Volume 13 Number 6 December 1975

Food and Cosmetics Toxicology

An International Journal published for the
British Industrial Biological Research Association

CONTENTS

Continued on inside back cover

ISSN 0015–6264

FCTXAV 13(6) 603–680 (1975)

Pergamon Press OXFORD LONDON NEW YORK PARIS

FOOD AND COSMETICS TOXICOLOGY

An International Journal published for the British Industrial Biological Research Association

Publishing Offices

Pergamon Press Limited, Headington Hill Hall, Oxford OX3 0BW, England
Pergamon Press Inc., Maxwell House, Fairview Park, Elmsford, New York 10523, U.S.A.

Advertising Office

Pergamon Press Limited, Headington Hill Hall, Oxford OX3 0BW, England

Published bi-monthly

Annual Subscription Rates (1976)

For Libraries, Research Establishments and all other multiple-reader institutions $110
Specially reduced rates for individuals: In the interests of maximizing the dissemination of the research results published in this important international journal we have established a two-tier price structure. Individuals, whose institution takes out a library subscription, may purchase a second or additional subscription for their personal use at the much reduced rate of US $25.00 per annum.
For members of BIBRA £4

Microform Subscriptions and Back Issues

Current subscriptions and back files on microfilm as well as back issues in the regular editions of all previously published volumes are available from our sole distributors, Microforms International Marketing Corporation Inc. (MIMC) at the most convenient address: *Cowper House, Olney, Bucks. England/Fairview Park, Elmsford, New York 10523, U.S.A.*

All subscription enquiries other than those above should be addressed to:

The Subscriptions Fulfilment Manager, Pergamon Press Limited, Headington Hill Hall, Oxford OX3 0BW

PERGAMON PRESS LIMITED

HEADINGTON HILL HALL
OXFORD OX3 0BW, ENGLAND

MAXWELL HOUSE, FAIRVIEW PARK
ELMSFORD, NEW YORK 10523, U.S.A.

Fd Cosmet. Toxicol. Vol. 13, p. i. Pergamon Press 1975. Printed in Great Britain

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.

I.C.T. 13/6—A

Fd Cosmet. Toxicol. Vol. 13, p. 603. Pergamon Press 1975. Printed in Great Britain

Obituary

Dr. J. M. Barnes

The loss of one of the world's leading toxicologists is a cause for sorrow, especially at a time when such scientific distinction, coupled with objectivity, is in short supply. The late John Barnes was an outstanding scientist; but he was much more, a well-balanced, thoroughly honest, unpretentious and sincere man, for whom there was universal respect and admiration in the world of toxicology.

My own acquaintance with him goes back to the early days of the Experimental Pathology Club, where his keen and inquiring mind provided the stimulus for excellent discussions. I still recall the meeting devoted to alkyltin compounds, and the heated debate on the issue of the carcinogenicity of iron dextran. John Barnes was a model of scientific objectivity and integrity on these as on all other occasions.

When BIBRA was founded, Dr. Barnes was in a key position, as one of the appointees of the D.S.I.R. on the Council and by far the most experienced toxicologist serving in that capacity. He proved to be a tower of strength, paving the way for the acquisition of the site at Carshalton, providing space in his Unit for the embryonic Biochemistry Section of BIBRA's research activities, and affording help and encouragement as a member of the Research Policy Co-ordinating Committee. Despite his important role in BIBRA's affairs, he remained diffident and unobtrusive, permitting the fledgling Association to pursue its own policy, while attempting to ease its inevitable growing pains.

When BIBRA's laboratories were finally erected and occupied, we really began to appreciate our good fortune in having such congenial, friendly and helpful neighbours in the MRC Toxicology Unit. John Barnes left no stone unturned to assist us. The excellent Toxicology Library was accessible to us, as was the cafeteria. Far more than material facilities, the scientific stimulus provided by him, and by his colleagues, was a tremendous boon. Not only were individuals ever co-operative, but the seminars held at the MRC Unit provided excellent scientific fare. When *Food and Cosmetics Toxicology* was launched, he gave it all his support, and was a member of the Editorial Board for some years. He continued his help to the end.

I shall leave it to others to recount the many scientific achievements which have brought distinction and world-wide recognition to John Barnes and to the MRC Toxicology Unit. Here it would be appropriate to record, on behalf of my former colleagues at BIBRA and myself, our sympathy to his family and his fellow members of the MRC Unit, as well as our condolences to the public that he served so long and so well.

Leon Golberg

Fd Cosmet. Toxicol. Vol. 13, pp. 605–609. Pergamon Press 1975. Printed in Great Britain

Research Section

INDUCTION OF NEUROGENIC AND LYMPHOID NEOPLASMS BY THE FEEDING OF THRESHOLD LEVELS OF METHYL- AND ETHYLNITROSOUREA PRECURSORS TO ADULT RATS

A. KOESTNER and R. H. DENLINGER*
Department of Veterinary Pathobiology, The Ohio State University, Columbus, Ohio 43210, USA
and
W. WECHSLER
Max-Planck Institut für Gehirnforschung, Köln, Bundesrepublik Deutschland

(*Received 4 May 1975*)

Abstract—Marginally low doses of harmless precursors of methyl- and ethylnitrosourea (MNU and ENU) added to the food and drinking-water of Sprague–Dawley rats resulted in tumours of the nervous system in 15–45% of the animals, depending upon the dose of methyl- or ethylurea (MU or EU) added to the drinking-water. The sodium nitrite was kept constant at a level of 0·3% in the food pellets to maintain an optimal ratio for combination with MU or EU, since *ad lib.* feeding did not ensure the simultaneous presence of both the nitrite and the alkylurea in the stomach. With the highest dose of 0·03% MU there was also a 25% increase in lymphomas. Except for a diffuse spongioblastoma and an orbital neurinoma of the optic nerve, all the neurogenic neoplasms observed were similar to those previously produced with MNU or ENU in the same strain of rat. The hazard of possible consumption of harmless precursors and the intragastric synthesis of potent nitrosourea carcinogens will have to be considered in any programme of cancer prevention.

INTRODUCTION

The remarkable carcinogenic and particularly the neuro-oncogenic effects of methylnitrosourea (MNU) and ethylnitrosourea (ENU) in rats (100% tumour production with optimal doses) has prompted numerous investigations designed to determine possible human exposure to these compounds or their precursors. While it is easy to avoid unprotected exposure to MNU and ENU in chemical laboratories, it is more difficult to avoid contact with harmless precursors, if they are available in the immediate environment and their transformation into active carcinogens occurs under natural conditions. If it were possible to establish that a wide range of naturally occurring precursors of nitrosoureas were capable of tumour production in animals following their intragastric transformation into carcinogens, the possibility of human exposure would have to be considered.

Sander (1970) succeeded in producing malignant tumours in rats by oral application of *N,N*-dimethylurea and nitrite and established the formation of dimethylnitrosourea from precursor substances under the influence of gastric acid. Mirvish & Chu (1973) substantiated this finding by demonstrating by chemical analysis the formation of nitrosourea in the stomach. Several investigators have produced tumours in rats by feeding relatively high doses of MNU precursors to young adult animals (Mirvish, Greenblatt & Choudari Kommineni, 1972; Sander, 1970; Sander & Bürkle, 1969). Low doses of five different amines with nitrate or nitrosoamino acids and nitrites failed to induce tumours in rats (Garcia & Lizinsky, 1973). The purpose of this investigation was to test the neuro-oncogenic effect of threshold levels of MNU and ENU precursors in Sprague–Dawley rats of the CD strain.

EXPERIMENTAL

Specific-pathogen-free Sprague–Dawley rats (CD) were divided when 30 days old into four experimental and three control groups, as indicated in Table 1. Experimental animals were given a custom-prepared diet of Purina Lab Chow 5010C containing 0·3% (w/w) sodium nitrite, and drinking-water containing methylurea (MU) or ethylurea (EU) dissolved at concentrations of 0·03 or 0·01% (w/v). Control rats received either regular Purina Lab Chow 5010C and 0·03% alkylurea or the nitrite diet and tap-water. All diets were provided *ad lib.* The rats were housed individually, observed daily for signs of neoplasia and weighed weekly for the duration of the experiment.

All rats were autopsied shortly after their spontaneous death or after they were killed when moribund. All surviving animals were killed after a 2-yr treatment period. The brain, spinal cord and major peripheral nerves were examined for macroscopically

*Present address: Battelle-Columbus Laboratories, 505 King Avenue, Columbus, Ohio 43201.

Table 1. *Experimental design indicating dose levels of MNU and ENU precursors*

Group*	Concn (%) in drinking-water		Dietary concn of $NaNO_2$ (%)
	MU	EU	
	Experimental groups		
A	0·03	—	0·3
B	0·01	—	0·3
C	—	0·03	0·3
D	—	0·01	0·3
	Control groups		
E	—	—	0·3
F	0·03	—	—
G	—	0·03	—

MU = Methylurea EU = Ethylurea
$NaNO_2$ = Sodium nitrite
*Each experimental group consisted of ten males and ten females and each control group of five males and five females.

detectable neoplasms. The tissues were fixed in neutral buffered 10% formalin, and serial blocks of the brain and all gross lesions, including lesions from non-neural organs, were embedded in paraffin, sectioned at 6 μm and stained with haematoxylin and eosin. Wilder's reticulum stain and Masson's trichrome stain were used to distinguish between neuroectodermal and mesenchymal components of neurogenic tumours. Brain sections prepared from serial blocks were examined microscopically for small neoplasms (microtumours) not detectable on gross examination.

RESULTS

Incidence and location of tumours and survival time

Tumours of the nervous system were found only in rats in groups A, B and C, receiving 0·03 or 0·01% MU or 0·03% EU in the drinking-water concomitantly with 0·3% sodium nitrite in the solid food (Table 2). Tumours of the nervous system developed in 45% of the animals receiving the higher dose of MU, and in 20% of those receiving the higher dose of EU and in 15% of those on the low dose of MU, but none occurred in the rats receiving the low dose of EU or in any animals receiving nitrite alone or the alkylureas alone. In addition, 25% of the group on the high dose of MU had malignant lymphomas or leukaemia. Lymphoid neoplasia did not occur in groups B (0·01% MU and nitrite) or C (0·03% EU and nitrite) but was demonstrated in one animal in each of the groups D (0·01% EU + nitrite) and E, F and G (controls).

The incidence of non-neurogenic tumours increased with the survival time and, with the exception of the lymphoma incidence, was unrelated to the treatment with the carcinogenic precursors. Since the survival time was generally longer in the controls, they had a higher incidence of non-neurogenic tumours. Group A was an exception because of the treatment-related lymphomas. The non-neurogenic tumours most frequently represented were mammary tumours (mostly fibroadenomas), affecting in the various groups 60–100% of the females as well as an occasional male. Another frequently occurring tumour was the chromophobe adenoma of the pituitary gland, the incidence ranging from 15 to 55% with no indication of treatment dependence (e.g. 15% in the group on the high dose of MU and nitrite and 55% in the control group). Many pituitary adenomas reached remarkable dimensions. One adenoma measured 3 × 2 × 2 cm and occupied some 66% of the base of the brain, compressing central portions of the brain, particularly the hypothalamus (Fig. 1). Such neoplasms were pleomorphic, with a high rate of mitosis but little invasion.

Classification of tumours of the nervous system

The classification of neurogenic tumours is summarized in Table 3. Twelve of the tumours occurred in the central and five in the peripheral nervous system. Eleven of the former tumours occurred in the brain and one in the spinal cord. Three of the five neurinomas involved the spinal roots and two involved cranial nerves (one the trigeminal and one the optic nerve).

Tumours of the central nervous system

The mixed gliomas and oligodendrogliomas and the gliosarcoma were prefentially located in the periventricular zones of the lateral ventricles and the subcortical white matter of the cerebral hemispheres. Early neoplastic proliferation consisted almost exclus-

Table 2. *Tumour incidence in rats following feeding of MNU and ENU precursors*

Group	No. of rats* with: Neurogenic tumours in CNS	Neurogenic tumours in PNS	Non-neurogenic tumours: Leukaemia or lymphoma	Pituitary	Mammary	Others	Mean survival time (days)	Percentage of rats with neurogenic tumours	Percentage of rats with non-neurogenic tumours
A	5	4	5	3	6	10	460 ± 138	45	90
B	3	—	—	8	8	7	590 ± 102	15	75
C	3	1	—	5	7	5	627 ± 76	20	55
D	—	—	1	11	10	2	597 ± 123	—	90
E	—	—	1	4	3	6	661 ± 60	—	80
F	—	—	1	5	3	2	612 ± 125	—	80
G	—	—	1	4	4	2	665 ± 67	—	90

*No. of rats affected out of the total of 20 in each experiment group (A–D) and ten in each control group (E–G)

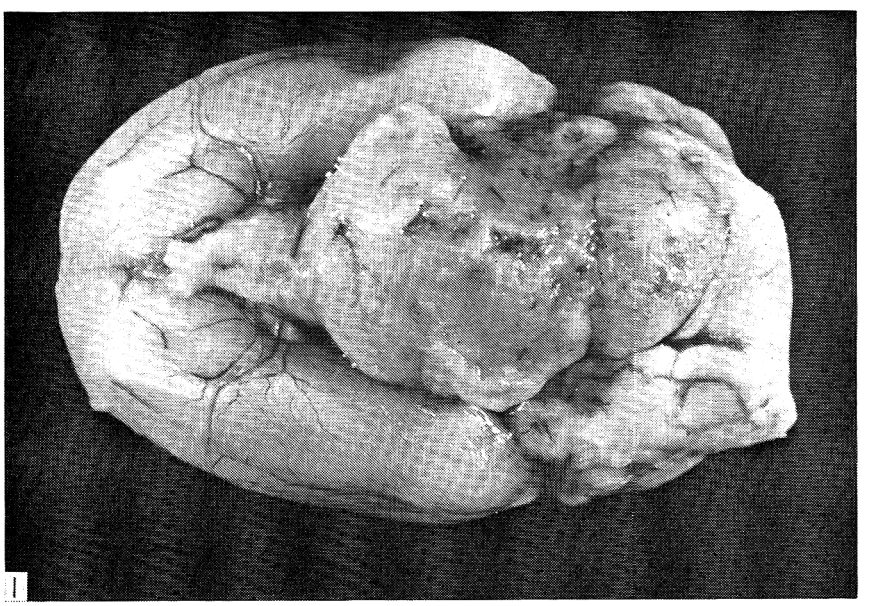

Fig. 1. Pituitary adenoma in rat of group A.

Facing p. 606

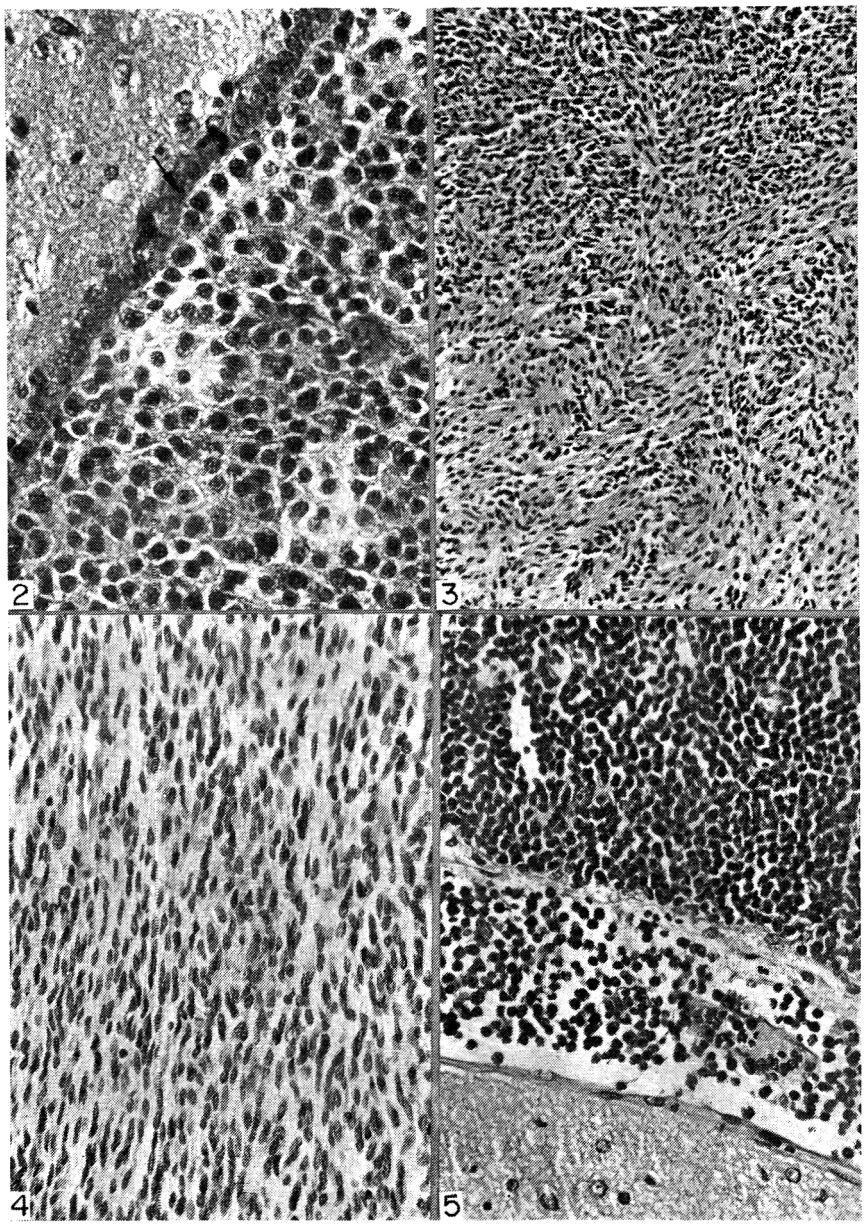

Fig. 2. Ependymoma of lateral ventricle in rat of group A, showing the ventricle packed with neoplastic cells which show characteristic line-up and a tendency to rosette formation (ependymal lining arrowed). Haematoxylin and eosin ×315.

