogenetic and mutagenic effects in cultures of bacteria (Dupuy & Usciati, 1966; Molin & Ehrenberg, 1964), plant cells (Holsten, Sugii & Steward, 1965; Swaminathan, Chopra & Bhaskaran, 1962; Swaminathan, Nirula, Natarajan & Sharma, 1963) and mammalian cells (Berry, Hills & Trillwood, 1965; Hills & Berry, 1967), as well as in higher organisms such as Drosophila (Chopra, 1965; Rinehart & Ratty, 1965; Schubert, 1969) following the use of nutrient media containing irradiated components. However, it is a matter of practical experience that a mutagenic risk to man cannot be assumed from findings in simple test organisms, but that such a conclusion should be based only on studies with mammals. A direct measure of mutation frequency in mammals can be made by determining the occurrence of dominant lethals at mid-term pregnancy (Bateman, 1958).

Moutschen-Dahmen, Moutschen & Ehrenberg (1970) have suggested that the induction of dominant lethal mutations is responsible for the increased pre-implantation embryonal death of mouse ova caused by the feeding of irradiated food. Likewise, extracts from irradiated potatoes have also been claimed to induce dominant lethal mutations in male mice (Kopylov, Osipova & Kuzin, 1972). On the other hand, several investigations have failed to reveal any evidence of mutagenic hazard associated with the consumption of an irradiated diet, as measured by the dominant lethal test. Thus, Levinsky & Wilson (1975) could not verify the earlier findings of Kopylov *et al.* (1972) on the mutagenicity of irradiated potato extracts in mice, and irradiated diet was not found to result in any increase in dominant lethal mutations in either rats (Eriksen & Emborg, 1972; Renner, Grünewald & Ehrenberg-Kieckebusch, 1973) or mice Renner *et al.* 1973).

The findings of an investigation designed to evaluate the possible induction of dominant lethals in Swiss male mice fed an irradiated whole diet are reported in this paper.

EXPERIMENTAL

Animals. Male and female mice of the inbred Swiss strain bred in the animal house of this Research Centre were used. From the age of 4–5 wk, groups of males were fed on one of three diets for 8 wk, after which they were all given the stock ration and kept for mating with virgin untreated females (9–11 wk old).

Diets. The three diets fed were the animal-house stock ration (negative control group), an unirradiated test diet (control group) and the same test diet irradiated at 2·5 Mrad. The stock ration (protein content 16%) consisted of wheat (70%), Bengal gram (*Cicer arietinum*; 20%), fish meal (5%), yeast powder (4%), shark-liver oil (0·75%) and sesame oil (0·25%). The test diet was composed of wheat flour (53·5%), Bengal gram (16%), skimmed-milk powder (10%), shrimps (4%), vegetables (2·5%), sesame oil (6%), sucrose (6%) and sodium chloride (2%) and had a protein content of 18%. This diet was cooked at 1·1 kg/cm^2 pressure for 30 min, dried in an air dryer at 60–65°C to about 10% moisture and divided into two batches, one serving as the control and the other being exposed to γ-radiation in a food-package irradiator (with cobalt-60 as the γ-ray source) at a dose rate of 4–4·5 krad/min.

Experimental procedure. In general, the procedure recommended by Epstein & Röhrborn (1971) was followed for the assessment of dominant lethals. Each male was caged with two or three virgin females, which were replaced at weekly intervals. The sequential mating with groups of females was continued for 4 wk. The female mice were autopsied 10–11 days after their separation from the males and their uterine contents were examined for live and dead implantations, the latter comprising dead embryos and deciduomas. The main criteria used for the assessment of mutagenicity were the numbers of dead implantations expressed per pregnant female and as a percentage of all implantations. Pre-implantation lethality was determined by comparing the total implantation rates in different groups and an estimate of total lethality (pre- and post-implantation deaths) was made on the basis of live implantations per

Table 1. *Reproduction indices for matings following feeding of males for 8 wk on irradiated and non-irradiated diets*

		Values for female rats mated with males† fed on														
							Test diet									
		Stock ration					Non-irradiated (control)					Irradiated				
Parameter	Mating wk...	1	2	3	4	1–4‡	1	2	3	4	1–4‡	1	2	3	4	1–4‡
Total females mated		85	95	69	103	352	107	97	46	104	354	94	120	51	114	379
No. of pregnant females		52	64**	50	65	231	75	59	35	80	249	71	68**	37	93*	269
Pregnancy rate (%)		61·3	67·3	72·5	63·1	66·1 (2·51)	70·1	60·8	76·1	76·9	71·0 (3·72)	75·5	56·7	72·6	81·6	71·6 (5·31)
Total implantations		484	557	446	623	2110	660	564	319	749	2292	674	618	329	835	2456
No. of implantations/pregnancy		9·30 (0·23)	8·70 (0·27)	8·92 (0·28)	9·59 (0·21)	9·13 (0·20)	8·80 (0·30)	9·56* (0·20)	9·11 (0·34)	9·36 (0·22)	9·21 (0·16)	9·49 (0·24)	9·09 (0·19)	8·89 (0·37)	8·98 (0·18)	9·11 (0·13)
Dead implantations§:																
per pregnancy		0·54 (0·19)	0·47 (0·12)	0·56 (0·12)	0·55 (0·13)	0·53 (0·02)	0·49 (0·11)	0·48 (0·09)	0·60 (0·20)	0·53 (0·12)	0·53 (0·03)	0·56 (0·12)	0·46 (0·10)	0·43 (0·11)	0·69 (0·10)	0·54 (0·06)
% of total implantations		5·79	5·39	6·28	5·78	5·81	5·61	4·97	6·58	5·61	5·69	5·94	5·02	4·86	7·67	5·87
Live implantations/pregnancy		8·77 (0·32)	8·23 (0·29)	8·36 (0·30)	9·03 (0·25)	8·60 (0·18)	8·31 (0·31)	9·09 (0·26)	8·51 (0·40)	8·84 (0·24)	8·69 (0·17)	8·93 (0·28)	8·63 (0·21)	8·46 (0·42)	8·29 (0·20)	8·58 (0·14)
Females with dead implantations:																
total no.		14	22	19	25	80	21	22	13	27**	83	24	23	14	39**	100
% of pregnancies		26·9	34·4	38·0	38·5	34·5 (2·68)	28·0	37·3	37·1	33·8	34·1 (2·17)	33·8	33·8	37·8	41·9	36·8 (1·94)

†No. of males in different groups: stock ration, 35; non-irradiated test diet, 44; irradiated test diet, 47.
‡Combined results for all matings in wk 1–4.
§Deciduomas plus dead embryos.
Values in parenthesis indicate ± SEM. Values marked with a single asterisk differ significantly ($P < 0.05$) from the corresponding figure for the stock ration, while pairs of asterisks indicate the groups showing maximum deviation: **$P > 0.05$ by χ^2 test.

pregnant mouse (Edwards & Searle, 1963; Röhrborn, 1970). Pregnancy rates were calculated and the incidence of females with dead implantations was also recorded.

Statistical methods. The statistical evaluation of the data was based on the relevant control values for each mating period. Data were analysed by a computerized programme using the analysis of variance, t test and the χ^2 test (Alder & Roesoler, 1968), taking $P = 0.05$ as the limit of significance. Furthermore, the overall populations of mice belonging to different groups were subjected to the Kolmogorov–Smirnov test for homogeneity testing (Massey, 1951).

RESULTS

Effect on pregnancy

There was no variation with respect to different weekly matings in the pregnancy rates of females mated with males fed on stock ration (Table 1). A somewhat larger variability, as indicated by the relatively higher value of standard error, was observed in the group fed on the irradiated diet. In this group, a reduction in the pregnancy rate was observed for mating wk 2, but this was not statistically significant ($P > 0.05$ by χ^2 analysis). The overall mean pregnancy rates during the whole test period as well as the weekly pregnancy rates were comparable in the groups fed the irradiated and control test diets.

Effect on total implantations

Total implantations including live foetuses and dead implantations were scored for each pregnant mouse and the means were determined for all the pregnant females mated in a given week in each group. As can be seen from Table 1, there were no significant differences ($P > 0.05$) between the total implantation rates in animals fed on irradiated and unirradiated test diets. The numbers of total implantations in the treated group did not indicate any increase in pre-implantation loss at any part of the test period. In fact the only significant difference ($P < 0.05$) observed was in the values for wk 2 matings between groups fed on stock ration and on unirradiated test diet, and this difference was not considered to be of any special consequence.

Effect on dead and live implantations

Following mating during the first 2 wk, the numbers of dead implantations were similar among the different groups (Table 1). The animals fed on irradiated diet showed a higher variability than the other groups in the incidence of dead implantations in different weeks, as indicated by the size of the standard error. In the wk 4 mating, this group showed a higher, although statistically insignificant ($P > 0.05$), embryonal death rate compared with the test control group. However, in the wk 3 mating, a reverse effect of similar magnitude was observed, indicating that these values fell within the range of normal experimental variation. The mean dead-implantation rates for the whole test period were similar ($P > 0.05$) in different groups of females mated with males fed on different diets.

There is thus no evidence of induced dominant lethal mutations in males fed on the irradiated whole diet. Similarly, the live-implantation rate, which gives a measure of total pre- and post-implantation lethality (Röhrborn, 1970), remained comparable ($P > 0.05$) among different groups of mice during the whole test period (Table 1).

Incidence of dominant lethals in females

The overall mean incidence of females with dead implantations for the whole test period was of the same order in all the three groups (Table 1). The maximum difference between groups was observed in the values for the wk 4 mating in the groups on the

Table 2. *Cumulative percentage distribution of mice with different numbers of dead implantations*

Diet	Total no. of		Percentage of mice with dead implantations totalling					
	Pregnant females	Females with dead implantations	0	1	2	3	4	⩾5
Stock ration	231	80	65·37 (151)	91·34 (60)	96·97 (13)	98·27 (3)	98·27 (—)	100·00 (4)
Non-irradiated test diet (control)	249	83	66·67 (166)	90·36 (59)	95·98 (14)	98·00 (5)	98·39 (1)	100·00 (4)
Irradiated test diet	269	100	62·83 (167)	87·36 (66)	95·91 (23)	98·51 (7)	99·26 (2)	100·00 (2)

Kolmogorov–Smirnov test used for comparison of distribution of dead implantations in different groups showed no significant differences.

unirradiated and irradiated test diets, but again, this was not significant ($P > 0.05$) when assessed by the χ^2 test.

The Kolmogorov–Smirnov test (Massey, 1951) showed overall homogeneity in the mouse population in the different groups in respect of the distribution pattern of dead implantations (Table 2).

DISCUSSION

The results of this study show some divergence from, as well as agreement with, those reported by Moutschen-Dahmen *et al.* (1970) in mice and by Eriksen & Emborg (1972) in rats. As was reported by these investigators, there was no indication that the irradiated diet had any effect on post-implantation loss. Furthermore, the present investigation showed no effects on the numbers of females with dead implantations or on pregnancy itself that could be attributed to the feeding of irradiated diet. The overall population of mice proved to be homogeneous as revealed by a test for goodness of fit.

Our data on pre-implantation loss, determined by comparing the total implantation rates, failed to demonstrate any evidence of increased lethality in the group of mice fed the irradiated test diet. This is in contradiction to the finding of Moutschen-Dahmen *et al.* (1970), who computed pre-implantation loss as the difference between corpora lutea and total implantations. It is pertinent in this context that counts of corpora lutea are relatively imprecise in mice and, because of the occurrence of spontaneous variation within the control population of mice (Bateman, 1958; Röhrborn, 1968), pre-implantation loss as such has been shown to lack statistical homogeneity (Machemer & Hess, 1971). Again the observations of Moutschen-Dahmen *et al.* (1970) were confined to mating in 1 wk only whereas, in the present study, mating was continued for 4 wk. The latter approach, in addition to revealing normal variations that may occur in different weeks during such studies, also involves a larger number of mice. It should also be borne in mind that whereas post-implantation lethality has been shown to result from genetic factors, the exclusively genetic origin of pre-implantation loss is doubtful (Bateman, 1958; Bateman & Epstein, 1971; Röhrborn, 1968).

Strain differences in the pharmacological response of mice to drugs (Meier & Fuller, 1966) and in the effects of radiation on the reproductive capacity of female mice (Ehling, 1964) are well known. The dominant lethal assay has also been found to show inter-strain differences in the response of mice to chemical mutagens (Cattanach & Pollard, 1971; Generoso & Russell, 1969; Petersen, Legator & Figge, 1972). Such differences have, in fact, been considered responsible for the contradictory results obtained by Kopylov *et al.* (1972) and Levinsky & Wilson (1975) in their studies on dominant lethal mutations in mice fed extracts of irradiated potatoes. Our findings of an absence of induction of dominant lethal mutations following the feeding of an irradiated diet are in agreement with reports of studies in different strains of rats (Eriksen & Emborg, 1972; Renner *et al.* 1973) and mice (Levinsky & Wilson, 1975; Renner *et al.* 1973).

Among other experimental variables that could have contributed to the differences in the observed effects are the dose of radiation used and the nature of the dietary component irradiated. Comparatively low doses of radiation (12–15 krad) have been used in some studies (Kopylov *et al.* 1972; Levinsky & Wilson, 1975), whereas sterilizing doses in the range of 4·5–5 Mrad have been used in others (Eriksen & Emborg, 1972; Moutschen-Dahmen *et al.* 1970; Renner *et al.* 1973). Some of these investigations have been concerned with specific components of the diet, such as potato extract (Kopylov *et al.* 1972; Levinsky & Wilson, 1975) or milk powder (Renner *et al.* 1973), while others have involved assessment of irradiated whole diets (Eriksen & Emborg, 1972; Moutschen-Dahmen *et al.* 1970). Since radiation-processed foods would replace at most only a fraction of an individual's total diet, feeding a wholly irradiated diet to experimental animals enhances the safety factor inherent in the evaluation; an additional increment in the safety factor may be obtained by using a higher dose than the one contemplated in practice. This philosophy underlies the investigations involving whole diets subjected to a sterilizing dose of radiation.

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REFERENCES

Alder, H. L. & Roesoler, E. B. (1968). *Introduction to Probability and Statistics*. p. 288. W. H. Freeman & Co., San Francisco.

Bateman, A. J. (1958). Mutagenic sensitivity of maturing germ-cells in the male mouse. *Heredity* **12,** 213.

Bateman, A. J. & Epstein, S. S. (1971). Dominant lethal mutations in mammals. In *Chemical Mutagens. Principles and Methods for Their Detection*. Vol. 2. Edited by A. Hollaender. p. 541. Plenum Press, New York.

Berry, R., Hills, P. R. & Trillwood, W. (1965) Demonstration of a cytotoxic agent in gamma irradiated carbohydrate solutions. *Int. J. Radiat. Biol.* **9.** 559.

Cattanach, B. M. & Pollard, C. E. (1971). Mutagenicity tests with cyclohexylamine in the mouse. *Mutation Res.* **12,** 472.

Chopra, V. L. (1965). Tests on *Drosophila* for the production of mutations by irradiated medium or irradiated DNA. *Nature, Lond.* **208,** 699.

Dupuy, P. et Usciati, M. (1966). Influence d'extraits de pommes de terre irradices sur la croissance de microorganisms. *Radiat. Bot.* **6,** 499.

Edwards, R. G. & Searle, A. G. (1963). Genetic radiosensitivity of specific post-dictyate stages in mouse oöcytes. *Genet. Res.* **4,** 389.

Ehling, U. H. (1964). Strain variation in reproductive capacity and radiation response of female mice. *Radiat. Res.* **23,** 603.

Epstein, S. S. & Röhrborn, G. (1971). Recommended procedures for testing genetic hazards from chemicals, based on the induction of dominant lethal mutations in mammals. *Nature, Lond.* **230,** 459.

Eriksen, W. H. & Emborg, C. (1972). The effect on preimplantation death of feeding rats on radiation-sterilized food. *Int. J. Radiat. Biol.* **22,** 131.

Generoso, W. M. & Russell, W. L. (1969). Strain and sex variations in the sensitivity of mice to dominant-lethal induction with ethyl methanesulfonate. *Mutation Res.* **8,** 589.

Hills, P. R. & Berry, R. J. (1967). Cytotoxicity of carbohydrates heavily irradiated in solution. *Nature, Lond.* **215,** 309.

Holsten, R. D., Sugii, M. & Steward, F. C. (1965). Direct and indirect effects of radiation on plant cells: Their relation to growth and growth induction. *Nature, Lond.* **208,** 850.

Kopylov, V. A., Osipova, I. N. & Kuzin, A. M. (1972). Mutagenic effect of extracts from gamma-irradiated potato tubers on the sex cells of male mice. *Radiobiologiya* **12,** 58.

Levinsky, H. V. & Wilson, M. A. (1975). Mutagenic evaluation of an alcoholic extract from γ-irradiated potatoes. *Fd Cosmet. Toxicol.* **13,** 243.

Machemer, L. & Hess, R. (1971). Comparative dominant lethal studies with phenylbutazone, thio-TEPA and MMS in the mouse. *Experientia* **27,** 1050.

Massey, F. J. (1951). The Kolmogorov-Smirnov test for goodness of fit. *J. Am. statist. Ass.* **46,** 68.

Meier, H. & Fuller, J. L. (1966). Response to drugs. In *Biology of the Laboratory Mouse*. Edited by E. L. Green. p. 447. The Blakiston Division, McGraw-Hill, New York.

Molin, N. & Ehrenberg, L. (1964). Antibacterial action of irradiated glucose. *Int. J. Radiat. Biol.* **8,** 223.

Moutschen-Dahmen, M., Moutschen, J. & Ehrenberg, L. (1970). Pre-implantation death of mouse eggs caused by irradiated food. *Int. J. Radiat. Biol.* **18,** 201.

Petersen, K. W., Legator, M. S. & Figge, F. H. (1972). Dominant-lethal effects of cyclohexylamine in C57 Bl/Fe mice. *Mutation Res.* **14,** 126.

Renner, H. W., Grünewald, Th. u. Ehrenberg-Kieckebusch, W. (1973). Mutagenitätsprüfung bestrahlter Lebensmittel mit dem "dominant lethal test". *Humangenetik* **18,** 155.

Rinehart, R. R. & Ratty, F. J. (1965). Mutation in *Drosophila melanogaster* cultured on irradiated food. *Genetics* **52,** 1119.

Röhrborn, G. (1968). Mutagenicity tests in mice. *Humangenetik* **6,** 345.

Röhrborn, G. (1970). The dominant lethals: Method and cytogenic examination of early cleavage stages. In *Chemical Mutagenesis in Mammals and Man*. Edited by F. Vogel and G. Röhrborn. p. 148. Springer-Verlag, New York.

Schubert, J. (1969). Mutagenicity and cytotoxicity of irradiated foods and food components. *Bull. Wld Hlth Org.* **41,** 873.

Swaminathan, M. S., Chopra, V. L. & Bhaskaran, S. (1962). Cytological aberrations observed in barley embryos cultured on irradiated potato mash. *Radiat. Res.* **16,** 182.

Swaminathan, M. S., Nirula, Satya, Natarajan, A. T. & Sharma, R. P. (1963). Mutations: Incidence in Drosophila melanogaster reared on irradiated medium. *Science, N.Y.* **141,** 637.

Fd Cosmet. Toxicol. Vol. 13, pp. 437–440. Pergamon Press 1975. Printed in Great Britain

QUELQUES EFFETS A MOYEN TERME DU LINDANE SUR LES ENZYMES MICROSOMALES DU FOIE CHEZ LE RAT

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Résumé—Nous incorporons dans le régime de rats mâles du lindane à raison de 120 ou 240 ppm. Nous mesurons après 4 ou 8 semaines de traitement l'activité de plusieurs enzymes microsomales. Les substrats de ces enzymes sont à la fois de type I ou II, hydro- ou liposolubles, subissant une oxydation ou une réduction. Le lindane est un inducteur de ces enzymes. Nous constatons que l'évolution de l'induction et son amplitude, au cours du temps de traitement, varient avec le substrat utilisé. Ces modifications enzymatiques sont associées à des augmentations du poids du foie et des reins et pourraient être le reflet d'un mécanisme de détoxication.

Abstract—The activities of several microsomal enzymes have been determined in male rats fed a diet containing 120 or 240 ppm lindane for 4 or 8 wk. The substrates were of type I or II, water- or liposoluble, undergoing oxidation or reduction. Lindane acted as an inducer of these enzymes and it was shown that the development and extent of the induction during the treatment period varied with the substrate used. These enzymatic changes were associated with increases in the weights of the liver and kidneys and may have been a reflection of a detoxication mechanism.

INTRODUCTION

Le lindane (isomère gamma de l'hexachlorocyclohexane) est surtout utilisé pour la protection des semences et des sols contre divers parasites souterrains. Ce pesticide a une toxicité modérée pour les mammifères. A long terme, la dose sans effet nocif (*no-effect level*) se situerait entre 25 et 50 ppm dans l'alimentation chez le rat (Herbst et Bodenstein, 1972).

On sait que de très nombreuses substances ont la propriété d'induire (parfois d'inhiber) les enzymes responsables du métabolisme des molécules exogènes et de certains constituants physiologiques (Albrecht, 1972; Conney, 1967). En particulier, il est bien établi que divers insecticides organochlorés induisent les enzymes microsomales liées au cytochrome *P*-450 (Albrecht et Manchon, 1974; Fouts, 1970; Street, 1969). Ainsi, une dose unique de 60 mg de lindane/kg accèlère les métabolismes du paraoxon, de la phénacétine, de la nikéthamide et de l'hexobarbital dans un surnageant postmitochondrial de foie chez le rat (Ghazal, Koransky, Portig, Vohland et Klempau, 1964). Plus récemment, Chadwick, Cranmer et Peoples (1971) ont montré que le traitement quotidien de rats au sevrage avec 2 mg de lindane *per os* pendant 14 jours (soit probablement environ 15 mg/kg/jour) induit la *O*-déméthylase du *p*-nitroanisole et l'hydrolyse oxydative de l'EPN (*O*-éthyl-*O*-(4-nitrophényl) phénylphosphonothioate) dans un homogénat total de foie.

Les expériences que nous présentons dans cet article constituent un travail préliminaire pour une étude précise de l'influence du lindane sur le métabolisme des substances exogènes dans les microsomes hépatiques. On sait l'importance de l'état nutritionnel sur l'intensité de ce métabolisme (Sosa-Lucero, de la Iglesia, Lumb et Feuer, 1973); nos animaux reçoivent une alimentation équilibrée dont nous connaissons la composition. Le choix des substrats, dont nous étudions le métabolisme, repose sur diverses considérations. Les substrats des enzymes microsomales sont classés en deux types suivant le genre de modification qu'ils exercent sur le spectre d'absorption du *P*-450 (Remmer, Schenkman, Estabrook, Sasame, Gillette, Narasimhulu, Cooper et Rosenthal, 1966). Nous utilisons donc à la fois des substrats de type I (aminopyrine) et de type II (aniline). D'autre part, un travail antérieur nous suggérait que l'orientation du métabolisme, sous l'effet d'un traitement, pouvait dépendre du caractère de solubilité de la molécule exogène (Albrecht, Manchon et Lowy, 1973). Nous choisissons des substrats hydrosolubles (aminopyrine, Bordeaux S) et liposolubles (BHT, Jaune de Beurre). Enfin, nous étudions des réactions d'oxydation (aminopyrine, aniline, BHT) et de réduction (Bordeaux S, Jaune de Beurre).

METHODES EXPERIMENTALES

Animaux et traitement

Nous travaillons avec des rats mâles de souche Wistar CF provenant d'un élevage à flore contrôlée. Nous recevons les animaux au sevrage à 18–19 jours. Pendant une semaine, nous leur donnons une provende commerciale (biscuits "Extralabo") puis durant deux semaines un régime semisynthétique équilibré que nous préparons au laboratoire contenant 18,2% (w/w) de protéines (Albrecht et Manchon, 1973). Les animaux sont ensuite traités pendant 4 ou 8 semaines par du lindane. Nous incorporons alors le pesticide dans le régime équilibré à raison de 120 ou 240 ppm de lindane pur. Ces quantités sont calculées de

manière que chaque rat ingère environ 5 ou 10% de la DL_{50} *per os* par jour. Le produit contient 99% de lindane; les impuretés sont constituées par divers isomères de l'HCH dont le principal est l'alpha (Société Pepro, Lyon).

Après 16–18 h de jeûne, nous pesons et sacrifions les animaux par décapitation. Les foie, coeur, reins et surrénales sont prélevés, lavés et pesés. Nous homogénéisons le foie, avec trois volumes de saccharose (0,25 M). refroidi à l'"Ultra Turrax", pendant 45 sec. Nous centrifugeons à 12500 *g* pendant 15 min à +2°C. Nous recueillons le liquide surnageant qui est exempt de mitochondries. Nous obtenons ensuite des microsomes par coprécipitation avec des ions Ca^{2+} et centrifugations, selon la technique qu nous avons décrite (Albrecht, Pélissier, Manchon et Rospars, 1973). Nous déterminons la teneur en protéines de cette préparation en appliquant la méthode de Lowry, Rosebrough, Farr et Randall (1951).

Activité enzymatiques

Toutes les mesures d'activités enzymatiques sont effectuées en double ou en triple.

Oxygénases microsomales. Nous mesurons l'activité des oxygénases après incubation de 15 min avec agitation d'un milieu contenant: 83,5 mM-tampon PO_4H_2Na/PO_4HK_2 à pH 7,4; 8,35 mM-nicotinamide; 6,66 mM-Cl_2Mg; 2,5 mM-glucose-6-phosphate; 1 mM-NADP; 1,2 UI de glucose-6-phosphate déshydrogénase/ml ou "fraction soluble" correspondant à 17 mg foie/ml. Les concentrations des substrats sont 1,33 mM pour l'aminopyrine et le BHT et 5 mM pour l'aniline. La quantité de microsomes hépatiques équivaut à 25 mg foie/ml. Nous incubons à 37°C avec une vive agitation pendant 15 min.

La *N*-déméthylation de l'aminopyrine est déterminée par la formation de 4-aminoantipyrine (Gilbert et Golberg, 1965). Nous mesurons l'activité de l'oxydase du BHT par l'apparition de BHT-alcool (Gilbert et Golberg, 1967). Nous évaluons l'hydroxylation de l'aniline par la formation de *p*-aminophénol (Imai, Ito et Sato, 1966).

Azoréductases du Bordeaux S et du Jaune de Beurre. Nous mesurons l'activité de l'azoréductase après incubation de surnageant postmitochondrial (12500 *g*) dans un milieu contenant: 50 mM-tampon PO_4H_2Na/PO_4HK_2 à pH 7,5; 20 mM-nicotinamide; 6.66 mM-Cl_2Mg; 2,5 mM-glucose-6-phosphate; 0,1 mM-NADP; 30,8 mM-KCl; 1 mM-EDTA; 0,3 UI de glucose-6-phosphate déshydrogénase/ml. Les concentrations de colorants sont 0,2 mM pour le Bordeaux S et 0.02 mM pour le Jaune de Beurre. La quantité de surnageant postmitochondrial équivaut à 33,3 mg foie/ml (Bordeaux S) ou 6,7 mg/ml (Jaune de Beurre). L'incubation à 37°C est menée pendant 15 min en enceinte ouverte pour le Jaune de Beurre et 30 min en anaérobiose stricte pour le Bordeaux S. Après défécation par de l'acide trichloracétique, nous mesurons la quantité de colorant disparu par spectrophotométrie à 520 nm.

Réductase NADPH-cytochrome c. Nous déterminons l'activité de cette enzyme selon la technique que nous avons décrite (Albrecht et Manchon, 1973). La quantité de microsomes équivaut à 1,1 mg foie/ml.

Analyse statistique

Les tableaux qui présentent nos résultats sont établis par des méthodes dérivées de l'analyse de la variance (Schwartz, 1966). Ils indiquent des résultats moyens suivis d'une valeur estimée de l'écart type sur la moyenne calculé à partir de la variance commune à l'ensemble des groupes de l'expérience. Pour diminuer au mieux la variance résiduelle nous utilisons la méthode des blocs complets avec répétition. Le calcul du poids relatif moyen des organes et de leur écart-type est obtenu par analyse de la covariance. Nous comparons les moyennes des rats traités au lindane à celles des témoins de la manière suivante:

(1) Comparaison des deux moyennes (120/240 ppm) chez les traités et

(2) si ces moyennes ne diffèrent pas significativement ($P > 0,05$), nous comparons l'ensemble des animaux traités (120 + 240 ppm) aux témoins.

RESULTATS

Caractéristiques des animaux

Quatre semaines d'administration de lindane abaissent légèrement la croissance pondérale des rats (Tableau 1), particulièrement chez ceux qui reçoivent la plus forte quantité (11% du poids au sacrifice). Le poids relatif du foie est augmenté de manière à peu

Tableau 1. *Poids corporel et poids relatif des organes des rats nourris avec un régime contenant du lindane*

Paramètre	Durée du traitement (semaines)	Nombre d'animaux...	Valeurs† pour des rats traités au lindane (ppm) 0 (témoins)	120	240
			12	6	6
Poids corporel (g)	4		297,3 ± 7,21	292 ± 10,2	265 ± 10,2*
	8		376 ± 10,4	374 ± 14,8	360 ± 14,8
Poids relatif du foie (g/100 g)	4		4.16 ± 0.070	4.47 ± 0.100*	4.80 ± 0.100**
	8		3,66 ± 0,079	3,99 ± 0,111*	4.34 ± 0,111**
du coeur (g/100 g)	4		0.32 ± 0.006	0.33 ± 0,084	0.32 ± 0.084
	8		0.30 ± 0.005	0.32 ± 0.067	0,31 ± 0,067
des reins (g/100 g)	4		0.92 ± 0,020	1.06 ± 0.029**	1.21 ± 0.029**
	8		0,80 ± 0,077	0,94 ± 0,109**	1,06 ± 0,109**
des surrénales (mg/100 g)	4		8,66 ± 0,490	8,80 ± 0,693	9,04 ± 0,693
	8		7,79 ± 0,401	8,91 ± 0,568	8,84 ± 0,568
Concn des protéines (g/100 g foie)	4		3,23 ± 0,089	3,41 ± 0,125	3,11 ± 0,125
	8		4,87 ± 0,116	4,59 ± 0,164	5,03 ± 0,164

†Moyenne, écart-type sur la moyenne calculé à partir de la variance commune; les valeurs marquées avec des astérisques diffèrent significativement des témoins: *$P < 0,05$; **$P < 0,01$.

Tableau 2. *Activité des oxygénases microsomales des rats nourris avec un régime contenant du lindane*

Enzymes	Durée du traitement (semaines)	Nombre d'animaux...	Activité enzymatique† chez les rats traités au lindane (ppm) 0 (témoins)	120	240
			12	6	6
			nmoles de 4-aminoantipyrine/100 mg protéines microsomales/min		
N-Déméthylase de l'aminopyrine	4		23,6 ± 2,85	51.8 ± 4,03**	59,0 ± 4,03**
	8		17,4 ± 1,09	27,0 ± 1,54**	35,5 ± 1,54**
			nmoles de BHT-alcool/100 mg protéines microsomales/min		
Oxydase du BHT	4		228 ± 10,9	271 ± 15,4*	306 ± 15,4**
	8		145,5 ± 5,16	184,2 ± 7,30**	195,7 ± 7,30**
			nmoles de *p*-aminophénol/100 mg protéines microsomales/min		
Hydroxylase de l'aniline	4		24,0 ± 2,38	36.3 ± 3,36**	42,5 ± 3,36**
	8		16,7 ± 1,25	28,2 ± 1,77**	27,5 ± 1,77**

†Moyenne, écart-type sur la moyenne calculé à partir de la variance commune; les valeurs marquées avec des astérisques diffèrent significativement des témoins: $*P < 0{,}05$; $**P < 0{,}01$.

près identique après 4 et 8 semaines pour chacune des doses. Nous remarquons que cette hypertrophie est fonction de la quantité de lindane ingérée pour une durée de traitement donnée: après 4 semaines, l'augmentation est de 7% avec 120 ppm et 15% avec 240 ppm; après 8 semaines respectivement 9 et 19%. Nous retrouvons les mêmes effets sur le poids relatif des reins (à 4 semaines, 15% avec 120 ppm et 32% avec 240 ppm; à 8 semaines: 17% avec 120 ppm et 32% avec 240 ppm).

Le lindane ne paraît modifier sensiblement ni le poids du coeur et des surrénales ni la concentration des protéines microsomales du foie (Tableau 1).

Activités enzymatiques

La *N*-déméthylase de l'aminopyrine (Tableau 2) est très fortement induite par le traitement au pesticide. Après 4 semaines, l'augmentation est de 135% et ne diffère pas suivant les doses. Après 8 semaines, l'induction s'infléchit d'autant plus que la quantité de lindane du régime est faible (120 ppm, 55%; 240 ppm, 104%).

Nous notons que l'oxydase du BHT (Tableau 2) est induite de manière à peu près semblable après 4 (27%) et 8 semaines (31%) de traitement. L'augmentation d'activité enzymatique ne dépend pas sensiblement de la dose.

Nous observons également une induction de l'hydroxylase de l'aniline (Tableau 2): 4 semaines, 64%; 8 semaines, 67%. Comme pour l'oxydase du BHT, l'augmentation d'activité ne paraît pas liée à la quantité de lindane dans le régime.

L'accélération du métabolisme de ces substances exogènes est associée à une induction de la réductase NADPH-cytochrome *c* microsomale (Tableau 3): 4 semaines, 23%; 8 semaines, 24%. L'augmentation serait peut être encore plus importante avec la dose élevée de lindane.

Le pesticide induit l'azoréductase du Bordeaux S (Tableau 3) seulement après 4 semaines de traitement (30%).

L'azoréductase du Jaune de Beurre serait peu influencée par le lindane (Tableau 3). Nous remarquons cependant une diminution (18%) après 8 semaines avec la dose la plus faible; ce résultat devrait être confirmé.

DISCUSSION

A moyen terme, le lindane incorporé dans le régime à des doses correspondant à 10% (240 ppm) et 5% (120 ppm) de la DL_{50} modifie plusieurs caractères biologiques chez le rat.