Fig. 3 Diffuse periventricular spongioblastoma of basal ganglia and hemispheres in rat of group B, depicting spread of spindeloid cells along nerve tracts. Haematoxylin and eosin ×125.

Fig. 4. Differentiated neurinomas of the spinal nerve root (at T9) in a rat of group A. Haematoxylin and eosin ×315.

Fig. 5. Malignant lymphoma infiltrating the meninges of a rat from group A. Haematoxylin and eosin ×315.

Table 3. *Classification of the neurogenic tumours induced in rats by administration of MNU and ENU precursors*

Diagnosis	Incidence of specific tumour: In group A	B	C	Total
Mixed glioma	3*	—	2	5
Oligodendroglioma	1*	—	1	2
Spongioblastoma	—	1	—	1
Glio-ependymoma	1	—	—	1
Ependymoma	1	1	—	2
Gliosarcoma	—	1	—	1
Neurinoma	4	—	1	5
Total...	10	3	4	17

*A mixed glioma and the oligodendroglioma in Group A occurred in the same animal.

ively of neoplastic oligodendrocytes. The predominant cell types in the mixed gliomas were neoplastic oligodendrocytes and astrocytes. The astrocytic cell population was usually interspersed among the more numerous smaller oligodendroglioma cells. The tumour cells in these cases were moderately well differentiated, with a low mitotic index. The gliosarcoma occurred in the spinal cord and was the only cord tumour observed in this study. The tumour was highly invasive and only marginal strips of uninvaded spinal cord were recognized in cross sections. The predominant cell types were a spindle cell and a plump, pleomorphic glial cell. The spindle cell, which formed criss-crossing, somewhat palisading cell bundles, produced some collagen and reticulin fibres. The glial cell type was larger than the spindle cell and had a round to slightly oval vesicular nucleus. There was no specific arrangement of these cells but they most closely resembled neoplastic astrocytes. The two ependymomas and the glioependymona occurred in lateral ventricles and projected into the ventricular lumen (Fig. 2). The cells lined up in cords and rosettes and were moderately well differentiated. The mitotic index was low. The gliomatous portion of the glioependymoma consisted of oligodendroglioma cells.

One brain tumour occurred in the midline and extended along the septum pelucidum bilaterally into the limbic system. The tumour extended up to the third ventricle and spread along nerve tracks from the thalamus into the basal ganglia (Fig. 3). The neoplastic cells were short spindle-shaped cells arranged rather densely in a streamlined fashion. At the margin, individual and small groups of these cells spread across the tumour border into the well-preserved brain parenchyma, seemingly overgrowing the local neurons. This tumour was diagnosed as a diffuse spongioblastoma.

Tumours of the peripheral nervous system

The three neurinomas of the spinal nerve roots and two neurinomas of cranial nerves were well differentiated neurinomas (Fig. 4) similar to those produced in rats by MNU (Denlinger, Koestner & Swenberg, 1973). All peripheral-nerve neurinomas arose from thoracic nerve roots, while one of the cranial-nerve neurinomas arose from the trigeminal nerve and the other from the optic nerve within the left orbit, invading and partially destroying the eye.

Lymphoma–leukaemia complex

Of the nine neoplasms in this category, five were diagnosed as malignant lymphomas and four as myelogenous leukaemias. One malignant lymphoma invaded the meninges (Fig. 5).

Survival period

The average survival times of the rats in the various experimental and control groups are listed in Table 2. The animals in Group A (high-dose MU and sodium nitrite) had a shorter average survival time primarily because of the high incidences of leukaemia and brain tumours, which usually resulted in death before the termination of the experiment (2 yr). The average survival time of animals with neurogenic tumours was 532 days (range, 363–691) while the average survival time in the three control groups with no neurogenic tumours was 646 days (range, 276–714 days).

DISCUSSION

The experiments described in this report demonstrate that repeated consumption of low doses of harmless precursors of MNU and ENU are capable of inducing in adult Sprague–Dawley rats tumours of the nervous system comparable with those produced by direct administration of nitrosourea compounds. Induction of neurogenic and lymphoid tumours in group A was interpreted as being the result of *in vivo* synthesis of MNU and ENU, since none of the precursors (MU, EU or sodium nitrite) administered alone resulted in tumours of the nervous system.

Administration of MU and nitrite resulted in a higher incidence of neurogenic tumours than administration of EU and nitrite at the same dose levels. Even the lower dose of MU (0·01%) produced neurogenic tumours in 15% of the rats, while only 20% of the rats exposed to the high dose of EU and sodium nitrite (0·03%) and none of the group on the low dose of EU developed neurogenic neoplasms. The lower incidence of tumours induced with the ethyl compared with the methyl compound was consistent with the lower neuro-oncogenic capacity of ENU in adult rats. Acid-catalysed chemical nitrosation of EU is apparently lower than nitrosation of MU (Mirvish & Chu, 1973; Montesano & Magee, 1971). Threshold values of tumour induction with ENU precursors appear to lie above the low dose (0·01%), while the threshold level of the MNU precursors is below the low dose used in these experiments.

No attempt was made to ensure the simultaneous ingestion of MU or EU and nitrite. Although two doses of MU and EU were used, the sodium nitrite content of the food pellets was kept constant at 0·3% to avoid limiting the availability of nitrite for the intragastric synthesis of MNU and ENU.

The tumour types and their localization in the nervous system correspond in general to those reported for adult rats treated with MNU (Druckrey, Ivankovic & Preussmann, 1965; Jänisch & Schreiber, 1969; Koestner, Swenberg & Wechsler, 1972; Swenberg,

Koestner & Wechsler, 1972; Wechsler, Kleihues, Matsumoto, Zülch, Ivankovic, Preussmann & Druckrey, 1969). There were differences, however, the most striking being the occurrence of three cerebral ependymomas in the precursor-treated rats. Ependymomas are extremely rare in MNU-treated rats, but are frequently encountered in the spinal cord of rats exposed transplacentally to ENU. Other tumours not encountered previously were the diffuse spongioblastoma and the orbital neurinoma of the optic nerve.

The gliomas and neurinomas induced with the precursors were similar to those induced with MNU (Swenberg *et al.* 1972). Of the extraneural tumours, only lymphomas and leukaemias had a significantly higher incidence in the group on nitrite and the highest MU dose level than in the other groups. One lymphoma involved the cerebral meninges. All other extraneural tumours, such as those of the pituitary, mammary gland and kidney, must be considered unrelated to the treatment, since they corresponded in location and incidence to the spontaneous tumours found in CD rats of comparable age.

Feeding or gastric administration of EU and sodium nitrite to pregnant rats has also been reported by several investigators (Ivankovic & Preussmann, 1970; Osske, Harzok & Schneider, 1972; Ramadan & Wechsler, 1975). All succeeded in producing neurogenic tumours in the offspring, similar to those reported previously with transplacental application of ENU (Druckrey *et al.* 1965; Koestner, Swenberg & Wechsler, 1971; Koestner *et al.* 1972; Wechsler *et al.* 1969).

Our experiments further substantiate reports by others that MNU and ENU are readily synthesized *in vivo* from precursors under the influence of gastric acid. They are then absorbed and carried to the nervous system and other target organs as intact molecules and transformed into active carcinogens by spontaneous cleavage of the alkyl and acyl radicals within the susceptible target cells.

How do these findings relate to human neoplasms? There is at present no indication that nitrosoureas are aetiologically associated with human cancer, but since these substances are capable of producing tumours, including those of the nervous system, in various animal species, man cannot be excluded. This report demonstrates that minimal oral doses or harmless precursors of nitrosamides are capable of inducing neoplasms in rats. The nitrite level used was approximately 15 times the maximal amount permitted by the FDA (Food and Drug Administration of the USA) for human consumption and was 10–30 times the level needed on a 1:1 molar basis for combination with alkylureas. The nitrite dose could have been reduced 10–30 times if the method of administration had been intragastric intubation, which would have ensured the simultaneous presence in the stomach of both sodium nitrite and methylurea. In that case, the nitrite level would have been below the standard level permitted as a food additive by FDA regulations. In addition to the nitrite consumed as a food additive, man is exposed to nitrates which may be reduced to nitrites by bacteria, especially when the acidity of the stomach is reduced.

Fortunately, MU and EU are not known to occur in food or elsewhere in nature, but at least 15 other ureas occur in various natural products, and many drugs and herbicides contain urea groups (Sander, 1971). Ureides are formed by the hydrolytic de-imidation of quinidine-compounds catalysed by bacterial enzymes or by carbamoylation of primary amines. Secondary amines occur ubiquitously in nature in the protein metabolism of plants, animals and microorganisms (Sander, 1971), while sulphanylurea, nitrofurazone and phenylureas are widely used as herbicides. It must, therefore, be considered possible that consumption of such precursors with nitrite constitutes a hazard for man and further studies should be directed towards the clarification of these problems.

Acknowledgement—This work was supported in part by grants CA11224 and GM1052 from The National Institutes of Health.

REFERENCES

Denlinger, R. H., Koestner, A. & Swenberg, J. A. (1973). An experimental model for selective production of neoplasms of the peripheral nervous system. *Acta neuropath.* **23**, 219.

Druckrey, H., Ivankovic, S. u. Preussmann, R. (1965). Selektive Erzeugung maligner Tumoren im Gehirn und Rückenmark von Ratten durch N-Methyl-N-Nitrosoharnstoff. *Z. Krebsforsch.* **66**, 389.

Garcia, H. & Lizinsky, W. (1973). Studies of the tumorigenic effect in feeding of nitrosamino acids and of low doses of amines and nitrite to rats. *Z. Krebsforsch.* **79**, 141.

Ivankovic, S. u. Preussmann, R. (1970). Transplazentare Erzeugung maligner Tumoren nach oraler Gabe von Äthylharnstoff und Nitrit an Ratten. *Naturwissenschaften* **57**, 460.

Jänisch, W. u. Schreiber, D. (1969). *Experimentelle Geschwülste des Zentralnervensystems.* Gustav Fischer, Jena.

Koestner, A., Swenberg, J. A. & Wecksler, W. (1971). Transplacental production with ethylnitrosourea of neoplasms of the nervous system in Sprague–Dawley rats. *Am. J. Path.* **63**, 37.

Koestner, A., Swenberg, J. A. & Wechsler, W. (1972). Experimental tumors of the nervous system induced by resorptive N-nitrosourea compounds. In *Progress in Experimental Tumor Research.* Vol. 17. Edited by F. Homburger. p. 9. S. Karger, Basel.

Mirvish, S. S. & Chu, Cecilia (1973). Chemical determination of methylnitrosourea and ethylnitrosourea in stomach contents of rats, after intubation of the alkylureas plus sodium nitrite. *J. natn. Cancer Inst.* **50**, 745.

Mirvish, S. S., Greenblatt, M. & Choudari Kommineni, V. R. (1972). Nitrosamide formation *in vivo*: Induction of lung adenomas in Swiss mice by concurrent feeding of nitrite and methylurea or ethylurea. *J. natn. Cancer Inst.* **48**, 1311.

Montesano, R. & Magee, P. N. (1971). Evidence of formation of N-methyl-N-nitrosourea in rats given N-methylurea and sodium nitrite. *Int. J. Cancer* **7**, 249.

Osske, G., Harzok, R. u. Schneider, J. (1972). Diaplazentare Tumorinduktion durch endogen gebildeten N-Äthyl-N-Nitrosoharnstoff bei Ratten. *Arch. Geschwulstforsch.* **40**/3, 244.

Ramadan, M. A. & Wechsler, W. (1975). Transplacental induction of neurogenic tumors in BDIX rats by intragastric administration of ethylnitrosourea precursors. *Z. Krebsforsch.* In press.

Sander, J. (1970). Induktion maligner Tumoren bei Ratten durch orale Gabe von N-N'-Dimethylharnstoff und Nitrit. *Arzneimittel-Forsch.* **20**, 418.

Sander, J. (1971). Untersuchungen über die Entstehung cancerogener Nitrosoverbindungen im Magen von Versuchstieren und ihre Bedeutung für den Menschen. I. Mitteilung. *Arzneimittel-Forsch.* **21**, 1572.

Sander, J. u. Bürkle, G. (1969). Induktion maligner Tumoren bei Ratten durch gleichzeitige Verfütterung von Nitrit und sekundären Aminen. *Z. Krebsforsch.* **73**, 54.

Swenberg, J. A., Koestner, A. & Wechsler, W. (1972). The induction of tumors of the nervous system with intravenous methylnitrosourea. *Lab. Invest.* **26**, 74.

Wechsler, W., Kleihues, P., Matsumoto, S., Zülch, K. J., Ivankovic, S., Preussmann, R. & Druckrey, H. (1969). Pathology of experimental neurogenic tumors chemically induced during prenatal and postnatal life. *Ann. N.Y. Acad. Sci.* **159**, 360.

Fd Cosmet Toxicol. Vol. 13, pp. 611–617. Pergamon Press 1975. Printed in Great Britain

STUDIES ON THE METABOLISM OF DIMETHYLNITROSAMINE IN THE RAT. II. THE EFFECTS OF PHENOBARBITONE AND 20-METHYLCHOLANTHRENE ON THE *IN VITRO* AND *IN VIVO* METABOLISM AND ACUTE TOXICITY OF DIMETHYLNITROSAMINE IN YOUNG AND MATURE RATS

J. C. PHILLIPS, CHRISTINE E. HEADING, B. G. LAKE, S. D. GANGOLLI and A. G. LLOYD

British Industrial Biological Research Association, Woodmansterne Road, Carshalton, Surrey, SM5 4DS, England

(*Received 21 November 1974*)

Abstract—Following ip administration of ^{14}C-labelled dimethylnitrosamine (DMN) to rats, $^{14}CO_2$ excretion was found to proceed approximately twice as fast in the young animal as in the mature rat of either sex. In contrast, the hepatic DMN demethylase activity and a number of biochemical parameters of the microsomal mixed-function oxidase system were similar in both age groups. Pretreatment with phenobarbitone or 20-methylcholanthrene increased hepatic DMN demethylase activity and other parameters of microsomal enzymes in both groups of rats, but did not alter the $^{14}CO_2$ excretion rates significantly in the intact animals, except in mature females. Neither did SKF 525-A, a microsomal-enzyme inhibitor, affect this *in vivo* index of DMN metabolism. Parallel studies on aminopyrine revealed an *in vitro* and *in vivo* induction of metabolism in rats pretreated with phenobarbitone, while SKF 525-A markedly inhibited $^{14}CO_2$ excretion from [^{14}C]aminopyrine administered to rats. Acute toxicity studies showed that whereas phenobarbitone afforded some protection in mature female rats but not in young males, 20-methylcholanthrene treatment rendered both groups more susceptible to DMN toxicity. These results are discussed in terms of the role of hepatic DMN demethylase in determining the *in vivo* metabolism and acute toxicity of DMN in the rat.

INTRODUCTION

It is generally recognized that dimethylnitrosamine (DMN) requires biological activation to exert its hepatotoxic and carcinogenic effects in a wide variety of mammalian species, including the rat (Magee & Barnes, 1967). The liver has been shown to be the organ principally involved in the metabolism of DMN and its eventual excretion as CO_2 (Knecht, 1966; Magee & Vandekar, 1958) and *in vitro* studies have indicated that the postulated metabolic degradation of DMN via a demethylation step to monomethylnitrosamine (the putative unstable toxic intermediate) and formaldehyde occurs in the hepatic endoplasmic reticulum (Magee & Lee, 1963). The enzyme mediating this process, DMN demethylase, which requires molecular oxygen and NADPH, has been considered to be associated with the microsomal mixed-function oxidase system dependent on the haemoprotein, cytochrome *P*-450 (Brouwers & Emmelot, 1960; Mizrahi & Emmelot, 1962; Omura, Sato, Cooper, Rosenthal & Estabrook, 1965).

There is, however, little information correlating the hepatic DMN demethylase activity with the metabolism of DMN to CO_2 in the intact animal. Metabolic studies on [^{14}C]DMN had shown that the $^{14}CO_2$ excretion rate from the young animal was almost twice that in the mature rat (Phillips, Lake, Heading, Gangolli & Lloyd, 1975).

In this paper we present our findings on the hepatic DMN demethylase activity and a number of biochemical parameters of the microsomal mixed-function oxidase system in the liver of young and mature rats of both sexes, and additionally the effects of phenobarbitone and 20-methylcholanthrene pretreatment on these *in vitro* and *in vivo* indices of DMN metabolism. Results are also included of parallel investigations into the effect of phenobarbitone pretreatment on the metabolism of ^{14}C-labelled aminopyrine, a model compound metabolized by the hepatic microsomal mixed-function oxidase system, and of SKF 525-A, a known inhibitor of microsomal enzymes (Anders & Mannering, 1966), on the *in vivo* metabolism of DMN and aminopyrine. Additionally, the acute toxicity of DMN, assessed as the LD_{50}, has been determined in rats treated with phenobarbitone or with 20-methylcholanthrene. A preliminary communication on part of this work has appeared (Lake, Heading, Phillips, Gangolli & Lloyd, 1974b).

EXPERIMENTAL

Chemicals. Phenobarbitone sodium was obtained from British Drug Houses Ltd., Poole, Dorset, and 20-methylcholanthrene from Eastman Kodak Ltd., Kirby, Liverpool, while 2-diethylaminoethyl 2,2-diphenylvalerate hydrochloride (SKF 525-A) was a

gift from Smith, Kline and French Ltd., Welwyn Garden City, Herts. DMN, purchased from Ralph N. Emanuel Ltd., Wembley, Middlesex, and ^{14}C-labelled DMN, prepared from [^{14}C]dimethylamine by the method of Dutton & Heath (1956), were analysed by the gas-liquid chromatographic method of Palframan, McNab & Crosby (1973) and found to be 99% pure. [Dimethylamine-^{14}C]aminopyrine (specific activity 9·6 mCi/mmol) and sodium [^{14}C]formate (specific activity 61·3 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks.

Animals and treatment. Wistar albino rats of both sexes obtained from Scientific Agribusiness Consultants (International) Ltd., Occold, Suffolk, were used in these studies. The young (5-wk-old) animals weighed 100 g and the 16-wk-old mature female and male rats weighed 210 and 300 g respectively. The animals were allowed free access to Spiller's Laboratory Small Animal Diet (No. 1) and water and were housed at 20 ± 1 C. Groups of rats received daily ip injections of a 0·9% (w/v) NaCl solution of phenobarbitone sodium (100 mg/kg body weight) or 20-methylcholanthrene (20 mg/kg body weight), dissolved in corn oil, for 4 days. Corresponding control animals were similarly treated with 0·9% (w/v) NaCl solution or corn oil in doses of 5 ml/kg body weight.

In vivo *investigations*

Rate of $^{14}CO_2$ excretion. Animals treated ip either with [^{14}C]DMN (5 mg/kg), [^{14}C]aminopyrine (15·6 mg/kg) or [^{14}C]formate (9·2 mg/kg) were placed in 'Metabowl' cages and the $^{14}CO_2$ in the respired air was measured at intervals by the method previously described (Phillips *et al.* 1975). The maximum rate of $^{14}CO_2$ excretion occurred 1·5–3 hr after [^{14}C]DMN or [^{14}C]formate administration and 2–4 hr after [^{14}C]aminopyrine treatment.

Acute toxicity study. The LD_{50} of DMN was calculated by the method of Weil (1952) in young male and mature female rats and in groups of animals pre-treated with phenobarbitone or 20-methylcholanthrene. Deaths were recorded for 10 days after dosing with DMN.

In vitro *investigations*

These studies were carried out on livers obtained from rats killed by cervical dislocation. The tissue was homogenized in 4 vols 0·154 M-KCl solution containing 50 mM Tris (pH 7·4), and the postmitochondrial and microsomal fractions were separated by differential centrifugation at 10,000 *g* (av.) for 20 min and 105,000 **g** (av.) for 60 min respectively. Microsomal protein was measured according to Lowry, Rosebrough, Farr & Randall (1951) and the cytochrome *P*-450 content by the method of Omura & Sato (1964). DMN demethylase activity was determined by the method previously described (Lake, Heading, Phillips, Gangolli & Lloyd, 1974a). Also assayed were ethylmorphine *N*-demethylase (Holtzman, Gram, Gigon & Gillette, 1968) and aniline 4-hydroxylase (Nakanishi, Masamura, Tsukada & Matsumura, 1971).

RESULTS

Hepatic DMN demethylase and in vivo *metabolism of DMN*

Livers obtained from young and mature rats of both sexes were examined for DMN demethylase activity and also for a number of parameters of the microsomal mixed-function oxidase system. The results (Table 1) showed that the levels of DMN demethylase activity in all four groups were similar. Additionally, it was found that the relative liver weights, microsomal protein and cytochrome *P*-450 contents and aniline 4-hydroxylase activities were comparable in all the animals. The hepatic ethylmorphine *N*-demethylase activity was significantly higher in male rats than in females, a finding in agreement with the observations of Sladek & Mannering (1969).

Table 1. *Hepatic DMN demethylase activity, some parameters of the liver microsomal mixed-function oxidase system and $^{14}CO_2$ excretion rates following ip administration of ^{14}C-labelled DMN or formate in young and mature rats*

Parameter	Values for rats aged			
	5 wk		16 wk	
	Male	Female	Male	Female
Relative liver weight (g/100 g body weight)	3·9 ± 0·2	4·0 ± 0·1	3·9 ± 0·2	3·9 ± 0·1
Microsomal protein (mg/g liver)	30·4 ± 1·3	36·8 ± 0·06	33·2 ± 2·2	32·9 ± 1·1
Cytochrome *P*-450 (ΔE/g liver)	2·0 ± 0·1	2·3 ± 0·1	2·1 ± 0·2	1·4 ± 0·1
DMN demethylase (μmol/hr/g liver)	2·2 ± 0·1	1·3 ± 0·04	1·6 ± 0·2	1·5 ± 0·2
Ethylmorphine *N*-demethylase (μmol hr/g liver)	14·6 ± 0·7	9·8 ± 0·6	17·4 ± 1·4	8·1 ± 0·5
Aniline 4-hydroxylase (μmol hr/g liver)	1·4 ± 0·1	1·4 ± 0·04	1·8 ± 0·1	1·8 ± 0·1
$^{14}CO_2$ excretion rate (% dose/hr) from				
[^{14}C]dimethylnitrosamine	30·0 ± 3·7	33·1 ± 3·5	14·0 ± 1·4	17·0 ± 2·3
[^{14}C]formate	23·7 ± 0·2	—	—	23·6 ± 1·1

Values are expressed as the mean ± SEM for groups of three ($^{14}CO_2$ excretion rate) or six (other parameters).

In contrast to the observed similarity in the hepatic DMN demethylase activity in young and mature rats, the results of our previously reported studies on the *in vivo* metabolism of DMN (Phillips *et al.* 1975) showed that the rate of $^{14}CO_2$ excretion following administration of [^{14}C]DMN to the young rats was almost twice that found in the older animals. On the other hand, the $^{14}CO_2$ excretion rates from [^{14}C]formate administered to young male and mature female rats were similar (Table 1). Preliminary experiments using [^{14}C]formaldehyde gave similar results, in agreement with the findings of Neely (1964). In subsequent work, [^{14}C]formate was used for ease of handling and standardization of the doses administered.

Effects of phenobarbitone and 20-methylcholanthrene pretreatments

Studies in vitro. Phenobarbitone pretreatment induced the hepatic DMN demethylase activity in young and mature animals of both sexes (Table 2) and also increased the relative liver weight and the other parameters of the mixed-function oxidase system examined in the treated rats. However, the inductive effect on the DMN demethylase activity was not as marked as the increases in the cytochrome *P*-450 content and ethylmorphine *N*-demethylase activity.

Similarly, animals treated with 20-methylcholanthrene showed an induction in the levels of hepatic DMN demethylase activity in both the young and mature groups (Table 3). This compound also enhanced the relative liver weight, cytochrome *P*-450 content and aniline 4-hydroxylase activity. The ethylmorphine *N*-demethylase activity remained unchanged in the treated female rats and was depressed in the male animals, as shown previously by Sladek & Mannering (1969).

Studies in vivo. The effects of phenobarbitone and 20-methylcholanthrene pretreatments on the $^{14}CO_2$ excretion rate following the administration of [^{14}C]DMN to rats are shown in Table 4. Phenobarbitone treatment did not alter the rate of $^{14}CO_2$ excretion significantly in young animals or in the mature male rat, but in the treated mature female rat the $^{14}CO_2$ excretion rate was increased to 175% of the corresponding control value. There was no significant change in the rate of $^{14}CO_2$ excretion in 20-methylcholanthrene-treated rats in any of the four groups. Parallel studies conducted on the $^{14}CO_2$ excretion rate from [^{14}C]aminopyrine administered to young

Table 2. *Effect of phenobarbitone pretreatment on hepatic DMN demethylase activity and some indices of the liver microsomal mixed-function oxidase system in the rat*

	Values (expressed as % of control value) for rats aged			
	5 wk		16 wk	
Parameter	Male	Female	Male	Female
Relative liver weight	138***	130***	128**	127**
Microsomal protein	138***	122***	122*	117**
Cytochrome *P*-450	390***	291***	348***	200***
DMN demethylase	250***	185***	212***	173*
Ethylmorphine *N*-demethylase	531***	496***	340***	353***
Aniline 4-hydroxylase	235***	257***	244***	133*

Values are means for groups of six rats and all differ significantly (Student's *t* test) from the control value, as indicated by asterisks: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 3. *Effect of 20-methylcholanthrene treatment on hepatic DMN demethylase activity and some indices of the liver microsomal mixed-oxidase system in the rat*

	Values (expressed as % of control value) for rats aged			
	5 wk		16 wk	
Parameter	Male	Female	Male	Female
Relative liver weight	129***	120**	128**	105
Microsomal protein	104	119**	106	104
Cytochrome *P*-450	250***	230***	158***	186***
DMN demethylase	177***	173*	133*	141*
Ethylmorphine *N*-demethylase	79·3**	96·4	72·6**	93·4
Aniline 4-hydroxylase	138***	157**	100	129*

Values are means for groups of six rats and those marked with asterisks differ significantly (Student's *t* test) from the control value: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 4. *Effect of phenobarbitone and 20-methylcholanthrene pretreatments on the* in vivo *metabolism of DMN and the effect of phenobarbitone on the metabolism of aminopyrine*

Treatment	Rate of $^{14}CO_2$ excretion (% of dose of labelled compound/hr) in rats aged			
	5 wk		16 wk	
	Male	Female	Male	Female
[^{14}C]DMN preceded by				
0·9% Saline (control)	30·0 ± 3·7	33·1 ± 3·5	14·0 ± 1·4	17·0 ± 2·3
Phenobarbitone	26·9 ± 2·4	33·6 ± 5·1	16·1 ± 2·2	29·8 ± 0·9**
Corn oil (control)	20·9 ± 2·8	30·4 ± 0·9	24·4 ± 3·1	21·3 ± 1·2
20-Methylcholanthrene	26·5 ± 2·8	24·8 ± 6·5	25·5 ± 1·6	23·4 ± 1·9
[^{14}C]Aminopyrine preceded by				
0·9% Saline (control)	16·5 ± 0·45	—	—	—
Phenobarbitone	19·3 ± 0·15***	—	—	—

Values are means for groups of four rats and those marked with asterisks differ significantly (Student's t test) from the control value: $^{**}P < 0.01$; $^{***}P < 0.001$.

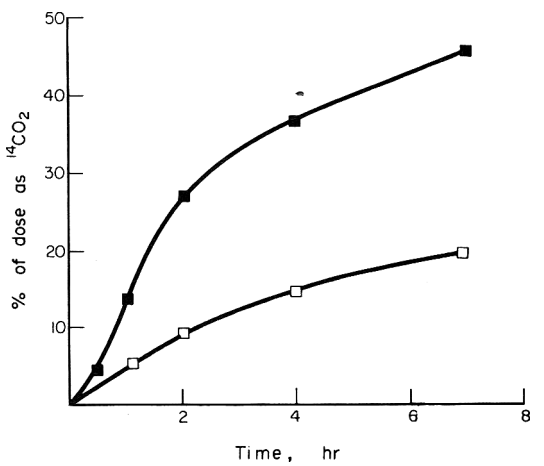

Fig. 1. Rate of excretion of $^{14}CO_2$ following the administration of [^{14}C]aminopyrine to young male rats (■) and to animals pretreated with SKF 525-A (□). Each point represents the mean for three animals.

male rats treated with phenobarbitone showed a significant increase to 115% compared with control animals (Table 4). Furthermore the hepatic aminopyrine *N*-demethylase activity was increased to approximately 200% of the control level.

Effect of SKF 525-A on the in vivo *metabolism of DMN and aminopyrine*

The administration of SKF 525-A (50 mg/kg body weight) 1 hr before the ip injection of [^{14}C]aminopyrine to rats markedly inhibited the initial rate of $^{14}CO_2$ excretion to 20% of that in the untreated group (Fig 1). However, this dose of SKF 525-A produced no significant change in the rate of $^{14}CO_2$ excretion from [^{14}C]DMN-treated rats. At a higher dose level (200 mg/kg) SKF 525-A caused some measure of inhibition and the $^{14}CO_2$ excretion rate was 85% of that of the untreated control.

Effect of enzyme induction on acute toxicity of DMN

The acute toxicity of DMN, assessed as the LD_{50}, was determined in young male and mature female

Table 5. *LD_{50} of DMN in young male and mature female rats and in animals pretreated with phenobarbitone or 20-methylcholanthrene*

Pretreatment	LD_{50} (mg DMN/kg body weight)
Males aged 5 wk	
0·9% Saline (control)	71·0 (63·6 – 78·7)
Phenobarbitone	63·2 (54·2 – 73·7)
Corn oil (control)	60·8 (50·9 – 72·6)
20-Methylcholanthrene	42·8* (39·1 – 46·7)
Females aged 16 wk	
0·9% Saline (control)	60·8 (50·9 – 72·5)
Phenobarbitone	78·1* (66·9 – 89·9)
Corn oil (control)	67·9 (56·9 – 81·1)
20-Methylcholanthrene	40·9* (36·7 – 45·6)

To determine the LD_{50} under each set of conditions, DMN was administered in logarithmically graded doses to four groups of five rats. Values are given with 95% confidence limits in parentheses, and those marked with an asterisk differ significantly ($P < 0.05$ by Student's t test) from the corresponding control value.

rats and in animals pretreated with phenobarbitone or 20-methylcholanthrene. The results (Table 5) did not reveal any significant difference in the LD_{50} of DMN between the young and mature animal. Phenobarbitone pretreatment reduced the acute toxicity of DMN in the mature female rat, while no change was observed in the young male. Animals pretreated with 20-methylcholanthrene were found to be more susceptible to the acute toxicity of DMN, the LD_{50} values in the treated rats being significantly lower than those in the control groups.

DISCUSSION

The mechanism postulated by Druckrey, Schildbach, Schmähl, Preussmann & Ivankovic (1963) for the mammalian metabolism of nitrosamines has found general acceptance. According to this scheme, DMN is metabolized primarily in the liver, by the microsomal mixed-function oxidase, DMN demethylase, to monomethylnitrosamine and formaldehyde. The unstable monomethyl compound is thought to decompose readily to diazomethane or carbonium ions, the putative alkylating species, and the formaldehyde is further oxidized to CO_2. Clearly, central to this hypothesis is the role of hepatic DMN demethylase in metabolizing DMN in the body. Thus the activity of this enzyme, measured in terms of the formaldehyde produced, should reflect the *in vivo* metabolism of DMN more accurately than does the $^{14}CO_2$ excretion rate.

The results of our studies on the relationship between these two indices of DMN metabolism in the rat revealed marked differences. Whereas the $^{14}CO_2$ excretion rate from young animals treated with labelled DMN was almost twice that found in mature rats of both sexes, the levels of hepatic DMN demethylase activity were similar in both age groups. Furthermore, it was found that the $^{14}CO_2$ excretion rate from [^{14}C]formate or [^{14}C]formaldehyde in the young rat was comparable to that in the mature animal.

In the evaluation of these findings, the $^{14}CO_2$ excretion rate merits further consideration as an index of DMN metabolism. Our earlier results (Phillips *et al.* 1975) showed, in agreement with those reported by Heath (1962), that the accumulation of respired $^{14}CO_2$ proceeded with the decline in the concentration of labelled DMN in the circulating blood of the rat. However, the metabolic processes involved in the oxidation of formaldehyde to CO_2 are complex and ill-defined. The mediation of formaldehyde dehydrogenase (Strittmatter & Ball, 1955) and formate dehydrogenase (Mathews & Vennesland, 1950) and the possible participation of peroxisomal catalase (de Duve, 1969) have been invoked in the biotransformation of formaldehyde via formate to CO_2 in the mammalian system. Notwithstanding the complexities of the intermediate enzyme reactions involved, analogous studies on the metabolism of methanol in the rat have established that the oxidative processes mediating the biotransformation of formaldehyde to CO_2 were not rate-limiting with respect to the metabolism of the alcohol, and further, that the $^{14}CO_2$ excretion rate was a valid index of the overall metabolism of labelled methanol in the whole animal (Mannering, Van Harken, Markar, Tephly, Watkins & Goodman, 1969; Tephly, Parks & Mannering, 1964). The close parallelism in certain aspects of the metabolic fate of methanol and of DMN in the rat therefore indicates that the formaldehyde-to-CO_2 pathway was not a rate-limiting factor in the *in vivo* metabolism of DMN.