Avec nos conditions expérimentales, nous confirmons que le lindane est un inducteur des "oxygénases à fonction mixte" dans les microsomes hépatiques. Il accélère le métabolisme à la fois d'un substrat de type I (aminopyrine) et de type II (aniline). Il augmente la vitesse d'oxydation de molécules liposoluble (BHT) et hydrosoluble (aminopyrine). Cependant, l'évolution au cours du temps de l'induction des oxygénases diffère suivant les substrats. Alors que l'augmentation d'activité de la *N*-déméthylase de l'aminopyrine est

Tableau 3. *Activité de la réductase NADPH-cytochrome c et des azoréductases des rats nourris avec un régime contenant du lindane*

Enzymes	Durée du traitement (semaines)	Nombre d'animaux...	Activité enzymatique† chez les rats traités au lindane (ppm) 0 (témoins)	120	240
			12	6	6
			µmoles de cytochrome réduit/100 mg protéines microsomales/min		
Réductase NADPH-cytochrome c	4		7,41 ± 0,471	8,50 ± 0,665	9,70 ± 0,665**
	8		4,24 ± 0,254	4,76 ± 0,359	5,74 ± 0,359**
			nmoles de colorant metabolisé/100 mg protéines microsomales/min		
Azoréductase du Bordeaux S	4		155 ± 15,8	187 ± 22,4	216 ± 22,4*
	8		92,2 ± 8,81	89 ± 12,5	105 ± 12,5
Azoréductase du Jaune de Beurre	4		173 ± 13,3	209 ± 18,9	202 ± 18,9
	8		119,5 ± 5,88	97,8 ± 8,32*	128,3 ± 8,32

†Moyenne, écart-type sur la moyenne calculé à partir de la variance commune; les valeurs marquées avec des astérisques diffèrent significativement des témoins: $*P < 0{,}05$; $**P < 0{,}01$.

maximum avant 4 semaines de traitement puis décroît d'autant plus vite que la quantité de lindane est faible. l'induction de l'oxydase du BHT ou de l'hydroxylase de l'aniline demeure à peu près constante entre 4 et 8 semaines; de plus elle est peu dépendante de la quantité d'insecticide.

Nos résultats concernant la réduction des azoïques sont moins clairs. Le lindane ne serait inducteur de l'azoréductase du Bordeaux S qu'à court terme et peut-être inhibiteur de l'azoréductase du Jaune de Beurre.

Nous remarquons que la réductase NADPH-cytochrome *c*, bien qu'induite par le traitement au lindane, ne paraît pas pouvoir expliquer à elle seule l'accélération du métabolisme des substrats exogènes dans les microsomes. Ainsi, la formation d'amino-antipyrine, à partir de l'aminopyrine, est doublée alors que la réduction du cytochrome c^{3+} par le NADPH est seulement augmentée de 30%. Le lindane modifie vraisemblablement d'autres composants du système microsomal de transport d'électrons. Nous nous proposons d'ailleurs d'étudier ultérieurement plus finement le mécanisme de l'induction enzymatique par le lindane. Selon Chadwick *et al.* (1971) et Kolmodin-Hedman (1972), l'insecticide augmente la quantité de *P*-450 dans les microsomes du foie mais il pourrait aussi induire la réductase NADPH *P*-450, augmenter la liaison des substrats au *P*-450 etc.

D'autre part, il serait intéressant de déterminer la quantité minimum de lindane faisant apparaître une modification de l'activité des enzymes microsomales. S'agit-il effectivement d'une dose bien inférieure à la *no-effect level* comme semble l'indiquer le travail de Kolmodin-Hedman (1972)?

Le lindane augmente le poids relatif du foie. Nous confirmons ainsi les observations de Fitzhugh, Nelson et Frawley (1950); mais contrairement à ces auteurs, nous notons aussi une augmentation du poids relatif des reins. Nous constatons que ces hypertrophies dépendent de la quantité d'insecticide ajoutée dans le régime, donc de la quantité journalière ingérée. Le phénomène doit être précoce car les augmentations de poids évoluent peu entre 4 et 8 semaines pour chacune des doses.

Nous pensons que l'on peut considérer l'hypertrophie du foie, provoquée par le lindane, comme un mécanisme de détoxication. En effet, on observe une prolifération du réseau endoplasmique sans altération histopathologique (Herbst et Bodenstein, 1972). L'hypertrophie est associée à une induction des "oxygénases à fonction mixte" des microsomes sans qu'il y ait de modification de l'activité de la glucose-6-phosphatase (résultat non publié). Selon Gilbert et Golberg (1965) ces faits caractérisent bien une hépatomégalie par hyperfonctionnement. De plus, Chadwick *et al.* (1971) et Chadwick et Freal (1972) montrent que l'administration continue de lindane conduit à une accélération de son propre métabolisme par oxydation.

Si l'on admet donc que l'augmentation du poids du foie constitue une adaptation physiologique, on remarquera qu'à court terme (moins de 4 semaines de traitement) cette réponse est encore modulée pour des quantités de lindane aussi importantes que 5–10% de la DL_{50} par jour.

REFERENCES

Albrecht, R. (1972). Quelques aspects des processus biochimiques intervenant au niveau des enzymes microsomales hépatiques. *Thérapie*, **27**, 751.

Albrecht, R. et Manchon, Ph. (1973). Influence de la teneur en vitamines hydrosolubles du régime et d'un traitement par un barbiturique sur quelques effets des colorants azoïques. *Ann. Nutr. Aliment.* **27**, 191.

Albrecht, R. et Manchon, Ph. (1974). Influence de l'environnement sur le métabolisme des molécules exogènes dans les microsomes du foie. *Ann. Nutr. Aliment.* **28**, 351.

Albrecht, R., Manchon, Ph. et Lowy, R. (1973). Influence de la teneur en protéines du régime sur quelques effets du Bordeaux S et du Jaune de Beurre chez le rat. *Fd Cosmet. Toxicol.* **11**, 383.

Albrecht, R., Pélissier, M. A., Manchon, Ph. et Rospars, J. P. (1973). Obtention de microsomes par coprécipitation avec Ca^{2+} et mise en évidence d'induction enzymatique par le phénobarbital. *C.r. Séanc. Soc. Biol.* **167**, 1552.

Chadwick, R. W., Cranmer, M. F. & Peoples, A. J. (1971). Comparative stimulation of γHCH metabolism by pretreatment of rats with γHCH, DDT and DDT + γHCH. *Toxic. appl. Pharmac.* **18**, 685.

Chadwick, R. W. & Freal, J. J. (1972). Comparative acceleration of lindane metabolism to chlorophenols by pretreatment of rats with lindane or with DDT and lindane. *Fd Cosmet. Toxicol.* **10**, 789.

Conney, A. H. (1967). Pharmacological implications of microsomal enzyme induction. *Pharmac. Rev.* **19**, 317.

Fitzhugh, O. G., Nelson, A. A. & Frawley, J. P. (1950). The chronic toxicities of technical benzene hexachloride and its alpha, beta and gamma isomers. *J. Pharmac. exp. Ther.* **100**, 59.

Fouts, J. R. (1970). Some effects of insecticides on hepatic microsomal enzymes in various animal species. *Revue can. Biol.* **29**, 377.

Ghazal, A., Koransky, W., Jr., Portig, J., Vohland, H. N. u. Klempau, I. (1964). Beschleunigung von Entgiftungsreaktionen durch verschiedene Insecticide. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **249**, 1.

Gilbert, D. & Golberg, L. (1965). Liver response tests. III. Liver enlargement and stimulation of microsomal processing enzyme activity. *Fd Cosmet. Toxicol.* **3**, 417.

Gilbert, D. & Golberg, L. (1967). BHT oxidase. A liver-microsomal enzyme induced by the treatment of rats with butylated hydroxytoluene. *Fd Cosmet. Toxicol.* **5**, 481.

Herbst, M. et Bodenstein, G. (1972). La toxicologie du lindane. In *Lindane*. Edited by E. Ulman. p. 25. Verlag K. Schillinger, Freiburg.

Imai, Y., Ito, A. & Sato, R. (1966). Evidence for biochemically different types of vesicles in the hepatic microsomal fraction. *J. Biochem. Tokyo* **60**, 417.

Kolmodin-Hedman, B. (1972). Effets biologiques observés chez des travailleurs exposés professionnellement à divers insecticides chlorés. Symposium international du lindane. p. 62. CIEL, Bruxelles.

Lowry, O. H., Rosebrough, Nira J., Farr, A. L. & Randall, Rose J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265.

Remmer, H., Schenkman, J., Estabrook, R. W., Sasame, H., Gillette, J., Narasimhulu, S., Cooper, D. Y. & Rosenthal, O. (1966). Drug interaction with hepatic microsomal cytochrome. *Molec. Pharmac.* **2**, 187.

Schwartz, D. (1966). Méthodes statistiques à l'usage des médecins et des biologistes. Ed. Flammarion, Paris.

Sosa-Lucero, J. C., de la Iglesia, F. A., Lumb, G. & Feuer, G. (1973). Nutritional influences on drug metabolism. *Revue can. Biol.* **32** suppl., 69.

Street, J. C. (1969). Organochlorine insecticides and the stimulation of liver microsome enzymes. *Ann. N.Y. Acad. Sci.* **160**, 274.

Fd Cosmet. Toxicol. Vol. 13, pp. 441–443. Pergamon Press 1975. Printed in Great Britain

SHORT PAPERS

THE CARCINOGENICITY OF AFLATOXIN M_1 IN RAINBOW TROUT

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Summary—Three groups, each initially of 250 trout, were fed a diet containing 5·8 ppb ($b = 10^9$) aflatoxin B_1 (AFB_1) or 5·9 or 27·3 ppb aflatoxin M_1 (AFM_1) for 16 months. A control group of 500 trout received the basal diet. In the early part of the study, a relatively high mortality rate resulted from underfeeding and infections. Toxin administration had no effect on the serum and liver enzymes studied and growth was unaffected up to month 12, although at month 15 there was a significant decrease in body weight in the two groups given AFM_1. Autopsy of fish killed after 5, 9 and 12 months revealed ceroid degeneration of the liver in all four groups but no tumours or preneoplastic changes. Autopsy of survivors at month 16, however, revealed six with hepatocellular carcinoma and 11 with hyperplastic nodules in the 48 trout fed 5·8 ppb AFB_1 and one with hepatocellular carcinoma and three with hyperplastic nodules in the 48 fed 27·3 ppb AFM_1, but no evidence of such lesions was seen in the 49 fed 5·9 ppm AFM_1 or in the 51 from the control group.

Introduction

Aflatoxins are known to be carcinogenic in many animal species (International Agency for Research on Cancer, 1972) and aflatoxin B_1 (AFB_1) seems to be the most carcinogenic form. There are also indications that aflatoxin causes tumours in man (Alpert, Hutt, Wogan & Davidson, 1971; Keen & Martin, 1971; Peers & Linsell, 1973; Shank, 1971; Shank, Bhamarapravati, Gordon & Wogan, 1972; van Nieuwenhuize, Herber, de Bruyn, Meyer & Duba, 1973).

Allcroft & Carnaghan (1963) detected in the milk of cows fed AFB_1-contaminated peanut-meal a factor that caused the same toxic effects as the known aflatoxins. Holzapfel, Steyn & Purchase (1966) isolated this factor from the urine of sheep fed with contaminated peanut-meal; it proved to consist of the hydroxy products of AFB_1 and aflatoxin B_2 (AFB_2) and these were called aflatoxins M_1 and M_2 (AFM_1 and AFM_2). de Iongh, Vles & de Vogel (1965) found AFM_1 in extracts of cultures of *Aspergillus flavus*, and AFM_1 was also detected in the urine of monkeys fed a diet contaminated with radio-labelled AFB_1 (Shanta, Sreenivasamurthy & Parpia, 1970). In addition, AFM_1 has been detected in human urine after ingestion of contaminated peanuts (Campbell, Caedo, Bulatao-Jayme, Salamat & Engel, 1970).

AFM_1 has been shown to be carcinogenic for rainbow trout (Sinnhuber, Lee, Wales, Landers & Keyl, 1970; Sinnhuber, Lee, Wales & Landers, 1974), but Butler (1971) found no tumours in rats fed for 2 yr with AFM_1-contaminated food. AFM_1 has been detected in milk and milk products (Hanssen, 1973; Kiermeier & Mücke, 1972; Neumann, Kleinpaul & Terplan, 1972; Purchase & Vorster, 1968; Verhülsdonk, Paulsch, van Gansewinkel en Deyll, 1973; Verhülsdonk, Paulsch & van Gansewinkel, 1973). It seemed important therefore to confirm the findings of Sinnhuber *et al.* (1970 & 1974), and because of the difficulty of obtaining large amounts of AFM_1, the experiment described here was similarly carried out in rainbow trout.

Experimental

Test material. AFM_1 was produced by adding aliquots of 200 ml distilled water to quantities of 400 g parboiled rice contained in 3-litre flasks, heating the mixtures for 15 min at 80°C and then sterilizing for 15 min at 120°C. Then 10 ml of a spore suspension of *A. parasiticus* NRRI 2999 (10^6–10^7 spore/ml) was added to the rice in each flask. After shaking, the flasks were put in an incubator (24°C), where they were rolled (4 r/min) and aerated. After incubation for 7 days, the cultures were extracted with chloroform and cleaned by column chromatography on silica gel and Sephadex. The final yield was about 20 mg ($M_1 + M_2$)/kg rice.

Animals and diets. Rainbow trout, about 4 months old and weighing about 25 g, were obtained from a hatchery in Vaessen and fed on Trouvit, a food of known composition supplied by Trouw (Putten, The Netherlands). Diets for three groups of 250 trout were prepared by adding 10 μg AFB_1/kg (10 ppb), 10 ppb AFM_1 and 50 ppb AFM_1, respectively, to the basic diet, moistening with distilled water and adding glycerol before pelleting. The pellets were dried for 48 hr at 35–40°C. A considerable loss of aflatoxin resulted from this process, so that the mean levels in the diets fed to the three test groups were 5·8 ppb AFB_1 and 5·9 and 27·3 ppb AFM_1. A fourth (control) group of 500 trout was fed Trouvit without added mycotoxin.

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Table 1. *Histopathological findings in the livers of rainbow trout fed diets containing AFB_1 or AFM_1 for 16 months*

Type of lesion	Incidence* of liver lesions in groups fed diet containing			
	No aflatoxin (control)	5·8 ppb AFB_1	5·9 ppb AFM_1	27·3 ppb AFM_1
No. of trout examined...	51	48	49	48
Vacuolization	0	0	0	1
Focal necrosis	0	1	0	1
Cirrhosis	0	1	0	1
Ceroid degeneration:				
slight	16	14	24	15
moderate	9	17	16	13
strong	3	4	5	3
Bile-duct proliferation:				
oval-cell type	0	8	0	5
tubular type + cholangiofibrosis	0	1†	0	0
Eosinophilic nodules	0	5	0	1
Basophilic nodules	0	5	0	2
Mixed lesions‡	0	1	0	0
Hepatocellular carcinoma	0	6	0	1

*No. of trout affected out of total no. examined.
†This fish also showed hypertrophy of the gall bladder.
‡Basophilic nodules containing cholangiomatous elements.

Experimental design and conduct. Each group was kept in a stainless-steel tank (about 400-litre capacity) in well-water kept at 15°C and containing more than 7 mg oxygen/kg at a flow rate of 8 litre/min. During the experiment, oxygen shortage resulted from the considerable growth of the fish. In spite of extra aeration of the tanks, it was necessary to reduce the number of fish in each group, and after 13 months each group was reduced to 50 animals, distributed between two tanks.

After 3 and 5 months and after each of months 7–12, the median weight of the trout was determined, and after 15 months the animals were weighed individually. After 16 months, the activities of hepatic alkaline phosphatase and glucose-6-phosphatase and of serum glutamic–pyruvic transaminase and glucose-6-phosphate dehydrogenase were determined in between two and eight animals from each group. Histopathological examination of the liver, kidneys and spleen was carried out after 5, 9, 12 and 16 months. At 16 months all the survivors were killed and examined, while at 5, 9 and 12 months, respectively, at least 25, 10 and 15 fish/group were studied. The tissues were fixed in Bouin's fixative and paraplast sections (5 μm) were prepared and stained with haemalum and eosin. All grossly detectable liver lesions were subjected to histological examination.

Results

During the first months of the experiment, some deaths were caused by infections with Chilodonella and *Costia necatrix*, but after this was successfully treated some deaths resulted from an accidental deficiency in food administration.

There were no significant differences in the weights of the fish up to month 12. After 15 months, the mean weight ($\pm$SEM, with group sizes in parenthesis) of the control group and the groups given 5·8 ppb AFB_1 and 5·9 and 27·3 ppb AFM_1 were, respectively, 1002 ± 319 (51), 883 ± 342 (48), 817 ± 214 (49) and 786 ± 241 (48). The two latter figures differed significantly from the control value ($P < 0{\cdot}01$ Student's t test). Administration of AFB_1 and M_1 had no effect, however, on the activities of the liver and serum enzymes tested. There was a considerable variation in the values, although fish with equal weights were selected for the enzyme determinations. All the histopathological examinations revealed a high incidence of ceroid degeneration in the liver, but no tumours or preneoplastic changes were detected at 5, 9 or 12 months. The results of the hepatic examinations at 16 months are summarized in Table 1. The hyperplastic and neoplastic lesions of the liver were classified according to Simon, Dollar & Smuckler (1967) and Ashley & Halver (1968), on the basis of the following descriptions:

Eosinophilic nodules. Groups of enlarged liver cells, usually more eosinophilic, forming nodules; nuclei are the same as in normal liver cells; there is no compression of adjacent tissue.

Basophilic nodules. Groups of small cells with basophilic cytoplasm forming nodules; the nuclei are usually small and sometimes show some variation in size; there is no compression of adjacent areas (Fig. 1).

Mixed lesion. Nodules consisting of small basophilic hepatocytes, with some variation in cell and nuclear size; in addition to the hepatocytes, this lesion contains proliferated bile ducts of the tubular- and/or oval-cell type; there is considerable cellular atypia. This lesion was considered to be malignant.

Hepatocellular carcinoma. Lesions consisting of small basophilic hepatocytes, usually showing some variation in nuclear and cell size; compression of adjacent tissue sometimes occurs; infiltrative growth is common but not always present. The presence of nuclear anisochromasia was the criterion most used to distinguish this lesion from basophilic nodules.

Table 1 shows that 5·8 ppb AFB_1 in the diet induced liver tumours in trout after the feeding of the diet for 16 months. No hyperplastic or neoplastic lesions were seen in the fish of the control group or of the group given 5·9 ppb AFM_1. The table also shows that AFM_1 at a dietary level of 27·3 ppb induced liver lesions, including one carcinoma (Fig. 2), suggesting that this compound is also carcinogenic for trout. The bile-duct lesions found at 16 months in the groups given AFB_1 or the higher level of AFM_1 were also thought to have been induced by the administered compound.

In a small proportion of the fish, the spleen showed an irregular surface and in these cases it was generally enlarged and congested. This lesion was found in the control and test groups and was therefore considered to be unconnected with the mycotoxin treatments.

Discussion

Comparing these results with those of Sinnhuber *et al.* (1970 & 1974), who used the Mt. Shastra strain known to be very sensitive to tumour induction, it is obvious that the trout strain used is very important. These authors found that AFM_1 was carcinogenic even when a dietary level of 4 ppb was used. Furthermore the lesions developed earlier and some were present after only 4 months. The lesions were all called hepatomas and were not subdivided as they were in this study.

In the early part of this study, the trout were accidentally given insufficient food, and this may have been responsible for their susceptibility to infections

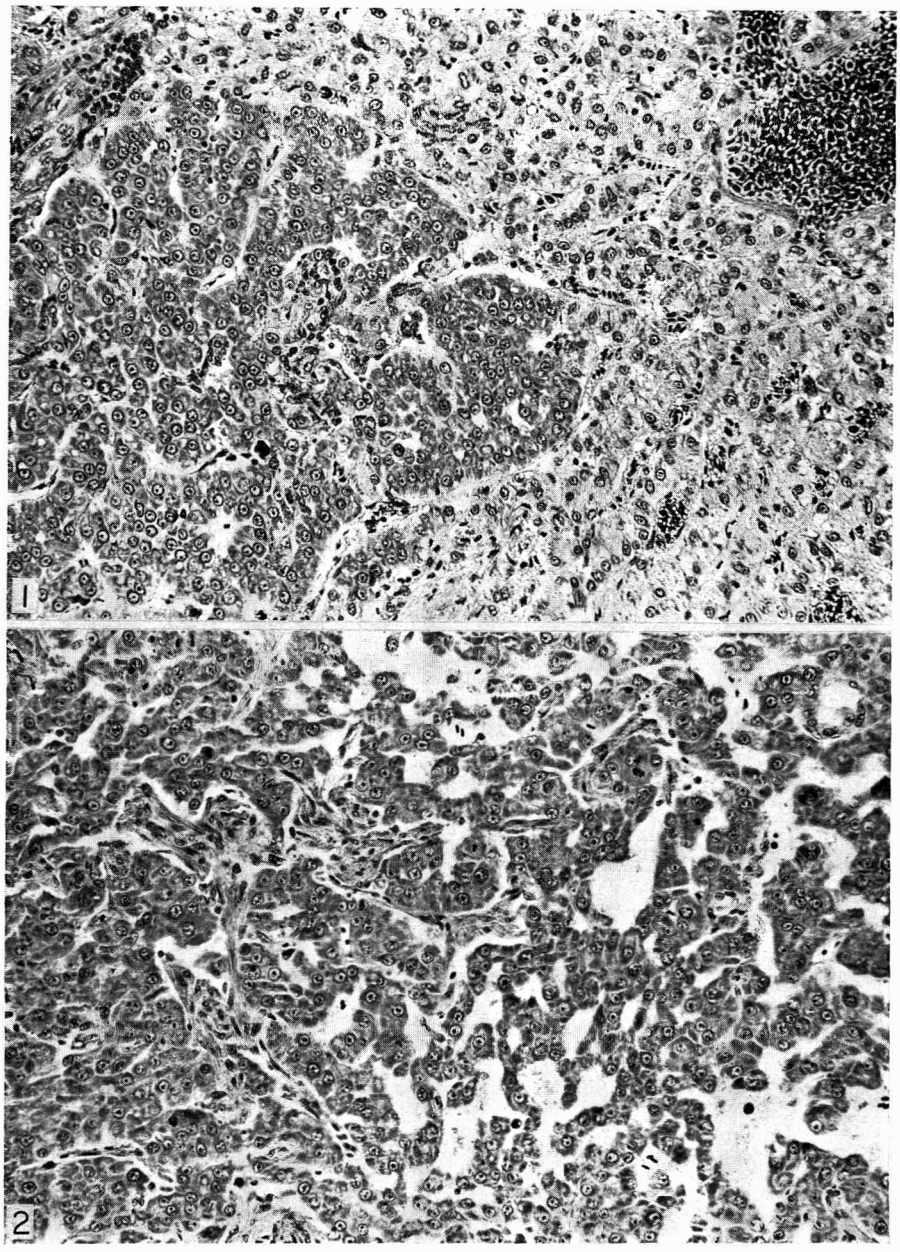

Fig. 1. Section of liver from a trout fed 5·8 ppb AFB_1 in the diet for 16 months, showing the border of a basophilic nodule and parenchymal cells of normal architecture. Haematoxylin and eosin × 200.

Fig. 2. Section of liver from a trout fed 27·3 ppb AFM_1 in the diet for 16 months, showing a hepatocellular carcinoma, with trabecular and adenomatous structures, nuclear pleomorphism and mitoses. Haematoxylin and eosin ×200.

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and for the ceroid degeneration in the liver. Other factors that may have influenced the results of this experiment were the way of keeping the animals, the kind of food, the temperature and the water-flow rate.

At the end of the experiment (at 16 months), some animals showed bile-duct proliferation, a well-known response to aflatoxin intoxication, and the nodules observed could be considered as precancerous lesions. From Table 1, it can be seen that eosinophilic nodules, basophilic nodules or mixed lesions were induced in 11 animals of the 5·8 ppb AFB_1 group, in none of the 5·9 ppb AFM_1 group and in three of the 27·3 ppb AFM_1 group. This suggests that the tumour incidence might have been higher if this experiment had lasted more than 16 months.

Sinnhuber *et al.* (1970 & 1974) found 12 tumours with 4 ppb AFB_1 and eight with 4 ppb AFM_1, in groups of 20 trout. In the current experiment, no tumours or nodules were found in 48 trout fed 5·9 ppm AFM_1, six tumours and 11 hyperplastic nodules were found in 49 trout given the 5·8 ppb AFB_1 diet and one tumour and three hyperplastic nodules in 48 trout on the 27·3 ppb AFM_1 diet. The results of Sinnhuber and his colleagues and those of this experiment thus indicate that AFM_1 is a less potent carcinogen for trout than AFB_1. These results are in accordance with those recently found in rats by Wogan & Paglialunga (1974).

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REFERENCES

Allcroft, Ruth & Carnaghan, R. B. A. (1963). Groundnut toxicity: An examination for toxin in human food products from animals fed toxic groundnut meal. *Vet. Rec.* **75**, 259.

Alpert, M. E., Hutt, M. S. R., Wogan, G. N. & Davidson, C. S. (1971). Association between aflatoxin content of food and hepatoma frequency in Uganda. *Cancer* **28**, 253.

Ashley, L. M. & Halver, J. E. (1968). Hepatoma induction. V. Experiments. In *Trout Hepatomagenesis.* Supplement to final report. Cooperative project NCI-FS-64-14. p. 1.

Butler, W. H. (1971). The toxicity of aflatoxin. In *Mycotoxins in Human Health.* Edited by I. F. H. Purchase. p. 141. The Macmillan Press Ltd., London.

Campbell, T. C., Caedo, J. P., Jr., Bulatao-Jayme, J., Salamat, L. & Engel, R. W. (1970). Aflatoxin M_1 in human urine. *Nature, Lond.* **227**, 403.

de Iongh, H., Vles, R. O. & de Vogel, P. (1965). The occurrence and detection of aflatoxin in food. In *Mycotoxins in Foodstuffs.* Edited by G. N. Wogan. p. 235. The M.I.T. Press, Cambridge.

Hanssen, E. (1973). Control of aflatoxins in the food-industry. *Pure appl. Chem.* **35**, 239.

Holzapfel, C. W., Steyn, P. S. & Purchase, I. F. H. (1966). Isolation and structure of aflatoxins M_1 and M_2. *Tetrahedron Lett.* no. 25, p. 2799.

International Agency for Research on Cancer (1972). IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man. Vol. 1. p. 145. IARC, Lyon.

Keen, P. & Martin, P. (1971). The toxicity and fungal infestation of foodstuffs in Swaziland in relation to harvesting and storage. *Trop. geogr. Med.* **23**, 35.

Kiermeier, F. u. Mücke, W. (1972). Über den Nachweis von Aflatoxin M in Milch. *Z. Lebensmittelunters. u. -Forsch.* **150**, 137.

Neumann, D., Kleinpaul, A. u. Terplan, G. (1972). Zum Vorkommen von Aflatoxin M_1 in Trocken-Milchprodukten. *Arch. Lebensmittelhyg.* **23**, 128.

Peers, F. G. & Linsell, C. A. (1973). Dietary aflatoxins and liver cancer—A population based study in Kenya. *Br. J. Cancer* **27**, 473.

Purchase, I. F. H. & Vorster, L. J. (1968). Aflatoxin in commercial milk samples. *S. Afr. med. J.* **42**, 219.

Shank, R. C. (1971). Dietary aflatoxin loads and the incidence of human hepatocellular carcinoma in Thailand. In *Mycotoxins in Human Health.* Edited by I. F. H. Purchase. p. 245. The Macmillan Press Ltd., London.

Shank, R. C., Bhamarapravati, N., Gordon, J. E. & Wogan, G. N. (1972). Dietary aflatoxins and human liver cancer. IV. Incidence of primary liver cancer in two municipal populations of Thailand. *Fd Cosmet. Toxicol.* **10**, 171.

Shantha, T., Sreenivasamurthy, V. & Parpia, H. A. B. (1970). Urinary metabolites of ^{14}C aflatoxin in some laboratory animals. *J. Fd Sci. Technol.* **7**, 135.

Simon, R. C., Dollar, A. M. & Smuckler, E. A. (1967). Descriptive classification on normal and altered histology on trout livers. In *Trout Hepatoma Research Conference Papers.* Edited by J. D. Halver and I. A. Mitchel. Research Report 70 from the Bureau of Sport Fisheries and Wildlife. p. 18.

Sinnhuber, R. O., Lee, D. J., Wales, J. H., Landers, M. K. & Keyl, A. C. (1970). Aflatoxin M_1, a potent liver carcinogen for rainbow trout. *Fedn Proc. Fedn Am. Socs exp. Biol.* **29**, 568.

Sinnhuber, R. O., Lee, D. J., Wales, J. H. & Landers, M. K. (1974). Aflatoxin M_1, a liver carcinogen for rainbow trout. In press.

van Nieuwenhuize, J. P., Herber, R. F. M., de Bruyn, A., Meyer, P. B. en Duba, W. C. (1973). Aflatoxinen. Epidemiologisch onderzoek naar carcinogeniteit bij langdurige "low level" expositie van een fabriekspopulatie. *T. soc. Geneesk.* **51**, 754.

Verhülsdonk, C. A. H., Paulsch, W. E. en van Gansewinkel, B. J. (1973). Het Aflatoxine M_1 Gehalte in de Nederlandse Consumptiemelk. National Institute of Public Health Report 113/73 LCLO.

Verhülsdonk, C. A. H., Paulsch, W. E., van Gansewinkel en Deyll, W. E. (1973). Het Voorkomen van Aflatoxine M_1 in Kalvermelkvoeders. National Institute of Public Health Report 155/73 LCLO.

Wogan, G. N. & Paglialunga, S. (1974). Carcinogenicity of synthetic aflatoxin M_1 in rats. *Fd Cosmet. Toxicol.* **12**, 381.

Fd Cosmet. Toxicol. Vol. 13, pp. 445–447. Pergamon Press 1975. Printed in Great Britain

EFFECT OF SUBACUTE ORAL ADMINISTRATION OF ZINC ETHYLENEBIS(DITHIOCARBAMATE) ON THE THYROID GLAND AND ADENOHYPOPHYSIS OF THE RAT

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Summary—The thyroid gland and adenohypophysis were studied after twice weekly oral administration of zinc ethylenebis(dithiocarbamate) (zineb) by gavage to rats for 4·5 months in doses equivalent to 1/10 or 1/100 LD_{50}. Compared with the control group, the thyroids of the rats treated with the higher dosage level of zineb were enlarged and composed of numerous microfollicles lined with columnar cells. Succinic dehydrogenase and cytochrome oxidase activities were raised in the columnar cells, while the PAS-positive granules were reduced. Furthermore, the colloid within the thyroid follicles stained less intensely with the PAS stain. The changes in the ultrastructure of the thyroid were consistent with long-term thyrotropic-hormone (TTH) stimulation. An increased number of basophilic cells, containing PAS-positive granules, was observed in the adenohypophysis, again in the rats on the higher level of treatment. In the same group, increases up to 80% were detected in the uptake of ^{131}I by the thyroid, 5 days after the last exposure to zineb. A high TTH level was recorded in the blood plasma of both zineb-treated groups.

Introduction

The toxic effects of dithiocarbamate compounds and their metabolites on the endocrine glands have been demonstrated in several studies (Smith, Finnegan, Larson, Sahyoun, Dreyfuss, Haag, 1953; Clayton, Hood, Barnes & Borgmann, 1957; Ivanova-Chemishanska, Markov & Dashev, 1971; Ivanova-Chemishanska & Mosheva-Izmirova, 1968; Ivanova-Chemishanska, Valcheva & Takeva, 1973; Seifter & Ehrlich, 1948; Graham & Hansen, 1972; Ulland, Weisburger, Weisburger, Rice & Cypher, 1972). Our previous work revealed the specific antithyroid action of zinc ethylenebis(dithiocarbamate) (zineb), manganese ethylenebis(dithiocarbamate) (maneb) and zinc manganese ethylenebis(dithiocarbamate) (mancozeb) as early as 24 hr after a single oral dose equivalent to half of the LD_{50}. This antithyroid action was indicated by a reduction in the rate of ^{131}I uptake (Ivanova-Chemishanska & Mosheva-Izmirova, 1968; Ivanova, Sheytanov & Mosheva-Izmirova, 1967). Daily treatment with 1/10 LD_{50} for 30 days resulted in similar but less marked changes accompanied by hypertrophy and hyperplasia of the thyroid gland (Ivanova-Chemishanska *et al.* 1971).

The present communication attempts to clarify some aspects of the thyroid gland-adenohypophysis interaction after long-term oral administration of zineb.

Experimental

The study was performed on male Wistar rats of mixed stock (body weight 200 g) divided into three groups, each of 35 animals. The two experimental groups were given a 10% aqueous suspension of zineb by stomach tube twice weekly for 4·5 months in doses of 1/10 or 1/100 LD_{50} (9·6 g/kg, determined in male rats). These two groups and the third (control) group were fed on a commercial chow.

At the end of the test period, the rats were killed and the tissues of the thyroid gland and the adenohypophysis were fixed in 4% neutral formaldehyde for 6 hr and then processed for staining with periodic acid–Schiff (PAS) reagent. Cryostat sections of the thyroid were stained for succinic dehydrogenase and cytochrome oxidase activities after brief fixation (4 min) in an absolute acetone–ethanol (1:1, v/v) mixture. PAS-stained sections of the thyroid were studied morphometrically and Student's *t* test was used for the statistical evaluation of the results.

Electron microscopy of the thyroid gland of four rats from each group followed fixation of the tissue in 2% Millonig-buffered osmium tetroxide solution, pH 7·3, and its embedding in Durcupan ACM Fluka (Fluka AG, Buchs, Switzerland). Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEM 100 B electron microscope.

Table 1. *Uptake of radioactive iodine by the thyroid of rats given zineb for 4·5 months by oral application*

Zineb dose level (% of LD_{50})	Time after injection (hr)...	Uptake of ^{131}I (% of administered dose)		
		2	4	24
0		11·2 ± 0·7	13·0 ± 3·9	20·7 ± 1·1
1		10·8 ± 0·7	14·3 ± 0·8	20·1 ± 2·2
10		16·2 ± 0·8*	23·3 ± 1·3*	29·3 ± 1·6*

Values represent the means ± SEM for determinations carried out on groups of ten rats, 5 days after administration of the final dose of zineb. Those marked with an asterisk differ significantly (Student's *t* test) from the corresponding control value: $*P < 0·05$.

Blood levels of thyrotropic hormone (TTH) were determined according to Querido, Kasennar & Lamayer (1953) as modified by Milanov (1968) the day after the last zineb dose was given. The rate of iodine uptake by the thyroid was measured in ten animals 5 days after the final zineb dose by injecting ip 1 μCi ^{131}I and determining the neck counts 2, 4 and 24 hr later. The data obtained were expressed as a percentage of the dose of ^{131}I administered.

Results

The data obtained from the ^{131}I test are represented in Table 1. Increased values of ^{131}I uptake were observed only in the animals that had received the higher doses of zineb (1/10 LD_{50}).