In the light of these observations, our demonstrations, on the one hand, of a $^{14}CO_2$ excretion rate in young animals twice that in mature rats and, on the other, of a similarity in the levels of hepatic DMN demethylase activity in the two age groups, suggest that the observed discrepancy in these two indices of DMN metabolism was not explicable in terms of this hepatic enzyme activity being a rate-limiting step in the overall metabolism of DMN in the whole animal. Thus the key role of this enzyme in mediating and determining the *in vivo* metabolic transformation of DMN along the postulated degradative pathway becomes questionable. A consideration of factors such as the relative liver weight, microsomal protein and cytochrome *P*-450 contents, likely to influence the *in vivo* capacity of hepatic DMN demethylase activity in the two age groups did not show any marked differences. These and the other biochemical parameters of the hepatic mixed-function oxidase system examined were comparable in both the young and mature rat, a finding in agreement with that reported by Basu, Dickeron & Parke (1971).

Further evidence supporting the conclusion that the *in vivo* metabolism of DMN was not ascribable solely to the mediation of the microsomal demethylase enzyme associated with the mixed-function oxidase system was obtained from the studies on the effects of the two inducers and one inhibitor of microsomal enzymes on the rate of metabolism of DMN in the two age groups of rats. Our results on phenobarbitone- and 20-methylcholanthrene-treated rats, showing an inductive effect on the hepatic DMN demethylase activity with no significant change in the $^{14}CO_2$ excretion rates, are comparable with the findings of McLean & Day (1974), who described a similar lack of correlation between the hepatic DMN demethylase activity and the rate of DMN disappearance from the plasma in rats treated with phenobarbitone and benzo[*a*]pyrene. Equally significant was the finding that SKF 525-A, an inhibitor of microsomal-enzyme activity, did not exert a measurable influence on the $^{14}CO_2$ excretion rate after [^{14}C]DMN administration to rats.

In contrast to these results, studies on aminopyrine, a model substrate of the hepatic microsomal mixed-function oxidase system, showed that phenobarbitone treatment not only induced predictably the hepatic aminopyrine demethylase activity but also increased the rate of $^{14}CO_2$ excretion from labelled aminopyrine administered to rats. This *in vivo* inductive effect agrees with the findings reported both in the rat and in man (Hepner & Vesell, 1974; Lauterburg & Bircher, 1973). Furthermore, SKF 525-A markedly inhibited the *in vivo* metabolism of aminopyrine in the rat.

In the studies on the acute toxicity of DMN, the measure of protection afforded by phenobarbitone

treatment in mature female rats (in which, exceptionally, an induction of *in vivo* metabolism was observed) but not in young males, and the finding that 20-methylcholanthrene pretreatment rendered both young male and mature female rats more sensitive to the acute toxicity of DMN are results as equivocal as those obtained by McLean & Verschuuren (1965) in their study on the protective effects of phenobarbitone and DDT in the young rat. These findings stress the limitations encountered in providing an adequate and meaningful interpretation of the relationship between the metabolism of DMN and the toxicity of the compound in terms of the level of hepatic DMN demethylase activity and of factors influencing the microsomal mixed-function oxidase system in the rat.

Preliminary investigations into the possible involvement of alternative metabolic pathways have shown that the metabolic degradation of DMN to formaldehyde by rat-liver preparations involved a multicomponent enzymic process (Lake *et al.* 1974a) and further that the *in vivo* metabolism of DMN in the rat was profoundly inhibited by pyrazole and 3-amino-1,2,4-triazole, compounds known to interfere with alcohol metabolism (Phillips, Heading, Lake, Gangolli & Lloyd, 1974). These and related findings lend support to the hypothesis that the metabolism of DMN may involve the formation of hydroxylated intermediates which act as substrates for alcohol dehydrogenase and catalase (Schoental, 1973). This may be analogous to the situation with long-chain alkylnitrosamines (Blattmann & Preussmann, 1973). The results of studies directed towards the elucidation of this problem will constitute the subject of subsequent publications.

Acknowledgement—This work was conducted in the context of a research project financially supported by the Ministry of Agriculture, Fisheries and Food. We gratefully acknowledge the advice and helpful criticism of the director, Dr. R. F. Crampton.

REFERENCES

Anders, M. W. & Mannering, G. J. (1966). Inhibition of drug metabolism. I. Kinetics of the inhibition of the N-demethylation of ethylmorphine by 2-diethylaminoethyl 2,2-diphenylvalerate HCl (SKF 525-A) and related compounds. *Molec. Pharmacol.* **2**, 319.

Basu, T. K., Dickerson, J. W. T. & Parke, D. V. W. (1971). Effect of development on the activity of microsomal drug-metabolizing enzymes in rat liver. *Biochem. J.* **124**, 19.

Blattmann, L. u. Preussmann, R. (1973). Struktur von Metaboliten carcinogener Dialkylnitrosamine im Rattenurin. *Z. Krebsforsch.* **79**, 3.

Brouwers, J. A. J. & Emmelot, P. (1960). Microsomal N-demethylation and the effect of the hepatic carcinogen dimethylnitrosamine on amino acid incorporation into the proteins of rat livers and hepatomas. *Expl Cell Res.* **19**, 467.

de Duve, C. (1969). Evolution of the peroxisome. *Ann. N.Y. Acad. Sci.* **168**, 369.

Druckrey, H., Schildbach, A., Schmähl, D., Preussmann, R. u. Ivankovic S. (1963). Quantitative Analyse der carcinogenen Wirkung von Diäthylnitrosamine. *Arzneimittel-Forsch.* **13**, 841.

Dutton, A. H. & Heath, D. F. (1956). The preparation of [^{14}C]dimethylamine and [^{14}C]dimethylnitrosamine. *J. chem. Soc.* p. 1892.

Heath, D. F. (1962). The decomposition and toxicity of dialkylnitrosamines in rats. *Biochem. J.* **85**, 72.

Hepner, G. W. & Vesell, E. S. (1974). Assessment of human hepatic function by breath analysis. *Gastroenterology*, **66**, 709.

Holtzman, J. L., Gram, T. E., Gigon, P. L. & Gillette, J. R. (1968). The distribution of the components of mixed-function oxidase between the rough and the smooth endoplasmic reticulum of liver cells. *Biochem. J.* **110**, 407.

Knecht, Margarete (1966). Untersuchungen über eine mikrosomale *N*-Demethylase in verschiedenen Organen der Ratte in vitro. *Z. Naturf.* **21b**, 799.

Lake, B. G., Heading, Christine E., Phillips, J. C., Gangolli, S. D. & Lloyd, A. G. (1974a). Some studies on the metabolism *in vitro* of dimethylnitrosamine by rat liver. *Biochem. Soc. Trans.* **2**, 610.

Lake, B. G., Heading, Christine E., Phillips, J. C., Gangolli, S. D. & Lloyd, A. G. (1974b). Studies on the effects of phenobarbitone and 20-methylcholanthrene pretreatments on the metabolism and toxicity of dimethylnitrosamine in the rat. *Biochem. Soc. Trans.* **2**, 882.

Lauterburg, B. & Bircher, J. (1973). Hepatic microsomal drug-metabolizing capacity measured *in vivo* by breath analysis. *Gastroenterology* **65**, 556.

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.

McLean, A. E. M. & Day, Pauline A. (1974). The use of new methods to measure: The effect of diet and inducers of microsomal enzyme synthesis on cytochrome P-450 in liver homogenates, and on metabolism of dimethyl nitrosamine. *Biochem. Pharmac.* **23**, 1173.

McLean, A. E. M. & Verschuuren, H. G. (1969). Effects of diet and microsomal enzyme induction on the toxicity of dimethyl nitrosamine. *Br. J. exp. Path.* **50**, 22.

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

Magee, P. N. & Lee, K. Y. (1963). Experimental toxic liver injury by some nitrosamines. *Ann. N.Y. Acad. Sci.* **194**, 916.

Magee, P. N. & Vandekar, M. (1958). Toxic liver injury. The metabolism of dimethylnitrosamine *in vitro*. *Biochem. J.* **70**, 600.

Mannering, G. J., Van Harken, D. R., Makar, A. B., Tephly, T. R., Watkins, W. A. & Goodman, J. I. (1969). Rose of the intracellular distribution of hepatic catalase in the peroxidative oxidation of methanol. *Ann. N.Y. Acad. Sci.* **168**, 265.

Mathews, M. B. & Vennesland, Birgit (1950). Enzymic oxidation of formic acid. *J. biol. Chem.* **186**, 667.

Mizrahi, I. J. & Emmelot, P. (1962). The effect of cysteine on the metabolic changes produced by two carcinogenic N-nitrosodialkylamines in rat liver. *Cancer Res.* **22**, 339.

Nakanishi, S., Masamura, E., Tsukada, M. & Matsumura, R. (1971). Kinetic studies for aniline hydroxylase after prolonged ethanol treatment. *Jap. J. Pharmac.* **21**, 303.

Neely, W. B. (1964). The metabolic fate of formaldehyde-^{14}C intraperitoneally administered to the rat. *Biochem. Pharmac.* **13**, 1137.

Omura, T. & Sato, R. (1964). The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. biol. Chem.* **239**, 2370.

Omura, T., Sato, R., Cooper, D. Y., Rosenthal, O. & Estabrook, R. W. (1965). Function of cytochrome P-450 of microsomes. *Fedn. Proc. Fedn Am. Socs exp. Biol.* **24**, 1181.

Palframan, J. F., McNab, J. & Crosby, N. T. (1973). An evaluation of the alkali flame ionisation detector and the Coulson electrolytic conductivity detector in the analysis of N-nitrosamines in food. *J. Chromat.* **76**, 307.

Phillips, J. C., Heading, Christine E., Lake, B. G., Gangolli, S. D. & Lloyd, A. G. (1974). Studies *in vivo* on the inhibition of dimethylnitrosamine metabolism in the rat. *Biochem. Soc. Trans.* **2**, 885.

Phillips, J. C. Lake, B. G., Heading, Christine E., Gangolli, S. D. & Lloyd, A. G. (1975). Studies on the metabolism of dimethylnitrosamine in the rat. I. Effect of dose, route of administration and sex. *Fd Cosmet. Toxicol.* **13**, 203.

Schoental, R. (1973). The mechanisms of action of the carcinogenic nitroso and related compounds. *Br. J. Cancer* **28**, 436.

Sladek, N. E. & Mannering, G. J. (1969). Induction of drug metabolism. II. Qualitative differences in the microsomal *N*-demethylating systems stimulated by polycyclic hudrocarbons and by phenobarbital. *Molec. Pharmacol.* **5**, 186.

Strittmatter, P. & Ball, E. G. (1955). Formaldehyde dehydrogenase, a glutathione-dependent enzyme system. *J. biol. Chem.* **213**, 445.

Tephly, T. R., Parks, R. E. & Mannering, G. J. (1964). Methanol metabolism in the rat. *J. Pharmac. exp. Ther.* **143**, 292.

Weil, C. S. (1952). Tables for convenient calculation of median effective dose (LD_{50} or ED_{50}) and instructions in their use. *Biometrics* **8**, 249.

Fd Cosmet. Toxicol. Vol. 13, pp. 619–627. Pergamon Press 1975. Printed in Great Britain

SAFETY EVALUATION OF YEAST GROWN ON HYDROCARBONS. IV. TWO-YEAR FEEDING AND MULTIGENERATION STUDY IN RATS WITH YEAST GROWN ON PURE *n*-PARAFFINS*

A. P. DE GROOT, HENRIETTE C. DREEF-VAN DER MEULEN, H. P. TIL and V. J. FERON
Central Institute for Nutrition and Food Research, TNO, Utrechtseweg 48, Zeist, The Netherlands

(*Received 14 April 1975*)

Abstract—Yeast grown on pure *n*-paraffins was fed at dietary levels of 10, 20 or 30% to groups of 30 male and 30 female rats in a 2-yr study and to groups of ten male and 20 female rats in a reproduction study over three generations. Each study included two control groups, one on feed similar to the yeast diets but with soya-bean meal substituted for the yeast, and the other on the Institute's stock diet. In the 2-yr study, the feeding of the yeast had no adverse effect on mortality, rate of body-weight gain, haematology, urine composition or kidney-function tests. The activities of glutamic–oxalacetic transaminase and alkaline phosphatase in the serum of yeast-fed rats were relatively high but within the normal range. The feeding of the yeast did not affect the incidence or severity of histopathological changes nor the type and incidence of tumours. The multigeneration study revealed no effects on fertility, the number of young or the growth and mortality of the young during lactation. In a 90-day study on rats from the third generation, no changes attributable to yeast-feeding were apparent in any of the parameters investigated. It was concluded that the type of yeast examined did not exert a harmful effect in rats at dietary levels up to 30%.

INTRODUCTION

The present world-wide shortage of food has greatly stimulated research into the development of industrial processes for the production of proteins. A large number of industries is engaged in developing procedures for the mass production of protein by the growth of various kinds of micro-organisms on a variety of substrates. The oil companies have based their efforts mainly on the fermentation of hydrocarbons or their derivatives by yeasts or bacteria.

The British Petroleum Company Ltd. has developed two processes for yeast production by fermentation of paraffins. One process uses, as a feedstock, the petroleum fraction, gas oil (boiling range 250–400°C), which contains 10–15% of C_{15}–C_{30} *n*-paraffins, while in the other process, previously purified *n*-paraffins are used as the substrate. The gas oil process requires a purification step consisting of exhaustive solvent extraction of the yeast harvested, to eliminate residual substrate. In the case of cultivation on purified alkanes, the substrate is almost completely metabolized by the yeast. Because of this and also because of the purity of the substrate, no solvent treatment is necessary. As a result of the final purification, the yeast produced by the gas oil process has a considerably lower lipid content (*c.* 1·5% against *c.* 9·0%) and a slightly higher protein content (*c.* 68% against *c.* 63%) than the yeast grown on pure *n*-paraffins.

The yeasts obtained by each of these two processes have been submitted to long-term safety evaluation studies in laboratory animals. Feeding studies in rats for a 1-yr period have been reported in this journal (de Groot, Til & Feron, 1970a,b). The results obtained with yeast grown on gas oil in a 2-yr feeding and multigeneration study in rats have also been summarized in this journal (de Groot, Til & Feron, 1971). The present paper deals with a similar study conducted with yeast grown on pure *n*-alkanes.

EXPERIMENTAL

Materials. The yeast was provided by the British Petroleum Company Ltd. It was manufactured in the pilot plant of the Biological Research Division, BP Refinery Ltd., Grangemouth, Scotland, according to the *n*-paraffin process, which has been described elsewhere (Bennett, Hondermarck & Todd, 1969). During the 2-yr experimental period, six batches of paraffin-yeast were used in succession, having been stored at room temperature. The material contained 4·2–7·8% moisture, 60·0–65·0% crude protein (N × 6·25), 6·4–10·0% lipids, 5·5–6·4% ash, 0·010–0·030% calcium and 1·3–1·6% phosphorus. Protein quality assays in rats showed a true digestibility of 88–93, a net protein utilization of 57–64 and a protein efficiency ratio of 1·80–2·21 (standardized with a casein value of 2·50). By supplementation with methionine the net protein utilization was raised to 75–92 and the protein efficiency ratio to 2·93–3·23.

Animals. Weanling rats of both sexes from the Institute's colony (Wistar-origin) were randomly bred and kept under conventional conditions. They were housed in a room kept at 24–26°C, with a relative humidity of 50–60%.

Diets. The yeast was incorporated into the diets at levels of 0 (control), 10, 20 and 30%, mainly at

*These studies were carried out in co-operation with the British Petroleum Company Ltd., London.

the expense of soya-bean oil meal in the basal diet. In view of the unusually high protein level in these diets (*c.* 27%), the Institute's stock diet was used as an additional control. The percentage composition of the diets and the analytical data have been given before (de Groot, *et al.* 1970b). Fresh batches were made every 2–3 wk and stored at room temperature.

Experimental design and conduct

Long-term feeding study. Each of the diets was fed to 35 males and 35 females caged in groups of five for 2 yr. Diets and tap-water were offered *ad lib.* A regular check was made on the general condition and behaviour of the animals. Individual body weights were recorded biweekly in the first 12 wk and once every 4 wk thereafter. Food consumption of groups of ten rats was measured at intervals during periods of 2 wk.

At wk 13, 26, 39, 52, 78 and 102, blood was collected from the tail-tip of ten rats/sex/group and examined for glucose and urea-nitrogen and for haematological values comprising haemoglobin content, packed cell volume and counts of erythrocytes, total leucocytes and the individual types of leucocytes. Serum activities of glutamic–pyruvic transaminase, glutamic–oxalacetic transaminase and alkaline phosphatase were determined in blood samples from the orbital plexus of ten rats/sex/group at wk 52 and 104. Urine analyses, including appearance, pH, glucose, protein, occult blood, ketones and microscopy of the sediment, were carried out on pooled samples from ten rats/sex/group at wk 13, 26, 39, 52, 78 and 104. At the same stages, kidney function was tested in ten rats/sex/group by measuring phenol-red excretion, specific gravity and glutamic–oxalacetic transaminase in the urine.

At wk 53, ten rats/sex/group were sacrificed for interim observations on organ weights and pathological changes. At wk 104, all survivors were killed by decapitation. During autopsy, an examination was made for any macroscopic abnormality and the major organs were weighed. Samples of these organs, a variety of other organs and any other tissue that appeared to be abnormal were taken and fixed in 10% buffered formalin. Rats that died during the study and those killed when moribund were also autopsied, but tissue samples were preserved only if autolysis was not too advanced. All tissues were processed in the usual way for paraffin embedding and sections were stained with haematoxylin and eosin, and with additional stains where appropriate. Bone-marrow smears from at least ten rats/sex/group were air-dried and stained with May-Grünwald-Giemsa. The following tissues from all rats of the two control groups and of the group fed 30% yeast were examined microscopically: heart, kidneys, liver, spleen, brain (three sites), gonads, pituitary, thyroid, parathyroids, adrenals, thymus, lung, trachea, salivary glands, gastro-intestinal tract (six sites), pancreas, urinary bladder, skeletal muscle, spinal cord, femoral nerve, skin, bone-marrow (smear and section of sternum), axillary and mesenteric lymph nodes, exorbital lachrymal gland and aorta. The mammary gland, uterus, prostate, testis, seminal vesicle and coagulating gland were also examined in the appropriate sex.

Reproduction study. Weanling rats (50 males and 100 females) were divided into five groups of ten males and 20 females to constitute the parent generation ($P = F_0$). The five diets (as used in the 2-yr study) and tap-water were provided *ad lib.* Body weights were recorded once every 2 wk until wk 12. At wk 12 and 20, all rats were mated in groups of five males and ten females on the same diet, to produce two successive litters (F_{1a} and F_{1b} respectively). After a mating period of 3 wk, the females were caged individually until after the litters had been weaned. The numbers of pups in each litter and the total weight of each litter were recorded at days 1, 10 and 20. Litters containing more than eight siblings were randomly culled to eight on day 1. All the young of the F_{1a} litters were discarded at weaning. When the F_{1b} litters reached weaning, ten males and 20 females of each diet group were selected from as many different litters as possible and fed on the same diet as their parents. The procedure described for the F_0-generation was followed with the F_{1b} animals to produce rats of the F_{2a} and F_{2b} generation. This procedure was repeated with the F_{2b} offspring to obtain F_{3a} and F_{3b} litters. After they had weaned their second litters, the mothers were killed and each uterus was treated with ammonium sulphide solution for staining and the counting of implantation sites.

When the F_{3b} generation was weaned, ten rats/sex/group were selected from ten different litters and kept on the appropriate diet for 90 days. Individual body weights were recorded weekly. The food consumption of each group was measured during wk 1–4 and 11–12. Haematological values were recorded at wk 12. Kidney-function tests and urine examinations were made at wk 13. Thereafter the rats were killed by decapitation and blood was collected for measurement of serum enzymes, total protein and albumin. At autopsy, the rats were examined grossly and ten different organs from each rat were weighed. A wide range of organs and tissues was fixed in formalin. Detailed histological examination was carried out on

Table 1. *Cumulative mortality of rats fed yeast at dietary levels of 0–30% for 2 yr*

Dietary level (%)	Initial size of group†	Total number of deaths at wk						
		36	48	60	72	84	96	104
Males								
0‡	35	0	0	0	3	7	8	10
0§	35	0	0	1	2	4	6	7
10	35	0	0	1	2	3	3	4
20	35	0	1	1	2	3	3	7
30	35	0	0	0	0	2	4	7
Females								
0‡	35	0	0	0	0	3	4	9
0§	35	1	1	1	1	2	4	5
10	35	0	0	1	3	3	5	7
20	35	0	0	0	1	2	2	2*
30	35	0	0	0	1	1	4	7

†After 12 months ten males and ten females of each group were killed for pathological examination.
‡Soya control.
§Stock diet control.
The value marked with an asterisk differs significantly (χ^2 test) from the control group on the soya diet: $*P < 0.05$.

Table 2. *Mean values of body weight and food consumption for rats fed yeast at dietary levels of 0–30% for 2 yr*

Dietary level (%)	Body weight (g) at wk				Food consumption (g/rat/day) during wk			
	52	76	88	104	60 + 61	72 + 73	88 + 89	96 + 97
Males								
0†	418	461	472	465	16·0	15·0	16·7	16·8
0‡	437*	474	496	489	17·3	16·2	15·2	15·9
10	424	468	473	477	16·9	16·4	16·7	16·7
20	413	445	455	441	14·9	14·7	16·1	15·7
30	418	461	464	467	15·8	17·9	16·1	17·6
Females								
0†	244	270	284	293	12·2	13·6	13·6	13·3
0‡	259*	290	299	312	11·8	10·9	13·2	12·4
10	248	294*	312*	327	12·7	12·6	12·5	15·0
20	246	280	295	328	10·9	11·9	12·0	12·5
30	252	282	299	307	11·0	11·1	12·7	12·7

†Soya control.
‡Stock diet control.
Values are the means for groups of 25 rats. Those marked with an asterisk differ significantly (Student's t test) from controls on the soya diet: $*P < 0.05$.

Table 3. *Terminal haematological values in rats fed yeast at dietary levels of 0–30% for 2 yr*

Sex and dietary level (%)	Hb (g/100 ml)	HC (%)	RBC (10^6/mm^3)	Leucocytes				
				Total (10^3/mm^3)	Differential (%)			
					L	N	E	M
Males								
0†	13·9	46·3	7·1	13·2	72·7	24·5	2·6	0·2
0‡	14·5	48·7	7·5	12·2	78·4	18·5	3·1	0·0
10	14·2	47·5	7·4	14·7	71·3	24·6	4·1	0·0
20	14·2	47·6	7·4	13·0	72·0	24·9	3·1	0·0
30	13·3	45·1	6·8	13·5	71·3	26·2	2·4	0·1
Females								
0†	13·6	45·1	6·4	12·1	73·3	24·9	1·8	0·0
0‡	14·2	45·2	6·1	14·9	71·4	24·0	4·6	0·0
10	14·1	45·6	6·5	11·9	73·7	25·2	1·1	0·0
20	14·3	47·1	6·8	11·1	78·7	19·4	1·9	0·0
30	14·0	45·3	6·4	10·6	71·3	25·4	3·3	0·0

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

†Soya control.
‡Stock diet control.
Values are the means for groups of ten rats. None of the above values nor those determined at wk 78 differed significantly from the control values.

all rats fed the 30% yeast diet or the soya control diet. Microscopic examination of the rats fed stock diet or diets with 10 or 20% yeast was restricted to the liver and thyroid.

RESULTS

Long-term feeding study

General condition and mortality. No abnormalities were seen in the appearance or behaviour of the rats. The general condition of the rats remained good during the first 72 wk. Thereafter symptoms of ageing developed in many animals and mortality increased rapidly. The total number of deaths varied considerably among the groups, both in males and females, but there was no correlation between the survival rate and the dietary level of yeast (Table 1). Autopsies of rats that died or were killed *in extremis* generally revealed pulmonary infections, neoplasias or other pathological processes commonly associated with ageing.

Growth and food intake. Mean body weights and food intake figures during yr 2 are shown in Table 2. Results obtained during yr 1 have already been published (de Groot *et al.* 1970b). Body weights of the male rats fed yeast diets were similar to those of the controls on the soya diet, but were slightly lower than those of the stock-diet controls. Females fed yeast diets were slightly heavier than control females on the soya diet but were about as heavy as those on the stock diet. The food-intake figures

Table 4. *Terminal blood chemistry of rats fed yeast at dietary levels of 0–30% for 2 yr*

Dietary level (%)	Blood sugar (mg/100 ml)	BUN (mg/100 ml)	SGPT (μmol/ litre/min)	SGOT (μmol/ litre/min)	SAP (BLU)	TSP (g/100 ml)	Albumin/ globulin ratio
			Males				
0†	77	9·8	42	131	4·8	6·6	1·32
0‡	71	9·8	38	124	3·8	6·5	1·52
10	74	11·6	55**	137	6·6**	6·9	1·02
20	76	10·8	51	138	6·9*	6·7	1·17
30	75	13·4	53*	121	6·6*	6·5	1·19
			Females				
0†	79	14·8	40	143	5·7	7·1	1·39
0‡	71	13·3	35	130	3·8**	7·3	1·85
10	74	13·5	48	145	4·3*	6·8	1·87
20	77	12·1	51**	147	5·7	7·3	2·39
30	75	12·3	48*	141	4·9	7·0	1·86

BUN = Blood urea nitrogen SGPT = Serum glutamic–pyruvic transaminase SGOT = Serum glutamic–oxalacetic transaminase SAP = Serum alkaline phosphatase TSP = Total serum protein (biuret) BLU = Bessey-Lowry units

†Soya control.

‡Stock diet control.

Values are the means for groups of 15 rats. Those marked with asterisks differ significantly (Student's *t* test) from controls on the soya diet: $*P < 0{\cdot}05$; $**P < 0{\cdot}01$.

showed considerable differences both between groups and between different stages, but consistent group differences did not occur.

Haematology. The results of the haematological investigations of samples collected at six different stages during the 2-yr period did not reveal any treatment-related differences between test and control animals. Table 3 shows results for wk 102.

Clinical chemistry. Slightly increased SGPT-values were found in both sexes of all the groups fed yeast (Table 4) and SAP-values were increased in yeast-fed males. There was, however, no trend towards higher values with increasing levels of yeast. Therefore these findings are not attributed to the ingestion of the yeast. Other clinical blood findings showed no significant differences among the groups.

Urine examinations. The composition of pooled urine samples collected at the different stages showed no changes attributable to the feeding of the yeast. The results of the kidney-function tests (Table 5) were within the range of values considered normal for the strain of rat.

Organ weights. The organ-to-body weight ratios (Table 6) showed lower values for the ovaries of the groups on yeast diets than for those of the soya control group, but such differences were not apparent between the test groups and the group on the stock diet. Some other significant decreases in organ weights in the yeast groups were considered to be chance effects because they did not occur in the group on the highest dose level.

Pathological observations. There were no notable gross pathological changes that could be related to the yeast treatment. The many lesions present in most of the old animals were recognized as those commonly associated with the process of ageing. The organs most frequently affected were the kidney, liver and lung in both sexes and the lachrymal gland of males. The incidence of non-neoplastic histopathological changes in the group on the highest treatment level and in the two control groups is shown in Table 7. There were no indications that the feeding of the yeast had influenced the incidence or severity of the lesions observed.

The incidence of histologically confirmed neoplasms is tabulated in Table 8. The total number of tumours observed in this study was relatively low for the strain of rat used. The most common tumours encountered were phaeochromocytoma in the adrenals, lymphoreticular lung tumours and fibroadenomas of the mammary gland. These and other types of neoplasia occurred in a random manner with no appar-

Table 5. *Terminal urine analyses of rats fed yeast at dietary levels of 0–30% for 2 yr*

Dietary level (%)	Phenol-red excretion (% after 1 hr)	Specific gravity†	GOT activity‡
	Males		
0§	68·3	1·0537	16·4
0‖	58·3	1·0575	16·1
10	65·4	1·0503	12·6
20	61·7	1·0585	13·7
30	61·0	1·0581	14·4
	Females		
0§	78·4	1·0649	13·2
0‖	70·6	1·0702	15·2
10	72·6	1·0541	15·6
20	74·5	1·0681	15·2
30	68·6	1·0616	15·9

†Measured in urine samples collected during the last 16 hr of a 24-hr period of deprivation of food and water.

‡Glutamic–oxalacetic transaminase activity expressed in Reitman-Frankel units.

§Soya control.

‖Stock diet control.

Values are the means for groups of ten rats. None of the values differed significantly (Wilcoxon test) from the controls on the soya diet. Values at wk 78 similarly showed no significant differences from the soya controls with the exception of that for phenol-red excretion in males on the 20% yeast diet (37·9 *v.* 52·1% in controls; $P < 0{\cdot}05$).

Table 6. *Relative organ weights of rats fed yeast at dietary levels of 0–30% for 2 yr*

Dietary level (%)	Terminal body weight (g)	No. of survivors	Relative organ weight (g/100 g body weight)								
			Heart	Kidneys	Liver	Spleen	Brain	Gonads	Pituitary	Thyroid	Adrenals
					Males						
0†	464	15	0·358	0·63	3·13	0·156	0·43	0·73	0·0028	0·0075	0·0101
0‡	486	18	0·340	0·61	2·94	0·148	0·41	0·60*	0·0024	0·0064	0·0099
10	476	22	0·343	0·63	2·94	0·156	0·43	0·74	0·0024*	0·0070	0·0098
20	449	17	0·352	0·66	3·14	0·157	0·45	0·71	0·0025	0·0068	0·0098
30	469	18	0·366	0·66	3·04	0·152	0·44	0·70	0·0030	0·0060	0·0095
					Females						
0†	295	15	0·389	0·64	3·26	0·207	0·62	0·022	0·0059	0·0088	0·0176
0‡	317	21	0·365	0·63	3·34	0·185	0·58	0·017**	0·0056	0·0086	0·0178
10	321	17	0·358	0·60	3·04	0·180	0·57*	0·016**	0·0060	0·0085	0·0177
20	307	22	0·354	0·63	3·03*	0·195	0·61	0·017**	0·0060	0·0078	0·0169
30	308	17	0·385	0·67	3·38	0·175	0·60	0·017*	0·0059	0·0077	0·0170

†Soya control.
‡Stock diet control.
Values are the means for the numbers of rats shown. Those marked with asterisks differ significantly (Student's t test) from controls on the soya diet: $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$.

Table 7. *Non-neoplastic histopathological changes in rats fed yeast at dietary levels of 0 or 30% for 2 yr*

Histopathological findings	Diet... No. of rats examined....	No. of rats affected					
		Males			Females		
		Soya	Stock	30% yeast	Soya	Stock	30% yeast
		21	25	23	22	24	23
Kidney							
Nephrosis		13	11	9	2	4	4
Calcareous deposits		1	0	1	6	2	4
Parasite in pelvis		0	0	1	0	0	0
Cortical cyst		0	0	1	0	0	0
Cortical infarct		0	0	1	0	0	0
Unilateral hydronephrosis		0	1	1	0	1	0
Liver							
Vacuolization of hepatocytes		4	5	1	1	0	1
Necrosis		0	1	0	0	1	0
Bile-duct proliferation		4	3	4	4	1	1
Pericholangitis		2	3	2	2	2	1
Peliosis-like change		0	0	0	0	2	1
Hepatitis		1	0	0	3	0	1
Hyperplastic nodule		1	0	1	0	0	0
Lung							
Chronic respiratory disease*		12	11	11	14	18	12
Grey-spot disease		2	3	1	2	2	1
Calcification of arteries		8	6	4	0	4	1
Pancreas							
Transformation of exocrine acinar cells into duct-like structures		5	2	3	0	0	0
Hyperplastic islet		1	0	0	0	0	0
Thyroid							
Diffuse proliferation of light cells		5	4	3	4	3	1
Ultimobranchial remnants		2	4	1	2	1	1
Heart							
Focal myocarditis		3	3	6	0	2	0
Endocarditis		2	2	0	0	0	0
Sublingual salivary gland							
Metaplasia of excretory ducts		3	6	3	2	7	4
Exorbital lachrymal gland							
Prosoplasia of glandular epithelium into Harderian type acini		8	10	6	0	0	0
Urinary bladder							
Trichosomoides parasite		6	11	3	2	3	0
Proteinaceous plug		0	2	2	0	0	0
Adrenal							
Haemorrhagic cyst in cortex		3	1	0	2	2	3
Cortical focal fatty change		1	1	0	2	0	0
Pituitary							
Colloid in pars distalis		1	1	3	0	0	0
Uterus							
Endometritis/pyometra					2	9	4
Testis							
Atrophy		5	11	6			
Calcification of arteries		0	0	1			

*Lungs containing lymphoreticular tumours are excluded.

ent relationship between number, location or type of tumour and diet composition.

Reproduction study

The body weights of the parent rats in successive generations were generally slightly higher in the groups on the yeast diets than in those in the soya control group, but were similar to those of the controls on the stock diet. Data summarizing the reproduction and lactation performances of the F_0, F_1 and F_2 generations are shown in Table 9. Yeast feeding had no effect on fertility, the number or weight of the young born, or the gain in body weight of the young during the lactation period. Preweaning mortality showed considerable variation both among groups and among different generations. Relatively high mortality occurred in the 30%-yeast group when the second litters of the F_1 generation were reared. No increased mortality was noticed in the litters of this group, however, after any of the previous or following matings. The ratio between the number of im-

plantation sites and the number of pups born (resorption quotient) in the various groups was only slightly above 1·0, indicating uniformly low mortality *in utero*.