The morphological examination of the thyroid gland also showed marked changes only in this group. The glands were enlarged and histologically numerous microfollicles were observed. The average diameter of the follicles in the experimental animals was 29·0 ± 1·9 μm, whereas in the control rats it was 40·0 ± 2·1 μm ($P < 0·01$). The height of the follicular cells tended to increase, from 8 μm in the control animals to 12 μm in the experimental. Most of the thyroid cells were columnar and had vesicular nuclei; the cytoplasm was vacuolated, with a reduced number of PAS-positive granules. The PAS-staining of the luminar colloid substance was weak. Epithelial cords formed microfollicles in the interstitial spaces (Fig. 1). An increase in succinic dehydrogenase and cytochrome oxidase activities was detected histochemically.

Two types of follicular cells were observed by electron microscopy. The first type was a low cell with flattened nucleus and indistinct inner structure. The rough endoplasmic reticulum was well developed and its cisternae were enlarged to a varying extent. The Golgi lamellae were also dilated and looked optically empty. They were surrounded by numerous vesicles. Most cells of this type contained numerous dense bodies which were often collected in one region of the cell. As a rule the cells lacked microvilli, apical granules and vesicles. The mitochondria appeared normal. Numerous colloid droplets were observed in these cells and very often large irregular masses of colloid-like substances were visible in the cytoplasm. The cells underwent intensive desquamation and cellular debris filled the follicular lumina (Fig. 2).

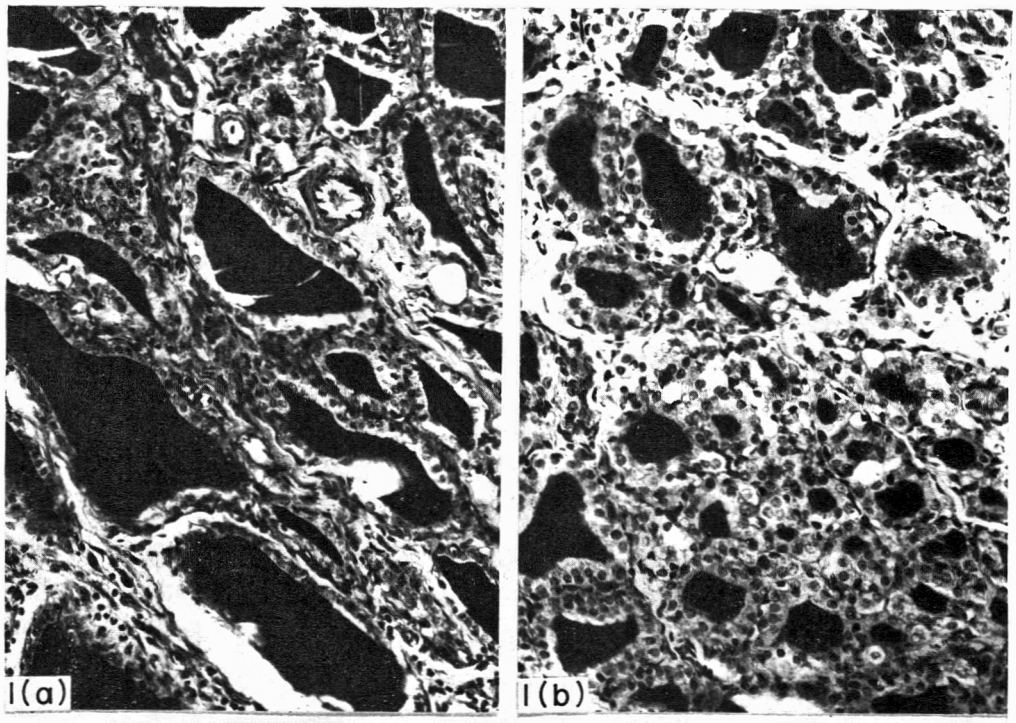
The second cell type was columnar. Numerous microvilli were present on the free surface, and many granules and vesicles occupied the apical cytoplasm. The endoplasmic cisternae were widened and contained substance of moderate density (Fig. 3). The Golgi stacks were made up of three or four parallel fragmented cisternae surrounded by numerous Golgi vesicles. The colloid droplets were small in size and apparently somewhat reduced in number. The colloid substance in the vicinity of such cells was of very low density. This type of cell was also subject to desquamation.

An increased incidence of PAS-positive granules was observed in the cytoplasm of the basophil cells of the adenohypophysis of the rats that had been given the higher dose level of zineb. They were localized in the periphery of the cells and their morphological features were comparable with the PAS-positive granules of the TTH-secreting cells. The basophil cells themselves were increased in number (Fig. 4). No morphological changes were found in the hypophysis of the animals given the lower dose level of zineb.

The determination of the blood levels of TTH at the end of the experiment demonstrated statistically significant ($P < 0·01$) increases in the values for both experimental groups, the mean values ± SEM for groups of ten rats being 9·26 ± 0·28 and 8·34 ± 0·24 ng TTH/ml plasma for the zineb-treated groups given the higher and lower dosage levels, respectively, compared with 4·35 ± 0·15 ng/ml for the control group.

Discussion

This study, like previous ones, showed the antithyroid action of the dithiocarbamates. These compounds cause a marked reduction in the iodine uptake of the gland and this can be detected 24 hr after oral treatment (Ivanova-Chemishanska *et al.* 1967; Ivanova-Chemishanska & Mosheva-Izmirova, 1968). In subacute experiments, the decreased iodine uptake of the thyroid has been shown to be accompanied by hypertrophy and hyperplasia of the parenchyma and signs of an increase in functional activity (Ivanova-Chemishanska *et al.* 1971). The morphological changes were much more evident in chronic conditions, when the gland became moderately enlarged. These changes were seen only with the higher dose level of zineb, and were in a distinct contrast to the findings in the control animals, although considerable variations in the normal ultrastructure of the gland have been shown to exist (Wissig, 1960). Under these conditions, the number of microfollicles increased, the thyroid cells were very often columnar and their cytoplasm was poor in mucopolysaccharide granules. Succinic-dehydrogenase and cytochrome-oxidase activities were raised. The rough endoplasmic reticulum was thickened in both subacute and long-term studies and the contents of its cisternae had a density similar to



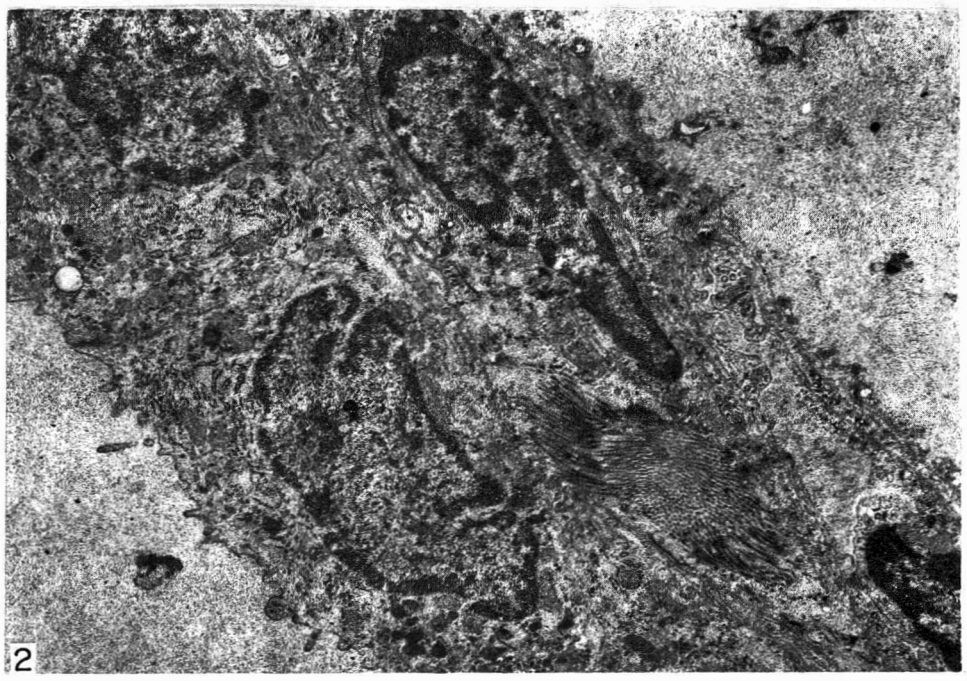
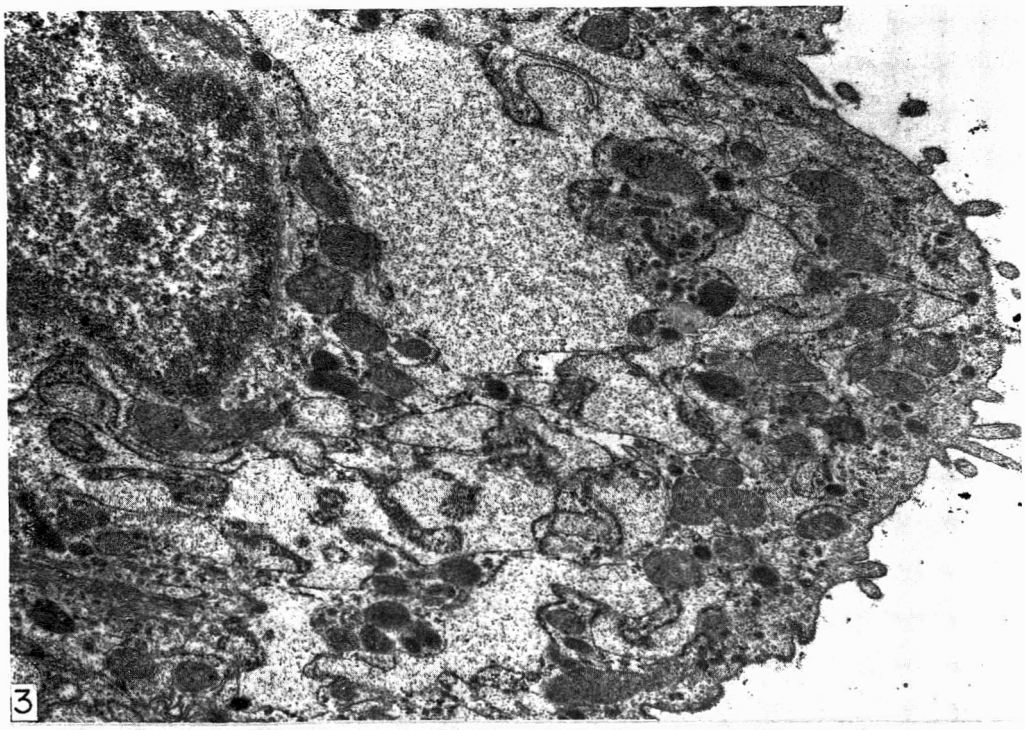


Fig. 1. Section of thyroid gland from a control rat (a) and from a rat treated with the higher dose of zineb (b), the latter showing microfollicles with reduced colloid substance. PAS ×240.

Fig. 2. Two neighbouring follicles, separated by a capillary and by connective tissue, from the thyroid of a zineb-treated (high dose) rat, showing completely desquamated epithelial cells in one follicle and a wall made up of low cells in the other. ×11,500.



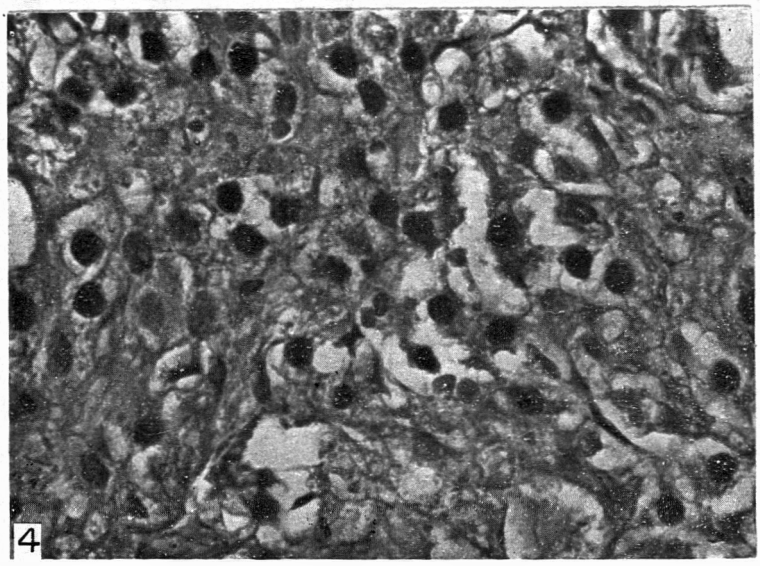


Fig. 3. Ultrastructure of the thyroid gland froma rat treated with zineb at the high dose level, showing the cytoplasm of a columnar cell with markedly dilated endoplasmic reticulum and a Golgi zone. The colloid substance in the lumen is of very low density. × 20,700.

Fig. 4. Section of adenohypophysis from a rat treated with zineb (high dose), showing increased number of basophil cells with PAS-positive granules. PAS × 480.

that of the luminar colloid. These findings are similar to the changes in the thyroid gland occurring after long-term TTH stimulation (Seljelid, Helminen & Thies, 1971). The changes in the Golgi apparatus suggest that enhanced thyroglobulin synthesis occurred. Numerous studies have shown that the Golgi complex is the site of addition of glycosyl groups to proteins (Beams & Kessel, 1968; Droz, 1966 & 1967; Favard, 1969; Favard-Sereno, 1971; Neutra & Leblond, 1966; Peterson & Leblond, 1964; Spiro, 1970).

Our unpublished observations on the administration of another pesticide of the same group (zinc propylenebis(dithiocarbamate); Antracol) for 4·5 months showed a decrease in the iodine uptake immediately after the end of the treatment. The typical rebound phenomenon, manifested by an increase in ^{131}I uptake 5 days after the end of the zineb administration, demonstrated the pronounced but reversible thyrostatic effect of this compound and also indicated the increased functional state of the gland (Studer & Greer, 1967). This phenomenon resembles the increased concentration rates in patients in the first days following a long treatment with thyrostatics, such as methylthiouracil (Iff, Burger, Studer & Wyss, 1967).

Previous studies suggested that the changes observed in the thyroid gland resulted from prolonged TTH stimulation. The increased values of TTH demonstrated in the blood plasma of both experimental groups in the present study, as well as the histological findings in the basophil cells of the adenohypophysis, suggest the participation of the anterior pituitary in the pathogenesis of the thyroid disorder. Most probably the changes observed in both the thyroid and pituitary glands were a compensatory response to the antithyroid effect of the pesticide. Further observations are necessary to clarify whether the thyroid–adenohypophysis interaction is influenced by a direct action of dithiocarbamate compounds on the glands or by an effect on the function of the cells secreting thyrotropin-releasing factor.

REFERENCES

Beams, H. W. & Kessel, R. G. (1968). The Golgi apparatus: Structure and function. *Int. Rev. Cytol.* **23,** 209.

Clayton, J. W., Hood, D., Barnes, J. K. & Borgmann, A. R. (1957). Chronic oral toxicity of manganese ethylenebisdithiocarbamate. *Am. ind. Hyg. Ass. Abstr. Ind. Hlth Conf.*

Droz, B. (1966). Elaboration de glycoproteins dans l'appareil de Golgi des cellules hépatiques chez le rat; étude radioautographique en microscopie électronique après injection de ^{3}H-galactose. *C.r. hebd. Séanc. Acad. Sci., Paris* **262,** 1766.

Droz, B. (1967). L'appareil de Golgi comme site d'incorporation du galactose-^{3}H dans les neurones ganglionaires spinaux chez le rat. *J. Microsc.* **6,** 419.

Favard, P. (1969). The Golgi apparatus. In *Handbook of Molecular Cytology.* Edited by A. Lima de Faria. p. 1130. North Holland Publ. Co., Amsterdam.

Favard-Sereno, Colette (1971). Cycles sécrétoires successifs au cours de l'élaboration des enveloppes de l'ovocyte chez le Grillon (insecte, orthoptère). Rôle de l'appareil de Golgi. *J. Microsc.* **11,** 401.

Graham, S. L. & Hansen, W. H. (1972). Effects of short-term administration of ethylenthiourea upon thyroid function of the rat. *Bull. env. contam. & Toxicol. (U.S.)* **7,** 19.

Iff, H. W., Burger, A., Studer, H. & Wyss, F. (1967). Thyrotrophin in rat and human serum after withdrawal of carbimazole. *Am. J. Physiol.* **213,** 250.

Ivanova, L., Sheytanov, M. & Mosheva-Izmirova, N. (1967). Changes in the functional state of the thyroid gland upon acute intoxication with certain dithiocarbamates—Zineb and Maneb. *C.r. Acad. bulg. Sci.* **20,** 1011.

Ivanova-Chemishanska, L., Markov, D. V. & Dashev, G. (1971). Light and electron microscopic observations on rat thyroid after administration of some dithiocarbamates. *Envir. Res.* **4,** 201.

Ivanova-Chemishanska, L. & Mosheva-Izmirova, N. (1968). Early changes in the absorbility of ^{131}I by the thyroid in acute experimental intoxication with dithan M-45. *C.r. Acad. bulg. Sci.* **21,** 965.

Ivanova-Chemishanska, L., Valcheva, V. & Takeva, Z. (1973). Effect of chronic peroral poisoning with Zineb on the reproductive capacity of white rats. In *Acta Medica Sociologica.* 1st Ed. p. 407. Medicina i Fizkultura, Sofia.

Milanov, S. (1968). Dosage of the thyrotropic hormone (TTH) in clinical practice. (In Bulgarian) *Vatr. Bol.* **3,** 333.

Neutra, Marian & Leblond, C. P. (1966). Synthesis of the carbohydrate of mucus in the Golgi complex as shown by electron microscope radioautography of goblet cells from rats injected with glucose-H^{3}. *J. Cell Biol.* **30,** 119.

Ovtracht, L. (1967). Ultrastructure des cellules sécrétrices de la glande multifide de l'Escargot. *J. Microsc.* **6,** 773.

Peterson, Marian R. & Leblond, C. P. (1964). Uptake by the Golgi region of glucose labeled with tritium in the 1 or 6 position, as an indicator of synthesis of complex carbohydrates. *Expl Cell Res.* **34,** 420.

Querido, A., Kasennar, A. A. H. & Lamayer, L. D. F. (1953). Assay of thyrotropic hormone by means of ^{131}I uptake in thyroid glands of iodocasein-treated mice. *Acta endoc., Buc.* **12,** 335.

Seifter, J. & Ehrlich, W. E. (1948). Goitrogenic compounds: Pharmacological and pathological effects. *J. Pharmac. exp. Ther.* **92,** 303.

Seljelid, R., Helminen, H. J. & Thies, G. (1971). Effect of long-term suppression and stimulation of rat thyroid with special reference to lysosomes. *Expl Cell Res.* **69,** 249.

Smith, R. B., Jr., Finnegan, J. K., Larson, P. S., Sahyoun, P. F., Dreyfuss, M. L. & Haag, H. B. (1953). Toxicologic studies on zinc and disodium ethylene bisdithiocarbamates. *J. Pharmac. exp. Ther.* **109,** 159.

Spiro, R. G. (1970). Glycoproteins. *A. Rev. Biochem.* **39,** 599.

Studer, H. & Greer, M. A. (1967). Thyroid function during the rebound phase following the discontinuation of antithyroid drugs. *Endocrinology* **80,** 52.

Ulland, B. M., Weisburger, J. H., Weisburger, Elizabeth K., Rice, J. M. & Cypher, R. (1972). Thyroid cancer in rats from ethylene thiourea intake. *J. natn. Cancer Inst.* **49,** 583.

Wissig, S. L. (1960). The anatomy of secretion in the follicular cells of the thyroid gland. I. The fine structure of the gland in the normal rat. *J. biophys. biochem. Cytol.* 7, 419.

Fd Cosmet. Toxicol. Vol. 13, pp. 449–457. Pergamon Press 1975. Printed in Great Britain

MONOGRAPHS

Monographs on Fragrance Raw Materials*

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BALSAM, CANADIAN

Description and physical properties: EOA Spec. no. 214. One of the chief constituents of this oil is 1-β-phellandrene (Guenther, 1952).
Occurrence: A liquid oleoresin found in the bark of the tree *Abies balsamea* (L.) Mill. (Fam. Pinaceae).
Preparation: The balsam is collected by breaking the blisters on the bark of the balsam tree. It is purified and clarified by filtration.
Uses: In public use since the 1930s. Use in fragrances in the USA amounts to less than 5000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·02	0·002	0·005	0·05
Maximum	0·15	0·02	0·03	0·2

Analytical data: Infra-red curve, RIFM no. 72-146.

Status

Fir balsam, Canadian was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1163). The Council of Europe (1974) included fir balsam, Canadian in the list of flavouring substances temporarily admitted for use, possibly with a limitation on the active principle in the final product.

Biological data

Acute toxicity. Both the acute oral LD_{50} value in rats and the acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Shelanski, 1973).

Irritation. Fir balsam, Canadian applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was not irritating (Shelanski, 1973). Tested at 2% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1973).

Sensitization. A maximization test (Kligman, 1966, modified) was carried out on 25 volunteers. The material was tested at a concentration of 2% in petrolatum and produced no sensitization reactions (Kligman. 1973).

References

Council of Europe (1974). Natural Flavouring Substances, Their Sources, and Added Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List N(1), Series 2, no. 3, p. 32. Strasbourg.
Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2115. *Fd Technol., Champaign* **19**(2), part 2, 155.
Guenther, E. (1952). *The Essential Oils.* Vol. VI, p. 232. D. Van Nostrand, Inc., Princeton, New Jersey.
Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.
Kligman, A. M. (1973). Report to RIFM, 10 July.
Shelanski, M. V. (1973). Report to RIFM, 30 January.

*The latest of the previous sets of these monographs appeared in *Food and Cosmetics Toxicology* 1975, **13**, 91.

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FIR NEEDLE OIL, CANADIAN

Synonym: Balsam fir oil.
Description and physical properties: *Food Chemicals Codex* (1972). The chief constituent of Canadian fir needle oil is 1-α-pinene (Guenther, 1952).
Occurrence: Found in the leaves of *Abies balsamea* (L.) Mill. (Fam: Pinaceae) (Guenther, 1952).
Preparation: By steam distillation of the needles and twigs of *A. balsamea* (*Fenaroli's Handbook of Flavor Ingredients*, 1971).
Uses: In public use since the 1930s. Use in fragrance in the USA amounts to less than 35,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·003	0·005	0·1
Maximum	0·3	0·03	0·1	1·0

Status

Fir needle oil, Canadian was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1163). The Council of Europe

(1974) included fir needle oil, Canadian in the list of flavouring substances temporarily admissible for use, possibly with a limitation on the active principle in the final product. The *Food Chemicals Codex* (1972) has a monograph on fir needle oil, Canadian.

Biological data

Acute toxicity. Both the acute oral LD_{50} value in rats and the acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Wohl, 1974).

Irritation. Fir needle oil, Canadian applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was not irritating (Wohl, 1974). Applied undiluted to the backs of hairless mice, it was not irritating (Urbach & Forbes, 1974). Tested at 10% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1974).

Sensitization. A maximization test (Kligman, 1966, modified) was carried out on 22 volunteers. The material was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Epstein, 1974).

Phototoxicity. No phototoxic effects were reported for fir needle oil, Canadian (Urbach & Forbes, 1974).

References

Council of Europe (1974). Natural Flavouring Substances, Their Sources, and Added Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List N(1), Series 2, no. 3, p. 32. Strasbourg.

Epstein, W. L. (1974). Report to RIFM, 20 May.

Fenaroli's Handbook of Flavor Ingredients (1971). Edited by T. E. Furia & N. Bellanca. p. 114. Chemical Rubber Co., Cleveland, Ohio.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2114. *Fd Technol., Champaign* **19**(2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 322. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.

Guenther, E. (1952). *The Essential Oils.* Vol. VI, p. 232. D. Van Nostrand, Inc., Princeton, New Jersey.

Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.

Urbach, F. & Forbes, P. D. (1974). Report to RIFM, 12 April.

Wohl, A. S. (1974). Report to RIFM, 2 April.

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FIR NEEDLE OIL, SIBERIAN

Description and physical properties: EOA Spec. no. 50. The chief constituent of fir needle oil, Siberian is *l*-bornyl acetate (Guenther, 1952).

Occurrence: Found in the needles and twigs of *Abies sibirica* Ledeb (Fam: Pinaceae).

Preparation: By steam distillation of the needles and twigs of *Abies sibirica* Ledeb.

Uses: In public use since the 1900s. Use in fragrances in the USA amounts to about 35,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·03	0·003	0·005	0·1
Maximum	0·3	0·03	0·1	0·25

Status

Fir needle oil, Siberian was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1163). The Council of Europe (1974) included fir needle oil, Siberian in the list of flavouring substances temporarily admitted for use, possibly with a limitation on the active principle in the final product. The *Food Chemicals Codex* (1972) has a monograph on fir needle oil, Siberian.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 10·2 g/kg (6·7–13·7 g/kg) (Moreno, 1971). The acute dermal LD_{50} value in rabbits exceeded 3 g/kg (Moreno, 1971).

Irritation. Fir needle oil, Siberian applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1971). Tested at 2·5% in petrolatum, it produced a mild irritation after a 48-hr closed-patch test on human subjects (Kligman, 1971).

Sensitization. A maximization test (Kligman, 1966, modified) was carried out on 25 volunteers. The material was tested at a concentration of 2·5% in petrolatum and produced no sensitization reactions (Kligman, 1971).

References

Council of Europe (1974). Natural Flavouring Substances, Their Sources, and Added Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List N(1), Series 2, no. 5, p. 32. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2905. *Fd Technol., Champaign* **19**(2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 323. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.

Guenther, E. (1952). *The Essential Oils.* Vol. VI, p. 214. D. Van Nostrand, Inc., Princeton, New Jersey.

Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.

Kligman, A. M. (1971). Report to RIFM, 2 April.

Moreno, O. M. (1971). Report to RIFM, 24 March.

GERANIUM OIL MOROCCAN

Description and physical properties: EOA Spec. no. 160. The main constituents of geranium oil Moroccan are geraniol and citronellol (*Fenaroli's Handbook of Flavor Ingredients*, 1971).

Occurrence: Found in the leaves and stems of *Pelargonium roseum* wild and allied sorts (*P. graveolens* Ait and *P. graveolens* × *P. terebinthaceum*) (Fam.: Geraniaceae).

Preparation: By steam distillation of the leaves and stems of the fresh plant.

Uses: In public use since the 1920s. Use in fragrances in the USA amounts to approximately 100,000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·002	0·005	0·05	0·3
Maximum	0·3	0·03	0·3	1·0

Status

Geranium oil was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (GRAS). The Council of Europe (1974) included geranium oil in the list of substances, spices and seasonings deemed admissible for use, with a possible limitation of the active principle in the final product.

Biological data

Acute toxicity. The acute dermal LD_{50} value in guinea-pigs was reported as >5 g/kg (Moreno, 1974).

Irritation. Undiluted geranium oil Moroccan applied to the backs of hairless mice was not irritating (Urbach & Forbes, 1974). Applied full strength to intact or abraded rabbit and guinea-pig skin for 24 hr under occlusion, it was slightly irritating (Moreno, 1974). Tested at 10% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1974).

Sensitization. A maximization test (Kligman, 1966, modified) was carried out on 26 volunteers. The material was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Epstein, 1974).

Phototoxicity. No phototoxic effects were reported for geranium oil Moroccan (Urbach & Forbes, 1974).

References

Council of Europe (1974). Natural Flavouring Substances, Their Sources, and Added Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List N(1), Series 1(b), no. 324, p. 87. Strasbourg.

Epstein, W. L. (1974). Report to RIFM, 19 August.

Fenaroli's Handbook of Flavor Ingredients (1971). Edited by T. E. Furia, & N. Bellanca. p. 122. Chemical Rubber Co., Cleveland, Ohio.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2508. *Fd Technol. Champaign* **19**(2), part 2, 155.

Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.

Moreno, O. M. (1974). Report to RIFM, 22 May.

Urbach, F. & Forbes, P. D. (1974). Report to RIFM, 1 May.

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GERANYL ISOBUTYRATE

Synonym: *trans*-3,7-Dimethyl-2,6-octadienyl isobutyrate.

Structure:

$$CH_3 \cdot C(CH_3):CH \cdot [CH_2]_2 \cdot C(CH_3):CH \cdot CH_2 \cdot OCO \cdot CH(CH_3)_2.$$

Description and physical properties: A colourless oily liquid.

Occurrence: Reported to be found in the essential oils of Japanese hops and valerian (*Fenaroli's Handbook of Flavor Ingredients*, 1971).

Preparation: From geraniol and isobutyric acid or by any other suitable means.

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	0·03	0·003	0·01	0·12
Maximum	0·15	0·015	0·05	0·6

Analytical data: Gas chromatogram, RIFM no. 72-151; infra-red curve, RIFM no. 72-151.

Status

Geranyl isobutyrate was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1974) included geranyl isobutyrate, at a level of 15 ppm, in the list of artificial flavouring substances that may be added to foodstuffs without hazard to public health.

Biological data

Acute toxicity. Both the acute oral LD_{50} value in rats and the acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Shelanski, 1973).

Irritation. Geranyl isobutyrate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was mildly irritating (Shelanski, 1973). Tested at 10% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1973).

Sensitization. A maximization test (Kligman, 1966, modified) was carried out on 25 volunteers. The material was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Kligman, 1973).

References

Council of Europe (1974). Natural Flavouring Substances, Their Sources, and Added Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List 1, no. 306, p. 189, Strasbourg.

Fenaroli's Handbook of Flavor Ingredients (1971). Edited by T. E. Furia & N. Bellanca. p. 412. Chemical Rubber Co., Cleveland, Ohio.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2513. *Fd Technol., Champaign* **19**(2), part 2, 155.

Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.

Kligman, A. M. (1973). Report to RIFM, 12 February.

Shelanski, M. V. (1973). Report to RIFM, 30 January.

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2-*n*-HEPTYL CYCLOPENTANONE

Synonym: α-Heptyl cyclopentanone.

Structure: 

Description and physical properties: *Givaudan Index* (1961).

Occurrence: Has apparently not been reported to occur in nature.

Preparation: By catalytic reduction of heptylidene cyclopentanone (Arctander, 1969).

Uses: In public use since the 1940s. Use in fragrances in the USA amounts to less than 1000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·2
Maximum	0·1	0·01	0·03	1·0

Analytical data: Gas chromatogram, RIFM no. 72-147; infra-red curve, RIFM no. 72-147.

Status

2-*n*-Heptyl cyclopentanone is not included in the listings of the FDA, FEMA (1965) or the Council of Europe (1974), nor in the *Food Chemicals Codex* (1972).

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as > 5 g/kg (Shelanski, 1973). The acute dermal LD_{50} value in rabbits was reported as 5 g/kg (Shelanski, 1973).

Irritation. 2-*n*-Heptyl cyclopentanone applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was irritating (Shelanski, 1973). Tested at 10% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1973).

Sensitization. A maximization test (Kligman, 1966, modified) was carried out on 25 volunteers. The material was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Kligman, 1973).

References

Arctander, S. (1969). *Perfume and Flavor Chemicals (Aroma Chemicals)*. Vol. 1, no. 1534. S. Arctander, Montclair, New Jersey.

Council of Europe (1974). Natural Flavouring Substances, Their Sources, and Added Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. *Fd Technol., Champaign* **19**(2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. National Academy of Sciences–National Research Council Publ. 1406. Washington, D.C.

Givaudan Index (1961). *Specifications of Synthetics and Isolates for Perfumery*. 2nd ed., p. 44. Givaudan-Delawanna, Inc., New York.

Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.

Kligman, A. M. (1973). Report to RIFM, 9 May.

Shelanski, M. V. (1973). Report to RIFM, 30 January.

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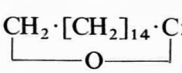
HEXADECANOLIDE

Synonyms: Dihydroambrettolide; cyclohexadecanolide; 16-hydroxyhexadecanoic acid lactone.

Structure: 

Description and physical properties: An opaque crystalline mass.

Occurrence: Has apparently not been reported to occur in nature.

Preparation: By the persulphuric acid (or other peracid) oxidation of cyclohexadecanone.

Uses: In public use since the 1950s. Use in fragrances in the USA amounts to less than 1000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·001	–	0·001	0·04
Maximum	0·01	–	0·003	0·4

Analytical data: Gas chromatogram, RIFM no. 74-206; infra-red curve, RIFM no. 74-206.

Status

Hexadecanolide is not included in the listings of the FDA, FEMA (1965) or the Council of Europe (1974), nor in the *Food Chemicals Codex* (1972).

Biological data

Acute toxicity. Both the acute oral LD_{50} value in rats and the acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Moreno, 1974).

Irritation. Hexadecanolide applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1974). Tested at 4% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1974).

Sensitization. A maximization test (Kligman, 1966, modified) was carried out on 25 volunteers. The material was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1974).

References

Council of Europe (1974). Natural Flavouring Substances. Their Sources, and Added Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. *Fd Technol., Champaign* **19**(2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. National Academy of Sciences–National Research Council Publ. 1406. Washington, D.C.

Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screeening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.

Kligman, A. M. (1974). Report to RIFM, 12 September.

Moreno, O. M. (1974). Report to RIFM, 26 August.

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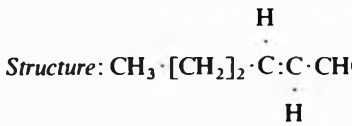
HEXEN-2-AL

Synonyms: *trans*-2-Hexenal; β-propyl acrolein.



Structure: $CH_3 \cdot [CH_2]_2 \cdot C{:}C \cdot CHO$.

Description and physical properties: A colourless liquid.

Occurrence: Reported to be found in the distillation waters of numerous plants; also identified among the constituents of a dozen essential oils (Gildemeister & Hoffman, 1963).

Preparation: From interaction of butyraldehyde acetal with vinyl ether followed by hydrolysis or by any other suitable means.

Uses: In public use since the 1950s. Use in fragrances in the USA amounts to approximately 1000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·003	0·04
Maximum	0·1	0·01	0·02	0·4

Analytical data: Gas chromatogram, RIFM no. 72-159; infra-red curve, RIFM no. 72-159.

Status

Hexen-2-al was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (21 CFR 121.1164). The Council of Europe (1974) included hexen-2-al in the list of artificial flavouring substances that may be added temporarily to foodstuffs without hazard to public health.

Biological data

Acute toxicity. The acute oral LD_{50} value in rats was reported as 0·85 g/kg (0·65–1·05 g/kg) (Moreno, 1973). The oral LD_{50} values for *trans*-2-hexenal were reported as 780–1130 and 1550–1750 mg/kg in rats and mice, respectively, and the ip LD_{50} values were reported as 100–200 mg/kg in both species (Gaunt, Colley, Wright, Creasey, Grasso & Gangolli, 1971). The acute dermal LD_{50} value in rabbits was reported as 0·60 g/kg (0·37–0·83 g/kg) (Moreno, 1973).