In the 3 months during which the rats from the F_{3b} generation were observed, growth and food intake were normal, while in both sexes the food efficiencies of the groups on the yeast diets were slightly more favourable than those of the two control groups. Haematological and biochemical values collected at wk 12–13 were comparable with those found in controls, except for a slightly increased alkaline phosphatase activity in the serum of males in the 30%–yeast group. Terminal kidney-function tests and urine examinations and the organ-to-body weight ratios of ten different organs were within normal limits. Gross and microscopic examinations revealed no yeast-related pathological changes.

Table 8. *Incidence and type of tumours in rats fed yeast at dietary levels of 0 or 30% for 2 yr*

Site and type of tumour	No. of rats affected					
	Males			Females		
Diet....	Soya	Stock	30% Yeast	Soya	Stock	30% Yeast
No. of rats examined....	21	25	23	22	24	23
No. of rats with tumours....	13	14	17	15	16	16
Lung						
Malignant lymphoreticular tumour	5	5	2	0	4	5
Adenoma	0	1	1	0	0	0
Epidermoid carcinoma	0	1	0	0	0	0
Thyroid						
Light cell adenoma						
small (hyperplasia?)	0	0	2	2	4	2
medium-sized	1	0	0	0	1	0
large	1	0	1	0	0	0
Anaplastic carcinoma	1	0	0	0	0	0
Pituitary						
Adenoma						
small (hyperplasia?)	0	0	1	0	0	0
medium-sized	0	0	1	2	1	1
large	0	0	1	0	2	0
Carcinoma	0	1	0	0	0	0
Adrenal						
Phaeochromocytoma						
small (hyperplasia?)	4	2	4	0	0	0
medium-sized	4	5	2	1	0	0
large	1	0	5	0	0	0
Mammary gland						
Fibroadenoma	0	0	0	4	6	2
Adenoma	0	0	1	3	1	2
Cystadenoma	0	0	0	0	1	2
Squamous-cell carcinoma	0	0	0	0	1	0
Liver						
Mesenchymal tumour	0	0	1	0	0	0
Abdomen						
Lymphoreticular tumour	1	0	0	3	0	0
Malignant mesenchymal tumour	0	1	0	0	0	0
Spleen						
Haemangioma	0	0	1	0	0	0
Brain						
Meningioma	1	0	0	0	0	0
Oligodendroglioma	0	0	1	0	0	0
Oligodendroblastoma	0	0	1	0	0	0
Subcutis						
Fibroma	1	0	0	0	0	0
Sarcoma	0	1	0	1	0	0
Salivary gland						
Carcinoma	0	0	1	0	0	0
Forestomach						
Papilloma	0	1	0	0	0	0
Ovary						
Granulosa-cell tumour				0	0	1
Leukaemia	2	2	1	2	0	1
Total number of primary tumours...	23	20	28	18	21	16

Table 9. *Reproduction data of F_0–F_3 generations of rats fed yeast at dietary levels of 0–30%*

Dietary level (%)	Percentage of females with litter*	Average no. of rats/litter at birth	Mean body weight (g) of young at day			Percentage of young dead at day		Resorption quotient†
			1	10	20	10	20	
			F_0, First mating					
0‡	95	9·5	6·7	20·1	36·5	3·4	3·4	
0§	100	10·7	6·5	20·6	38·3	2·6	2·6	
10	100	9·8	6·6	20·6	37·1	0	0	
20	95	9·3	6·8	20·4	39·2	2·1	2·1	
30	95	9·8	6·7	19·2	35·5	0	2·1	
			F_0, Second mating					
0‡	100	9·9	6·3	19·8	38·9	0	0	1·08
0§	100	11·3	6·2	21·8	39·8	2·1	2·1	1·07
10	100	11·0	5·9	19·3	38·6	2·6	2·6	1·07
20	100	10·6	5·9	18·6	38·2	2·8	3·5	1·08
30	80	11·6	6·2	20·5	40·7	4·0	4·0	1·10
			F_{1b}, First mating					
0‡	100	9·1	6·3	18·7	35·1	0	0	
0§	90	10·6	6·3	20·0	39·4	6·8	6·8	
10	100	9·6	6·3	17·9	35·8	6·3	6·3	
20	100	9·7	6·2	17·6	34·2	5·8	7·1	
30	95	11·3	6·1	18·3	35·7	4·1	4·1	
			F_{1b}, Second mating					
0‡	100	9·1	6·2	19·2	40·3	2·3	3·8	1·08
0§	95	12·0	6·4	20·6	41·8	0·7	0·7	1·02
10	100	9·5	6·4	18·6	39·2	6·4	7·1	1·03
20	100	9·7	6·4	18·8	40·1	4·4	6·1	1·03
30	95	11·4	6·4	18·7	39·5	12·5	17·8	1·02
			F_{2b}, First mating					
0‡	95	9·1	6·5	18·2	34·6	0	0·7	
0§	100	10·8	6·3	19·6	37·3	5·0	5·0	
10	100	10·3	6·4	18.1	33·6	1·3	1·3	
20	100	8·9	6·5	19·0	36·7	0	0	
30	100	9·3	6·4	19·4	36·3	0·7	2·0	
			F_{2b}, Second mating					
0‡	100	11·1	6·4	18·3	27·1	3·2	9·6	1·10
0§	100	13·0	6·1	20·0	39·6	2·5	2·5	1·04
10	100	10·9	6·2	17·8	36·0	0·7	0·7	1·05
20	90	10·1	6·5	19·8	40·5	2·3	3·1	1·07
30	95	10·9	6·3	18·7	37·3	3·0	6·8	1·10

*No. of females mated in each group was 19 or 20.
†No. of implantation sites/no. of young born.
‡Soya control.
§Stock diet control.

DISCUSSION

The feeding of the yeast at dietary levels up to 30% for 2 yr did not result in any significantly adverse effects on body-weight gain and food efficiency, mortality, haematology, serum chemistry, urinary cell excretion, renal function or reproduction and lactation performance, or in any of the criteria applied to the offspring when the same diets were fed over three generations of rats. Male and female rats from the F_{3b} generation were continued on their diets to produce further generations, but with only one mating in each generation. At present 16 successive generations have been reared without any impairment of reproduction or lactation performance.

The histological changes observed in the 2-yr study were consistent with those expected in ageing rats. The incidence, type and severity of the liver lesions observed did not account for the somewhat raised activities of alkaline phosphatase in the serum of rats fed yeast diets.

Most of the tumours found in rats fed 30% yeast also occurred with a similar incidence in control rats and could not be attributed to treatment. Some types of tumour found in only one rat fed on a yeast-containing diet and not in controls have been found occasionally in rats of our strain in previous studies. Therefore the pathological changes observed were considered to be spontaneous, age-dependent and unrelated to the ingestion of the yeast.

The results of the present 2-yr feeding and reproduction studies conducted with a yeast grown on pure *n*-paraffins confirm the lack of toxic effects found in a previous 1-yr study using the same yeast and the same diets (de Groot *et al.* 1970b). They are also in agreement with the results of similar studies conducted previously with a different yeast produced by the gas oil process (de Groot *et al.* 1971). A major difference in composition between the two types of yeast is the considerably higher lipid content of the yeast examined in the present studies (8·2 against

1·2%). The lipids of micro-organisms grown on hydrocarbons may contain odd-numbered fatty acids in amounts considerably higher than those normally found in foods and feeds (Bird & Molten, 1972). Uneven fatty acids are broken down in the same sequence as even fatty acids, except that they yield a terminal unit of propionyl CoA, rather than acetyl CoA. Propionyl CoA can be converted to succinyl CoA which may enter the citric acid cycle (Green & Allman, 1968). Nearly 33% of the lipids present in the type of yeast used in this study were found to consist of odd-chain fatty acids, mainly $C_{15:0}$ and $C_{17:1}$ with a small proportion of $C_{13:0}$, $C_{15:1}$ and $C_{17:0}$. Yeast diets with a calculated dietary level of 0·8% odd-chain fatty acids were not associated with any unfavourable effect in the present long-term studies. This agrees with the lack of toxicity in short-term studies in rats fed diets containing *c.* 10% odd fatty acids (VanItallie & Khachadurian, 1969; Saxena, Vendelmans-Starrenburg & Vles, 1972).

The level of residual paraffin in the yeast examined averaged nearly 0·5%. Although a small part of ingested paraffin is absorbed, no adverse effects were noticeable. This is consistent with the toxicological evaluation of food-grade mineral oil by the Joint FAO/WHO Expert Committee on Food Additives (1970).

These studies have thus provided no evidence that the type of yeast examined has any harmful potential when fed to rats at dietary levels up to 30% for 2-yr and over three generations.

REFERENCES

Bennett, I. C., Hondermarck, J. C. & Todd, J. R. (1969). How BP makes protein from hydrocarbons. *Hydrocarb. Process.* **48** (3), 104.

Bird, C. W. & Molton, P. M. (1972). The production of fatty acids from hydrocarbons by microorganisms. In *Topics in Lipid Chemistry*. Vol. 3. Edited by F. G. Gunstone. p. 125. Elek Science, London.

de Groot, A. P., Til, H. P. & Feron, V. J. (1970a). Safety evaluation of yeast grown on hydrocarbons. I. One-year feeding study in rats with yeast grown on gas oil. *Fd Cosmet. Toxicol.* **8**, 267.

de Groot, A. P., Til, H. P. & Feron, V. J. (1970b). Safety evaluation of yeast grown on hydrocarbons. II. One-year feeding study in rats with yeast grown on pure *n*-paraffins. *Fd Cosmet. Toxicol.* **8**, 499.

de Groot, A. P., Til, H. P. & Feron, V. J. (1971). Safety evaluation of yeast grown on hydrocarbons. III. Two-year feeding and multigeneration study in rats with yeast grown in gas oil. *Fd Cosmet. Toxicol.* **9**, 787.

Green, D. E. & Allmann, D. W. (1968). Fatty acid oxidation. In *Metabolic Pathways. Vol. II Lipids, Steroids, and Carotenoids*. 3rd Ed. Edited by D. M. Greenberg. p. 1. Academic Press, New York.

Joint FAO/WHO Expert Committee on Food Additives (1970). Toxicological Evaluation of Some Extraction Solvents and Certain Other Substances. *F.A.O. Nutr. Mtg Rep. Ser.* no. 48A, WHO/Food Add./70.39.

Saxena, S. C., Vendelmans-Starrenburg, A. & Vles, R. O. (1972). Effects of feeding medium chain triglycerides to rats for 13 weeks. *Nutr. Metabol.* **14**, 362.

VanItallie, T. B. & Khachadurian, A. K. (1969). Rats enriched with odd-carbon fatty acids: Maintenance of liver glycogen during starvation. *Science, N.Y.* **165**, 811.

Fd Cosmet. Toxicol. Vol. 13, pp. 629–632. Pergamon Press 1975. Printed in Great Britain

TERATOGENIC EVALUATION OF LEAD COMPOUNDS IN MICE AND RATS

G. L. KENNEDY, D. W. ARNOLD and J. C. CALANDRA

Industrial BIO-TEST Laboratories, Inc., Northbrook, Illinois 60062, USA

(*Received 13 April 1975*)

Abstract—Groups of pregnant albino mice and rats were treated by gavage with doses up to 714 mg lead acetate/kg or 10 mg tetraethyllead (TEL)/kg. The compounds were administered daily during the period of rapid organogenesis (days 5–15 of pregnancy for mice and days 6–16 for rats). Maternal toxicity was observed and foetal resorption and general retardation of development were encountered at the higher dosage levels. Neither lead compound caused any congenital malformations. Foetuses derived from lead-exposed females were examined grossly and for internal structural and skeletal development but no teratogenic response was evident, even at dose levels at which frank signs of maternal toxicity were observed. It is concluded that lead, as the acetate or as tetraethyllead, is not teratogenic to the mouse or rat.

INTRODUCTION

Exposure of the pregnant human female to high levels of lead has apparently resulted in abortion (Taussig, 1936; Wilson, 1966). It has been demonstrated that lead crosses the human placenta in cases of high-level exposure (Barltrop, 1968). The evaluation of teratogenic potential in animal models has received a great deal of attention. In hamsters, the injection of lead salts produced changes in the sacral and, to a less marked degree, the tail vertebrae without any increase in foetal mortality (Ferm & Carpenter, 1967). No evidence for a teratogenic response in cows (Shupe, Binns, James, & Keeler, 1967) or sheep (James, Lazar & Binns, 1966) has been reported. In the rabbit, the feeding of up to 546 ppm lead throughout gestation was found to be both non-teratogenic and non-embryotoxic (Jessup, 1967). The same workers found no lead-induced alterations in rats exposed to up to 1000 ppm lead (as lead acetate) in a three-generation reproduction study (Jessup, 1969). McClain & Becker (1972) treated rats with tetraethyllead (TEL) at doses up to 30 mg/kg for short periods during gestation and found delayed ossification and general retardation of growth. No specific teratological findings were noted in that study and the observations referred to were seen only in maternal animals that exhibited severe organolead toxicity and were severely debilitated. Single iv doses of lead nitrate were found to cross the placenta and were embryotoxic but not teratogenic to the rat (McClain & Becker, 1975).

In this experiment, lead acetate and TEL were evaluated to determine their teratological potential at maximally tolerated doses in two rodent species, the mouse and the rat. Doses were administered over the entire period of rapid organogenesis in both species.

EXPERIMENTAL

Materials. Lead acetate (certified ACS grade) was obtained from J. T. Baker Chemical Co., Phillipsburg, N.J., and TEL (98% pure) was supplied by the Houston Chemical Co., Freeport, Texas.

Animals and diet. The animals used were CD-1 random-bred albino mice and COBS random-bred albino rats impregnated at the Charles River Breeding Laboratories, Wilmington, Mass. The day of sperm-positive vaginal examination was designated gestation day 0 and the animals were shipped to these laboratories on that day. For acute studies, non-pregnant rodents of the same age were obtained from the same supplier. All animals were housed individually in hanging stainless-steel rodent cages and were maintained on a standard pelleted feed, obtained from Ralston-Purina, St. Louis, Mo., and water provided *ad lib.*

Experimental design. Female animals were divided into groups of 20 and were treated with the test compounds by oral intubation. Lead acetate was given as an aqueous solution and TEL as a solution in corn oil. Concurrently maintained control animals were treated with either distilled water on corn oil in volumes equivalent to those received by the animals on the highest dose level. Mice were treated from gestation day 5 to 15 and rats from day 6 to 16.

Preliminary acute range-finding studies were conducted in mice and rats to determine tolerable doses. Single graded oral doses of each material were given to groups of ten animals and 14-day LD_{50} values were calculated (Litchfield & Wilcoxon, 1949). Dose levels selected for use in the teratological study were 0, 7·14, 71·4 and 714 mg lead acetate/kg body weight and 0, 0·01, 0·1, 1·0 and 10 mg TEL/kg body weight for both species. Because of the toxicity observed at the highest dose levels (714 mg lead acetate/kg and 10 mg TEL/kg), treatment at these levels was discontinued after three daily doses. Animals were weighed daily to facilitate accurate dosing on a mg/kg basis.

Animals were killed by cervical dislocation just before natural delivery (day 18 for mice and day 20 for rats) and the uterine horns were examined for implantation sites, resorption sites and viable foetuses.

Table 1. *Acute oral toxicity of tetraethyllead*

Species	Dose (mg/kg)	No. dead/no. tested	Reactions (onset-duration, hr)
Mouse	35·12	0/10	None
	52·67	2/10	Hypoactivity (6–24)
	79·01	10/10	Hypoactivity (6-until death) Tremors (5–24 or until death) Hypoactivity (2-until death)
	118·5	10/10	Tremors (3–12 or until death) Clonic convulsions (2–3 to 5)
Rat	35·12	1/10	None
	52·67	5/10	Hypoactivity (6–24)
	79·01	10/10	Hypoactivity (6-until death) Tremors (5–24 or until death) Clonic convulsions (2–5 or until death)

A complete external examination of each foetus was conducted and, following removal of excess amniotic fluid, each was weighed. Foetuses were divided into two equal groups (randomly assigned in each litter) for evaluation of skeletal development (Hurley, 1965) or of internal development (Wilson, 1966).

RESULTS AND DISCUSSION

In acute toxicity studies. LD_{50} values for lead acetate in both rats and mice were greater than 2 g/kg. In both species, doses of 600 mg/kg or more resulted in hypoactivity, the degree and duration being proportional to the dose. LD_{50} values for TEL were 65 ± 3·5 mg/kg for mice and 53 ± 8 mg/kg for rats (Table 1). Schroeder, Avery & Cross (1972) found the oral LD_{50} of TEL in rats to be 14·18 mg/kg using peanut oil as the vehicle. The reason for this difference is not clear, but values in the study described here are based on groups of ten rather than four animals. Further, the steep response seen in the work of Schroeder *et al.* (0/4 deaths at 13·0 mg/kg and 4/4 deaths at 16·9 mg/kg) may indicate a more genetically homogeneous test model. McClain & Becker (1972) encountered little maternal mortality at a dose level of 30 mg TEL/kg, a finding in line with the results reported here.

Animals given up to 0·10 mg TEL/kg or 71·4 mg lead acetate/kg showed no evidence of a pharmacotoxic response to the chemicals and displayed normal weight gains during pregnancy. Female mice treated with 1·0 mg TEL/kg gained 25% less weight during gestation than did the controls, and the response was more noticeable in rats (with a weight gain 70% less than that of the controls). Both species displayed hypoactivity, tremors and convulsions along with severe body-weight loss after three daily doses of 10 mg TEL/kg, and subsequent dosing was discontinued. Treatment of rats and mice with 714 mg lead acetate/kg resulted in hypoactivity, severe diarrhoea and sharply reduced body weights, forcing discontinuation of treatment after three doses.

Reproductive parameters, including the number of females examined and the numbers of implantation sites, resorption sites, foetuses and terata observed are presented in Tables 2 (mice) and 3 (rats). The number of pregnancies was sharply reduced with 10 mg TEL/kg and with 714 mg lead acetate/kg. In both species, many females in these groups were observed to be full of brown fluid without any trace of foetal tissues or remnants thereof.

An increase in foetal resorption occurred in both species with exposure to 1·0 mg TEL/kg or 714 mg lead acetate/kg, the number of foetuses being corres-

Table 2. *Reproduction and teratological data for mice exposed to lead compounds during gestation*

Treatment	Dose of: Compound (mg/kg)	Dose of: Lead (mg/kg)	No. of pregnant females examined	No. of: Implantation sites*	No. of: Resorption sites*	No. of: Foetuses*	No. of: Abnormal foetuses†
Water (control)	—	—	16	195 (12·2)	7 (0·4)	188 (11·8)	1 (0·5)
Corn oil (control)	—	—	16	186 (11·6)	12 (0·8)	174 (10·9)	2 (1·1)
TEL	0·01	0·006	15	187 (12·5)	11 (0·7)	176 (11·7)	0
	0·10	0·064	20	256 (12·8)	18 (0·9)	238 (11·9)	3 (1·3)
	1·00	0·64	15	185 (12·3)	32 (2·1)	153 (10·2)	0
	10·0	6·40	5	58 (11·6)	8 (1·6)	50 (10·0)	0
Lead acetate	7·14	3·90	11	152 (13·8)	9 (0·8)	143 (13·0)	0
	71·4	39·0	16	215 (13·4)	12 (0·7)	203 (12·7)	0
	714·0	390·0	3	38 (12·7)	7 (2·3)	31 (10·3)	1 (3·2)

*Figures in parentheses refer to mean/female.
†Figures in parentheses refer to percentage of total foetuses.

Table 3. *Reproduction and teratological data for rats exposed to lead compounds during gestation*

Treatment	Dose of Compound (mg/kg)	Dose of Lead (mg/kg)	No. of pregnant females examined	No. of Implantation sites*	No. of Resorption sites*	No. of Foetuses*	No. of Abnormal foetuses†
Water (control)	—	—	19	241 (12·7)	17 (0·6)	224 (11·8)	1 (0·4)
Corn oil (control)	—	—	16	196 (12·2)	12 (0·8)	184 (11·5)	2 (1·0)
TEL	0·01	0·006	15	188 (12·5)	10 (0·7)	178 (11·9)	1 (0·5)
	0·10	0·064	20	256 (12·8)	18 (0·9)	238 (11·9)	1 (0·4)
	1·00	0·64	16	205 (12·8)	37 (2·3)	168 (10·5)	0
	10·0	6·40	7	77 (11·0)	8 (1·2)	69 (9·9)	0
Lead acetate	7·14	3·90	19	258 (13·6)	15 (0·8)	243 (12·8)	2 (0·8)
	71·4	39·0	16	214 (13·4)	13 (0·8)	201 (12·6)	0
	714·0	390·0	3	46 (11·5)	10 (2·5)	36 (9·0)	0

*Figures in parentheses refer to mean/female.
†Figures in parentheses refer to percentage of total foetuses.

pondingly reduced in these groups. No increase in foetal resorption nor decrease in viable foetuses was observed at dose levels up to 0·10 mg TEL/kg or 71·4 mg lead acetate/kg.

No evidence for a teratogenic response to lead was obtained from the gross examination of mice from the lead-treated groups. Three of 362 control foetuses (0·8%) displayed external anomalies, consisting of one exencephaly, one runt and one cleft palate. Three abnormal young, all displaying torsion of the rear limbs, were recovered from one litter in the group on 0·10 mg TEL/kg. One foetus in the group on 714 mg lead acetate/kg displayed exencephaly. All other foetuses were grossly normal. Internal evaluation revealed no significant structural deviations in the lead-exposed mouse foetuses. Skeletal examination revealed slight delays in ossification in foetuses recovered from females exposed to either 1 or 10 mg TEL/kg or to 714 mg lead acetate/kg. No specific skeletal anomalies were detected in any of the animals. Supernumerary ribs and incomplete or non-ossified sternal sections were observed in all groups, including the controls, with the same relative frequency.

In the rat study, three grossly abnormal foetuses were recovered from the 408 foetuses from the control group, one displaying severe stunting, one anophthalmia and one cleft palate. Among the lead-treated groups, one foetus in the group on 0·01 mg TEL/kg was non-viable, one in the group on 0·10 mg TEL/kg had malrotation of a hind limb, and two foetuses in the group given 7·14 mg lead acetate/kg were non-viable. All other foetuses were grossly normal, indicating the lack of a teratogenic response. As was the case in mice, no internal structural deviations were seen in the lead-treated rats. Retarded skeletal development (general lack of ossification) was observed in increased numbers of offspring of females exposed to either 10 mg TEL/kg or 714 mg lead acetate/kg. Foetuses obtained from dams exposed to 1·0 mg TEL/kg or less or to 71·4 mg lead acetate/kg or less showed normal skeletal development. In these groups, incidental findings, such as incomplete ossification of the sternum and supernumerary ribs, were observed with the same relative frequency as in the control groups. There were no specific skeletal abnormalities that could be correlated with exposure to lead.

Growth of foetuses (Table 4), as indicated by terminal body weights, was reduced in both species among animals exposed to 1·0 mg TEL/kg or more and 714 mg lead acetate/kg.

It is concluded that no teratogenic response is pro-

Table 4. *Body weights of foetuses recovered from lead-treated dams*

Treatment	Dose of Compound (mg/kg)	Dose of Lead (mg/kg)	Mean foetal weight (g) Mouse Male	Mouse Female	Rat Male	Rat Female
Water (Control)	—	—	1·1	1·0	4·3	4·2
Corn oil (Control)	—	—	1·1	1·1	4·1	4·0
TEL	0·01	0·006	1·1	1·1	4·3	4·3
	0·10	0·064	1·0	1·0	4·1	4·1
	1·00	0·64	0·8*	0·8*	3·8*	3·6*
	10·0	6·40	0·4*	0·4*	3·2*	3·0*
Lead acetate	7·14	3·90	1·1	1·1	4·1	4·1
	71·4	39·0	1·1	1·0	4·0	4·1
	714·0	390·0	0·7*	0·6*	3·2*	3·2*

Figures marked with an asterisk differ significantly (*$P < 0·05$) from the corresponding control values.

duced in rats and mice following *in utero* exposure to maximally tolerated doses of either lead acetate or TEL. Embryotoxicity is observed at levels that result in severe maternal toxicity.

REFERENCES

Barltrop, D. (1968). The transfer of lead to the human foetus. In *Mineral Metabolism in Paediatrics*. Blackwell Scientific Publications, Oxford.

Ferm, V. H. & Carpenter, S. J. (1967). Developmental malformations resulting from the administration of lead salts. *Expl mol. Path.* **7**, 208.

Hurley, L. S. (1965) Demonstration A-Alizarin staining of bone (revised). Supplement of Teratology Workshop Manual, Berkeley, Cal. p. 121.

James, L. F., Lazar, V. A. & Binns, W. (1966). Effects of sublethal doses of certain minerals on pregnant ewes and foetal development. *Am. J. vet. Res.* **27**, 132.

Jessup, D. C. (1967). Lead Acetate, Teratology Study—Rabbits. US National Technical Information Service Report PB-201, p. 139.

Jessup, D. C. (1969). Lead Acetate, Three-generation Reproduction Study—Rats. US National Technical Information Service Report PB-201, p. 138.

Litchfield, J. T., Jr. & Wilcoxon, F. (1949). A simplified method of evaluating dose-effect experiments. *J. Pharmac. exp. Ther.* **96**, 99.

McClain, R. M. & Becker, B. A. (1972). Effects of organolead compounds on rat embryonic and fetal development. *Toxic. appl. Pharmac.* **21**, 265.

McClain, R. M. & Becker, B. A. (1975). Teratogenicity, fetal toxicity, and placental transfer of lead nitrate in rats. *Toxic. appl. Pharmac.* **31**, 72.

Schroeder, T., Avery, D. D. & Cross, H. A. (1972). The LD_{50} value of tetraethyl lead. *Experientia* **28**, 425.

Shupe, J. L., Binns, W., James, L. F. & Keeler, R. F. (1967). Lupine, a cause of crooked calf diseases. *J. Am. vet. med. Ass.* **151**, 198.

Taussig, F. J. (1936). *Abortion, Spontaneous and Induced*. pp. 111 & 354. Kimpton, London.

Wilson, J. T. (1966). Effects of abnormal lead content of water supplies on maternity patients. The use of a simple industrial screening test in ante-natal care in general practice. *Scott. Med. J.* **11**, 73.

Fd Cosmet. Toxicol. Vol. 13, pp. 633 638. Pergamon Press 1975. Printed in Great Britain

OBSERVATIONS ON THE ORAL ADMINISTRATION AND TOXICITY OF VINYL CHLORIDE IN RATS*

V. J. FERON, A. J. SPEEK, MARIANNE I. WILLEMS, D. VAN BATTUM and A. P. DE GROOT

Central Institute for Nutrition and Food Research (CIVO) TNO, Utrechtseweg 48, Zeist, The Netherlands

(*Received* 1 *April* 1975)

Abstract—Various possibilities were studied for administering vinyl chloride monomer (VCM) orally to rats. Upon storage, solutions of VCM in soya-bean oil appeared to be stable with respect to their VCM content and the fatty acid composition of the oil. In addition no oligomers of vinyl chloride or reaction products of VCM with components of the oil were detected. Stomach intubation of such solutions was considered an acceptable method of oral administration of VCM to rats in short-term toxicity studies. Within a period of 4 hr following intragastric intubation of VCM in soya-bean oil (300 mg VCM/kg body weight), over 92% of the VCM administered was recovered from the gases excreted (mainly exhaled?) by the animals. Eructation did not appear to be involved in the excretion of VCM.

VCM dissolved in soya-bean oil was administered by gavage to male and female rats at levels of 0 (controls), 30, 100 and 300 mg/kg body weight, once daily on 6 days/wk for a period of 13 wk. Several haematological, biochemical and organ weight values differed to a statistically significant degree from those of the controls, but these differences were considered to have only minor, if any, toxicological significance. A slight increase in liver-to-body weight ratio occurred in males and females on the highest dose level. This increase was not accompanied by liver damage, as was evident from histological examination, enzyme histochemistry and electron microscopy. The no-effect level in this 90-day study was conservatively placed at 30 mg VCM/kg body weight, but was probably higher since the effects occurring at 100 and 300 mg/kg body weight were of doubtful toxicological significance.

Indications were obtained that the feeding of rats on diets containing polyvinyl chloride (PVC) powder with a high VCM content is a more practical method for the long-term oral exposure of rats to VCM than is stomach intubation of VCM in oil. VCM was almost completely released from PVC powder during passage through the digestive tract.

INTRODUCTION

Vinyl chloride monomer (VCM) is used in the manufacture of the resin, polyvinyl chloride (PVC). Industrial exposure to VCM has been associated with several disorders, including acro-osteolysis (Dinman, Cook, Whitehouse, Magnuson & Ditcheck, 1971; Harris & Adams, 1967; Lange, Jühe, Stein & Veltman, 1974; Markowitz, McDonald, Fethiere & Kerzner, 1972) and non-malignant liver diseases (Kramer & Mutchler, 1972; Marsteller, Lelbach, Müller, Jühe, Lange, Rohner & Veltman, 1973; Suciu, Drejman & Valaskai, 1967) and, only recently, also with angiosarcoma of the liver (Block, 1974; Creech & Johnson, 1974; Lee & Harry, 1974) and tumours of the brain and lungs (Monson, Peters & Johnson, 1974). Degenerative changes in the liver, kidneys, bone and brain were detected in rats and rabbits following exposure to VCM inhalation (Basalaev, Vazin & Kochetkov, 1972; Jaeger, Reynolds, Conolly, Moslen, Szabo & Murphy, 1974; Torkelson, Oyen & Rowe, 1961; Viola, 1970). Moreover, VCM inhalation was found to induce tumours in both rats and mice (Maltoni & Lefemine, 1974; Viola, Bigotti & Caputo, 1971).

Certain formulations of PVC are used widely as food-packaging materials. Residual VCM present in the extruded polymeric product was shown to be liable to migration into PVC-packed foods and drinks, especially distilled spirits (Randolph, 1973). Since no data on the oral toxicity of VCM appeared to be available, studies were initiated to examine the subacute toxic properties of this compound when administered orally to rats. Oral administration is greatly hampered by the fact that VCM is a gas at room temperature (b.p. *c.*–13·8°C) and therefore preliminary experiments were carried out to find an acceptable way of administering the compound orally to rats.

Administration in the drinking-water was considered in this respect but was rejected because of the rather poor solubility of VCM in water (Hardie, 1964) and, more significantly, because of the difficulties to be expected in handling the solutions and in estimating the quantities of VCM actually ingested by the animals.

As VCM is lipophilic, we investigated the possibility of administering VCM as a solution in edible oil either by gastric intubation or by incorporation into the diet. Another possibility studied was the addition to the diet of a PVC powder containing an unusually high concentration of VCM.

The present report describes tentative experiments on the oral administration of VCM and presents some observations on the fate of VCM in rats. In addition, the results are given of a subacute toxicity

*This study was sponsored by a group of co-operating European industries, including Verband Kunststofferzeugende Industrie e.V. (Federal Republic of Germany), Shell Nederland Chemie, Dutch State Mines, Akzo Zout Chemie Nederland B.V. and Dow Chemical Europe S.A.

study in rats given VCM dissolved in soya-bean oil by gavage for 13 wk.

EXPERIMENTAL

Materials. VCM, obtained from Akzo Zout Chemie, Rotterdam, The Netherlands, in pressurized stainless-steel cylinders, had the following specification: M = 62·50; m.p. = −153·8°C; b.p. = −13·37°C; density = 0·9106; n_D = 1·3700. PVC powder, specially prepared to contain approximately 1800 ppm VCM, was supplied by Shell Nederland Chemie, Pernis, The Netherlands, in closed steel barrels.

Animals and diets. Male and female Wistar-derived rats from the Institute's colony were used. The Institute's stock diet and tap-water were offered *ad lib.*

Solutions of VCM in soya-bean oil. These were prepared either by bubbling VCM gas through the oil or by injecting liquid VCM into the oil. The stability of the solutions with regard to their VCM content was tested in storage experiments under various conditions either by weighing the vessels containing the solutions or by gas chromatography. For each gas-chromatographic determination, 1 g VCM-containing oil was mixed with 10 ml *N,N*-dimethylacetamide containing toluene as an internal standard. The mixture (1 μl) was injected on a glass column (length 2 m, i.d. 3 mm) filled with 10% DC 200 on Gas Chrom Q, 100–200 mesh. The temperature was 90°C.

The VCM solutions were also examined for polymerization products of VCM and for reaction products of VCM with unsaturated fatty acid moieties of the oil. The presence of oligomers of VCM was checked by injecting 1 μl of a mixture of 1 ml oil and 10 ml *p*-xylene or *N,N*-dimethylacetamide directly on to two different gas chromatographic columns and comparing the chromatograms with those obtained from pure soya-bean oil diluted and chromatographed in the same way. The column used for the oil diluted with *p*-xylene was: length 2 m, i.d. 3 mm, filled with Porapak Q, 80–100 mesh, temperature 230°C. For the oil diluted with *N,N*-dimethylacetamide the DC 200 column mentioned above was used.

The fatty acid composition of the solutions in soya-bean oil was determined according to the IUPAC (Oils and Fat Section) methods 2D19 (preparation of methyl esters) and 2D25 (gas chromatography of fatty methyl esters).