Subacute toxicity. trans-2-Hexenal was fed to rats at dietary levels of 0 (control), 260, 640, 1600 or 4000 ppm for 13 wk (Gaunt *et al.* 1971). The group given 4000 ppm showed a slight (but not statistically significant) reduction in growth rate associated with a reduced intake of a diet shown to be less palatable than the control diet. The treatments had no effect on haematology or serum and urine analyses. The only consistent abnormality was an increase in the ovary weights of all treated female rats, but this was unaccompanied by any change in ovarian histology. This effect was not dose-related and in further studies (Gaunt *et al.* 1971), in which female rats were fed *trans*-2-hexenal at 4000 ppm of the diet for 13 wk and female rabbits were given daily oral doses of 200 mg/kg for 13 wk, no effect on ovary weight was found. A mild anaemia, increased stomach weights and acute gastric ulceration were seen in the treated rabbits, but it was considered likely that these effects were due to the high local concentrations of the test compound resulting from oral intubation. The no-untoward-effect level for *trans*-2-hexenal in the rat study was 1600 ppm of the diet (approximately 80 mg/kg/day).

Irritation. Hexen-2-al applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was moderately irritating (Moreno, 1973). Tested at 4%

in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1973).

Sensitization. A maximization test (Kligman, 1966, modified) was carried out on 25 volunteers. The material was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1973).

References

Council of Europe (1974). Natural Flavouring Substances, Their Sources, and Added Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List 2, no. 2007, p. 281. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2560. *Fd Technol., Champaign* **19**(2), part 2, 155.

Gaunt, I. F., Colley, J., Wright, M., Creasey, Margaret, Grasso, P. & Gangolli, S. D. (1971). Acute and short-term toxicity studies on *trans*-2-hexenal. *Fd Cosmet. Toxocol.*, **9**, 775.

Gildemeister, E. u. Hoffman, F. (1963). *Die Ätherischen Öle.* Vol. IIIc. p. 38. Akademie Verlag, Berlin.

Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.

Kligman, A. M. (1973). Report to RIFM, 9 October.

Moreno, O. M. (1973). Report to RIFM, 23 July.

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cis-3-HEXENYL ACETATE

Synonym: *cis*-3-Hexen-1-yl acetate.

Structure:

$$CH_3 \cdot CH_2 \cdot \underset{H}{C} : \underset{H}{C} \cdot [CH_2]_2 \cdot OCO \cdot CH_3.$$

Description and physical properties: EOA Spec. no. 269.

Occurrence: Reported to occur in tea leaves and *Achillea fragrantissima.*

Preparation: By the acetylation of *cis*-3-hexenol.

Uses: In public use since the 1950s. Use in fragrances in the USA amounts to approximately 2000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·05
Maximum	0·15	0·015	0·02	1·0

Status

cis-3-Hexenyl acetate was granted GRAS status by FEMA (1971).

Biological data

Acute toxicity. Both the acute oral LD_{50} value in rats and the acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Wohl, 1974).

Irritation. cis-3-Hexenyl acetate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was not irritating (Wohl, 1974). Tested at a concentration of 10% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1974).

Sensitization. A maximization test (Kligman, 1966, modified) was carried out on 22 volunteers. The material was tested at a concentration of 10% in petrolatum and produced no sensitization reactions (Epstein, 1974).

References

Epstein, W. L. (1974). Report to RIFM, 20 May.

Flavoring Extract Manufacturers' Association (1971). Survey of flavoring ingredient usage levels. No. 3171. *Fd Technol., Champaign* **24**(5), 25.

Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.

Wohl, A. J. (1974). Report to RIFM, 2 April.

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n-HEXYL ETHYL ACETOACETATE

Synonyms: Ethyl-2-hexyl acetoacetate; hexyl acetoacetic ester.

Structure:

$$CH_3 \cdot CO \cdot CH([CH_2]_5 \cdot CH_3) \cdot OCO \cdot CH_2 \cdot CH_3.$$

Description and physical properties: A colourless slightly oily liquid.

Occurrence: Has apparently not been reported to occur in nature.

Preparation: By the alkylation of ethyl acetoacetate with hexyl bromide or by any other suitable method.

Uses: In public use since the 1950s. Use in fragrances in the USA amounts to approximately 1000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·005	0·05
Maximum	0·1	0·01	0·02	0·4

Analytical data: Gas chromatogram, RIFM no. 72-148; infra-red curve, RIFM no. 72-148.

Status

n-Hexyl ethyl acetoacetate is not included in the listings of the FDA, FEMA (1965) or the Council

of Europe (1974), nor in the Food Chemicals Codex (1972).

Biological data

Acute toxicity. Both the acute oral LD_{50} value in rats and the acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Shelanski, 1973).

Irritation. *n*-Hexyl ethyl acetoacetate applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was not irritating (Shelanski, 1973). Tested at 4% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Kligman, 1972).

Sensitization. A maximization test (Kligman, 1966, modified) was carried out on 25 volunteers. The material was tested at a concentration of 4% in petrolatum and produced no sensitization reactions (Kligman, 1972).

References

Council of Europe (1974). Natural Flavouring Substances. Their Sources, and Added Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. *Fd Technol., Champaign* **19**(2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. National Academy of Sciences–National Research Council Pybl. 1406, Washington, D.C.

Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.

Kligman, A. M. (1972). Report to RIFM, 22 November.

Shelanski, M. V. (1973). Report to RIFM, 30 January.

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RUE OIL

Description and physical properties: EOA Spec. no. 90. The main constituent of rue oil is methyl nonyl ketone (Guenther, 1949).

Occurrence: Found in the plants *Ruta montana* L., *R. gravolens* L. and *R. bracteosa* L. (Fam: Rutaceae).

Preparation: By steam distillation from the fresh blossoming plants.

Uses: In public use before the 1900s. Use in fragrances in the USA amounts to less than 1000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·003	0·04
Maximum	0·05	0·005	0·01	0·15

Analytical data: Gas chromatogram, RIFM no. 74-114; infra-red curve, RIFM no. 74-114.

Status

Rue oil was granted GRAS status by FEMA (1965) and is approved by the FDA for food use (GRAS). The Council of Europe (1974) included rue oil in the list of flavouring substances temporarily admitted for use, possibly with a limitation on the active principle in the final product. The *Food Chemicals Codex* (1972) has a monograph on rue oil.

Biological data

Acute toxicity. Both the acute oral LD_{50} value in rats and the acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Moreno, 1974). The acute oral LD_{50} (g/kg) of volatile oils of Ruta species to white mice was 2·54 for leaves and 3·73 for fruit of *R. graveolens*, 3·99 for leaves of *R. bracteosa* and 2·07 for leaves of *R. divaricata* (Srepel, 1964; Srepel & Akacic, 1962).

In guinea-pigs and rabbits, large oral doses of commercial rue oil produced dyspnoea, diarrhoea, torpor, sometimes haematemesis and loss of weight. The most important lesions were granular fatty hepatitis and parenchymatous nephritis (Patoir, Patoir & Bédrine, 1938).

Anthelmintic activity. The anthelmintic activity *in vitro* was proportional to the concentration in the oil of methyl nonyl ketone, the major constituent. LC_{50} (g/100 ml) values of *R. graveolens*, *R. bracteosa* and *R. divaricata* were 0·094–0·155 for *Tubiflex rivulorum* (worm), 0·076–0·128 for *Hirudo medicinalis* (leech) and 0·063–0·120 for *Ascaris suilla* (nematode). The LT_{100} value for *Anguillula aceti* (nematode) ranged from 10 min at 200 mg/100 ml to 45 min at 20 mg/100 ml (Srepel, 1962).

Irritation. Undiluted rue oil applied to the backs of hairless mice was not irritating (Urbach & Forbes, 1974). Applied full strength to intact or abraded rabbit skin for 24 hr under occlusion, it was slightly irritating (Moreno, 1974). When tested at 1% in petrolatum on human subjects by a 48-hr occluded-patch test, rue oil was not irritating (Kligman, 1974). Rue oil may harm the mucous membranes and irritate the skin (Arctander, 1960), producing erythema and vesication after frequent dermal contact (*Merck Index*, 1968).

Human toxicity. Taken internally, rue oil may produce haemorrhages (Arctander, 1960). Ingestion of large quantities of rue oil causes epigastric pain, nausea, vomiting, confusion, convulsions, and death; abortion may also result (*Merck Index*, 1968).

Sensitization. A maximization test (Kligman, 1966, modified) was carried out on volunteers, using 1% rue oil in petrolatum. No sensitization reactions were produced (Kligman, 1974).

Phototoxicity. Distinct phototoxic effects were reported for two samples of undiluted rue oil (Urbach & Forbes, 1974a). Various concentrations of rue oil in methanol were also tested for phototoxicity in mice, positive results being obtained with concentrations of 100, 50, 25, 12·5, 6·25 and 3·125%, borderline (positive and negative) results with 1·56% and negative results with 0·78% (Urbach & Forbes, 1974b). Rue oil has been included in a list of plants reported to cause phototoxicity (Pathak, Daniels & Fitzpatrick, 1962).

References

Arctander, S. (1960). *Perfume and Flavor Materials of Natural Origin.* no. 563. S. Arctander, Elizabeth, New Jersey.

Council of Europe (1974). Natural Flavouring Substances, Their Sources, and Added Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. List N(1) Series 2, no. 412, p. 102. Strasbourg.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. No. 2995. *Fd Technol., Champaign* **19**(2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. p. 705. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.

Guenther, E. (1949). *The Essential Oils.* Vol. III, p. 378. D. Van Nostrand, Inc., New York.

Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.

Kligman, A. M. (1974). Report to RIFM, 5 June.

Merck Index (1968). *An Encyclopedia of Chemicals and Drugs.* 8th ed., pp. 761 & 925. Merck & Co., Rahway, New Jersey.

Moreno, O. M. (1974). Report to RIFM, 23 January.

Patoir, A., Patoir, G. et Bédrine, H. (1938). Note sur l'action de l'essence de Rue sur l'organisme animal. *C.r. Séanc. soc. Biol.* **127**, 1324.

Pathak, M. A., Daniels, F. & Fitzpatrick, T. B. (1962). The presently known distribution of furocoumarins (psoralens) in plants. *J. invest. Derm.* **39**, 225.

Srepel, B. & Akacic, B. (1962). Ispitivanje anthelmintickog djelovanja etericnih ulja roda Ruta. *Acta pharm. jugosl.* **12**, 79.

Srepel, B. (1964). Prüfung eines weniger bekannten ätherischen Öles des Kroatischen Küstengebietes. *Bull. scient. Cons. Acads RPF Yougosl.* **9**(4/5), 109.

Urbach, F. & Forbes, P. D. (1974a). Report to RIFM, 22 January & 1 May.

Urbach, F. & Forbes, P. D. (1974b). Report to RIFM, 6 December.

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ANISYLIDENE ACETONE

Synonyms: Methyl-*p*-methoxycinnamylketone; 4-(*p*-methoxyphenyl)-3-buten-2-one.

Structure: $CH_3 \cdot O \cdot C_6H_4 \cdot CH{:}CH \cdot CO \cdot CH_3$.

Description and physical properties: White or yellowish leafy crystals.

Occurrence: Has apparently not been reported to occur in nature.

Preparation: From anisaldehyde and acetone by condensation using a suitable catalyst.

Uses: In public use since the 1950s. Use in fragrances in the USA amounts to less than 1000 lb/yr.

Concentration in final product (%):

	Soap	*Detergent*	*Creams, lotions*	*Perfume*
Usual	0·01	0·001	0·01	0·06
Maximum	0·1	0·01	0·1	0·2

Status

Anisylidene acetone is not included in the listings of the FDA, FEMA (1965) or the Council of Europe (1974), nor in the *Food Chemicals Codex* (1972).

Biological data

Acute toxicity. Both the acute oral LD_{50} value in rats and the acute dermal LD_{50} value in rabbits exceeded 5 g/kg (Wohl, 1974).

Irritation. Anisylidene acetone applied full strength to intact or abraded rabbit skin for 24 hr under occlusion was not irritating (Wohl, 1974). Tested at a concentration of 2% in petrolatum, it produced no irritation after a 48-hr closed-patch test on human subjects (Epstein, 1974).

Sensitization. A maximization test (Kligman, 1966, modified) was carried out on 22 volunteers. The material was tested at a concentration of 2% in petrolatum and produced two sensitization reactions out on the 22 tested (Epstein, 1974).

Antitumour activity. In vitro studies indicated weak antitumour activity for anisylidene acetone (Doré, 1973).

Metabolism. When the side chain of a mixed ketone contains a double bond, both the keto group and the double bond are potentially reducible *in vivo*. In a related material, methyl styryl ketone ($C_6H_5 \cdot CH{:}CH \cdot CO \cdot CH_3$), the keto group appears to be more readily reduced than the double bond, with reduction via $C_6H_5 \cdot CH{:}CH \cdot CH(OH) \cdot CH_3$ to the completely reduced carbinol compound $C_6H_5 \cdot [CH_2]_2 \cdot CH(OH) \cdot CH_3$, which is found as the main product (Fischer & Bielig, 1940). The ether link is relatively stable in substituted anisoles, such as anethole (p-$CH_3 \cdot OC_6H_4 \cdot CH{:}CH \cdot CH_3$), containing a potential carboxyl group attached to the aromatic ring (Williams, 1959).

References

Council of Europe (1974). Natural Flavouring Substances, Their Sources, and Added Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. Strasbourg.

Doré, J. C., Sekera, A., Redeuilh, G. et Viel, C. (1973). Chimiothérapie antitumorale et synthèses dans le domaine des antitumoraux d'origine naturelle. IV. Remplacement du NO_2 de B-nitrostyrènes actifs par divers substituants électroattracteurs. *Chimie Thér.* **8**, 75.

Epstein, W. L. (1974). Report to RIFM, 20 May.

Fischer, F. G. u. Bielig, H. J. (1940). Über die Hydrierung ungesättigter Stoffe im Tierkörper. (Biochemische Hydrierungen VII.) *Hoppe-Seyler's Z. physiol. Chem.* **266**, 73.

Flavoring Extract Manufacturers' Association (1965). Survey of flavoring ingredient usage levels. *Fd Technol., Champaign* **19**(2), part 2, 155.

Food Chemicals Codex (1972). 2nd ed. Prepared by the Committee on Specifications, Food Chemicals Codex, of the Committee on Food Protection. National Academy of Sciences–National Research Council Publ. 1406, Washington, D.C.

Kligman, A. M. (1966). The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. *J. invest. Derm.* **47**, 393.

Williams, R. T. (1959). *Detoxication Mechanisms. The Metabolism and Detoxication of Drugs, Toxic Substances and Other Organic Compounds.* 2nd ed., p. 340. Chapman & Hall Ltd., London.

Wohl, A. J. (1974). Report to RIFM, 2 April.

Fd Cosmet. Toxicol. Vol. 13, pp. 459–464. Pergamon Press 1975. Printed in Great Britain

Review Section

BOOK REVIEWS

Natural Flavouring Substances, Their Sources, and Added Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. Council of Europe, Strasbourg. Maisonneuve S. A., Sainte-Ruffine, 1974. pp. 463. 160 Fr.

First issued in 1970 under the title *Natural and Artificial Flavouring Substances* (*Cited in F.C.T.* 1971, **9**, 251), this document has been considerably revised and enlarged. A single volume now incorporates both the French and English versions, and is bound in plastic rather than in the flimsy paper used for the first edition.

The broad categories of natural and artificial flavourings remain, classified in each case according to whether they are considered "admissible" or "temporarily admissible" or have not been fully evaluated, the last subdivision replacing the "not admissible at present" category of the previous version. Consecutive numbering has been introduced for artificial flavourings, with numbers 1–2000 reserved for acceptable flavourings, 2001–4000 for those that are temporarily acceptable and 4001–7000 for those not fully evaluated. There are now 692 artificial flavourings in the first category, 284 in the second and 246 in the third, compared with the previous totals of 611, 236 and 199, respectively. Additions have been made chiefly at the end of each list, rather than according to chemical class; this preserves the original order to a large extent, although deletions and occasional insertions elsewhere mean that few compounds have the same number as before.

Notable additions to the lists of admissible or temporarily admissible artificial flavourings are some of the γ- and δ-lactones, specifically ones devoid of double bonds of the type thought to be associated with carcinogenic potential and those for which chronic toxicity data have become available. Several of these were originally classified as of definite toxicity, a category that now includes only chloroform, ethylmethylphenyl glycidate and 2-oxo-(5 or 6)-hexyl-1,4-dioxan.

The new edition retains the maximum levels of use and acceptable daily intakes previously specified for acceptable artificial flavourings, with only a few changes. It is somewhat odd that similar limitations are given for the new compounds listed in the temporarily admissible series and the additional data required are not specified, in contrast to the procedure followed for the materials originally listed in this group.

No additions or deletions appear to have been made to the list of natural flavourings, but it is good to find that this list is now printed so that two open pages may be read as one sheet. This dispenses with the annoying juggling necessitated by the previous version, in which opposite pages faced contrarily in opposite directions. The preambles to each section have also stayed largely unchanged—and still include 1 July 1973 as the date by which data on temporarily admissible flavourings should be made available!

It is a great pity that the volume still contains no cumulative index, each category of artificial flavourings being listed separately in English and French at the end. It may also be remarked that some of the classifications of individual flavouring materials still seem somewhat arbitrary, perhaps because the relevant toxicological data existing in the literature have not been submitted to the Council. However, as long as it is treated as a guide rather than a bible, this publication should find a deserved place in the reference library of anyone concerned with the use of flavouring materials.

Toxicity of Pure Foods. By E. M. Boyd. Edited by C. E. Boyd. CRC Press, Ohio, 1973. pp. iii + 260. $35.00.

The concept apparently first propounded by Paracelsus more than four centuries ago that there is nothing without poisonous qualities and it is only the dose that makes a thing a poison is perhaps the central axiom of contemporary toxicology. While the chief value of this latest book from the CRC Press lies in its final explosion of the myth of 'non-toxic' natural foods, however, it lays little emphasis on the equally fallacious concept of 'pure' food in the context of man's daily diet.

Successive chapters describe the toxicological implications of an excessive intake of carbohydrates, fats and oils, proteins and vitamins in experimental animals. The studies reported are almost entirely acute and short-term investigations, and they demonstrate convincingly that for certain 'pure' foods the doses required to produce toxic or even lethal symptoms in animals are well within the limits of conceivable human consumption. To illustrate how tenuous is the line separating definitions of food additives and food itself, the authors have also included chapters on gum tragacanth, iron, silicates and tannic acid. The studies reported were prompted by evidence that while drug toxicity in experimental animals was augmented by many vitamin-deficient diets, the effect was not overcome by vitamin supplementation of the diet; the only common feature of these diets was their content of large amounts of purified or semipurified foodstuffs. Taking the question to what may be considered, in this context, the ultimate limits, the authors have included a chapter on the toxic effects of distilled water, which has been shown to produce minor evidence of toxicity in daily doses of 20 mg/kg in rats.

Overall, this book issues no threats to reasonably abstemious individuals, but may constitute a cautionary tale for those whose voracious appetites render them oblivious to the 'natural' food items in their

diet. At $35.00 the volume may be considered expensive for its mere 260 pages, but its presentation is good and it is easy to read.

Datensammlung zur Toxikologie der Herbizide. Series 1. Deutsche Forschungsgemeinschaft—Kommission für Pflanzenschutz-, Pflanzenbehandlungs- und Vorratsschutzmittel. Verlag Chemie GmbH, Weinheim, 1974. pp. 152. DM 58.

A toxicological working group of the commission of the Deutsche Forschungsgemeinschaft concerned with plant-protection, plant-treatment and storage-protection agents has produced a series of monographs on important herbicides, considered from the standpoints not only of occupational hazard and the hazard to the consumer from possible crop contamination but also of environmental protection. The data reproduced have been collected both from published literature and from the manufacturers of the materials concerned and the monographs should therefore present a comprehensive picture on the data available and provide some indication of where essential information is lacking.

The monographs are all arranged in a standard form, which facilitates their use. An introductory section, providing details of identification and physical properties, is followed by a series of sections dealing in turn with range of application, general functional characteristics, toxicity studies in animals, *in vitro* studies (where available), metabolism in animals and in plants, breakdown in soils and water, effects in man and in domestic, farm and wild animals, interactions with other materials, and therapy in cases of poisoning, culminating in a paragraph presenting an overall toxicological assessment and in a list of relevant references.

The first set of monographs issued covers amitrol, atrazine, barban, chlorbufan, chlorpropham, diallate, diuron, phenmedipham, propham and triallate, and 2-methyl-4-chlorophenoxyacetic acid, 4-(2-methyl-4-chlorophenoxy)butyric acid, dichlorprop, mecoprop, 2,4-D and 4-(2,4-dichlorophenoxy)butyric acid and their salts and esters. Separate appendices provide lists of commercial herbicidal preparations containing these compounds together with their trade-names and manufacturers, and the addresses of the latter.

To enable subsequent monographs to be incorporated in logical sequence, the first set has been published in loose-leaf form in a convenient PVC binder. The clear layout, and the fact that data are tabulated where possible, will help those without a profound knowledge of German to obtain useful information from these monographs.

Progress in Toxicology. Special Topics. Vol. 1. By G. Zbinden. Springer-Verlag, Berlin, 1973. pp. 88. $4.70.

This small volume, comprising a collection of essays on various aspects of drug toxicology, is the first of a series planned by the author. By dealing with "just a few aspects of toxicology at one time" and publishing "at frequent intervals", the author hopes to overcome the problems inherent in the production of a comprehensive textbook in a field as broad and as rapidly expanding as toxicology. Nevertheless the present volume covers a remarkably wide range of topics and does so with a degree of insight that may no doubt be attributed to the author's experience, formerly as a research director of a large American pharmaceutical company and currently as an academic and consultant to various regulatory agencies.

The first and longest essay considers the toxicological prerequisites for the study and release of new drugs. Currently accepted procedures are examined critically with reference to possible alternative protocols and the dangers of too rigid an approach to drug testing are emphasized. A useful inclusion here is an extensive check-list of the type of information that may be required before or during clinical trials or following the release of a new drug. The following two essays deal with the anticipation of adverse effects from a consideration of a compound's known chemical and biological properties and with the problem of assessing whether the effects of a drug in animal systems are of significance in relation to its use in man. Particular attention is paid to the influence on drug action of such factors as microsomal enzyme induction, binding to plasma proteins, ambient temperature and species and age differences. The importance of information derived from the clinical observation of adverse drug reactions is illustrated by a discussion of drug-induced allergic reactions and of oxalate metabolism in relation to the formation of kidney stones. Recent experience with hexachlorophene is used to emphasize the need for a continual reappraisal of toxicological data on compounds in general use. An interesting inclusion in this context is a brief review of vitamin toxicology, prompted by the possibility of hypervitaminoses arising both from the increasing therapeutic use of vitamins and from their increased consumption as dietary supplements. The final essay is concerned with the puzzling phenomenon that toxic reactions to a drug may occur more frequently in one country or continent than in another. Climate, nutritional practices, racial influences and prescribing habits are among the factors that may account for such differences. These are discussed with particular reference to the cases of subacute myelooptico-neuropathy (SMON) that occurred in Japan during the late 1950s and the 1960s.

Although set in typescript, the book is well produced, bearing in mind its modest price. Some 230 references are included and there is an adequate subject index. The author's style is informal but eminently readable and the text is not marred by linguistic lapses. While in no way a reference work on drug toxicology, this book—and presumably the subsequent volumes—should provide interesting reading not only for clinicians and research workers but also for newcomers to the field.

Carcinogenesis Testing of Chemicals. Proceedings: Conference on Carcinogenesis Testing in the Development of New Drugs, May 23–25, 1973, Washington, D.C. Edited by L. Golberg. CRC Press, Cleveland, Ohio, 1974. pp. xv + 144. $25.00.

Among the many problems that confront all sectors of the chemical industry, none is so disturbing as the discovery that some widely-used compound presents a carcinogenic hazard to exposed individuals. The problem has become particularly acute in the field

of new drug development, following the increasing awareness of actual or potential carcinogenicity for man of some drugs already in use and the reports of neoplasia produced in animals by numerous other widely-employed and valuable therapeutic agents. Consumer groups in the United States are clamouring for action by the FDA, to ensure that all compounds be fully tested for carcinogenic potential in animals before being given to human volunteers, even under the very limited circumstances of a Phase I study. It was this situation, and its grave implications for new drug development, that prompted the organization of a Conference in Washington, D.C., jointly sponsored by the FDA, the National Cancer Institute and the National Institute of General Medical Sciences. The volume under review presents a succinct account of the proceedings and conclusions of this Conference.

The Conference was intended to serve two purposes. The first was a stock-taking, an assessment of the present state of the art with regard to carcinogenesis testing of chemicals by established procedures. The second purpose was to bring together all the short-term procedures that have been developed, or are in the course of development, and to evaluate their merits and shortcomings as screening procedures for carcinogenicity. The arrangement of the Conference was unusual in that the first day was taken up by discussions. Fifteen Discussion Groups were organized, each under a Discussion Leader, and to each was assigned a specific topic, on which the Group had to produce a report and recommendations. The composition of these Discussion Groups included many of the leading experts in the particular areas under discussion. The remainder of the Conference followed more conventional lines, with the presentation of formal papers and Discussion Group reports.

The account of these proceedings constitutes a very valuable document. Not only does it provide a most complete and authoritative commentary on various aspects of carcinogenesis testing; it has also the distinction of including several unique position papers. Notable among the latter is Section 8, Interpretation of Test Results in Terms of Carcinogenic Potential to the Test Animal: The Regulatory Point of View. The author is Dr. W. D'Aguanno, of the Bureau of Drugs in the FDA, and his is a statement that provides much food for thought. An important controversy that surfaces in this volume is the question of the routine use of positive controls in the conduct of carcinogenesis bioassays. The Discussion Group led by Dr. J. H. Weisburger stressed the desirability in principle of including known carcinogens as positive control compounds, but pointed out the problems created by such an approach in practice. An Appendix to this report illustrates the essentiality of such positive controls in bioassays of the carcinogenicity of tobacco-smoke condensates, air-pollutant concentrates or a series of methylchrysenes. Among the recommendations of the Conference, as stated by the Chairman, was the conclusion that: "For the purpose of testing new drugs, it is likely that, in most instances, sound practical considerations will dictate a decision not to use positive controls."

Perhaps the most important section of the book deals with short-term tests for carcinogenesis. Introduced by a historical account of the evolution of such tests (by Dr. D. B. Clayson), it includes sections by such well-known authorities as Drs J. A. DiPaolo, F. J. de Serres, A. Freeman, B. Weinstein, H. C. Pitot and D. S. R. Sarma. Here is the most complete account of the subject available at present, and the impression left with the reader is one of great hope that useful procedures are evolving and are being validated by comparison with the standard bioassay.

Conspicuous by its absence is any discussion of the statistical aspects of carcinogenesis testing and the interpretation of results. This is a specialized area, bordering on what Alvin Weinberg has termed 'trans-science'. Although the Conference did have contributions from statisticians, they have obviously proved reluctant to commit themselves in print. With this exception, however, the volume under review is fully comprehensive. It has the advantage of an excellent index and can be strongly recommended to all concerned with the development, application and safety evaluation of chemical agents.

Cancer Experiments and Concepts. By R. Süss, V. Kinzel and J. D. Scribner. Springer-Verlag, Berlin, 1973. pp. xx + 285. DM 26.50.

The cancer problem, according to the authors of this somewhat unorthodox textbook, is both an integral part of medicine and a natural science in its own right. Above all, it is a challenge for the curious. Consequently, the reader is taken on a voyage of discovery and adventure through the dark and seemingly impenetrable jungle of experimental cancer research, in search of the crock of gold, the solution to carcinogenesis. An expeditionary force of physicists, biochemists, pathologists, virologists, toxicologists and other assorted scientists leads the way along innumerable dead ends and garden paths until eventually the bewildered reader is shown a glimmer of light in the distance. Whether this heralds the end of the journey or is just a clearing in the undergrowth, is a question for the future.

The book begins with a discourse on the growing prevalence of carcinogens in our environment and the increasing incidence of cancer. A brief history of experimental cancer research follows, highlighting the role played by polycyclic hydrocarbons. Activation through metabolism is discussed, with particular reference to the aromatic amines, and then quantitative aspects of carcinogenesis are investigated, followed by a discussion of the multiple-step hypotheses of skin-cancer induction.

The authors' strongly held view of oncology as an approach to biological problems from the direction of tumour research is uppermost when tissue-specific growth regulation and the role of cell organelles, especially membranes, in cellular "sociology" are discussed. An account of the work concerning tumour immunology is then given, and this leads into a discussion of the extensive field of cancers induced by viruses. One section is devoted to tissue culture investigations of DNA tumour viruses, and this is followed by an account of the role of genetics and the part played by DNA in carcinogenesis. Tumour chemotherapy is mentioned briefly and the last section of the book is devoted to an analysis of the validity or otherwise of certain widely held concepts in the

science of oncology. The text is brought to a close by a question and answer session—in fact an imaginary conversation which serves to reiterate many of the arguments covered in earlier parts of the book. An extensive morphological glossary, references and an index complete the volume.

The unusual layout of the book, involving many short sections with headings, and the unorthodox conversational style of the text serve the authors' aim of producing a book to be read for enjoyment. However, the text, originally in German, has unfortunately emerged from translation with too strong a transatlantic bias for the comfort of many British readers. This, together with the informal dialogue employed, has led to an occasional lack of clarity in the definition of certain concepts. Nevertheless, the book succeeds in demonstrating that oncology has in many ways become the melting-pot of methods in modern biological research. In the process, it has often strayed into areas far removed from the immediate problem of finding a cure for cancer. It is imperative that workers in these fascinating fields never lose sight of this basic goal.

Advances in Cancer Research. Vol. 18. Edited by G. Klein and S. Weinhouse. Academic Press, London, 1973. pp. ix + 409. £12.90.

The principal trends in current cancer research relate mainly to immunological processes in cancer and carcinogenesis *in vitro*. Both of these approaches figure prominently in this issue of a well-known series.

The immunological aspects of tumour induction by chemicals are extensively reviewed by R. W. Baldwin. The author presents concisely the evidence for the presence of antigens specific to tumour cells and the role these play in setting in motion the complex process involving macrophages, lymphocytes and serum factors which may result in the arrest or elimination of the malignant growth. As the story unfolds, the reader gets the impression that there is a considerable variation in the ability of tumours to provoke an immune response directed against them. Tumours caused by viruses appear, in general, to be fairly potent in this respect, while cancers caused by physical factors, for example by the subcutaneous implantation of plastics films in rodents, are weaker. Tumours induced by chemical agents seem to be of intermediate potency. The role played by immunosuppression in enhancing tumour growth is also examined, and there is little doubt that any procedure that reduces the immune response also enhances tumour growth. Unfortunately the effect that an enhancement of immunological reactions may have on tumour growth receives inadequate treatment, although the author does mention the possibility of an adverse effect.

Immunological reactions in cancer also receive attention from J. H. Kersey and his colleagues. These authors examine the problem from a purely practical angle. They review the experimental and clinical evidence linking immunodeficiency and cancer in reasonable detail and largely avoid drifting into speculation irrelevant to their topic. Their conclusion deserves to be quoted: "...all forms of primary and secondary immunodeficiency tend to favor development of lymphoreticular cancers...the increased susceptibility to malignancy in immunodeficiencies reflects a susceptibility to etiologic agents which can operate more effectively in the absence of immunocomponent mechanisms".

Although only one chapter is devoted to chemical carcinogens *in vitro*, C. Heidelberger has successfully produced an adequate review of this difficult yet important field. One of the main objectives of *in vitro* work is to effect the transformation of a normal to a 'malignant' cell, but despite considerable efforts in this direction, there is no clear evidence that success has been achieved. In this respect chemicals differ considerably from viruses, which readily transform normal cells *in vitro*. However, tissue-culture work has successfully elucidated some of the early effects of carcinogens on cells, such as binding to cellular macromolecules.

One of the main difficulties experienced by workers in this field is the contamination of tissue-culture cells by viruses. This may be unavoidable in tissues from rodents such as the mouse and hamster, which normally harbour tumour viruses, and therefore any experiment claiming that cells derived from these rodents have been transformed *in vitro* by chemical carcinogens is immediately suspect. Much work needs to be done before one can be confident that transformation in these two species is really the result of chemical interaction. Perhaps the author could have placed more emphasis on the need to look into the possibility of using cultured cells from species less susceptible to contamination by tumour viruses.

Another topic of interest is discussed by Yee Chu Toh. Naturally occurring cancers in both animals and man tend to exhibit a marked sex difference in occurrence, a difference not limited to tumours of the sex organs. Experimentally-induced tumours, particularly those induced by chemicals, tend to follow a similar pattern, and the author examines this sex difference in tumour incidence and distribution in the light of the influence exercised by sex hormones. This chapter contains some valuable information on intersex and interspecies differences in the metabolism of some important chemical carcinogens.

The remaining three chapters deal with topics as diverse as isozymes and cancer, the chemotherapy and immunology of gestational choriocarcinoma and the glycolipids of the tumour-cell membrane. These will appeal mainly to those particularly involved in these highly specialized fields.

On the whole the volume achieves the same high standard as its predecessors and keeps the reader abreast of advances in important and rapidly developing areas of cancer research.

Mercury, Mercurials and Mercaptans. Edited by M. W. Miller and T. W. Clarkson. Charles C. Thomas, Springfield, Illinois, 1973. pp. xvii + 386. $19.75.

This publication emerged from a conference held in 1971 at the University of Rochester, New York, on the interaction of mercury compounds with biological systems. The first four chapters consist of short reviews on various aspects of the toxicology of mercurials and the role of mercaptans in the biological activity of these compounds, and give the reader some background knowledge to promote appreciation of subsequent chapters. The remainder of the book is

devoted mainly to original papers on selected areas of current research, presented by specialists in the fields of the toxicity and metabolism of mercury compounds. The wide range of topics covered includes the action of mercury on transport processes in the kidney, complexes of mercury with bile and kidney proteins and the biotransformation of inorganic and organic mercurials by plants, algae and bacteria.

It is interesting to compare the approach of this volume with that of the excellent book *Mercury in the Environment. An Epidemiological and Toxicological Appraisal*, edited by L. Friberg and V. Vostal (*Cited in F.C.T.* **11**, 661). The latter, which did not of course arise from a symposium, is a mine of information, but the data are presented for the most part without further comment. This is in direct contrast to the publication under review, which is not comprehensive in its treatment of the subject, but instead focusses on important areas of research and explores them in detail. Transcripts of the discussions by participants in the symposium are included at the end of each contribution and are symptomatic of this reasoned approach. To one reader at least, these discussion sections appeared extremely valuable, analysing the significance of many points in the preceding paper and highlighting areas demanding further research.

Clearly, in both contents and treatment, this book is aimed at the scientist specializing in research in the field of mercury toxicity and such people will find it a very useful publication.

The Chemistry and Metabolism of Drugs and Toxins. An Introduction to Xenobiochemistry. By M. Briggs and Maxine Briggs. William Heinemann Medical Books Ltd., London, 1974. pp. xii + 386. £5.00.

A xenobiotic may be defined as any compound that cannot be utilized by the body for the production of energy or for the synthesis of new tissue. Such substances are foreign to the metabolic pathways of the organism and must be eliminated. A complex enzyme system exists in the endoplasmic reticulum of mammalian liver cells for transforming xenobiotics to more polar compounds which may subsequently be excreted. This process of biotransformation—which often involves hydroxylation—normally results in the detoxication of the foreign compound.

The monograph under review here consists of four lengthy and inadequately indexed chapters. The first is devoted to the metabolism of foreign compounds, but unfortunately the theoretical considerations of biotransformation and conjugation processes are poorly treated. However, the metabolic pathways of a large number of compounds are included. The second chapter deals with the biochemistry of drugs, and covers fairly well the processes of absorption, transport and excretion. Some space is also devoted to the mode of action of drugs and their side-effects. The two remaining chapters are concerned with natural anti-metabolites and with venoms, and are concentrated mainly on examples of toxic agents derived from microbial, plant and animal sources.

Over the past 10–20 years, several books have been published on the mechanisms of detoxication of foreign compounds. Excellent if somewhat dated examples are the well known "*Detoxication Mechanisms...*" by R. T. Williams, "*Selective Toxicity...*" by A. Albert and *The Biochemistry of Foreign Compounds* by D. V. Parke. While this new monograph covers a wide range of types of xenobiotic and considers many examples, it does not seem to justify its description as "an introduction to the new science of Xenobiochemistry". Although potentially useful as a reference work, it can scarcely be recommended either for basic theory or for general reading. At a cost of £5 in paperback, moreover, it is not good value for money.

Intestinal Enzyme Deficiencies and Their Nutritional Implications. Symposia of the Swedish Nutrition Foundation, XI. Edited by B. Borgström, A. Dahlqvist and L. Hambraeus. Almqvist & Wiksell, Stockholm, 1973. pp. 149. Sw. kr. 50.

This volume presents the proceedings of a symposium organized by the Swedish Nutrition Foundation and held in Stockholm in August 1972. The emphasis appears to be mainly on the clinical aspects of problems related to the nutritional consequences of intestinal enzyme deficiencies in man. There is a distinct bias towards studies on intestinal disaccharidases, the majority of papers being concerned with this subject. Demographic studies showing the significance of genetically determined factors in enzyme-deficiency conditions are discussed in a number of papers and the dietary management of children and adults suffering from carbohydrate malabsorption syndromes is also presented.

Three papers deal with the malabsorption of proteins and amino acids and two consider the problems of impaired fat absorption. Discussions on the papers are somewhat briefly reported and in many instances are not included at all. No index is provided. This volume may be of some limited value to nutritionists actively involved in a specialized field, but is unlikely to attract a wider circle of readers.

Introduction to Molecular Embryology. By J. Brachet. Heidelberg Science Library. Vol. 19. The English Universities Press Ltd., London, 1974. pp. xi + 176. £2.45.

For the intelligent layman marvelling over the infinite variety of human faculties, Professor Brachet's latest paperback will provide no startling revelations. As an informed portrayal of ideas surrounding molecular embryology, however, it will be a notable addition to the literature for the esoteric student. As the author concedes, the text owes much to his own students at the University of Brussels, and it follows that a similar audience will benefit most from its logical account of embryonic development.

Generations of scientists have striven to decipher the vastly complex code that organizes events at the molecular level in terms of sequential organ and tissue development, but all have been hampered by an inadequate knowledge of the mechanisms that regulate eukaryotic genetic activity. Nevertheless, Professor Brachet's book makes it abundantly clear that enough is now known about regulatory aspects of protein synthesis for theoretical models to be derived in an attempt to explain cell differentiation in genetic terms, and in the near future it should be possible

to test their validity and, if necessary, make appropriate modifications. Morphological studies of the development of structures such as cilia and flagella —which consist of proteins very similar to the contractile proteins of muscle—may promote the understanding of differentiation in more complex organs and tissues. Finally, the improvement of culture conditions, to rival those given by the placenta itself, could lead in the foreseeable future to a possible molecular analysis of mammalian development.

If there is one major criticism of this volume it must relate to the comparative dearth of cited references, of which only a handful describe literature published since 1970. In consequence, Professor Brachet has made only fleeting mention of certain important developments in molecular genetics, such as the experiments of H. Harris, who used cell-fusion techniques to probe the influence of cytoplasmic components on nuclear activity. On the credit side, however, is the balanced and logical development of the text, with descriptions of nucleic acid synthesis appearing alongside those of cell biology. If Professor Brachet's aim was to demonstrate that the proper study of cellular differentiation lies in the developing embryo itself, then he has succeeded in his task.

Organometallic Compounds: Methods of Synthesis, Physical Constants and Chemical Reactions. Edited by M. Dub. Vol. II. Compounds of Germanium, Tin and Lead Including Biological Activity and Commercial Application. First Supplement. By R. W. Weiss. 2nd ed. Springer-Verlag, Berlin, 1973. pp. xxiv + 1116. DM 112.90.

Those involved in research necessitating any detailed consideration of organic compounds of germanium, tin or lead will probably be familiar with the parent volume of which the book now under review is a very weighty offspring. Following the general trend for children to outgrow their parents, this first supplement to the second of a series of volumes on organometallic compounds covers literatures only for the period 1965–1968 but includes more references than the main volume, which covered the years from 1937 to 1964.

Basically the supplement, like the parent volume, is intended to provide data on methods of synthesis, physical constants, chemical reactions, biological activity and commercial application for all the known organic derivatives of germanium, tin and lead, an organometallic compound being defined in the strict sense of a compound with at least one metal–carbon bond. The decimal system used previously for the arrangement of compounds has been continued and expanded in the supplement and where previous reference has been made to a compound, the relevant main-volume page number is indicated. In most cases, specific data are presented, but where accurate reporting of information would necessitate the reproduction of considerable detail (such as in connexion with the effects of triethyltin compounds on specific aspects of metabolism or morphology), data are limited to references to the relevant information sources.

The reproduction of data from over 3000 original papers and patents inevitably involves considerable abbreviation and compression, and the result is a somewhat forbidding tome of closely printed tables, symbols and formulae. Moreover, it could be argued that all the data could be traced through *Chemical Abstracts*, which was used as the source of all the literature and patent references (although for the most part the original documents were studied for the data compilation). Nevertheless, the value of having scattered data brought together in this way is indisputable and a little practice enables the user to locate more easily the information he needs.

Carbohydrate Chemistry—VI. Plenary Lectures Presented at the VIth International Symposium on Carbohydrate Chemistry Held at Madison, USA, 14–18 August 1972. Edited by W. M. Doane. Butterworths, London, 1973. pp. 78. £2.35.

This volume contains five lectures presented at the plenary session of the Sixth International Symposium on Carbohydrate Chemistry held at Madison, USA, in August 1972.

The lectures, generally in the form of reviews, cover a wide range of topics from aspects of the pure chemistry of carbohydrates to the economics and technology of carbohydrate products. The scientific papers include a conformational study on the coordinated complexes formed by the interaction between sugars and metal ions, and the application of electrophoresis and proton magnetic resonance spectroscopy to these investigations. An excellent review is presented on the synthesis of fluorinated carbohydrates, a group of compounds of increasing importance in view of their selective cytotoxic effects. A brief article on the biosynthesis of chondroitin sulphate, exemplifying the formation of connective-tissue polysaccharides, is also included.

This slim volume is clearly intended for specialists in the field of carbohydrate chemistry and is unlikely to be of more than marginal interest to toxicologists. Since most carbohydrate chemists might be expected to have access to *Pure and Applied Chemistry*, in which the contents of the book also appeared, one must question the justification for this particular volume. Perhaps the increasing shortage of raw materials may encourage a more careful evaluation of proposed publications of this kind and combat the apparently growing tendency towards the uncritical reproduction of symposium papers irrespective of the potential value of their contents.

BOOKS RECEIVED FOR REVIEW

Ecological Aspects of Toxicity Testing of Oils and Dispersants. Edited by L. R. Beynon and E. B. Cowell. Applied Science Publishers Ltd., Barking, Essex, 1974. pp. viii + 149. £5.00.

Advances in Cardiology. Vol. 13. Comparative Pathology of the Heart. Edited by F. Homburger and I. Lucas. S. Karger, Basel; 1974. xii + 379. £37.80.

Fd Cosmet. Toxicol. Vol. 13, pp. 465–472. Pergamon Press 1975. Printed in Great Britain

Information Section

ARTICLES OF GENERAL INTEREST

MUTAGENICITY TESTING WITH BACTERIA

It is only in the last 20 or 30 years that the ability of many chemicals to cause genetic changes has been fully realized. The few chemicals initially recognized as having mutagenic effects, the nitrogen mustards for example, have now grown to many hundreds, and as techniques for their detection become more sensitive it seems certain that many hundreds more will be discovered. The genetic hazard of these chemicals to man is the subject of much debate, but the possibility must give cause for concern.

There are three types of genetic change that can be produced by mutagenic agents, namely chromosome breakage, non-disjunction and point mutation. The most subtle of these genetic changes, and yet the one likely to have the most far-reaching consequences for man, is point mutation, an alteration in one of the nucleotide bases of the DNA. This partially inactivates a gene and in turn results in a defective or inactive gene product. In contrast to non-disjunction and chromosome breakage, which lead to drastic and often lethal genetic consequences, point mutations are usually inherited and may pass unnoticed for many generations. This is because most mammalian genes occur in pairs in each cell and a mutation in a gene on one chromosome is usually masked by the wild-type gene on the other chromosome. Usually the mutation is only expressed in subsequent generations when chromosomes bearing mutations in the same gene are received from each parent. Clearly the likelihood of an offspring acquiring two chromosomes bearing a mutation in the same gene will depend on the size of the 'pool' of such mutations within a population. If such a 'pool' is building up, the genetic consequences for future generations may be dire.

Obviously before the genetic risks to man can be evaluated we must be able to identify mutagenic chemicals and quantify their effects. A multitude of procedures for screening chemicals for mutagenic activity has been developed, ranging from tests using animals to ones involving bacteria and viruses. *In vivo* testing in mammals probably has the greatest relevance to the human situation, but tests such as the specific locus test in the mouse (Cattanach, *Chemical Mutagens**, Vol. 2, p. 535) require enormous numbers of animals and are consequently prohibitively expensive and time-consuming for the routine testing of drugs and chemicals. In addition they are less sensitive than microbial tests. Other mammalian tests, including the dominant lethal assay (Bateman & Epstein, *ibid*, Vol. 2, p. 541), and tests using Drosophila (Abrahamson & Lewis, *ibid*, Vol. 2, p. 461) detect gross chromosomal damage rather than point mutations.

Bacterial tests offer many advantages over mammalian systems in that they are far cheaper, very rapid (the results are obtained usually within 2 days) and extremely sensitive. Indeed microbial tests probably represent the only system suitable for screening large numbers of chemicals for mutagenic activity. It has been suggested that the wide phylogenetic difference between bacteria and mammals precludes the use of the former in mutagen testing, but this objection may be countered by the fact that the target molecule of mutagens is DNA, which is substantially the same whether the cell is eukaryotic or prokaryotic.

Most of the bacterial tests for detecting mutagens utilize bacteria bearing a mutation in a gene involved in the synthesis of a particular amino acid; hence the bacteria (called auxotrophs) require an external supply of the amino acid for growth. Spontaneously, and at low frequency, some of the bacteria revert, or back-mutate, to the wild-type, able to grow on media lacking the amino acid. Mutagens can induce such back mutations and so an increase in the number of revertants over the number of spontaneous back mutations can be used as an index of the mutagenicity of the compound under test. The best known of these back-mutation systems are the histidine-requiring mutants of *Salmonella typhimurium* developed by Ames (*ibid*, Vol. 1, p. 267) and the tryptophan mutants of *Escherichia coli* described by Bridges (*Lab. Pract.* 1972, **21**, 413). In both systems, the sensitivity of the test has been increased by utilizing derivatives of the tryptophan or histidine auxotrophs deficient in one or more of the mechanisms by which the bacterial cells repair damage to DNA. The most useful of these are *uvr* strains.

As it stands, this test has several disadvantages, not the least of which is the considerable difference in permeability between bacterial and mammalian cells. In the bacterial test, a negative result may be due not to a lack of mutagenic activity on the part of a chemical but to its inability to penetrate the cell wall and membrane and reach the DNA within the bacterium. To combat this, Ames *et al.* (*Proc. natn. Acad. Sci. U.S.A.* 1973, **70**, 782) obtained 'deep rough' derivatives of *S. typhimurium* strains, the cell walls of which were deficient in lipopolysaccharide components and were thus more permeable to various mutagens, including dibenz[*a,h*]anthracene.

Perhaps the most important drawback of the bacterial test described above is that it does not take into account the major differences in metabolism between mammalian cells and bacteria (e.g. the cytochrome *P*-450 drug-metabolizing system of mam-

**Chemical Mutagens. Principles and Methods for Their Detection.* Vols 1 & 2. Edited by A. Hollaender. Plenum Press, New York, 1971.

malian liver, a system which has no counterpart in the bacterial cell). Thus a non-mutagenic chemical metabolized by such enzymes to an active mutagenic species will not be detected by the simple bacterial system. (Obviously the converse is also true—a mutagen inactivated by mammalian enzymes will give a 'false positive' when tested with bacteria.) Thus mutagens such as acetylaminofluorene and dimethylnitrosamine, which are not mutagenic *per se* but require activation by liver enzymes, do not revert the histidine or tryptophan auxotrophs. This problem has been partially overcome by two methods, the host-mediated assay and the activation of mutagens by liver homogenates.

In its simplest form, the host-mediated assay consists of injecting the bacterial tester strain (or in some cases a yeast) into the peritoneal cavity of a mouse or rat prior to intravascular or oral administration of the potential mutagen which, it is hoped, will be converted to the genetically active form and then act on the bacteria in the peritoneum (Gabridge & Legator, *Proc. Soc. exp. Biol. Med.* 1969, **130**, 831; Legator & Malling, *Chemical Mutagens*, Vol. 2, p. 569). After a suitable period of time, the micro-organisms are recovered from the peritoneum and the number of revertants is counted. This test has met with considerable antipathy for many reasons, one being its insensitivity. Large amounts (often near the LD_{50}) of substances such as dimethylnitrosamine are needed to obtain a significant increase in reversion frequency (Malling, *Mutation Res.* 1974, **26**, 465). In addition, the fact that the host animal is infected with a large dose of a pathogenic organism and is simultaneously given an enormous quantity of a drug or chemical casts doubt on the results, since the metabolic response of the animal is unlikely to be normal under such conditions. Furthermore, the active metabolites of many chemicals are known to be extremely short-lived (Miller, *Cancer Res.* 1970, **30**, 559); if produced in the liver they would almost certainly react with material *in situ*. It seems unlikely, therefore, that sufficient quantities of such metabolites would be active by the time they reached the peritoneum. It is perhaps for this reason that diethylnitrosamine is virtually inactive as a mutagen in the host-mediated assay at doses up to 400 mg/kg (Malling, *loc. cit.*).

There are several variations on the host-mediated assay. Ficsor & Muthiani (*Mutation Res.* 1971, **12**, 335), for example, have suggested that bacteria should be exposed to homogenates of organs removed from animals injected with potential mutagens. The method gave good results with streptozotocin, the only mutagen tested, but it must be noted that the latter is mutagenic *per se* to bacteria. It seems unlikely that short-lived mutagenic metabolites will be detected by this method. A further modification of the host-mediated assay, proposed by Malling & Frantz (*ibid* 1974, **25**, 179), appears to increase the sensitivity of the method considerably, at least for dimethylnitrosamine. It involves the iv injection of micro-organisms, a proportion of which become trapped in the liver and lungs of the animal. Treatment of the animal with dimethylnitrosamine, and subsequent recovery of the injected micro-organisms, resulted in a large increase in mutation frequency compared with the controls, although diethylnitrosamine was much less active. Whether the method is applicable to other types of mutagen remains to be established.

The second approach to the problem of mutagen activation, the use of liver homogenates or microsomal fractions, looks more promising (Ames *et al. Proc. natn. Acad. Sci.* U.S.A. 1973, **70**, 2281). This group showed that several compounds that do not normally mutate *S. typhimurium in vitro*, will do so when a rat-liver homogenate is incorporated into the agar plate. Unfortunately, this investigation was confined to a very restricted range of mutagens, mostly polycyclic aromatic hydrocarbons, so it is not certain how well the system responds to other types of mutagen, although dimethyl- and diethylnitrosamines are known to be genetically active by this method (Malling, *loc. cit.*). One of the major problems with this method is the choice of animal for use as the source of the liver extract. Malling & Frantz (*loc. cit.*) have shown that for dimethylnitrosamine there is a remarkable difference in metabolic activity not only between liver homogenates derived from the rat and mouse but also between those from different strains of mouse. Furthermore, the method does not take into account compounds activated by enzymes other than those associated with the liver. To be certain of detecting such mutagens, homogenates of other organs, such as the lung, intestinal epithelium and kidney, would also have to be used.

An important limitation on the use of bacteria in tests for mutagenic activity is their specificity in response to particular types of mutagen. Some strains are back-mutated only by certain types of frameshift mutagen, others by mutagens causing base pair substitutions (Ames *et al. Science, N.Y.* 1972, **176**, 47). No one strain will respond to all types of mutagen. It is for this reason that Ames *et al.* 1972, *loc. cit.*) proposed the use of a group of four strains of *S. typhimurium*, each of which detect different types of mutagen. To be certain of detecting all mutagens, it is likely that even more strains will have to be used, especially in the light of the recent discovery that nitrofuran derivatives, which have been shown to be potent carcinogens in rats and mice, did not mutate any of the four tester strains recommended by Ames *et al.* (1972, *loc. cit.*) although they were mutagenic to *E. coli* WP2 (Yahagi *et al. Cancer Res.* 1974, **34**, 2266). Thus, to ensure that all environmental mutagens are detected, we may have to face the daunting task of using many different strains of bacteria in combination with liver homogenates from a variety of animals—besides the possible use of homogenates of other organs. Clearly the number of permutations is enormous.

In theory the induction of forward mutations, i.e. mutations leading to loss of function of a particular gene, should be much less specific than back-mutation systems and hence should provide a more general test for mutagens. However, the few that have been proposed (Bridges *et al. Mutation Res.* 1973, **21**, 303; Mohn, *ibid* 1973, **20**, 7) have not been tested exhaustively against a wide variety of mutagens and, in addition, such tests suffer from the disadvantage that since the level of spontaneous mutants obtained is high, weak mutagens may go undetected.

Recently, the observation that bacteria deficient in DNA polymerase or lacking DNA-repair capacity are more sensitive than the parent strains to agents that

damage DNA has been exploited to provide a rapid plate test for mutagens, which involves measuring the inhibition of growth of parent and mutant strains (Kada *et al. ibid* 1974, **26**, 243; Slater *et al. Cancer Res.* 1971, **31**, 970). Longnecker *et al.* (*ibid* 1974, **34**, 1658) evaluated the test with a wide variety of mutagens, however, and concluded that it was not as sensitive as was first thought, especially with mutagens requiring metabolic activation. Moreover, the observation that for some repair-deficient strains the extent of killing by mutagens is unrelated to the number of induced mutations detracts somewhat from the value of the method (Hince & Neale, *Mutation Res.* 1974, **22**, 235).

In spite of the limitations discussed in this article, microbial mutagenicity tests are obviously the only means available for screening large numbers of chemicals and drugs for mutagenic activity, since in terms of speed and sensitivity they are vastly superior to *in vivo* tests in mammals. Clearly, both positive and negative results from microbial tests must be evaluated most carefully in the light of the recognized limitations, but such tests, in combination with other short-term tests for mutagenicity including those involving mammalian cells in culture, would provide a most effective primary screen for detecting mutagens. This approach has been suggested by Bridges (*ibid* 1974, **26**, 335), who has outlined a three-tier system of mutagenicity testing, in which this primary screening may be followed by *in vivo* tests using whole mammals and finally, in the light of the earlier results and potential levels of exposure, by experiments designed to give some quantitative indication of the potential risk to man. Obviously, for a full definition of the mutagenic potential of a chemical it is necessary to determine many more facts, such as the mutagen concentration at the site of action, the active metabolic products and their rate of absorption, metabolism and elimination.

[I. R. Rowland—BIBRA]

RENAL RESPONSE TO LEAD.....

The three heavy metals, lead (Pb), mercury and cadmium, are ingested in small amounts with many types of food and are currently the object of some concern to health authorities all over the world. Despite wide differences in their absorption and excretion rates and in the pathological changes they may induce, these metals possess at least one common feature, namely their ability to accumulate in the kidney and produce renal damage. The type of damage produced by each element has distinctive features, although some effects are common to all three. This article is the first of a short series on these metals and is concerned specifically with the renal changes associated with Pb intoxication.

Pb penetrates rapidly into the renal cells. Within 1 hour of iv injection of 100 μg ^{210}Pb as Pb acetate into rats, the Pb was identified in all the fractions of a renal homogenate (Castellino & Aloj, *Br. J. ind. Med.* 1969, **26**, 139). During the first 24 hours after a single injection, the amount of Pb in the nuclear and mitochondrial fractions increased at the expense of that in the microsomal fraction, and throughout the subsequent 8 days of the study the distribution remained relatively stable, with considerably higher levels in the two former fractions than in the microsomal and soluble fractions. Washing with 0·25 M-sucrose, alone or containing 0·001 M-ethylenediaminetetraacetic acid (EDTA), removed Pb less readily from the mitochondrial than from other fractions, indicating some degree of stable binding. Further insight into the distribution of Pb within the kidney cells has been provided by Barltrop *et al.* (*J. Lab. clin. Med.* 1971, **77**, 705). After injecting radioactive ^{203}Pb, these authors subdivided the original mitochondrial fraction of the kidney homogenate into mitochondrial and lysosomal fractions and found that 2 hours after treatment the mitochondria contained four times as much ^{203}Pb as the lysosomal fraction. It appears, however, that Pb is lost from the mitochondria more readily than from the lysosomes, since the concentration in both organelles was virtually the same 7 and 14 days after treatment. Furthermore, previous oral dosing with stable Pb acetate (300 mg/kg/day) for 7 consecutive days had little effect on the lysosomal uptake of ^{203}Pb, whereas the mitochondrial uptake of the isotope was reduced by 30–40%.

To some extent, these biochemical data have been reflected in the findings of an ultrastructural study by Murakami & Hirosawa (*Nature, Lond.* 1973, **245**, 153), who used electron-microscopic autoradiography to demonstrate the presence of ^{210}Pb within the mitochondria of the tubular epithelial cells of the kidney 30 minutes after administration of an ip injection containing this radioactive isotope.

The mitochondria from Pb-treated animals display other types of abnormality apart from these Pb inclusions. Usually elongated and rod-shaped, the mitochondria of the proximal renal tubules become oval or rounded and increase in volume (Goyer, *Lab. Invest.* 1968, **19**, 71). Cristae may be vesicular but more often they are shortened and marginal, while matrical granules become less homogeneous and tend to clump.

More significant, perhaps, is the information on the functional state of the mitochondria obtained by Goyer *et al.* (*ibid* 1968, **19**, 78) in a study designed to elucidate the meaning of the observed morphological changes. Mitochondria isolated from the proximal tubular cells of rats fed a diet containing 1% Pb acetate for 6 weeks were found to have a decreased rate of respiration and partially uncoupled oxidative phosphorylation. This functional defect was accentuated by *in vitro* ageing of the mitochondria at 0°C and was partially reversed by addition of EDTA. Moreover, the mitochondrial outer membranes, where some of the respiratory enzymes are located, were ruptured. Further evidence of an impairment in the respiratory-enzyme activity of mitochondria from

these Pb-intoxicated rats was obtained at a later stage of this work, when a significant reduction in cytochromes aa_3 and b was demonstrated (Rhyne & Goyer, *Expl mol. Path.* 1971, **14**, 386).

This mitochondrial defect may provide some explanation for the amino-aciduria seen as a result of Pb poisoning, since the transport of amino acids in the proximal renal tubule is an active, energy-requiring process (Goyer *et al. loc. cit.*). The amino-aciduria occurring in Pb poisoning has been characterized as "generalized" and is usually accompanied by glycosuria and hyperphosphaturia (Chisolm, *J. Pediat.* 1962, **60**, 1). It was investigated further by Goyer *et al.* (*Proc. Soc. exp. Biol. Med.* 1970, **135**, 767), who confirmed that in Pb-poisoned rats it is largely secondary to impaired function of the renal tubules. They demonstrated a one- to threefold elevation in the renal clearances of most amino acids; the increase in histidine clearance was exceptionally large, but changes in glycine and valine clearances were slight and tyrosine clearance actually decreased. This loss of amino acids is unlikely to be due entirely to the impairment of tubular reabsorption, however, and the formation of metal–amino acid complexes may be a contributory factor.

This excessive amino-aciduria develops within 3 weeks in rats fed 1% Pb acetate, and administration of Pb for longer periods tends to accentuate the renal tubular damage. Feeding of 1 or 2% Pb acetate for 10–40 weeks resulted in an increase in kidney weight and a decrease in the capacity of the kidney to synthesize glucose and metabolize pyruvate (Hirsch, *Toxic. appl. Pharmac.* 1973, **25**, 84). Excretion of *p*-aminohippurate during the first 2 hours after administration was higher than that in controls. Feeding of a diet containing 200 ppm Pb or less did not affect these parameters. Histological examination revealed vacuolar degeneration in the distal segment of the proximal renal tubules of rats subjected (either directly or, initially, via the milk of treated dams) to a diet containing 2% Pb acetate for the first 10 weeks of life. A 4% dietary level of Pb acetate caused, in addition, some cellular necrosis.

The renal response to Pb intoxication has been studied less intensively in other species than in the rat, but the data available indicate that broadly speaking similar changes occur. Levels of activity of lactate dehydrogenase and glucose-6-phosphate dehydrogenase were up to twice as high as normal in kidney homogenates of guinea-pigs given Pb nitrate in a daily oral dose of 120 mg/kg for 40–50 days (Secchi *et al. Clinica chim. Acta* 1970, **27**, 467). On the other hand, glutamate-dehydrogenase activity was reduced. These changes were the same as those found in guinea-pig kidney tissue rendered ischaemic by occlusion of the renal artery, and were thought to indicate an impairment of oxygen utilization and involvement of the mitochondria.

Histological evidence of renal tubular damage has also been observed in other species. Chicks given 1·0 or 0·5% Pb acetate in the diet for 3 weeks developed necrosis of the epithelium of the renal proximal tubules, and inclusion bodies—similar to those seen in rats—were demonstrated by electron microscopy in the nuclei of cells from this part of the nephron (Simpson *et al. Am. J. vet. Res.* 1970, **31**, 515). Similar inclusion bodies were seen in young dogs given 0·01% Pb acetate in the diet for 12 weeks from the age of 6 weeks, and foci of necrosis were seen in the proximal renal tubules of these animals (Stowe *et al. Archs Path.* 1973, **95**, 106). Cynomolgus monkeys exposed virtually continuously for 104 weeks to Pb chlorobromide dispersed in particulate form in the atmosphere developed a nephropathy characterized by dilatation of the proximal convoluted tubules and degeneration of their epithelial cells, which showed vacuolated cytoplasm, vesiculated nuclei, disrupted borders and the presence of intranuclear inclusions (Campbell *et al. J. Am. vet. med. Ass.* 1971, **159**, 1523).

[P. Grasso—BIBRA]

.....MERCURY.....

The second article in this series on the effects of heavy metals on the mammalian kidney is concerned with the actions of mercury (Hg).

Human experience

The nephrotoxic hazard from the use of Hg compounds either medicinally, as pesticides or in industry is well recognized, but the advantages possessed by many Hg-based compounds make the finding of adequate substitutes a difficult task. The renal changes produced in experimental animals by the administration of high doses of Hg compounds both in the short and long term are reasonably well established, but the picture in man is far less clear.

Several cases of extensive renal damage occurring in unexpected circumstances shed some light on the type of effect that may be induced in man, at least by phenylmercuric compounds, which are perhaps more commonly used medicinally than any other mercury derivatives. Four children and two adults were given chloramphenicol injections that contained Merthiolate, a phenylmercuric compound used extensively as an antiseptic agent, in a concentration 1000 times greater than the correct level (Axton, *Post-grad. med. J.* 1972, **48**, 417). Five of the patients died, although three of them were treated with 2,3-dimercapto-1-propanol (BAL), and at autopsy the kidneys were swollen and necrosis was apparent in the proximal tubular cells. Each of these fatal cases had received between 20 and 110 mg Hg/kg or approximately 70–330 mg Merthiolate/kg. The oral LD_{50} of Merthiolate in rats is reported as 60 mg/kg. In at least two cases, gross albuminuria and glycosuria were observed and the one surviving child, who had received a total dose of about 30 mg/kg, had traces of albumin in its urine for some time after cessation of the chloramphenicol treatment.

Albuminuria and glycosuria have also been associated with poisoning resulting from more prolonged

treatment with smaller amounts of phenylmercury, and are usually accompanied by other signs of tubular dysfunction, such as an enhanced excretion of amino acids and phosphate. This pattern of tubular dysfunction was observed in a 1·5-year-old girl whose gums had been treated topically with Glyceromerfen over a 12-month period because of teething difficulties. Altogether, eight bottles of the preparation, corresponding to 320 mg phenylhydrargyrum boricum, the phenylmercuric formulation, had been applied in this way (Rützler, *Schweiz. med. Wschr.* 1973, **103,** 678) and urinary excretion of Hg (mainly organic) rose to 60 μg/24 hours. Signs, of tubular dysfunction disappeared within 5 weeks of the cessation of treatment, although urinary Hg excretion was still raised after 3 months.

In some instances, prolonged albumin loss leads to the development of oedema and clinically the combination of these signs is known as the nephrotic syndrome. According to Barr *et al.* (*Am. J. clin. Path.* 1973, **59,** 36), there is strong evidence for an aetiological relationship between the use of skin-lightening creams containing ammoniated Hg and the development of the nephrotic syndrome, a problem to which reference was made in a recent issue (*Cited in F.C.T.* 1975, **13,** 407). High renal levels of Hg are normally found in such conditions, and this finding is important as a means of eliminating other possible causes of the nephrotic syndrome. However, this is not always a practicable proposition and Barr *et al.* (*Am. J. clin. Path.* 1973, **59,** 515) found that a satisfactory indication of Hg exposure could be obtained from Hg levels in the scalp hair, pubic hair and fingernails. The mechanism by which Hg produces the nephrotic syndrome is unknown, but a similar type of disease has been induced in rats by repeated sc injection of Hg compounds (Bariety *et al. Am. J. Path.* 1971, **65,** 293). Immunological studies revealed changes in the kidneys of these rats similar to those found in the kidneys of human cases of nephrotic syndrome.

Morphological studies

The renal damage produced by Hg compounds in the rat has continued to receive attention. In an experiment in which rats were given single sc injections of mercuric chloride ($HgCl_2$) in the 2–20 mg/kg range, Janssen (*Scand. J. Urol. Nephrol.* 1970, **4,** 157) observed that all rats given 10 mg $HgCl_2$/kg or more developed severe kidney damage, involving extensive necrosis of the proximal tubular cells, and died in uraemia. With a dose of 4 mg/kg or less, the only observable effect was polyuria. According to Janssen (*loc. cit.*) this investigation lends support to the theory that $HgCl_2$ poisoning affects both the glomerular filtration rate and the tubular reabsorption rate. The polyuria is thus seen as a discrepancy between these two functions.

Not all the doses of $HgCl_2$ that produce cell necrosis lead inevitably to uraemia and death. Recovery is possible and is surprisingly rapid. A single sc injection of 1·5 mg $HgCl_2$/kg produces necrosis of the middle convoluted and terminal straight portions of the proximal tubules of the rat kidney (Cuppage & Tate, *Am. J. Path.* 1967, **51,** 405). Residual cells divide rapidly, relining the tubule completely in 5 days, and this is followed by restitution of cytoplasmic organelles within the regenerating cells and complete structural and functional recovery of the nephron within 28 days. The mitotic index is at a peak 3 days after administration of the Hg (Cuppage *et al. Lab. Invest.* 1969, **21,** 449). Recent studies using both continuous and pulse labelling with tritiated thymidine have shown that the regenerating cells originate mainly from the zone of necrosis and progress through a cycle of three or four divisions until the tubule is completely relined (*idem, ibid* 1972, **26,** 122).

In contrast to the attention given to obvious disturbances produced in renal structure and physiology by high doses of Hg compounds, the changes produced by low doses have attracted comparatively little attention. Yet knowledge of such changes is important if we are to be in a position to assess the hazard from the low level of Hg in our environment. Fowler (*Science, N.Y.* 1972, **175,** 780) has attempted to fill this gap by feeding 28-day-old rats on diets containing 2 ppm Hg as methylmercuric chloride for 12 weeks, after which the animals were killed for autopsy. Large spherical masses were observed with the light microscope in the lumina of the pars recta segment of the proximal tubules of the kidneys. Ultrastructurally these consisted of a few ribosomes and a single compact bundle of smooth endoplasmic reticulum (SER), sometimes enveloping a microbody. It is thought that the blebs resulted from a process of exocytosis of non-functional SER, which may have been damaged by inorganic Hg released as a result of cleavage of methylHg by this organelle.

Unfortunately, the work of Fowler (*loc. cit.*) throws no light on the intracellular site of deposition of Hg or on other cell organelles. Verity & Brown (*Am. J. Path.* 1970, **61,** 57) found that between 3 and 24 hours after intramuscular injection of a single dose of 5 mg $HgCl_2$/kg, approximately 50% of the Hg was found in the cell sap and 33% in the nucleus, the remainder being shared equally between the "large granule fraction" and the microsomes. Activity of the lysosomal enzymes, β-glucuronidase, *N*-acetylglucosaminidase and acid phosphohydrolase, dropped abruptly 24–36 hours after injection but rose again to around the control levels by day 4. Glucose-6-phosphatase activity showed an abrupt fall at the same time as these lysosomal enzymes but remained low during the period of observation.

Detection of these profound enzyme changes in the tissues led to investigations into urinary enzyme activity as a possible marker of enzyme damage. Ellis *et al.* (*Chemico-Biol. Interactions* 1973, **7,** 101) monitored alkaline and acid phosphatases, β-glucosidase, β-galactosidase, *N*-acetyl-β-glucosaminidase and lactate dehydrogenase in the urine and serum of dogs in which renal tubular damage had been induced by a series of doses of $HgCl_2$. The levels of dosage used were 0·1 mg/kg/day for 7 days, 0·2 mg/kg/day on 2 days and a single dose of 2·0 mg/kg, all given iv, and 2·5 mg/kg/day given orally for 9 days. Alkaline- and acid-phosphatase activities in the urine were significantly increased even by the lowest dose level, which had no effect either on the serum enzymes (isocitrate dehydrogenase and glutamic–oxalacetic and glutamic–pyruvic transaminases) frequently used as indicators of tissue damage or on other parameters indicative of renal dysfunction. It thus appeared that these two urinary enzymes were more sensitive indi-

cators of renal damage in the dog than were conventional tests.

Biochemical investigations

In an effort to understand differences in reactivity and toxicity between the various organomercurials, particularly in relation to their effects on the kidney, Ellis & Fang (*Toxic. appl. Pharmac.* 1971, **20**, 14) studied the *in vivo* binding of Hg to the soluble proteins of this organ. Phenylmercuric acetate and mercuric acetate, each labelled with ^{203}Hg, were administered orally to rats in a dose corresponding approximately to 10% of the respective LD_{50}. A very large part of the ^{203}Hg was bound to a soluble protein with a molecular weight of 8000–13,000, while about 20% was bound to proteins with molecular weights in the 40,000–60,000 range or over 100,000. The rate of removal of Hg from all these protein fractions was biphasic, the first removal rate having a half-life of 19–22 hours and the second a half-life of 12–44 days.

It seems likely that these proteins are distributed throughout the various organelles, since Verity & Brown (*loc. cit.*) demonstrated the presence of Hg in different fractions accounting between them for virtually all of the cell organelles. The same authors provided confirmation of this assumption from their cited biochemical studies, which indicated that lysosomes and SER were involved, and from other studies indicating possible involvement of the mitochondria. At low concentrations *in vitro*, mercurials suppress oxidative phosphorylation in isolated rat-kidney mitochondria. Mitochondria isolated from the kidneys of rats treated with 3 mg Hg/kg (as $HgCl_2$) were found to be defective in coupled ATP synthesis, a defect that can be corrected by the addition of magnesium ion to the assay medium (Southard & Nitisewojo, *Biochem. biophys. Res. Commun.* 1973, **52**, 921). This work complements some results obtained by Janssen (*Scand. J. Urol. Nephrol.* 1970, **4**, 163), who found that the *in vitro* oxygen consumption of kidney slices from rats treated with 4 mg $HgCl_2$/kg was considerably lower than that of slices from untreated rats. Signs of cell necrosis were not detected until the oxygen consumption had dropped to some 50% of the normal value. Hg has also been found to affect the metabolism of lactate in the rat. The catabolic oxidation of [1-^{14}C]lactate occurs via two distinctive pathways, namely a decarboxylation reaction and the TCA cycle. *In vivo* and *in vitro*, both phenylmercuric acetate and mercuric acetate affected both pathways, the effect being more pronounced with a higher concentration or a larger dose (Ellis & Fang, *Toxic. appl. Pharmac.* 1973, **24**, 230). The correlation between dose and effect was roughly linear.

Although the picture of Hg nephrotoxicity is gradually unfolding, the story is by no means complete. Patient and meticulous collection of evidence indicates that Hg exercises an adverse effect on a number of cell organelles and one presumes that this contributes to the demise of affected cells, collectively identified by the histologist as proximal tubular necrosis. One must bear in mind that Hg also has adverse effects on other mammalian organs, so the story of its nephrotoxicity is but one chapter in the longer saga of Hg intoxication.

[P. Grasso—BIBRA]

.....AND LAST, BUT NOT LEAST, CADMIUM

The tendency of lead and mercury to accumulate preferentially in the mammalian kidney and their widespread occurrence in the environment are two features shared by a third heavy metal, cadmium (Cd). This element attracted public attention in connexion with the well-known Japanese "itai-itai" disease, which was traced to the prolonged ingestion of Cd (*Cited in F.C.T.* 1972, **10**, 249).

Morphological studies of the kidney

Administration of Cd chloride to Wistar rats at levels of 50 or 300 ppm Cd in the drinking-water for 40 or 24 weeks, respectively, resulted in a slight swelling of the epithelial cells of the proximal convoluted tubules and the formation of small vacuoles lying near the base of the cells (Nishizumi, *Archs envir. Hlth* 1972, **24**, 215). In addition, however, Itokawa *et al.* (*ibid* 1974, **28**, 149) observed desquamation of some of the epithelial cells and formation of tubular casts following the administration of 50 ppm Cd in the drinking-water (again as the chloride) for 17 weeks. This author also reported necrosis and partial hyalinization of the glomeruli. Combined administration of Cd sulphate by sc injection (1·4 mg $CdSO_4$ three times weekly) and orally (0·3% $CdSO_4$ in the drinking-water) produced histological changes similar to those that followed administration of the chloride (Berry, *Path. Biol., Paris* 1972, **20**, 401). The lesions apparent after treatment for 2 months consisted mainly of desquamation and loss of the brush border of some of the proximal tubular cells at the cortico-medullary junction. These changes became more pronounced after treatment for 3 months and were widespread in rats killed after 4 or 5 months. Hyaline casts and necrosis of epithelial cells were also observed.

Electron-microscopic studies

More striking and diverse changes were observed ultrastructurally. Nishizumi (*loc. cit.*) reported an increase and aggregation of smooth endoplasmic reticulum, an increase in microbodies and changes in the mitochondria consisting of vesiculation of the cristae and decreased matrical density. Rupture of the outer mitochondrial membrane was observed as a late event (week 40) in rats given 50 or 300 ppm Cd. The inner membrane was also involved at the higher level of treatment. The mitochondrial membranes remained unaffected throughout the 40-week treatment period in rats given 10 ppm Cd. Lysosomes and autophagic vacuoles showed both a dose- and time-related increase. This author did not observe any changes in

the glomerular or interstitial capillaries, but reported a thickening of the glomerular basement membrane.

Berry (*loc. cit.*) reported some interesting ultrastructural findings in the glomeruli. Numerous filaments displaying the banding that is characteristic of fibrin were observed in the capillary lumina, and were associated with platelets and erythrocytes. The platelets appeared to be free in the lumen and to have no connexion with the capillary wall. Some capillary lumina were blocked by the fibrin present in them. Changes similar to those found in the glomerular capillaries were found also in the interstitial capillaries. The endothelial cells contained granular deposits surrounded by a membrane and the podocytes had flattened processes.

The proximal tubular epithelial cells displayed electron-dense, amorphous deposits lying parallel to the basement membrane. These deposits were shown by the electron-probe technique to contain iron. The mitochondria contained deposits made up of needle-like filaments, which were shown to contain calcium and phosphorus. Cd was found only in the deposits within the proximal tubular lumen, along with calcium, phosphorus and sulphur.

In rabbits treated with Cd (160 ppm in the drinking-water for 200 days), the microscopic and ultrastructural changes in the kidney were broadly similar to those in the rat (Stowe *et al. Archs Path.* 1972, **94**, 389), but some additional lesions were identified. Histologically these consisted of early nuclear damage and an interstitial fibrosis, while ultrastructurally collagen deposition was apparent in the glomeruli.

Tests of renal function

The histological and ultrastructural changes in the kidney are reflected in alterations in renal function assessed by a number of test procedures. Gieske & Foulkes (*Toxic. appl. Pharmac.* 1974, **27**, 292) investigated changes in renal function in rabbits 3 days after administration of a single iv injection of a Cd chloride–mercaptoethanol mixture (10 μmol Cd and 350 μmol mercaptoethanol/kg). The glomerular filtration rate and *p*-aminohippurate clearance *in vivo* were severely depressed, the latter reflecting the damage to the proximal tubular epithelium. Reabsorption of nineteen amino acids, measured individually, was markedly inhibited, the site of this inhibition being the luminal membrane of the tubular cells. The relatively specific nature of the Cd-induced changes was indicated, however, by the finding that the maximum ability of the tubules to reabsorb filtered glucose was unaltered in the treated animals.

Aminoaciduria is a known complication of Cd toxicity in man and some authors claim that it is associated with hypercalciuria, impaired concentrating ability and impaired acid excretion (Gieske & Foulkes, *loc. cit.*). Goyer *et al.* (*Am. J. clin. Path.* 1972, **57**, 635) confirmed the presence of aminoaciduria in some Japanese workers engaged in smelting alloys of silver and Cd and exposed to a Cd concentration in the working environment of about 130 $\mu g/m^3$ over the 8-hour working day. The increased amino-acid excretion was modest and generally non-specific, the largest increases being noted in the cases of threonine, serine, glycine and alanine. Only three of the ten workers studied showed clear evidence of aminoaciduria and of these, the one exhibiting the most severe condition was thought to be a carrier of cystinuria. High protein excretion, however, was a common finding in the group, although the level of proteinuria did not correlate with the degree of increase in the urinary excretion of Cd.

Nomiyana *et al.* (*Toxic. appl. Pharmac.* 1973, **24**, 625) used the measurement of urinary enzymes in an endeavour to obtain a more complete picture of the renal damage produced by Cd intoxication. Seven rabbits given an sc injection of 6 mg Cd/kg/day (as Cd chloride) on nine successive days, except for a break of 1 day between the fourth and fifth doses, showed an increase in urinary excretion of acid phosphatase (AcPase), alkaline phosphatase (AlkPase) and lactic dehydrogenase on day 2. On day 5, there was a reduction in urine volume and, in four of the six animals, a transient increase in proteinuria. The tubular excretion rate for *p*-aminohippurate was subsequently reduced, and this decrease was maintained for at least 13 days after cessation of the Cd treatment, although excretion of the enzymes quickly returned to normal. When the experiment was repeated with 1·5 mg Cd/kg/day given for 45 days, similar but less marked changes in the pattern of renal excretion of AcPase, AlkPase and protein were observed and there was again a reduction in urine volume. Furthermore, urinary amino acids and glutamic–oxalacetic transaminase were increased on day 9 of treatment while the glomerular filtration rate, as measured by inulin clearance, and the rate of tubular *p*-aminohippurate excretion were reduced by day 21 of the treatment. These results, along with further results from stop-flow experiments, were thought to indicate that the glomeruli and proximal and distal tubules are all adversely affected by Cd.

Biochemical investigations

Considerable attention has been paid to the kind and type of macromolecules responsible for the binding of Cd in mammalian organs. The kidney has attracted particular interest in this connexion because of the marked accumulation of Cd in this organ. Kägi & Vallee (*J. biol. Chem.* 1961, **236**, 2435) isolated from the kidney cortex of horses a metal-binding protein, metallothionein, which they thought might be responsible for the binding. This protein has also been isolated from the cortex of the human kidney (Pulido *et al. Biochemistry, N.Y.* 1966, **5**, 1768). The site of this protein in the general cellular architecture is not well defined, however, although it seems to be well established that Cd is found mainly in association with the cytoplasmic soluble fraction. Shaikh & Lucis (*Archs envir. Hlth* 1972, **24**, 419) undertook the more precise location of the protein fraction responsible for the binding of Cd in the kidney and liver, and found that Cd was bound to proteins of 11,000–12,000 mol wt. They demonstrated that Cd remained firmly bound to this Cd-binding protein and suggested that it might represent a biochemical mechanism for the sequestration of toxic Cd ions.

Webb (*Biochem. Pharmac.* 1972, **21**, 2751) went a step further, pointing out that the proteins that bind Cd in the rat liver and kidney are not identical but have properties in common, such as an abundance of sulphydryl groups. They do not seem to be normal

components of these tissues but appear to be synthesized in response to the uptake of the foreign cation. If this mechanism occurs in man, it may have important implications in the assessment of hazard from the presence of Cd in the diet or other sections of the environment.

It seems, however, that any protection afforded by this protein is limited. We have indicated above that relatively low experimental levels of Cd interfere with both the morphology and the function of the kidney. Metabolic changes, too, are apparently produced by levels of this order and they do not appear to undergo rapid reversal. Thus Singhal *et al.* (*Science, N.Y.* 1974, **183**, 1094) found that daily administration of Cd chloride in an ip dose of 1 mg/kg/day for 45 days enhanced gluconeogenesis, as evidenced by significant increases in the activities of pyruvate carboxylase, phosphopyruvate carboxylase, hexosediphosphatase and glucose-6-phosphatase, the key rate-limiting enzymes involved in the biotransformation of non-carbohydrate precursors into glucose. When the measurements were repeated 28 days after discontinuation of treatment, these biochemical changes had not returned to control values.

Depression of sodium excretion, another adverse effect of Cd on the kidney, also persisted for a limited time after cessation of Cd treatment (Foulke *et al. J. pharm. Sci.* 1974, **63**, 563). Single doses of Cd acetate equivalent to 1 mg Cd/kg administered ip to rats reduced sodium excretion to about 40% of control values within 12 hours of administration and the values remained significantly below those of the controls for up to 7 days, but doses of 0·5 mg/kg and below given either as a single dose or by repeated injections were ineffective.

These biochemical studies throw some light on the pathways affected by Cd and it will be interesting to see whether future work can relate them to the morphological findings. The suggestion that protein-binding may act as a protective mechanism is particularly intriguing from a toxicological standpoint, but must be viewed in the light of the undoubted damage produced by Cd at both biochemical and pathological levels and, particularly, of the demonstration that some of these lesions do not appear to return to normal in a hurry. Attention must also be paid to the fact that Cd may have adverse effects also on other organs, particularly the liver and bone, as has been demonstrated by Stowe *et al.* (*loc. cit.*). Indeed the itai-itai disease demonstrates how easy it is to be misled if attention is concentrated on a single organ.

[P. Grasso—BIBRA]

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TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

COLOURING MATTERS

2868. Amaranth not teratogenic, embryotoxic or foetotoxic

Keplinger, M. L., Wright, P. L., Plank, J. B. & Calandra, J. C. (1974). Teratologic studies with FD & C Red No. 2 in rats and rabbits. *Toxic. appl. Pharmac.* **28,** 209.

Russian workers (Shtenberg & Gavrilenko, *Vop. Pitan.* 1970, **29** (2), 66) claimed that amaranth (FD & C Red No. 2) was gonadotoxic, foetotoxic and teratogenic in rats, but the lack of much experimental detail and the small numbers of animals used laid this verdict open to question. No evidence of teratogenicity was obtained in a subsequent FDA study in which amaranth was administered to rats by stomach-tube on days 0–19 of pregnancy, but there was an apparently dose-related increase in resorptions and foetal deaths, the increase being significant at levels above 15 mg/kg (Collins & McLaughlin, *Fd Cosmet. Toxicol.* 1973, **11,** 355; *Food Chemical News* 1972, **14** (16), 13). However, no such effects were evident in an FDA multigeneration feeding study in rats using dietary levels of up to 30,000 ppm (*Food Chemical News* 1974, **16** (12), 36) or in industry tests involving gavage of rats on days 6–15 of gestation, according to the standard protocol for teratogenicity testing. Results of this last study have now been published.

Amaranth administered by stomach-tube to pregnant rats in daily doses of 15, 50 or 150 mg/kg on days 6–15 of gestation was found to have no effect on maternal weight gain or the incidence of resorptions, and foetal viability, body weight and sex distribution and the incidence of external and internal defects were also unaffected. These parameters were similarly unaffected in rabbits given 1·5, 5 or 15 mg/kg daily in gelatin capsules on days 6–18 of gestation. At all levels there was an apparent decrease in the number of viable rabbit pups, but this was neither dose-related nor statistically significant. In both treated rabbits and negative controls there was a high and comparable incidence of supernumerary ribs and deficient ossification of the sternum. Treatment of other rats with sodium salicylate (200 mg/kg/day) and rabbits with thalidomide (37·5 mg/kg/day) confirmed that the animals used were susceptible to teratogenic effects.

The authors criticize the FDA study on the grounds that an unusually low number of resorptions was found in the negative control group, and that only one such group was used, as compared with three in both the rat and rabbit studies reported here. It is also thought possible that the different periods of dosage, or the different strains of rat (Sprague–Dawley in the FDA study, Charles River here) may have been responsible for the observed differences in effect.

[A recent Canadian study showed no effect on implantation or embryonic survival when amaranth was given to rats by gavage or in the diet on days 1–19 of pregnancy (Khera *et al. Fd Cosmet. Toxicol.* 1974, **12,** 507), suggesting that the period of administration is of little relevance. Similar negative results have been reported in a study conducted at the National Center for Toxicological Research (*Food Chemical News* 1974, **16** (12), 36). As a result of this accumulation of reassuring evidence, an *ad hoc* group of scientific consultants, appointed by the FDA, is expected soon to produce a favourable report on the colouring.]

2869. Carcinomas from Violet 6B

Ikeda, Y., Horiuchi, S., Imoto, A., Kodama, Y., Aida, Y. & Kobayashi. K. (1974). Induction of mammary gland and skin tumours in female rats by the feeding of Benzyl Violet 4B. *Toxicology* **2,** 275.

A Japanese paper last year reported the development of five mammary and four ear-duct "carcinomas" in 18 female rats who survived the feeding of 5% Violet 6B (Benzyl Violet 4B) in the diet for up to 1 yr, although no tumours were found in males (Uematsu & Miyaji, *J. natn. Cancer Inst.* 1973, **51,** 1337). Various shortcomings in this study prevented its general acceptance as a reliable index of the carcinogenic potential of Violet 6B, and similar conclusions were reached with regard to earlier studies conducted in the United States and Canada. A long-term mouse study, using dietary levels up to 3500 ppm, subsequently revealed no evidence of carcinogenicity or, indeed, any other adverse effects (Grasso *et al. Fd Cosmet. Toxicol.* 1974, **12,** 21), while in a 90-day study in rats a dietary level as high as 3% produced only weight changes in certain organs, without histopathological effects (Gaunt *et al. ibid* 1974, **12,** 11). However, another Japanese study now claims confirmation of the earlier findings of carcinogenicity in the rat.

Thirty-five female Sprague–Dawley rats (the same strain as was used in the first Japanese study) were fed Violet 6B at a dietary level starting at 1% and increased after 7 days to 3% and after a further 7 days to 5%, on which level the rats were maintained for the subsequent 12 months. The rats showed a significant decline in growth rate, although their food consumption was unaffected, and mortality increased from month 6, only two animals surviving the full experimental term. In contrast, all 35 control rats survived throughout. Of the rats fed Violet 6B, 22 developed a total of 38 tumours, of which 26 were mammary-gland carcinomas and 12 were squamous-cell carcinomas of the skin. Ten of the latter involved the ear duct, and the remaining two were found in the buccal and axillary regions. Mammary-gland tumours generally appeared earlier than squamous-cell carcinomas, the first tumour of each type being seen at wk 13 and 27, respectively. Hyperplasia of sebaceous

glands was observed in the skin at the site of development of both types of tumour. Despite the fact that both types were characterized as carcinomas, gross examination of the internal organs revealed no evidence of nodules suggestive of metastasis. No external tumours were seen in the control animals.

[This study is certainly more worrying than those reported previously, and presumably formed the basis of the recent US ban, on the grounds that the colouring used was certifiable FD & C Violet No. 1 (*Food Chemical News* 1973, **15** (4), 3). A possible explanation for the difference in results between this and the BIBRA mouse study may lie in the much higher dietary levels administered in the present case (5% compared with 0·35%), with consequent ingestion of much greater quantities not only of the colouring but also of its contaminants. It is to be hoped that an FDA study, undertaken as a result of the Japanese findings, will resolve the whole question.]

FLAVOURINGS, SOLVENTS AND SWEETENERS

2870. The metabolism of *d*-limonene

Igimi, H., Nishimura, M., Kodama, R. & Ide, H. (1974). Studies on the metabolism of *d*-limonene (*p*-mentha-1.8-diene). I. The absorption, distribution and excretion of *d*-limonene in rats. *Xenobiotica* **4**, 77.

Kodama, R., Noda, K. & Ide, H. (1974). Studies on the metabolism of *d*-limonene (*p*-mentha-1,8-diene). II. The metabolic fate of *d*-limonene in rabbits. *Xenobiotica* **4**, 85.

Although monoterpenes, of which the title compound is an example, are widely distributed in the natural environment and find extensive industrial use as flavouring agents in foods and cosmetics, little systematic information about their metabolic fate is available. The potential clinical use of *d*-limonene as an agent for dissolving cholesterol gallstones (Nishimura, *Jap. J. Surg.* 1972, **2**, 62) prompted this thorough study of the absorption, metabolism and excretion of *d*-limonene in rats and its metabolic fate in the rabbit.

In both studies, the animals were given ^{14}C-labelled *d*-limonene orally by stomach tube, in a single dose of the order of 800 mg/kg, and a number of urinary metabolites were isolated and identified. In both rat and rabbit urine, perillic acid, *p*-menth-1-ene-8,9-diol, perillic acid-8,9-diol and 8-hydroxy-*p*-menth-1-ene-9-yl-*β*-D-glucopyranosiduronic acid were found. However, at least two compounds present in rabbit urine, *p*-mentha-1,8-dien-10-ol and *p*-mentha-1,8-dien-10-yl-*β*-D-glucopyranosiduronic acid were not present in rat urine. A tentative scheme for the metabolism of *d*-limonene in the rabbit has been deduced to account for these metabolites.

The rat studies showed that *d*-limonene was rapidly absorbed from the gastro-intestinal tract, with blood levels of radioactivity reaching a maximum 2 hr after dosing and remaining high for 10 hr. A similar pattern was seen in the liver and kidneys. Elimination from the body was rapid; little activity was left at 48 hr, and autoradiography showed no significant accumulation at this time in any organ. The principal route of elimination was the urine and over 60% of the administered dose was eliminated by this route in 48 hr. Biliary excretion amounted to 25% of the dose within 24 hr, but as faecal excretion of radioactivity in non-cannulated animals was low, it appears that biliary metabolites were reabsorbed prior to elimination. The major biliary metabolite was identified as 8-hydroxy-*p*-menth-1-en-9-yl-*β*-D-glucopyranosiduronic acid, and the effect of this compound on the solubilization of cholesterol gallstones is under investigation.

ANTIOXIDANTS

2871. Propyl gallate: A clandestine sensitizer?

Kahn, G., Phanuphak, P. & Claman, H. N. (1974). Propyl gallate-contact sensitization and orally-induced tolerance. *Archs Derm.* **109**, 506.

Propyl gallate (PG) is extensively used as an antioxidant in fats to prevent the development of rancidity. It appears that no reports of contact sensitization to PG have appeared in the USA despite its use there since 1947, but evidence is presented that induced immunological tolerance may explain this absence of overt reactions.

Ten adult subjects applied 20% PG in 70% ethanol to the skin of the forearm daily for 24 days, or until redness or itching occurred. No subject developed signs of reaction during the first 14 days but during wk 3 and 4 five participants complained of pruritus and erythema. These effects were minimal in three cases and subsided within a few days, while in the other two the rash spread to the upper arm, neck and trunk within 3–4 days despite avoidance of further exposure to PG. Two of the mildly sensitized reactors and 25 non-sensitized controls were then treated with 2% PG and 2% lauryl gallate patch tests for 48 hr. Both sensitized persons had mild positive reactions to both esters while the controls showed no response.

In further tests, in guinea-pigs, intradermal injections of 0·1 ml of 5% PG in Freund adjuvant given three times on alternate days conferred strong sensitization lasting at least 3 months. The animals cross-reacted to lauryl gallate (weakly) but not to pyrogallol, gallic acid or methyl gallate. Three paintings of

guinea-pigs with 20% PG in 70% ethanol, each followed by occlusion for 24 hr, produced weak sensitization after 2 wk but high reactivity after 3 months. Tolerance to this procedure was seen, however, in guinea-pigs given PG orally for 7 consecutive days before intradermal injection and contact testing. The tolerance was specific and did not extend to dinitrochlorobenzene when this was used to induce concurrent sensitization.

AGRICULTURAL CHEMICALS

2872. Rapid metabolism of benomyl

Gardiner, J. A., Kirkland, J. J., Klopping, H. L. & Sherman, H. (1974). Fate of benomyl in animals. *J. agric. Fd Chem.* **22,** 419.

Benomyl (methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate) has been used for several years as a fungicide. Rats fed a dietary level of 2500 ppm were found to excrete methyl 5-hydroxy-2-benzimidazolecarbamate (5-HBC) in the urine (Gardiner *et al. J. agric. Fd Chem.* 1968, **16,** 1050). Those given [2-^{14}C]benomyl by gastric intubation in the present study also eliminated 5-HBC as the major urinary metabolite, in the form of three glucuronide and/or sulphate conjugates, and little if any (<5%) of the parent compound was excreted unchanged or as methyl 2-benzimidazolecarbamate (MBC). After 24 hr, 78·9% of the radioactivity was detected in the urine and 8·7% in the faeces, and a total of 98·9% had been eliminated by these routes after 72 hr. Tissue analyses revealed measurable quantities (0·2%) only in the fur, gut and gut contents and liver. MBC was eliminated by rats in an almost identical fashion, suggesting that it is an intermediate product in the conversion of benomyl to 5-HBC.

A dog given a capsule of radioactive benomyl similarly excreted about 99% in 72 hr, the faeces containing 83% and the urine 16%. Tissue residues were detectable only in the gut and its contents (0·13%) and in the liver (0·31%). About 82% of the faecal activity in the dog corresponded to MBC and only 12% to 5-HBC. Analysis of muscle, fat, liver and kidney from rats and dogs that had been fed 2500 ppm benomyl for 2 yr suggested an absence of marked accumulation; the highest residues were detected in the rat kidney, which contained 22 ppm 5-HBC in the males but only 2·8 ppm in females and in which small quantities of the isomeric 4-HBC and of benomyl and/or MBC were also evident.

Cows fed benomyl at dietary levels of 2, 10 or 50 ppm (providing 0·06, 0·3 or 1·4 mg/kg/day) for 32 days showed no adverse effects. In this case the urine contained up to 19 ppm 5-HBC and up to 1·9 ppm 4-HBC, and small quantities (not more than 0·06 ppm) of both metabolites were also detected in the milk of cows fed 50 ppm, but at a dietary level of 10 ppm only 5-HBC (0·01 ppm) was found in the milk. There were no detectable residues in the liver, kidney, fat or muscle at any stage, or in the excreta or milk 2 days after termination of treatment.

When chickens were fed 5 or 25 ppm dietary benomyl for 4 wk, 5-HBC was detectable in the excreta but not in the liver, breast muscle or fat, and none was found in eggs from birds given the lower level. Residues of 0·03–0·06 ppm were found in eggs from birds fed 25 ppm benomyl, but these were no longer evident after a 1-wk withdrawal period. No adverse effects were noted on feed consumption, body-weight gain or egg production.

2873. Male fertility and carbaryl

Thomas, J. A., Dieringer, C. S. & Schein, L. (1974). Effects of carbaryl on mouse organs of reproduction. *Toxic. appl. Pharmac.* **28,** 142.

Three-generation studies in rats and gerbils have shown that fertility is significantly reduced by the feeding of carbaryl at a level of 2000 ppm in the diet and that gerbils are more sensitive to carbaryl than are rats (*Cited in F.C.T.* 1972, **10,** 261).

The paper cited above is concerned with the effect of repeated oral doses of carbaryl on the reproductive tract of the male mouse. The animals were given a corn-oil suspension of carbaryl providing a dose of 8·5, 17 or 34 mg/kg daily by intubation for 5 days, followed on day 6 by a single injection of 10 μg [1,2-^{3}H]testosterone/kg, and the assimilation of the hormone by the prostate gland was studied. For this purpose, the mice were killed 5 min after the injection and the ^{3}H activity in the anterior prostate was determined. Carbaryl treatment produced no significant alteration in the weight of the testes or anterior prostate and there was only a slight and statistically insignificant increase in the prostate assimilation of testosterone. A single dose of 0·9 mg [^{14}C]carbaryl/kg given to normal mice resulted in only small amounts of radioactivity in the prostate gland, seminal vesicles and testes. The seminal plasma and epididymal fat pads also showed very low concentrations of radiolabel.

These findings indicate that, at the dose levels studied, carbaryl has no markedly toxic effects on the male reproductive tract of the mouse. Its distribution thus differs from that of DDT, which accumulates in substantial amounts in the male reproductive organs.

2874. Elimination of 2,4-D in man

Kohli, J. D., Khanna, R. N., Gupta, B. N., Dhar, M. M., Tandon, J. S. & Sircar, K. P. (1974). Absorption and excretion of 2,4-dichlorophenoxyacetic acid in man. *Xenobiotica* **4,** 97.

2,4-Dichlorophenoxyacetic acid (2,4-D) has been shown to be rapidly absorbed and excreted by the rat at low dose levels, although elimination of large doses was still not complete after 6 days (*Cited in*

F.C.T. 1967, **5**, 578). In man, adverse effects have been reported from occupational exposure to both 2,4-D and 2,4,5-trichlorophenoxyacetic acid (*ibid* 1971, **9**, 908), and a case of accidental poisoning has been described (*ibid* 1972, **10**, 262).

In the present study, six healthy male volunteers were given a single oral dose of 5 mg 2,4-D/kg, and blood samples were collected at varying periods up to 168 hr after treatment. A significant amount of 2,4-D was detected in the plasma after 1 hr, and the highest concentration (about 40 μg/ml) was reached in 7–24 hr. Plasma levels then declined steadily, although on average about 4 μg/ml was still present after 168 hr. The urine, collected for 96 hr after administration, contained 2,4-D after only 2 hr, and 76·5% on average had been eliminated by this route after 96 hr. In none of the samples was any metabolite of 2,4-D detected. None of the subjects complained of any ill-effects from the 2,4-D, and no changes were detected in blood pressure, pulse rate, haemoglobin content, or total or differential white cell count. The lack of metabolic transformation and rapid excretion suggested that cumulative toxicity from 2,4-D would be unlikely.

2875. DDT mutagenicity and the mouse

Johnson, G. A. & Jalal, S. M. (1973). DDT-induced chromosomal damage in mice. *J. Hered.* **64**, 7.

Clark, J. M. (1974). Mutagenicity of DDT in mice, *Drosophila melanogaster* and *Neurospora crassa*. *Aust. J. biol. Sci.* **27**, 427.

Large doses of DDT have been suspected of producing chromosomal damage in several species, and studies in the mouse have attracted some interest. In the first study cited above, single doses of 100–400 mg DDT/kg, dissolved in peanut oil, were injected ip into BALB/c mice, and bone-marrow cells were examined by karyotype analysis using a carbol fuchsin staining procedure.

Dose levels of 150 mg DDT/kg or more were found to induce significantly higher proportions of chromosomal aberrations than were found in control animals. This must be viewed in the context of an LD_{50} value of 550 mg DDT/kg by the ip route in this species. Deletions or sticky chromosomes were the commonest abnormalities but ring and metacentric chromosomes were occasionally observed. Since the induction of chromosomal damage is closely associated with a mutagenic effect in mammals, the authors concluded that DDT appeared to be a potential mutagen, at least in the mouse.

This possibility received further consideration in a later paper (Clark, cited above), which described a dominant lethal assay and a host-mediated assay using *Neurospora crassa* in mice, as well as studies of DDT mutagenicity in the fruit fly, *Drosophila melanogaster*. The test material in all these studies was technical-grade DDT containing 80% *p,p'*- and 19% *o,p'*-DDT.

Acute oral dosage of male mice with 150 mg DDT/kg/day for 2 days induced dominant lethal mutations in early spermatid and spermatocyte stages, and oral doses of 100 mg/kg given twice weekly for 10 wk produced a persistent increase in the numbers of dominant lethal mutations. The latter treatment caused a marked reduction in testis weight and in the numbers of cells in the seminiferous tubules and degeneration of some B-type spermatogonia. An increase in chromosomal anomalies, including breakage and stickiness, was detected in the spermatocytes following acute treatment with DDT. A similar increase in dominant lethality and chromosomal aberrations was demonstrated in DDT-treated fruit flies, but treatment of a fly population for 8 months caused no significant increase in the frequency of second-chromosome recessive lethal mutations. The effects of DDT on *N. crassa* both *in vitro* and in the peritoneal cavity of mice were inconclusive, but it appeared that any weak mutagenic effect of DDT on this organism was not potentiated by the metabolic or other influences of the mouse in the host-mediated assay.

While this study shows that DDT has an adverse effect on reproduction in the mouse and is weakly mutagenic in both the mouse and *D. melanogaster*, two species in which its metabolic routes differ, no firm conclusions can be drawn on the mechanism of this action or on the identity of the actual mutagen. It seems probable that DDT itself is the active compound, but the possible involvement of some metabolite or of a contaminant in the commercial DDT cannot be discounted.

[As in the case of potential DDT carcinogenicity, the significance of the available data to the question of incriminating DDT as a possible mutagen in man is far from clear. In both cases effects have been observed in mice, but a recent dominant lethal test on DDT in rats demonstrated only a marginally positive reaction (Palmer *et al. Fd Cosmet. Toxicol.* 1973, **11**, 53). Moreover, it could be argued that some toxic effect is inevitable at concentrations of DDT approaching the LD_{50} value.]

2876. Hexachlorobenzene across the placenta

Villeneuve, D. C., Panopio, L. G. & Grant, D. L. (1974). Placental transfer of hexachlorobenzene in the rabbit. *Envir. Physiol.* **4**, 112.

Hexachlorobenzene (HCB) ingestion has been associated with cutaneous porphyria in man, and porphyria, liver damage and phototoxicity have been described in HCB-fed rats (*Cited in F.C.T.* 1974, **12**, 283). Dose levels of 80 mg/kg or more in pregnant rats were associated with maternal neurotoxicity and a reduction in foetal weight, and even at lower levels there was an apparent dose- and time-related increase in the incidence of fourteenth and sternal defects in the foetus, although it was thought that these may have been indications of maternal stress rather than of a direct teratogenic effect (Khera, *Fd Cosmet. Toxicol.* 1974, **12**, 471). In the quail, HCB accumulated in the liver in a dose-dependent fashion, and males had higher brain residues than females, a finding consistent with HCB excretion via the egg (*Cited in F.C.T.* 1972, **10**, 263). HCB residues have also been demonstrated in human blood (*ibid* 1973, **11**, 913).

In the present study, oral dose levels of 0·1, 1·0 or 10 mg HCB/kg given to pregnant rabbits on days 1–27 of gestation had no adverse effects on maternal

body or organ weights, foetal body or brain weights or placental weights, and there were no increases in foetal deaths or deformities, resorptions or abortions. Foetal liver weight was decreased in the group given 0·1 mg/kg, but this was apparently fortuitous as no such effect was seen at higher levels. In the dams, tissue concentrations increased markedly with increasing dose level; the highest concentrations (350 ppm at 10 mg/kg) were found in the fat, followed in decreasing order by the liver, heart, kidney, brain, lung, spleen and plasma. A dose-dependent accumulation was also found in the foetus, in which the total body burden ranged from 0·025 mg with the 0·1-mg/kg dose level to 24·84 mg with 10 mg/kg. Foetal liver concentrations were greater than in the corresponding maternal organ and comprised about 70–80% of the total foetal body burden, the proportion being greatest at the lowest dose level. Brain concentrations, however, were generally lower in the foetus than in the dam. The pattern of accumulation was similar to that previously reported for polychlorinated biphenyls (Grant *et al. Envir. Physiol.* 1971, **1**, 61), except that the latter accumulated to a greater extent in maternal liver and less in foetal liver and, unlike HCB, the polychlorinated biphenyls were foetotoxic under the experimental conditions used.

2877. Percutaneous absorption of pesticides

Feldmann, R. J. & Maibach, H. I. (1974). Percutaneous penetration of some pesticides and herbicides in man. *Toxic. appl. Pharmac.* **28**, 126.

Twelve ^{14}C-labelled pesticides and herbicides, dissolved in acetone, were applied to the ventral forearm of human volunteers in an amount (4 $\mu g/cm^2$) equivalent to a thin film of 0·25% solution. The area of application was left uncovered and volunteers were asked to leave it unwashed for 24 hr. Absorption was assessed by measuring the percentage of the applied radioactivity excreted in the urine over a 5-day period and adjusting this to allow for incomplete excretion, the necessary correction factor being determined from the proportion of an iv dose excreted in the urine over the same period of time.

The ability of the kidney to excrete these compounds varied over a wide range, from just over 3% for aldrin and dieldrin, for example, to over 80% for malathion, baygon and 2,4-D. With regard to topical application, carbaryl was the compound found to be most completely absorbed and diquat the least, 74 and 0·3% of the doses of these two compounds being absorbed. The levels of absorption of the remaining compounds tested, which also included azodrin, ethion, guthion, parathion and lindane, ranged between 3 and 20%. There were marked individual variations in the skin absorption of any one compound under similar experimental conditions, standard deviations of 33–50% of the mean being obtained. It was established that very little of this variation was due to experimental error.

While these data have certain limitations as far as practical applications are concerned, they provide useful information on one factor to be taken into account in a comparison of the relative toxic potential of these compounds when applied to the skin surface.

MISCELLANEOUS CONTAMINANTS

2878. Cardiovascular lesions from a linoleate oxidation product

Cutler, M. G. & Schneider, R. (1974). Linoleate oxidation products and cardiovascular lesions. *Atherosclerosis* **20**, 383.

It is not yet known whether linoleate hydroperoxide may be involved in the pathogenesis of atheroma in man, or whether its presence in diseased arteries (Harland *et al. Biochim. biophys. Acta* 1973, **316**, 378) is merely a consequence of the structural lesions already present. To cast further light on the problem, an investigation into the toxic effects of linoleate hydroperoxide in the cardiovascular system of the rat and rabbit was undertaken.

A total dose of 353 mg sodium linoleate hydroperoxide was administered to rats in 20 sc injections given at intervals of 4–5 wk over 100 wk. In another series of experiments, rabbits were injected sc with either sodium linoleate, sodium linoleate hydroperoxide or degradation products of linoleate hydroperoxide in total doses of 750, 750–1000 or 750 mg, respectively, the injections being given at intervals of 3 wk. Control animals of both species received injections of physiological saline. Other rabbits received cholesterol in the diet twice weekly throughout the experimental period of 90 wk. All animals were killed 24 hr after the last injection.

Following treatment of rats with linoleate hydroperoxide, the lipid peroxide content of the liver was significantly increased compared with the saline-treated controls, but no effect was noted on the values obtained at the site of injection, or in the aorta or heart. In rabbits, a significant increase both in the liver and in the aorta resulted from this treatment. Linoleate administration produced no significant increase in the peroxide content of any of the organs examined, and after injection of the linoleate-hydroperoxide degradation products only the aorta showed a significant increase. The feeding of cholesterol was without significant effect on the peroxide value of the aorta, heart or liver.

Administration of linoleate hydroperoxide produced a significant increase in heart weight relative to body weight in rabbits. This effect was not seen in similarly treated rats nor in rabbits given the other treatments.

On histological examination, perivascular and/or interstitial fibrosis of the myocardium was found in 95% of the hearts of rats treated with linoleate hydroperoxide, compared with about 50% of the saline-treated controls. The coronary vessels of all the treated rats showed medial thickening, but no abnormalities were observed in the aorta. In rabbits, the incidence of myocardial fibrosis (perivascular and interstitial) was significantly increased in the groups that received linoleate hydroperoxide or cholesterol.

In the coronary vessels, intimal plaques occurred to a significant extent only in the rabbits fed cholesterol, while in the aorta they were visible to the naked eye in all rabbits given either linoleate hydroperoxide or cholesterol. The plaques contained deposits of lipid, fibrinogen and β-lipoprotein. The small intimal plaques seen microscopically in about 50% of the rabbits injected with linoleate hydroperoxide degradation products contained lipid, but fibrinogen and β-lipoprotein were not detected.

Total serum lipids were significantly raised only in the rabbits given dietary cholesterol, and in this group the serum levels of cholesterol and β-lipoprotein were also significantly increased. Injection of linoleate hydroperoxide resulted in a slight rise in serum cholesterol only and no significant changes in any of these levels resulted from treatment with linoleate or linoleate-hydroperoxide degradation products.

In view of the findings of lesions in the cardiovascular system of rats and rabbits treated with linoleate hydroperoxide, it is suggested that the compound may exert similar toxic effects in the heart and vessels of man and that its presence in atheromatous plaques could possibly increase the severity of the disease process.

THE CHEMICAL ENVIRONMENT

2879. Hypotension from sodium azide

Roberts, R. J., Simmons, A. & Barrett, D. A., II (1974). Accidental exposures to sodium azide. *Am. J. clin. Path.* **61**, 879.

Early work showed sodium azide to be a potent hypotensive agent, with cardiovascular activity similar to that of sodium nitrite (Graham, *J. Pharmac.* [*sic*] 1949, **4**, 1). It has now been reported that a worker in a haematology laboratory developed symptoms of tachycardia, hyperventilation and hypotension after accidental ingestion of a small quantity of Tris buffering solution, containing 4% sodium azide as a preservative. Similar symptoms of hypotension occurred in another incident in which blood-bank saline solution was accidentally used as a lavage solution for collecting gastric cytology specimens. Fortunately recovery in the first case was complete within 24 hr without specific therapy, and the symptoms in the second incident were successfully treated with supportive methods.

Certain laboratory agents containing 0·1% sodium azide bear a warning that they are not for internal use, but neither these nor products with higher concentrations carry any indication of the potential danger. As 1 ml of a 4% solution ingested by a 60-kg adult would provide a dose of 700 μg/kg and the hypotensive dose in man has been put at only 0·2–4·0 μg/kg, the potential toxicity of such products would appear to warrant greater publicity.

2880. Lead exposure from lubricating oils

van Peteghem, Th. & de Vos, H. (1974). Toxicity study of lead naphthenate. *Br. J. ind. Med.* **31**, 233.

Recent investigation of oils and greases used in a steel plant disclosed that several contained lead naphthenates to improve the performance of the lubricants under high pressure. The heat and friction cause dissociation of the lead from the naphthenate. Naphthenic salts are of comparatively low toxicity and the main source of concern was therefore the lead component.

Blood levels of lead and urinary δ-aminolaevulinic acid were measured in a number of employees in the plant, including ten subjects not exposed occupationally to lead. The maintenance technicians and assemblers who were investigated have to clean electrically driven motors and lubricate them with the lead-containing oils. Blood-lead levels, determined by absorption spectrometry, were found to be significantly higher in the workers exposed to the lead-containing lubricants than in those not exposed to lead occupationally, even when two extremely high results were excluded. Twelve of the 104 technicians examined had blood-lead levels in excess of 40 μg/100 ml, a level above normal but below the maximum acceptable concentration. Comparable results were obtained for urinary δ-aminolaevulinic acid determined by the method of Davis & Andelman (*Archs envir. Hlth* 1967, **15**, 53).

The authors concluded that although the increased lead level present caused changes in the haem cycle, the reactions were acceptable in terms of lead toxicity.

2881. Lead and the diet

Garber, B. T. & Wei, E. (1974). Influence of dietary factors on the gastrointestinal absorption of lead. *Toxic. appl. Pharmac.* **27**, 685.

Kello, D. & Kostial, K. (1973). The effect of milk diet on lead metabolism in rats. *Envir. Res.* **6**, 355.

The acceptable upper limit of lead (Pb) intake, especially by children, has been assessed at different figures, in accordance with variable data for the absorption and retention of ingested Pb (*Cited in F.C.T.* 1975, **13**, 277). That dietary factors have a considerable influence on the body load of Pb derived from the environment has long been known and the elucidation of some of these factors is of obvious value.

In the first paper cited above, the authors describe the oral administration of Pb acetate labelled with ^{210}Pb to mice in a single intubated dose of 0·2, 2·0 or 20 mg Pb/kg. The size of the dose had no significant effect on the fraction absorbed (about 5% in each case), and about 96% of the ingested dose was excreted within 24 hr. Prior starvation of the animals for 16 hr significantly increased Pb absorption in those given the 0·2 mg/kg dose, but absorption from

the 2·0 mg/kg dose was not affected, indicating that the presence of food in the stomach depresses absorption of traces of Pb. Five chelating agents, nitrilotriacetic acid (NTA), diethylenetriammepentaacetic acid (DTPA), ethylenediaminetetraacetic acid (EDTA), D-penicillamine and Na citrate, were then given orally in equimolar doses, in each case with 2 mg Pb/kg. The absorption of Pb was significantly increased by the presence of NTA or Na citrate, and also by orange juice (containing citrate), whereas DTPA and EDTA and milk, also administered with 2 mg Pb/kg, had no significant effect on absorption. Thus, Pb absorption from the gastro-intestinal tract, whether from traces or from larger quantities in the diet, depends upon the amount and chemical composition of the food taken with it.

The findings of the second paper cited indicate that, notwithstanding the above observation that milk has no significant effect on Pb absorption, this may not be true for a high-milk diet. Female rats were given a single oral or ip dose of ^{203}Pb chloride while being maintained on a normal diet or on one of three diets containing a high proportion of milk. The milk diets involved a higher daily intake of fat and protein, and a lower intake of carbohydrate, vitamin D and iron, compared with the control diet. The whole-body retention of orally administered ^{203}Pb determined after a 6-day interval was much higher in the rats on the milk diets, the ratios of the values for these groups to the control-diet value being approximately 5 for rat food plus cows' milk, 33 for an aqueous powdered-milk diet and 57 for a diet of cows' milk only. The corresponding ratio after an ip dose of ^{203}Pb did not exceed 1·30, irrespective of the diet. It is thus evident that a high-milk diet can enhance body retention of dietary Pb by increasing its absorption from the gastro-intestinal tract.

2882. Another dust causes problems

Pimental, J. C. (1973). A granulomatous lung disease produced by bakelite. A clinic-pathologic and experimental study. *Am. Rev. resp. Dis.* **108,** 1303.

Bakelite, a phenol-formaldehyde condensation product, is widely used industrially, particularly in electrical and telephonic equipment, and dates back to the earliest days of the plastics industry. Findings now presented suggest that the inhalation of bakelite dust can produce a diffuse respiratory disease with a clinical similarity to the extrinsic allergic alveolitis associated with farmer's lung, bagassosis and similar fungal syndromes (*Cited in F.C.T.* 1969, **7,** 668).

This paper describes the case histories of two patients, one a 43-yr-old cabinet maker, the other a 42-yr-old female factory worker, who were exposed to bakelite dust for 3 and 15 yr, respectively. The cabinet maker's illness was of sudden onset and progressed rapidly. He complained of weakness and dyspnoea on exertion and chest X-rays revealed disseminated reticulonodular lesions. Lung function was not significantly affected but lung-biopsy tissue showed sarcoid-type granulomas with many inclusions within their epithelioid and giant cells. These effects, and the radiographic changes, regressed to some extent without treatment following the removal of the patient from his occupational environment.

In the second case, a 6-yr history of weakness and loss of appetite, accompanied during the latter half of this period by bouts of fever with a persistent cough, led to progressive irreversible respiratory insufficiency and death. Bilateral diffuse pulmonary fibrosis was found at autopsy together with "honeycomb" lung and marked arteriosclerosis of the branches of the pulmonary artery. In some areas of the lungs, remnants of epithelioid granulomas were identified in an advanced stage of healing. Inclusions similar to those found in the other patient were seen in both the granulomas and fibrotic areas.

The presence of phenolic compounds within the inclusions was demonstrated, and a histochemical technique (based on reaction with cyclohexanol–2,6-dibromoquinone-chlorimine) was used to show that the bakelite dust within the pulmonary lesions of these patients was similar in nature to the bakelite dust that occurred in the factory environments.

Lung changes were investigated in guinea-pigs exposed to a high atmospheric concentration of bakelite dust 3 times/day for periods up to 325 days. Only slight macroscopic changes were observed, with five animals showing signs of emphysema, but microscopic examination revealed pulmonary lesions in 11 of the 18 guinea-pigs tested. In about 50%, the interalveolar septa showed new formation of reticular fibres and a cellular infiltration of histiocytes and fibroblasts, inclusions being found in histiocytes in the septa. In others, sarcoid-type epithelioid granulomas were also found, localized in the interalveolar septa, under the pleura and in the connective tissue around the vessels and bronchi and containing foreign-body giant cells. In inclusions found in both giant cells and some epithelioid cells in these granulomas, a positive reaction for phenol compounds was demonstrated by the histochemical technique described above.

Following a review of 1250 pulmonary resection specimens and 175 surgical lung biopsies, eight further cases of this syndrome were identified in workers who had been exposed for long periods to bakelite dust. From the data considered, the authors conclude that the disease can present itself in at least two distinct forms, one being an interstitial disease of the lung involving, in some cases, the formation of epithelioid granulomas, and the other a predominantly bronchial manifestation with initial signs resembling "chronic bronchitis" leading to the formation of bronchiectasis and pulmonary fibrosis.

2883. Another black mark for benzene?

Aksoy, M., Erdem, S., Dinçol, K., Hepyüksel, T. & Dinçol, G. (1974). Chronic exposure to benzene as a possible contributory etiologic factor in Hodgkin's disease. *Blut* **28,** 293.

The radiomimetic effect of benzene has previously been described (*Cited in F.C.T.* 1972, **10,** 271). The solvent has been incriminated in the finding of a higher rate of acute leukaemia among exposed workers (*ibid* 1971, **9,** 750), and concern has also been expressed over its apparent effect in increasing chromosomal aberrations in a way that failed to correlate with time of exposure or the age of the subject (*ibid* 1970, **8,** 601).

Aksoy *et al.* (cited above) have studied workers in Turkey who were exposed to benzene during the manufacture of slippers and shoes, and have identified 34 patients with pancytopenia, 26 with acute leukaemia and one with myeloid metaplasia. During a 5-yr period, they also found six workers who had been exposed to benzene for prolonged periods in various industries among a total of 94 patients admitted to an Istanbul clinic suffering from Hodgkin's disease. All six had been using benzene-containing adhesives in poorly ventilated premises where concentrations of 150–210 ppm benzene were generally present during working hours, with higher peaks at intervals when the adhesive was being applied. The mean duration of exposure was 11 yr (range 1–28 yr). The apparently disproportionate number of benzene-exposed workers among this group of patients with Hodgkin's disease raises the possibility of a causal relationship between this disease and benzene exposure. Nevertheless, the lack of reliable statistics showing the incidence of the disease in the general working population of Turkey precludes any firm conclusion.

2884. Metabolism of ethylbenzene

Kiese, M. & Lenk, W. (1974). Hydroxyacetophenones: Urinary metabolites of ethylbenzene and acetophenone in the rabbit. *Xenobiotica* **4**, 337.

Mandelic acid is a major urinary metabolite of inhaled ethylbenzene (EB) in man, although a much smaller proportion is excreted in this fashion after EB is absorbed through the skin (*Cited in F.C.T.* 1968, **6**, 105; *ibid* 1971, **9**, 753). When EB was given orally to rats, hydroxylation of the side chain produced 1- and 2-phenylethanols, and a small quantity was eliminated in the form of *p*-ethylphenol (*ibid* 1971, **9**, 301). In the rabbit, preliminary indications have recently been obtained of an additional metabolite of EB, tentatively identified as ω-hydroxyacetophenone (Kiese & Lenk, *Biochem. Pharmac.* 1973, **22**, 2265 & 2275), and this situation has now been further explored.

Ten male rabbits were each injected ip with 1 g EB, and their urine was collected for 48 hr and incubated with β-glucuronidase. In addition to 1-phenylethanol (2–10% of the administered dose), small quantities of ω-, *p*- and *m*-hydroxyacetophenones were identified and confirmed by nuclear magnetic resonance spectrometry in amounts corresponding to 0·04–0·11, 0·13 and 0·03%, respectively, of the dose administered. Acetophenone administered in similar fashion produced a 3·55% yield of 1-phenylethanol and somewhat greater quantities (0·5–0·95, 0·4 and 0·1% respectively) of the ω-, *p*- and *m*-hydroxyacetophenones.

These results suggest that, in the rabbit, acetophenone is an intermediate in the major pathway of EB metabolism to hippuric acid, and is converted by the side reactions of *p*- and *m*-hydroxylation to *p*- and *m*-hydroxyacetophenones. The major pathway apparently involves its ω-oxidation to ω-hydroxyacetophenone, which is then converted successively to phenylglyoxal, phenylglyoxylic acid and (by a process of decarboxylation and conjugation) hippuric acid. Mandelic acid and 1-phenylethanol would also be produced during the conversion of EB to acetophenone, while an accessory metabolic pathway, identified by previous workers, involves ω-oxidation of EB to phenylacetic acid and its conjugation to produce phenaceturic acid.

2885. Short tests on diaminodiphenylmethane

Gohlke, Rosemarie u. Schmidt, P. (1974). 4,4'-Diaminodiphenylmethan—histologische,fermenthistochemische und autoradiographische Untersuchungen im akuten und subakuten Versuch an Ratten mit und ohne zusätzliche Hitzebelastung. *Int. Arch. Arbeitsmed.* **32**, 217.

4,4'-Diaminodiphenylmethane (DDM), used industrially as a hardener for epoxy resins and as a raw material in the preparation of isocyanates, has been shown to be toxic in a range of animal species and its hepatotoxicity in man was demonstrated some years ago through the medium of an accidentally contaminated bag of flour (*Cited in F.C.T.* 1967, **5**, 120). There has also been some indication (*ibid* 1968, **6**, 809) that DDM may have carcinogenic properties, but the study that originally raised this question was on too small a scale to be conclusive. The work now reported was concerned not with the possible carcinogenicity of DDM but with its short-term effects on the liver of rats.

Male rats were given a single dose of 50–600 mg DDM/kg by gavage and were killed 24 hr later, or were given eight doses of 8–50 mg DDM/kg/day over a 10-day period and killed between 24 and 264 hr after treatment. In each study, some of the animals were subjected to an additional stress in the form of an ambient temperature of 35–37°C and a relative humidity of 50–70%, but this did not have any significant effects on the findings in the test animals. Both single and repeated doses caused bile-duct necrosis, a loss of glycogen and a marked increase in mitotic activity, associated with an increased incorporation of labelled thymidine into the nuclei of the hepatocytes and bile-duct epithelium. High doses (200–600 mg/kg) induced periportal necrosis of the liver parenchyma. Repeated doses of 8 mg/kg had little effect, however.

Histochemical studies on the livers of DDM-treated rats revealed a marked drop in the activities of several enzymes, notably succinic dehydrogenase, lactic dehydrogenase and acid phosphatase, together with a significant increase in glucose-6-phosphate dehydrogenase and alkaline phosphatase and dose- and time-related changes in glucose-6-phosphatase, compared with controls. Apart from the liver changes, the higher single doses caused, in some rats, lesions in the kidneys, heart muscle, brain and/or testes, characterized particularly by inter- and intracellular oedema and degeneration of the parenchyma, and some histological changes were also seen in the spleen, thyroid and adrenals.

This study thus yielded additional evidence that DDM can induce liver enlargement and necrosis, disrupt carbohydrate metabolism and increase the rate of mitosis, as well as having some extrahepatic effects, but was too brief to contribute to the question of possible carcinogenicity. Perhaps the long-term studies to be reported later by these authors will provide some clarification on this point.

2886. More solvent teratogenicity studies

Schwetz, B. A., Leong, B. K. J. & Gehring, P. J. (1974). Embryo- and fetotoxicity of inhaled carbon tetrachloride, 1,1-dichloroethane and methyl ethyl ketone in rats. *Toxic. appl. Pharmac.* **28**, 452.

We recently reviewed a paper describing dose-related foetal abnormalities that occurred following inhalation of up to 300 ppm chloroform by rats (*Cited in F.C.T.* 1975, **13**, 402). These results led the same group to investigate the sub-anaesthetic effects of other solvents commonly found in the working environment. Because it appeared that the hepatotoxic effects of chloroform might be implicated in the original findings, carbon tetrachloride (CCl_4) was tested as a compound with a high hepatotoxic potential, and 1,1-dichloroethane (DCE) was selected because of its lack of hepatotoxic effect. Methyl ethyl ketone (MEK) was included as a widely used nonchlorinated solvent. Female Sprague–Dawley rats were exposed to 300 or 1000 ppm CCl_4, 3800 or 6000 ppm DCE or 1000 or 3000 ppm MEK for 7 hr/day on days 6–15 of pregnancy. Control animals were exposed to filtered room air. Non-pregnant females were treated at the same time as the test animals and used for evaluation of changes in serum glutamic–pyruvic transaminase (SGPT) activity.

SGPT activity was raised throughout the exposure period in CCl_4-treated rats and gross changes were apparent in the liver on day 15, but these effects were not evident 6 days after the last exposure. The toxicity of CCl_4 also caused a reduction in maternal body weight but had no effect on conception or the number of implantations. The number of foetal resorptions was normal, but development of the foetuses was retarded. A significant increase in sternebral abnormalities was seen in foetuses exposed to 1000 ppm CCl_4, but this was regarded as a sign of retarded development rather than of teratogenicity. With DCE, maternal food consumption and weight gain were decreased, and increased relative liver weights were found 6 days after the end of treatment in the non-pregnant test animals. DCE appeared to have little embryotoxic effect, although foetal development was retarded. MEK, however, at the 3000 ppm exposure level caused a low incidence of true foetal abnormalities (acaudia, imperforate anus and brachygnathia) and a significant increase in malformations indicative of delayed foetal development. No maternal toxicity was apparent.

From this and their other results, the authors suggest that there is no correlation between the toxicity of these solvents to the dam and their effects on the foetus. They conclude from a comparison of their dose levels with the time-weighted threshold limit values (TLVs) proposed by the American Conference of Governmental Industrial Hygienists that, in relation to the respective TLVs (25, 10, 200 and 200 ppm), chloroform is more toxic to the developing embryo than CCl_4, DCE or MEK.

NATURAL PRODUCTS

2887. Another Aspergillus toxin

Wu, M. T., Ayres, J. C., Koehler, P. E. & Chassis, G. (1974). Toxic metabolite produced by *Aspergillus wentii*. *Appl. Microbiol.* **27**, 337.

Continuing the saga of mould-contamination of country-cured ham (*Cited in F.C.T.* 1975, **13**, 159), we come to *Aspergillus wentii* and the toxin it produces. Mycelium of a strain of *A. wentii*, initially isolated from country-cured ham and subsequently cultured on a yeast-extract sucrose (YES) medium, was highly toxic to mice when fed in ground mouse chow, a 1:9 (w/w) ratio of mycelium to chow killing all the treated mice in 4–6 days. Corn and rice contaminated with the mould were much less toxic, but chloroform extracts of the mycelium were particularly active, a 1:25 extract-chow mixture causing 100% mortality in 4–5 days. Lower dietary levels of the extract caused a more delayed death preceded by weakness and growth retardation.

Thin-layer chromatography of the chloroform extract produced thirteen bands, several of which (nos 1, 2, 4, 6, 7, 8 and 11) had a markedly adverse effect on the hatchability of chick embryos. Band no. 6 was the most toxic, reducing hatchability to zero from the 98% level achieved in chloroform-treated control eggs. Further chromatography of this fraction followed by crystallization yielded orange-red needle-shaped crystals (m.p. 285–286°C) with absorption maxima in chloroform solution at 270, 295 and 452 nm. The minimum amount of this component that completely inhibited hatching of chick embryos was 50 μg/egg, a 100% hatch being obtained with 10 μg/egg.

2888. Bracken and the mouse embryo

Yasuda, Y., Kihara, T. & Nishimura, H. (1974). Embryotoxic effects of feeding bracken fern (*Pteridium aquilinum*) to pregnant mice. *Toxic. appl. Pharmac.* **28**, 264.

A considerable amount of research has been devoted to the study of bracken since it was found to be carcinogenic in cows (*Cited in F.C.T.* 1968, **6**, 108) and several studies have concentrated on the identity and location of the carcinogenic factors in bracken (*ibid* 1975, **13**, 405). Various compounds, including urethane and other carbamates for example (*ibid* 1968, **6**, 412), have been shown to be teratogenic as well as carcinogenic in rodents, but although carcinogenic effects have been demonstrated in the offspring of mice fed on bracken extracts during pregnancy and lactation (*ibid* 1972, **10**, 882), it appears that no evidence of teratogenicity or embryotoxicity of bracken has been reported.

In the study cited above, eight female ICR-JCL mice were given a diet containing 33% dried bracken-fern powder from day 0 to day 17 of pregnancy and were

then killed. Two control groups, each of seven pregnant mice, were fed the basal diet or the basal diet containing 33% cellulose over the same period. Day 0 was the day on which a vaginal plug was found. Blood samples were taken just before death and used for the estimation of serum-protein levels. The numbers of implantation sites and foetal deaths were recorded, and the live foetuses were weighed, examined for gross external and internal malformations and then cleared, stained and examined for skeletal anomalies.

Compared with the other two groups, the bracken-fed animals showed a significant reduction in body weight in spite of a higher food intake. Reproduction data parameters were similar for all the dams, however. The numbers of implants, foetal mortality and incidence of external malformations showed no significant differences between the three groups, although the mean foetal weights in the bracken-fed group were significantly reduced. Examination of cleared foetuses showed an increase in rib anomalies together with retarded ossification and incomplete fusion of the sternebrae in the test group. The suggestion that the bracken-fed animals might be protein-deficient was rejected on the grounds that higher quantities of dietary protein were available to these animals than to the controls, and it was therefore concluded that bracken itself was responsible for the maternal and foetal toxicity observed.

However bracken has been associated with anti-thiamine activity in some species (*ibid* 1973, **11**, 163) and, since thiamine deficiency can cause growth retardation in rat embryos and maternal toxicity in rats (Brown & Snodgrass, *J. Nutr.* 1965, **87**, 353), it seems that this factor may have contributed to the effects observed.

[More conclusive information might have been obtained if larger numbers of animals had been used in each group and more than one dose level had been studied.]

2889. A haemolytic factor in kale

Smith, R. H., Earl, C. R. & Matheson, N. A. (1974). The probable role of *S*-methylcysteine sulphoxide in kale poisoning in ruminants. *Biochem. Soc. Trans.* **2**, 101.

The anaemia caused in cattle and goats by a high intake of kale (*Cited in F.C.T.* 1971, **9**, 759) also occurs to a less marked extent in sheep. The factor responsible for the haematological changes is not limited to any one variety of kale but its chemical identity has so far remained unknown.

The work cited above was aimed at elucidating the nature of this haemolytic factor, which was found to remain in the aqueous phase after exhaustive chloroform-extraction of largely deproteinized and clarified kale juice. This aqueous phase, which was highly toxic when administered to a goat, was fractionated by displacement chromatography. Fractions rich in acidic amino acids were obtained and when these were pooled and administered via a rumen cannula to goats fed on hay, the haematological changes observed were comparable to those in goats fed exclusively on kale.

Relatively high concentrations of *S*-methylcysteine sulphoxide (SMCSO) were present in both whole kale and the haemolytic fraction, and support for the possibility that this compound might be implicated in kale anaemia was derived from the finding of high concentrations of volatile organic sulphur compounds in the rumen of an acutely poisoned goat. Administration of SMCSO to a hay-fed goat again produced the type of haematological response seen with kale. The possibility that the active haemolysin was produced by rumen fermentation of SMCSO led to *in vitro* studies with rumen contents. Dimethyl disulphide was the main product of the early fermentation stage, while later the proportions of methanethiol and dimethyl sulphide increased, although the latter remained a relatively minor component. *S*-Methylcysteine, present to a smaller extent in kale, also gave rise mainly to methanethiol and dimethyl disulphide with a minor amount of dimethyl sulphide.

While it thus appears that SMCSO is the primary factor in the haemolytic activity of kale, it remains to be established whether dimethyl disulphide and/or methanethiol may be the actual toxin. Haemolytic activity has previously been associated with di-*n*-propyl and di-*p*-tolyl disulphides as well as with thiols.

2890. Toxins isolated from oriental mushrooms

Lin, J.-Y., Lin, Y.-J., Chen, C.-C., Wu, H.-L., Shi, G.-Y. & Jeng, T.-W. (1974). Cardiotoxic protein from edible mushrooms. *Nature, Lond.* **252**, 235.

A cardiotoxic protein, volvatoxin A, was previously isolated from the edible mushroom *Volvariella volvaceae* by Lin *et al.* (*Nature, Lond*, 1973, **246**, 524). The isolation of another cardiotoxic protein is now reported, this time from *Flammulina velutipes*, a mushroom widely eaten in the East and canned for export. This toxin, named flammutoxin, was present in the mushrooms at a level of 320 ppm and had an LD_{50} of 2·45 mg/kg.

Unlike volvatoxin A, from which two components (namely A1 and A2) were isolated, flammutoxin contained only one type of molecule, with a molecular weight of 22,000. The amino acid composition was similar to that of volvatoxin A2. Flammutoxin, like volvatoxin A, haemolysed human blood cells of group O, caused a 'writhing reaction' with a delay before onset and affected the electrocardiogram, depressing the ST segment and inverting the T wave. In addition, flammutoxin induced a sharp fall in blood pressure, caused swelling of Ehrlich ascites tumour cells and inhibited their respiration. These latter effects were demonstrated also with volvatoxin A2, but this fraction alone did not elicit the writhing reaction. Volvatoxin A1 exhibited none of the biological activity shown by volvatoxin A or A2, and it had no effect on the biological activity of flammutoxin.

2891. Absorption of iron from ingested liver

Naish, R., Kimber, C. L. & Deller, D. J. (1974). Liver iron: Changes induced by cooking and acid-peptic digestion. *Br. J. Haemat.* **26**, 459.

In all attempts to curb the high rate of iron-deficiency anaemia throughout the world, attention has to be paid not only to the total amount of iron in

the diet but also to the proportion of this total that can be absorbed from the human intestinal tract. Although the total iron content of staple foods has been well documented, some difficulty has been encountered in monitoring the changes induced in the physical and chemical state of dietary iron, and consequently in its absorbability, by the cooking of food and the process of digestion. The authors cited above have therefore studied the iron present in liver before and after cooking and/or enzymatic digestion and also its absorption after the different treatments. Guinea-pig liver was used for practical reasons and because its iron content and haem/non-haem iron distribution are comparable with those of the types of liver that constitute an important source of dietary iron.

When guinea-pig liver was 'cooked' by heating to 100°C for 10 min, no alteration was observed in the distribution of iron between the haem and non-haem fractions, but compared with the findings in raw liver there was an increase in the high-molecular-weight fraction of liver iron as demonstrated by dialysis and molecular sieving on Sephadex G-200 and G-50 columns. Incubation of the liver homogenate with pepsin for 2 hr at pH 1·6 similarly failed to affect the haem/non-haem distribution pattern, but reduced the molecular weight of the iron complexes. When cooking was followed by this acid-peptic digestion, the overall increase in low-molecular-weight iron complexes was greater than that observed during acid-peptic digestion alone.

Absorption studies were conducted in fasted female albino rats given intragastric doses of raw, cooked, digested, or cooked and digested homogenates containing ^{59}Fe-labelled ferric chloride. Each dose contained the equivalent of 20 μg elemental iron. Cooking combined with acid-peptic digestion of the liver increased the liver-iron absorption by about 75%, a finding ascribed to the high percentage of low-molecular-weight iron components. No such increase was found, however, with liver subjected only to *in vitro* peptic digestion.

2892. Garlic alters enzyme systems

George, K. C. & Eapen, J. (1974). Mode of action of garlic oil—Effect on oxidative phosphorylation in hepatic mitochondria of mice. *Biochem. Pharmac.* **23**, 931.

Augusti, K. T. & Mathew, P. T. (1974). Lipid lowering effect of allicin (diallyl disulphide-oxide) on long term feeding to normal rats. *Experientia* **30**, 468.

Garlic oil has been shown to be effective against insect pests, and both garlic oil and diallyl disulphide (DAD) have been found to inhibit protein synthesis in mosquito larvae (George *et al. Chemico-Biol. Interactions* 1973, **6**, 169). In an attempt to elucidate this action, the effect of garlic oil, DAD and dipropyl disulphide (DPD) on oxidative phosphorylation in the hepatic mitochondria of mice was investigated, using three substrates.

Glutamate oxidation was inhibited by 55 and 63%, respectively, by concentrations of 0·5 and 1·0 μl garlic oil in 3 ml reaction mixture, while phosphorylation was inhibited by 52 and 71% respectively. Succinate oxidation in the presence of 1·0 μl garlic oil/3 ml was reduced by 18% and phosphorylation by 68%. Ascorbate oxidation was not affected by garlic oil, but phosphorylation was reduced by 64 and 79%, respectively, by 0·5 and 1·0 μl garlic oil/3 ml. DAD (0·5 μl/3 ml) reduced phosphorylation of glutamate by 53%, of succinate by 35% and of ascorbate by 59%, whereas DPD had relatively little effect. Thus, inhibition and uncoupling by garlic oil and DAD appear to affect phosphorylation sites 1, 2 and 3. Respiration is more affected by these substances during oxidation of glutamate than during that of succinate or ascorbate, and phosphorylation is inhibited to a greater degree than is oxidation.

The second paper concerns the lipid-lowering effect of DAD oxide (allicin) which occurs at a level of about 0·15% in garlic. In young rats given an oral dose of allicin of 100 mg/kg/day for 2 months, lipid levels in serum and liver were significantly reduced, the reduction being more marked in the case of the liver lipids, but the proteins of serum and liver were not significantly affected. The change in lipids was due chiefly to a reduction in triglycerides and free cholesterol. The effect of allicin may be on lipid synthesis or catabolism, but interference with the biosynthesis of cholesterol and other lipid components is suggested by the capacity of allicin to combine with the sulphydryl group in coenzyme A, which is essential for the biosynthesis of fatty acids, cholesterol, triglycerides and phospholipids, and by the finding that the cholesterol-lowering effect is most pronounced on free cholesterol. The reported therapeutic value of garlic in atherosclerosis receives support from these findings.

2893. More on tannin carcinogenicity

Pradhan, S. N., Chung, E. B., Ghosh, B., Paul, B. D. & Kapadia, G. J. (1974). Potential carcinogens. I. Carcinogenicity of some plant extracts and their tannin-containing fractions in rats. *J. natn. Cancer Inst.* **52**, 1579.

Herbal remedies, beverages and food plants used by certain populations appear to have some connexion with a local prevalence of oesophageal cancer. It has been suggested that the tumorigenic agent in such commodities as sorghum and *Krameria ixina* may be the tannin (*Cited in F.C.T.* 1971, **9**, 898).

This seems amply borne out by the evidence of the paper cited above, which describes the treatment of rats with injections of extracts of whole herbs or of tannin-containing or tannin-free fractions. For up to 75 wk, the rats were given weekly sc injections of total aqueous extracts or fractionated extracts of *K. ixina* (without root), *K. triandra* root, *Acacia villosa* root and *Sorghum vulgare* seeds. The total extract and tannin-containing fraction of *K. ixina*, *K. triandra* and *A. villosa* were all carcinogenic. The acacia extracts were most potent and produced the first fibrous histiocytomas after 8 months. In contrast, a tannin-free extract of *K. ixina* produced tumours in only three of 60 rats. The total and tannin-containing extracts of *K. ixina* and *K. triandra* were less potent than those of acacia, and the appearance of the histiocytomas was more delayed. A cold-water extract of sorghum gave no indication of any carcinogenic potential.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

2894. Carcinogenicity of a *m*-dioxane derivative

Hoch-Ligeti, Cornelia, Argus, Mary F. & Arcos, J. C. (1974). Oncogenic activity of an *m*-dioxane derivative: 2,6-Dimethyl-*m*-dioxan-4-ol acetate (dimethoxane). *J. natn. Cancer Inst.* **53**, 791.

p-Dioxane has been found to induce nasal carcinoma in rats (*Cited in F.C.T.* 1971, **9**, 157). The carcinogenicity of a derivative of *m*-dioxane, 6-acetoxy-2,4-dimethyl-*m*-dioxane (dimethoxane; DMX) has now been investigated by the same group of workers. DMX is a broad-spectrum antimicrobial agent used in various aqueous solutions and emulsions, including cosmetic products.

In this study, 25 Wistar rats were given 1% DMX in their drinking-water over a period of 613 days, by which time they had received an average of 237 g. Fresh DMX solutions were prepared daily. The animals were killed 90 days after the end of treatment and all the major organs were examined histologically. A group of 14 control animals received tap-water instead of DMX solution. Altogether, 14 tumours (eight hepatomas, one renal carcinoma, two tumours of the skin and subcutaneous tissue, two lymphosarcomas and one leukaemia) were found among the DMX-treated animals. Five rats died during the experiment period and of these four had tumours. The liver tumours appeared to be similar, histologically, to those produced by nitrosamine derivatives, cycasin or dioxane. Most animals had purulent or granulomatous inflammation of the lungs, but no lung tumours were found. All test animals showed kidney damage consisting of distension of the tubules and hyperplasia of the glomerular capsular epithelium. In only one of the 14 controls was a tumour found, and this was a lymphosarcoma. As *m*- and *p*-dioxane are unstrained ring structures, these authors suggest that the carcinogenicity of dioxane and DMX is not due to alkylation following opening of the ring, as occurs with some strained epoxides, lactones and anhydrides. They postulate that the effects of dioxane may be due to the formation of carbonium ion(s) during metabolism, and suggest that this may also be the mechanism in the case of DMX.

[More information could possibly have been derived from this study if more than the one high dose level had been used. In view of the use of this material as an antibacterial agent, further data on this compound, its metabolism and its percutaneous absorption would be desirable.]

2895. Role of Peruvian balsam and perfumes in contact dermatitis

Ebner, H. (1974). Perubalsam und Parfüms. Untersuchungen über allergologische Beziehungen zwischen diesen Substanzen. *Hautarzt* **25**, 123.

During the 10 yr from 1963 to 1972, 309 patients —representing 5·22% of the total number with a contact allergy admitted to the University of Vienna skin clinic—were found to be sensitized to Peruvian balsam. From this group of 309, 36 women and 49 men were subsequently patch-tested with Peruvian balsam, with nine known group allergens and with ten perfumes commonly present in household products, toilet soaps and cosmetics. A summary of the results shows positive reactions to benzoin in 47 (55·2%) of those sensitized to Peruvian balsam, to wood tar in 49·4% (which included 61·1% of the women) and to one or more perfumes in 41·1% (52·7% of the women and 32·6% of the men). The higher incidence of perfume allergy in women was associated with their greater exposure to these materials in household materials and cosmetics and, in general, the study offers some explanation for the high sensitivity to Peruvian balsam found in housewives with chronic hand eczema. It seems that patients known to be allergic to Peruvian balsam are likely to react to perfumes and to benzoic acid and related preservatives in soaps and cosmetics.

[In this connexion, the figures of 7·9% for the incidence of contact sensitization to Peruvian balsam among patients with contact eczemas and of 2·9% for paraben sensitivity within the same group (*Cited in F.C.T.* 1973, **11**, 147) should perhaps be borne in mind.]

2896. No snag in whiter-than-white

Keplinger, M. L., Fancher, O. E., Lyman, F. L. & Calandra, J. C. (1974). Toxicologic studies of four fluorescent whitening agents. *Toxic. appl. Pharmac.* **27**, 494.

Optical brighteners are used to improve the appearance of fabrics by causing absorption of ultraviolet radiation and emission of visible light of short wave lengths. Some have been accused of provoking contact dermatitis (*Cited in F.C.T.* 1972, **10**, 439) but limited photosensitization tests have provided no evidence in support of allegations that there is a relationship between fluorescence and photosensitization (*ibid* 1974, **12**, 433). The four compounds examined in great detail in the study cited above were sodium salts of 2-(4-styryl-3-sulphophenyl)-2*H*-naphtho-[1,2,*d*]-triazole (I), 4,4'-bis-[(4-anilino-6-morpholino-1,3,5-triazin-2-yl)amino]stilbene-2,2'-disulphonic acid (II), 4,4'-bis-[(4-anilino-6-(*N*-methyl-2-hydroxycthylamino)-1,3,5-triazin-2-yl)amino]stilbene-2,2'-disulphonic acid (III) and 4,4'-bis-(2-sulphostyryl)biphenyl (IV).

The acute oral LD_{50} of each substance in mice and rats exceeded 5 g/kg. A 0·01% solution of I in 60% ethylene glycol did not irritate the skin of albino mice or rabbits, and 0·5 g of II occluded on to the shaved skin of rabbits for 24 hr was non-irritant. Skin application of II in aqueous suspension (in a dose of 5 or 10 g/kg) or as a paste in polyethylene glycol in a dose of 5 g/kg caused moderate erythema in the skin of albino rabbits; this disappeared by day 4 without signs of systemic toxicity. Repeated applications of 5% I or III in aqueous emulsion or 1% IV in aqueous suspension produced no local irritation or systemic toxicity in mice, rats and/or rabbits, and challenge with I in mice and rabbits 2 wk after subacute treatment produced no evidence of sensitization. Intradermal injection of IV did not sensitize guinea-pigs. Unlimited contact with the rabbit eye produced

mild irritation with II, extreme irritation with IV and moderate effects with the other two, but contact limited to a few seconds produced only mild irritation. Exposure to the dust at atmospheric levels of 2·9–5·5 g/m^3 for up to 4 hr produced only slight grooming, sneezing, inactivity and weakness in rats. The 96-hr LC_{50} values were 120–2000 ppm for trout and 86–1060 ppm for catfish, III proving the most toxic, probably because it was the only one in solution at the concentrations tested.

All compounds were devoid of teratogenicity when given to rabbits at dose levels of 10 and 30 mg/kg on days 6–16 of gestation and no mutagenic activity was detected in a dominant lethal study in male mice. In 90-day subacute oral toxicity trials, dogs, but not rats or monkeys, developed mild gastritis with 400 ppm I and peritonitis with 2000 ppm I. The latter effect was overcome by buffering the compound to pH 7. Neither II, III nor IV had any adverse effects in dogs fed 10,000 ppm or rats fed 5000 ppm for 90 days, and no evidence was obtained in the first 9 months of a continuing 2-yr feeding study of any toxic effects from I (neutralized), II, III or IV in rats fed up to 1000 ppm or dogs fed up to 2000 ppm. In human repeated-insult patch tests, 5% I, II or III in petrolatum and 5% IV in water failed to cause irritation or skin sensitization. Applications of II and III in detergent solutions were similarly devoid of irritant or sensitizing activity.

In view of the fact that detergent formulations generally contain optical whitening agents at levels below 1%, the margin of safety in the use of these compounds seems to be wide.

TOXICOLOGY

2897. Nitroreductase activity of intestinal bacteria

Zachariah, P. K. & Juchau, M. R. (1974). The role of gut flora in the reduction of aromatic nitro-groups. *Drug Metab. Dispos.* **2**, 74.

Recent years have seen a growing realization of the important role of the gut flora in many metabolic processes in mammals. We referred to this recently in connexion with nitrosamines (*Cited in F.C.T.* 1975, **13**, 409), but the metabolism—and therefore potential toxicity or effectiveness—of a wide variety of drugs and other compounds has been shown to be influenced by the presence of bacterial species in the intestine (*ibid* 1969, **7**, 261; *ibid* 1970, **8**, 120; *ibid* 1973, **11**, 679).

The authors cited above studied the reduction of aromatic nitro groups *in vivo* in rats and *in vitro* using homogenates of gut contents, gut wall and whole liver from rats. The reaction used was the conversion of *p*-nitrobenzoic acid (PNBA) to *p*-aminobenzoic acid (PABA). When incubated under 100% nitrogen, the nitroreductase activity of the gut contents was found to be 22·2 μmols PABA formed/g protein/hr, approximately twice as high as the specific activity of the gut-wall homogenate under similar incubation conditions and four times as high as that of the liver homogenate. Part of the activity found in the gut wall may have been due to bacterial contamination, which could not be eliminated completely. The nitroreductase system(s) of the bacterial flora were relatively resistant to inhibition by carbon monoxide (CO), inhibition of 90, 54 and only 22%, respectively, being demonstrated in hepatic, gut-wall and gut-content incubations under 100% CO compared with those under 100% nitrogen. Oxygen inhibited PNBA reduction by at least 90% in all three systems.

Testing of five isolated strains of intestinal bacteria associated the highest nitroreductase activity with *Escherichia coli* (specific activity 200 μmols/g protein/hr under nitrogen) and the lowest (87 μmols/g protein/hr) with *Aerobacter aerogenes*. Specific activities between 140 and 150 μmols/g protein/hr were demonstrated for *Proteus vulgaris*, *Salmonella typhimurium* and *Streptococcus faecalis*. Degrees of inhibition of these bacterial systems averaged 29 and 69% for CO and oxygen incubations respectively.

The whole-rat studies demonstrated the effect of oral administration of antibiotics (bacitracin, tetracycline and neomycin) on the urinary excretion of PABA following either oral or ip dosing with PNBA. Antibiotics given for 2 days before and 2 days after the PNBA caused an 80–90% decrease in the proportion of the PNBA dose excreted as PABA within 48 hr, irrespective of the route of PNBA administration. Incubation studies on gut contents and liver homogenates from antibiotic-treated rats showed that nitroreductase activity was virtually absent from the gut contents for at least 12 hr after antibiotic treatment, while hepatic nitroreductase activity was not detectably inhibited. It was thus confirmed that the gut flora plays a major part in the reduction of aromatic nitro groups *in vivo*, even when the nitro compound is administered parenterally.

PATHOLOGY

2898. Starch transplanted with the kidney

Elfenbein, I. B., McAlack, R. F., Mills, D. C. B., Munoz-Ghannam, E., Milder, J. E. & Coté, M. (1974). Starch emboli in transplanted kidneys. *Lancet* **II**, 1009.

Following our recent review of the hazards of starch in surgical gloves (*Cited in F.C.T.* 1975, **13**, 389), a new problem has been reported. Corn-starch emboli were found in the glomerular capillaries and arterioles of kidneys transplanted into three children when kidney biopsy was performed 90 min after transplantation. In each case the kidney had been kept in a cold, pulsatile perfusing machine before use. The corn-starch granules were identified as similar to those used for dusting surgical gloves. Neutrophilic granulocytes and platelet thrombi were associated with the starch granules, and partial degranulation of some platelets was observed in glomeruli free from

starch. Addition of starch to platelet-rich plasma in 0·15 M-sodium chloride showed no platelet aggregation up to 60 min, but the reconstituted plasma clotting-time, obtained by adding calcium chloride to the mixture, was reduced by 20–45%, indicating some activation of the platelets and/or clotting system.

The starch emboli persisted in the kidneys, some being still present after 7 wk. By 22 days all granules had been phagocytosed. Of the kidney transplants reported in this letter, one resulted in infarction due to a technical error, and a second in removal after 7 wk because of rejection and recurrence of the initial disease. There have been previous reports of corn starch emboli in renal transplants (Min *et al. Kidney Internat.* 1972, **2**, 291) and an experimental study has been conducted in rats (Lamb & Roberts, *J. clin. Path.* 1972, **25**, 876) but the situation regarding starch phagocytosis and the formation of platelet thrombi is far from clear and is undergoing further investigation. However, it seems that manipulation of organs outside the patient is another situation in which adverse effects may arise from corn starch in surgical gloves.

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SUMMARIES OF TOXICOLOGICAL DATA

TOXICOLOGICAL TESTS ON FLAVOURING MATTERS. II. PYRAZINES AND OTHER COMPOUNDS

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Introduction

Since our first publication (Posternak *et al. Fd Cosmet. Toxicol.* 1969, **7**, 405), twelve more flavouring substances have been screened for toxicity and the results have been submitted to the expert panel set up by the Flavor and Extract Manufacturers' Association of the United States (FEMA). Taking into account the conditions of intended use, the analogies that can reasonably be drawn and the consistent toxicological behaviour summarized in this report, the expert panel included these substances in the lists of the Generally Recognized as Safe (GRAS) substances published by Hall & Oser (*Fd Technol., Champaign* 1970, **24**(5), 25; *idem, ibid* 1972, **26**(5), 35).

At the time of testing (November 1966–August 1969), 2-acetylpyrrole and 3-ethyl-2,6-dimethylpyrazine were the only members of the group known to occur in natural food products, but three more substances (2-acetylpyridine, rose dihydroketone and acetylpyrazine) have since been found to occur naturally. The other substances studied are structurally related to naturally occurring flavouring substances and may be of interest for use in the compounding of new flavours. All are new as intentional flavouring ingredients.

Table 1 gives the code number, FEMA list number and chemical name of the test substance, together with its percentage purity, the name of known impurities, the LD_{50} in rats and the average dietary intake of the rats during the 12- or 13-wk test. These dietary intakes were calculated according to Oser *et al.* (*Fd Cosmet. Toxicol.* 1965, **3**, 563): "Single dosage levels for each substance were derived from the total estimated daily intake, calculated on a mg/kg body weight basis assuming 50 kg as the average body weight, and multiplying by 100. Thus a "no-effect" response at this dosage would indicate that the estimated maximum use level complied with the application of a 1:100 safety factor."

Average maximum concentrations likely, according to our experience, to be used in human food (Table 2) have been used for calculating the appropriate average dietary intakes for the rats, as listed in Table 1. These average dietary intakes were adjusted during the tests in order to compensate for the variation in the food consumption/body weight ratio during the growth of the rats. On a mg/kg body weight basis, the test animals thus received over 100 times the estimated maximum daily dietary intake of a 50-kg man. This calculation assumes that the consumer would eat high-average levels of all the food categories mentioned in Table 2, uniformly flavoured and without processing or other loss. Such flavour uniformity is never encountered in actual human feeding habits, so that the real safety factor for these tests is not one hundred but several hundred.

Toxicity tests

These tests were performed in two different laboratories. Compounds 155–158 (Study I, a and b) were tested in the toxicological laboratory of Firmenich & Cie, under the supervision of the first author of this report, and compounds 163–170 (Study II, a and b) in the laboratories of the Battelle-Geneva Research Centre. Unless otherwise specified, the materials and methods used were the same in both laboratories.

Design of experiments

Groups of 16 male and 16 female rats of the Charles River Sprague–Dawley C.D. strain (Study I) or of the Wistar CF/Gif Carworth strain (Study II) were housed in pairs of the same sex and fed *ad lib.* for 13 wk (or 12 wk in the tests on compounds 164–165) on a nutritionally adequate powdered diet made by Nafag, Gossau, Switzerland (Study I) or Altromin, Lippe, Germany (Study II). The basic diet was given alone to control groups, which were set up for each study, or was supplemented with one of the 12 flavouring substances under study (Table 1). All test chemicals were mixed with five parts of microcrystalline cellulose before being added to the rat diet. The

Table 1. *Details of flavouring matters tested*

Code no.	FEMA no.	Name	Purity (%)	Impurities	Oral LD_{50}* in rats (g/kg)	Mean dietary intake of rats in 13-wk study (mg/kg/day)	
						Males	Females
155	3208	2-Methyl-(3, 5 or 6)-methylthiopyrazine (mixture of isomers)	2,3- isomer: 70 2,6- isomer: 30 2,5- isomer: traces	—	1·97†	4·00	4·08
156	3202	2-Acetylpyrrole	98–99	3-acetylpyrrole	—‡	87·46	86·31
157	3186	4-Methyldiphenyl	99–100	—	2·57	8·74	8·72
158	3192	2-Methyl-5-methoxythiazole	98–99	1–2% unknown	1·25†	8·83	8·63
163	3251	2-Acetylpyridine	99	—	2·28	3·13	3·06
164	3140	2,6-Dimethylheptan-4-ol; di-isobutylcarbinol	99–100	—	4·35	11·33	11·06
165	3150	3-Ethyl-2,6-dimethylpyrazine	95	*c.* 5% 3,5-diethyl-2,6-dimethyl-pyrazine	0·504	12·69	12·33
166	3231	Pyrazinylmethyl-methylsulphide	99–100	—	2·15	1·66	1·63
167	3189	2-Methyl-3, 5 or 6-furfuryl-thiopyrazine (mixture of isomers)	2·3- isomer: 70 2,6- isomer: 30 2·5- isomer: traces	—	1·00	1·66	1·64
168	3243	*trans*-4-(2,6,6-Trimethyl-cyclohex-1-enyl)-but-2-ene-4-one; rose dihydro-ketone	99–100	traces of *cis* isomer	2·92	2·38	2·35
169	3126	Acetylpyrazine	99–100	—	> 3·00	8·25	8·15
170	3230	Pyrazinylethanethiol; pyrazine ethanethiol	99–100	—	0·158	16·56	16·30

*Calculated by the methods of Litchfield & Wilcoxon (*J. Pharmac. exp. Ther.* 1949. **96**, 99) or Weil (*Biometrics* 1952, **8**, 249).
†In ml/kg.
‡Not determined.

dietary levels of the chemicals were adjusted five times during Study I and once every week throughout the test period in Study II.

At the start of the experiments, the body weights of control and test animals of both sexes were in the range of 61–79, 60–79, 86–131 and 75–119 g in Studies Ia, Ib, IIa and IIb, respectively. Individual body weights were recorded weekly and food consumption was measured for each cage and calculated on a weekly basis. Efficiency of food utilization was calculated for pairs of rats (as housed). Haematological examinations and blood urea determinations were carried out on 50% of the animals at wk 7 and on all the animals at the end of the test. At autopsy, the liver and kidneys were weighed and sections of the following organs were stained with haemalum–eosin for histological examination: liver, kidneys, heart, lung, brain, hypophysis, thyroid, adrenals, pancreas, testes and epididymis or ovaries and uterus, submaxillary gland, spleen, stomach, large and small intestine, mesenteric lymph node, urinary bladder, sternal marrow, spinal cord and striated muscle.

The results were submitted to a statistical analysis including variance (final body weight, total food consumption and haematological results), covariance (final body weight in relation to food consumption and weight of liver and kidneys in relation to body weight) and the chi-squared distribution (differential

Table 2. *Average maximum concentrations of test compounds likely to be used in foods*

Code no. of flavouring	Maximum concentrations (ppm) likely to be used in							
	Beverages	Ice creams	Candy	Baked goods	Gelatin desserts	Chewing gum	Meat	Soups
155		2	2	4	2			
156	50	50	50		50			
157	5	5	5		5			
158	2			4			2	2
163	1	1			1		1	1
164			10			500		
165	7·5	7·5	7·5		7·5			
166	1	1	1		1			
167	1	1	1		1			
168	0·2	1	1	2	1	10		
169	5	5	5		5			
170	10	10	10		10			

Table 3. *Selected mean values, including statistically significant differences between test and control groups, in rats fed a basic or flavouring-supplemented diet for 3-months*

Study	Code no. of flavouring	Sex of group	Terminal body weight (g)	Food efficiency†	Weight of liver		Weight of kidneys	
					Absolute (g)	Relative (g/100 g body weight)	Absolute (g)	Relative (g/100 g body weight)
Ia	—	M	492·2	16·68	12·23	2·48	2·85	0·581
		F	259·4	11·21	6·17	2·58	1·68	0·647
	155	M	460·4**	16·29	12·14	2·64	2·83	0·615
		F	243·3	10·38**	6·57	2·70	1·70	0·698
	156	M	460·3**	15·75*	11·60	2·52	2·93	0·637
		F	246·1	10·47*	6·58	2·68	1·63	0·664
	157	M	458·4*	15·85	11·63	2·53	2·65	0·577
		F	256·6	10·46**	7·05	2·75	1·72	0·670
Ib	—	M	472·7	16·18	12·43	2·62	2·96	0·624
		F	249·1	10·70	6·60	2·65	1·72	0·688
	158	M	480·1	16·21	13·09	2·73	3·11	0·649
		F	256·4	11·01	7·05	2·75	1·77	0·688
IIa	—	M	426·4	14·86	15·66	3·68	2·32	0·548
		F	251·4	9·69	8·92	3·55	1·48	0·590
	163	M	416·4	14·85	13·98	3·33	2·18	0·523
		F	249·2	9·47	8·88	3·59	1·44	0·579
	164	M	409·3	14·11	14·94	3·53	2·30	0·563
		F	229·6**	8·64**	8·91	3·88***	1·45	0·634*
	165	M	396·7*	13·19**	13·56	3·41	2·18	0·554
		F	225·4***	8·19***	8·28	3·71	1·40	0·625*
IIb	—	M	401·8	15·05	10·62	2·55	2·19	0·545
		F	231·2	9·79	6·46	2·80	1·41	0·609
	166	M	387·4	14·82	10·74	2·77	2·23	0·576
		F	227·1	9·24	6·58	2·85	1·40	0·618
	167	M	389·5	14·29	10·83	2·77	2·22	0·569
		F	219·9	9·13	6·50	2·97	1·40	0·638
	168	M	382·1	13·69	10·48	2·74	2·23	0·584***
		F	227·2	8·85*	6·96**	3·07***	1·53*	0·674***
	169	M	397·1	14·73	10·96	2·77	2·23	0·563
		F	229·2	9·63	6·37	2·79	1·42	0·621
	170	M	397·4	13·96	10·84	2·73	2·39**	0·603***
		F	224·2	9·08	6·41	2·87	1·46	0·656*

†[Weight gained (g)/food consumed (g)] × 100.
Values are means for groups of 16 rats and those marked with asterisks differ significantly from the corresponding control value: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

leucocyte counts), all these analyses being followed by tests of differences between means.

Results

Body weight and food intake. Some differences (Table 3) between the control and test groups were found in body-weight gain, food intake and/or efficiency of food utilization. Slight reductions in body-weight gain were observed in male test groups fed with compound 155, 156 or 157, the difference from the controls being statistically significant but lying in every case below 10%. These results were reflected in a somewhat reduced efficiency of food utilization. Female rats given compound 164 showed a weight gain significantly lower than that of the controls (−12·1%) resulting in a decreased efficiency in food utilization (−10·8%). Compound 165 significantly slowed the rate of growth and caused a moderate reduction in efficiency of food utilization in both male and female animals. Lastly, although the gain in body weight was not influenced by the addition of compound 166, 167 or 168 to the diet, the food intake was significantly increased in the female test groups compared with the controls and, accordingly, the efficiency of food utilization decreased slightly.

Haematology. There were no major differences between test and control groups in the haemoglobin concentration or in the total and differential leucocyte counts. At wk 7, the erythrocyte counts revealed a transitory decrease in male and female groups receiving compound 156. A decrease in the haematocrit was noted at wk 7 in the female group receiving chemical 164. However, as these results fell within the limits of admissible biological variation observed in our laboratories for control populations of adult rats and, moreover, as no such differences appeared between control and test groups at the end of the treatments, the slight and transient modifications seen at wk 7 can be considered toxicologically insignificant.

Clinical chemistry. The studies on serum urea levels did not reveal any modification that could be considered biologically significant.

Organ weights. Most values fell within the normal limits of biological variance. The exceptions were a slight enlargement of livers and kidneys in females treated with compound 168 and of kidneys in males fed with compound 170. In these cases there were

statistically significant increases in the absolute as well as relative weights of the organs.

Organ histopathology. Non-specific inflammatory changes were seen in the livers or kidneys of a few animals. As these changes were rare and randomly distributed between test and control groups, it was assumed that they were not caused by the addition of the flavouring substances to the food. No compound-related pathology was observed.

Discussion

At no time during these 3-month toxicity studies did test and control animals differ in any way in their behaviour and physical appearance. No deaths occurred in any of the groups. No toxicologically significant modifications were observed either in the haematological or histological examinations. In view of these findings, the authors considered that there was no biological significance either in the moderate reduction in body weight gain and the accompanying slight decrease in efficiency of food utilization observed in the test groups receiving compound 155, 156 or 157, or in the slight increases in the weight of the livers of females receiving compound 168 and of the kidneys of animals on compound 168 or 170. Only the following differences between test and control groups exceeded 10% of the control values and could have been toxicologically relevant: the decrease in body-weight gain in females treated with compound 164 or 165, and the lower efficiency of food utilization in both male and female animals treated with 165 (Table 3). Since these findings were not associated with other modifications, such as histological lesions of toxic origin, it seems reasonable to assume that they did not represent what may properly be called 'toxic effects'.

Conclusion

The results of these studies do not indicate that any toxic effects were associated with the feeding of compounds 155–158, 163 or 166–170 to rats for 12 or 13 wk, in amounts exceeding the probable human intake by a factor of several hundred. The reduction in body-weight gain observed in the female rats receiving compound 164 and in both males and females fed compound 165 were most probably of no toxicological significance, since they were not associated with other toxicologically significant differences between test and control animals.

Fd Cosmet. Toxicol. Vol. 13, pp. 491–492. Pergamon Press 1975. Printed in Great Britain

LETTER TO THE EDITOR

THE CHANCE DISCOVERY OF OESTROGENIC ACTIVITY IN LABORATORY RAT CAKE

Sir,—The routine oestrogen assay used in this laboratory is based on the increase in weight of the uterus that occurs when a test substance is fed to weanling female mice. Pasture samples or their extracts are routinely added to ground commerical rat cake and, as a control, a group of mice is fed ground rat cake alone. Over a period of 8 months the mean uterus weight for control groups in 17 bioassays was 7·34 mg (95% confidence limits 6·90–7·80 mg; geometric mean), but an abrupt change to 17·2 mg was associated with feeding one particular batch of feed, suggesting contamination with an oestrogen. The rat cake was kept away from the laboratory where standard oestrogenic substances were used, so this source of contamination was excluded, and because of the stated composition of the diet in the manufacturers' literature, the presence of pasture oestrogens was not suspected. Routine multi-mycotoxin screening (Patterson & Roberts, In Proceedings of 2nd IUPAC Symposium on *Mycotoxins in Food*, Poland, 1974, in press, 1975; Roberts & Patterson, *J. Ass. off. analyt. Chem*, 1975, in press) failed to detect the presence of the oestrogenic Fusarium toxin, zearalenone (detection limit 1·0 ppm), and mycological examination was inconclusive.

Subsequently the biological activity of the suspect rat cake was compared with that of zearalenone (20–80 μg) and coumestrol (150–900 μg) standards added to fresh control rat cake (6 g). Results of these assays are presented in Table 1, which suggests that, with a greater activity associated with a second sample of rat cake (uterus weight 26·7 mg), the contaminant was not uniformly spread throughout the batch of feed.

Extracts used for mycotoxin screening by thin-layer chromatography (TLC) were also tested and found to be oestrogenic. These extracts were then fractionated by preparative TLC on silica gel GHR chromatoplates and eluted with methanol to give six components separated according to the presence of bands fluorescing under ultraviolet light (Table 1). The entire activity was associated with fraction 6, sub-fractions of which were prepared using the same TLC conditions (Scott *et al. Appl. Microbiol.* 1970, **20**, 839). As very small amounts of these sub-fractions were available for bioassay, the more sensitive intravaginal tetrazolium reductase assay of Martin (*J. Endocr.* 1964, **30**, 21) as modified by Drane & Saba (*J. agric. Sci., Camb.* 1968, **70**, 165) was used to locate oestrogenic activity. The bulk of activity was found to be present in sub-fraction 6b (R_F 0·50–0·56; R_F for zearalenone, 0·66), which was purified to give three further sub-fractions, of which only sub-fraction 6bII contained oestrogenic activity (Table 1). Finally, sufficient 6bII was collected from preparative chromatoplates to carry out a feeding trial (uterine weight bioassay) and its oral oestrogenic activity was confirmed. Prior to feeding, its ultraviolet spectrum in ethanol solution was compared with that of zearalenone and while the latter absorbed light maximally at 236, 274 and 316 nm, the rat-cake oestrogen absorbed only in a broad band at 274–280 nm with a shoulder at 225 nm.

Unlike zearalenone and some of the common phyto-oestrogens (isoflavones and coumestans), sub-fraction 6bII is not appreciably fluorescent, and because of its absorption characteristics it cannot be located as an absorbing spot on gel F_{254} chromatoplates. However it can be detected as a magenta-coloured spot fading overnight to olive green after spraying with the anisaldehyde–sulphuric acid reagent of Scott *et al.* (*loc. cit.*). Its reaction with the Folin–Ciocalteu reagent indicates that it is probably a phenol.

While further investigation is continuing to establish the chemical composition of the rat-cake oestrogen, an interim report seems of value since it indicates an unexpected source of error in biological work involving laboratory rodents. We were able to detect the presence of this oestrogen only because of the particular orientation of our work,

Table 1. *Oestrogenic activity and fractionation of laboratory rat diet*

Assay no.	Substance tested†	R_F limits	Weight of uterus (g) or reduced tetrazolium (OD units)	
			Mean‡	95% confidence limits
	Uterine weight assay			
1	Ground feed	—	26·7*	20·8–34·1
	Control	—	6·9	5·4–8·8
	Coumestrol (600 μg)	—	27·2	21·3–34·9
2	TLC extract	—	50·2*	40·0–63·2
	Control	—	7·1	5·8–8·6
3	TLC extract	—	16·6*	14·0–19·7
	Fraction 1	0·00–0·08§	6·3	5·3–7·5
	2	0·08–0·17§	7·2	6·1–8·6
	3	0·17–0·22§	5·7	4·8–6·8
	4	0·22–0·32§	7·7	6·5–9·1
	5	0·32–0·48§	7·7	6·5–9·1
	6	0·48–1·00§	13·7*	11·5–16·2
4	TLC extract	—	19·4*	15·0–25·1
	TLC extract (12 g)	—	31·2*	24·2–40·3
	Sub-fraction 6bII (20 g)	0·29–0·53‖	13·4*	10·4–17·3
	Control	—	6·0	4·7–7·8
	Zearalenone (80 μg)	—	17·1	13·2–22·0
	Martin intravaginal assay			
1	Fraction 6	0·48–1·00§	0·657*	0·434–0·995
		0·48–1·00§	0·423*	0·279–0·640
	Sub-fraction 6a	0·44–0·50§	0·332*	0·219–0·502
	6b	0·50–0·56§	0·781*	0·516–1·183
	6c	0·56–0·66§	0·182	0·120–0·275
	6d	0·66–0·76§	0·108	0·071–0·163
	6e	0·76–0·84§	0·184	0·122–0·163
	6f	0·84–1·00§	0·141	0·093–0·212
	Control	—	0·069	0·065–0·150
	Oestradiol (0·05 ng)	—	0·576	0·381–0·873
2	Sub-fraction 6b	0·50–0·56§	0·419*	0·294–0·599
	Further 6bI	0·00–0·29‖	0·176	0·123–0·251
	sub-fractions 6bII	0·29–0·53‖	0·577*	0·404–0·823
	6bIII	0·53–1·00‖	0·113	0·079–0·612
	Control	—	0·064	0·045–0·091
	Oestradiol (0·01 ng)	—	0·418	0·293–0·597

†Weight or equivalent weight of feedstuff: 6 g/mouse for the uterine weight assay and 100 mg/mouse for the intravaginal assay, except where stated otherwise.
‡Geometric mean.
§Preparative TLC using toluene–ethyl acetate–95% formic acid, 6:3:1, by vol. (Scott *et al. loc. cit.*) as developing solvent; fluorescent bands.
‖Acetone–chloroform, 7:93, v/v, used as developing solvent; acidic anisaldehyde spray.
Values marked with an asterisk differ significantly ($P < 0·001$) from the appropriate control.

but other biochemists or toxicologists would have been unaware of its presence and hence of its probable modifying effects on the metabolism of their 'control' rats. It is clearly impractical to screen each batch of rat cake for oestrogens, but this observation does at least indicate a possible reason for the occasional poor reproducibility of certain metabolic data.

Acknowledgement—We are indebted to Miss C. N. Hebert for the statistical analysis of the bioassay data.

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Contents continued]

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.

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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.

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