VCM in the diet. The possibility of administering VCM to rats in the diet was studied by mixing the diet with a solution of VCM in soya-bean oil, with liquid VCM (in a closed vessel) or with PVC powder containing a high level of VCM*. PVC powder was used either as obtained from Shell (containing about 1800 ppm VCM) or after raising its VCM content to approximately 4000 ppm by mixing the powder with a calculated amount of liquid VCM in a closed steel barrel. The stability of the diets with respect to their VCM content was examined by storing the diets in open glass vessels at room temperature and determining their VCM content at regular intervals by gas chromatography, in the first two cases by the method described above and in the case of the diet-incorporated PVC powder by mixing 5 g of the diet with 20 ml tetrahydrofuran, precipitating the PVC by adding 10 ml water, and injecting 1 μl of the supernatant on to a glass gas-chromatographic column (length 2 m, i.d. 3 mm) filled with Porapak Q 80–100 mesh (column temperature, 230°C, carrier 50 ml nitrogen/min, detection by FID).

Fate of VCM in oil administered orally or ip to rats. To study the fate of VCM after oral or ip administration to rats, four separate experiments were carried out.

(1) 1 ml of a 7·2% solution of VCM in soya-bean oil was given by gavage to each of two rats (body weight 240 g, dose level 300 mg/kg), which were placed immediately after treatment in a glass exposure cylinder normally used for inhalation toxicity studies. The cylinder was connected with a trapping system consisting of a drying tube, a trapping device containing diethyl ether and an air pump with a capacity of 1 litre/7·5 min. The trapping device was cooled in a Dewar flask containing a mixture of alcohol and dry ice. Every 7·5 min the trap was replaced by a fresh one and each time the amount of VCM in the ether was determined gas chromatographically.

(2) The experiment described under (1) was repeated with two other rats, in each of which the oesophagus was ligated under anaesthesia immediately after intubation of the VCM solution.

(3) Two rats were each injected ip with 1 ml of the VCM solution in oil.

(4) The VCM content was determined in blood obtained at intervals from the tip of the tail of one rat intubated intragastrically with 1 ml of a 10% VCM solution in soya-bean oil. For the VCM determination, 0·1 ml blood was extracted with 1 ml ether, and 5 μl ether extract was injected on a gas-chromatographic column (glass, length 2 m, i.d. 3 mm) filled with Porapak Q 80–100 mesh (column temperature, 230°C, carrier 50 ml nitrogen/min, detection by FID).

Subacute toxicity study of VCM administered in oil by gavage to rats. Solutions of 0, 1·0, 3·3 and 10% VCM in soya-bean oil were administered by stomach tube to groups of 15 male and 15 female weanling rats. The mean initial body weights were 44 g for both males and females. The rats in the various groups received approximate doses of VCM of 0, 30, 100 or 300 mg/kg body weight daily on 6 days/wk for a period of 13 wk. The doses were adapted to the mean body weights once every week. The animals were housed in wire-screen cages in groups of three in an air-conditioned inhalation chamber (temperature 22–24°C, relative humidity 60%).

Individual body weights were recorded weekly. The food intake of each group was measured in wk 1–4 and in wk 11 and 12. Haematological indices (blood clotting time, haemoglobin content, packed cell vol-

*Attempts were made unsuccessfully to encapsulate VCM in malto-dextrin (type Snowflake 01902, Corn Products Company, 'Sas van Gent' B.V. Nederland), by freeze- or oven-drying of emulsions of soya-bean oil containing 10% VCM in an aqueous solution of malto-dextrin and sodium caseinate (emulsifier).

ume and counts of erythrocytes and total and differential leucocytes) and biochemical blood values (sugar, urea nitrogen, total serum protein, serum albumin and activities of glutamic–oxalacetic transaminase (GOT), glutamic–pyruvic transaminase (GPT) and alkaline phosphatase) were recorded terminally in ten males and ten females of each group. Kidney function (specific gravity and activity of GOT in the urine) was examined in wk 13 in ten males and ten females of the control and top-dose group. Also in wk 13, urine examinations, including appearance, pH, glucose, protein, occult blood, ketones and microscopic constituents, were conducted upon pooled urine samples from ten males and ten females of each group.

In wk 14 all rats were killed by decapitation and examined for gross changes. The heart, kidneys, liver, spleen, brain, gonads, thymus, thyroid and adrenals were weighed. Samples of these and a range of other organs were preserved in 10% buffered formalin. Detailed microscopic examination was performed on all rats of the high-dose group and on the controls. Paraffin sections stained with haematoxylin–eosin were prepared from the weighed organs and from the lungs, salivary glands, trachea, thoracic aorta, skeletal muscle, axillary and mesenteric lymph nodes, ceruminous glands, pancreas, urinary bladder, sternum with bone marrow, prostate, epididymis, uterus, mammary glands, oesophagus, fore and glandular stomach, duodenum, ileum, caecum and colon. In rats given the other dose levels, only the liver was examined microscopically.

The following histochemical enzyme determinations were carried out* on cryostat sections of liver specimens (frozen in isopentane at −70°C) from five males and five females of each group: alkaline phosphatase (Gomori, 1939), acid phosphatase (Barka & Anderson, 1963; Gomori, 1939) and adenosine monophosphatase, adenosine triphosphatase and glucose-6-phosphatase (Wachstein & Meisel, 1957).

For electron microscopy, small pieces of liver (1 mm^3) from one male and one female control rat and from two males and two females from each test group were fixed in 1% osmium tetroxide buffered with 0·1 M-sodium cacodylate (pH 7·4) for 2 hr at 4°C. The cubes were dehydrated in alcohol and embedded in Epon resin. Sections (1 μm) were cut and stained with toluidine blue for light microscopy. Selected areas were cut on an LKB ultramicrotome and ultrathin sections were stained with uranyl acetate and lead citrate and examined in a AEI EM6B electron microscope†.

RESULTS

Stability of VCM solutions in soya-bean oil

When solutions of VCM in soya-bean oil were stored in open vials at room temperature, their VCM content diminished at a fairly low rate. For example, a 5·6% VCM solution had lost only 10% of its VCM after 1 hr, 16% after 4 hr and 29% after 8 hr. A 0·5% solution appeared to lose 25% of its VCM after a storage period of 24 hr, 50% after 4 days and 80% after 8 days.

Table 1. *Fatty acid composition of pure and VCM-containing soya-bean oils after storage at 37 C for a period of 8 days*

Fatty acid	Fatty acid content (%) of	
	Pure oil	Oil containing 10% VCM
Myristic	0·2	0·2
Palmitic	10·9	11·0
Stearic	3·6	3·8
Oleic	22·8	22·7
Linoleic	55·8	55·8
Linolenic	6·7	6·6

Comparison of the chromatograms of a 10% VCM solution in soya-bean oil stored in a closed vial for a period of 8 days at 37°C with those of pure soya-bean oil did not reveal any evidence of the formation of vinyl chloride oligomers or other reaction products of VCM with oil compounds during the storage of the VCM solutions. Neither was the fatty acid composition of soya-bean oil significantly altered by VCM present in the oil for a prolonged period of time (Table 1).

VCM in the diet

A large part of the VCM evaporated during the mixing of a VCM solution in soya-bean oil with the stock diet (Fig. 1) and during storage of this diet in

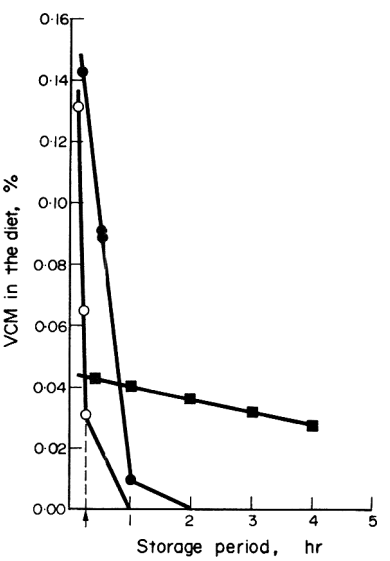

Fig. 1. Stability of VCM content of stock diet during storage at room temperature in open vessels, following mixing of the diet with soya-bean oil containing 3% VCM (9:1, w/w) in an open vessel (○), with soya-bean oil and liquid VCM (9:1:0·05, by weight) in a closed steel barrel (●) or with PVC containing 0·44% VCM (9:1, w/w) (■); ↑ indicates end of mixing period. The VCM determinations were carried out by gas-chromatography, using the method described on p. 634.

*By Dr. R. Kroes of the Laboratory of Pathology of the National Institute of Public Health, Bilthoven, The Netherlands.

†The ultrastructural studies on the liver were carried out by Mr. R. Hendy and Mr. M. Wright of the British Industrial Biological Research Association, Carshalton, England.

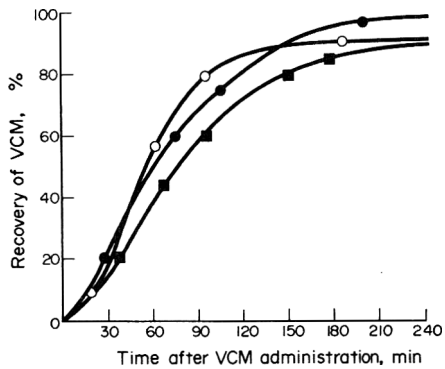

Fig. 2. Recovery of VCM from the atmosphere surrounding rats following administration of a dose of 300 mg VCM/kg body weight (as a 7·2% VCM solution in soya-bean oil) either by stomach tube to rats with an open (○) or ligated (■) oesophagus or by ip injection (●). A 1-μl aliquot of the ether used for trapping the VCM in the atmosphere was injected on to a glass gas-chromatographic column (length 2 m, i.d. 3 mm) filled with Porapak Q 80–100 mesh (column temperature 230°C, carrier 50 ml nitrogen/min, detection by FID).

an open vial the VCM content decreased to an undetectable level in a period of 1 hr. The rate of decrease of the VCM content of the diet was slightly lower following mixing of the diet with liquid VCM in a closed vessel (Fig. 1).

When PVC powder was stored at room temperature in an open vial, its VCM content decreased relatively slowly. For example, an initial VCM content of 1850 ppm decreased to 750 ppm after a storage period of 6 hr and to 85 ppm after 70 hr of storage. PVC powder spread in a very thin layer on plates in a drying oven at 50°C lost its VCM completely (VCM content less than 0·3 ppm) within a period of 2–4 hr but when larger amounts of PVC (e.g. 50 kg) were to be freed from VCM, it appeared necessary to keep the powder (in layers of 4–6 cm thick) in a vacuum oven at 60°C for a period of at least 60 hr.

A 9:1 mixture of stock diet and PVC powder containing a high level of VCM (e.g. 4400 ppm) lost VCM at a fairly slow rate when stored at room temperature in animal feeders. Such a diet still contained approximately 60% of the initial amount of VCM after a storage period of 4 hr (Fig. 1). The stability of the diet as to its VCM content was slightly improved (by roughly 10%) when the PVC powder was mixed with a 1% aqueous solution of guar gum or pregelatinized starch before addition to the diet.

When rats were fed a diet containing 10% PVC powder with a VCM content of 1850 ppm for a period of 2 days (receiving a fresh portion of the diet at 17·00 hr on days 1 and 2 and being killed the next morning at 09·00 hr), the VCM level of the contents of the colon and rectum was found to be virtually nil, indicating that VCM was released almost completely from the PVC powder during its passage through the digestive tract.

Fate of VCM given in oil by gavage or ip injection

Within 4 hr of the oral or ip administration of VCM at a dose level of 300 mg/kg body weight, over 92% of the administered VCM was recovered from the atmosphere surrounding the animals (Fig. 2). The VCM recovery curves were not significantly influenced by the way in which the compound was administered, which suggests *inter alia* that belching of VCM did not play a significant role in the excretion of the compound by rats. VCM was detectable in the tail blood only 2 min after intragastric intubation of a VCM solution in soya-bean oil. The VCM content of the blood reached its maximum (1·9 μg/ml) about 10 min after dosing and decreased to an undetectable level in the next 40 min.

Table 2. *Mean results of administering 0–300 mg VCM/kg body weight/day to rats by gavage on 6 days/wk for 13 wk, showing differences of possible toxicological significance between test and control animals*

Parameter affected	Values for males given VCM in doses (mg/kg) of				Values for females given VCM in doses (mg/kg) of			
	0	30	100	300	0	30	100	300
Total leucocytes (10^3/mm^3)	18·5	18·8	17·0	16·5	17·3	17·6	14·6*	14·8*
Blood sugar (mg/100 ml)	88	84	78**	77**	89	88	74***	79**
Serum: GOT (R-Fu)	228	217	224	201*	227	212	212	200
GPT (R-Fu)	53	49	48	45*	42	38	38	39
Urinary GOT (R-Fu)	30	NE	NE	22*	16	NE	NE	18
Liver weight (g/100 g body weight)	3·51	3·60	3·74	3·76*	3·32	3·34	3·51	3·71***
Adrenal weight (g/100 g body weight)	0·0158	0·0147	0·0143	0·0133*	0·0247	0·0241	0·0239	0·0244
Foci of hyperbasophilic hepatocytes†	0	0	1	1	0	0	1	1
RER in liver cells	Normal	NE	NE	Ht	Normal	NE	NE	Ht

GOT = Glutamic-oxalacetic transaminase GPT = Glutamic-pyruvic transaminase R-Fu = Reitman-Frankel units
NE = Not examined RER = Rough endoplasmic reticulum Ht = Hypertrophic

†No. of rats affected/group.

Values are the means of at least ten rats, except for the electron-microscope observations on the liver which were made on one control and two test animals. Those marked with asterisks differ significantly (Wilcoxon's test) from those of the controls: *$P < 0·05$; **$P < 0·01$; ***$P < 0·001$.

Toxicity of VCM administered in oil by stomach tube to rats

The gavage administration of VCM solutions in soya-bean oil at levels up to 300 mg/kg body weight did not cause any noticeable changes in appearance or behaviour, body-weight gain or food intake. The total number of white blood cells and the sugar content of the blood were slightly decreased by the intermediate and high dose levels (Table 2). The activities of serum GOT and GPT and of urinary GOT were decreased in males given the top dose (Table 2). There were no other significant changes in the haematological or biochemical indices and no treatment-related alterations were observed in the microscopic constituents of the urine.

The relative weight of the liver in males and females showed a tendency to increase with increasing doses of VCM but the difference from the controls was statistically significant only at the highest dose level (Table 2). In addition a dose-related decrease in the relative weight of the adrenals occurred in males, the difference from the controls being statistically significant again only in the high-dose group. The other organ weights recorded were closely comparable in all groups.

Minimal histological changes in the liver, seen as one or a few foci of 40–70 hyperbasophilic hepatocytes, occurred in some rats of the intermediate and high-dose groups (Table 2). The hyperbasophilic foci were not accompanied by architectural liver changes. Randomly distributed histopathological changes unrelated to treatment included small accumulations of reticulo-endothelial cells in the liver, focal tubular nephrosis, a slight degree of nephrocalcinosis, parasites (*Trichosomoides crassicauda*) in the renal pelvis and urinary bladder, early signs of chronic respiratory disease in the lungs, granulomatous pneumonic foci, medial hypertrophy of medium-sized pulmonary arteries, 'morphological' activation of the thyroid gland, focal myocarditis, transformation of pancreatic acini into duct-like structures, 'squamous' metaplasia of the epithelium lining excretory ducts of the sublingual salivary gland and slight prostatitis.

The histochemical studies of liver enzymes failed to reveal any difference between test and control animals either in the concentrations or distribution patterns of the various enzymes examined.

Electron microscopic examination of the liver showed hypertrophy of the endoplasmic reticulum in hepatocytes of animals given the highest dose. No difference from controls was observed with respect to other liver-cell constituents, and the capillary endothelial cells and the Kupffer cells were ultrastructurally indistinguishable from those of the controls.

DISCUSSION

Since solutions of VCM in soya-bean oil appeared to be stable when stored in closed vials for a prolonged period of time, they were considered suitable for the oral administration of VCM in toxicity studies in rats. However, the feeding of such VCM solutions in the diet was regarded as inadequate because the diet was found to lose its VCM in a very short time. Stomach intubation turned out to be an acceptable alternative, especially as there was no experimental evidence that eructation was involved in the excretion of VCM by the rats. Moreover, since the level of VCM in the blood reached its maximum within only 10 min of gastric intubation and by far the greater part of the administered VCM was recovered from the gases excreted (mainly exhaled?) within 4 hr, it was apparent that VCM given by gavage was readily absorbed from the gastro-intestinal tract.

From the toxicity study in which VCM was administered by stomach intubation, it appeared that several haematological and biochemical indices (white blood-cell counts, blood-sugar content and activities of serum GOT and GPT and urinary GOT) were lower to a statistically significant degree in the groups given the intermediate and high doses, or just in the group given the highest dose, than in the controls. In all cases, however, the values in the test groups were within the normal range and, moreover, the values obtained for the control animals were invariably very close to the upper limit of normality. Therefore, these differences between test and control animals were considered of minor, if any, toxicological significance.

Exposures to atmospheres containing VCM have been reported to result in malignant and non-malignant liver changes in several animal species and in man (Block, 1974; Falk, Creech, Heath, Johnson & Key, 1974; Kramer & Mutchler, 1972; Lange *et al.* 1974; Makk, Creech, Whelan & Johnson, 1974; Maltoni & Lefemine, 1974; Marsteller *et al.* 1973; Torkelson *et al.* 1961; Viola *et al.* 1971). In the present experiment on the oral toxicity of VCM, a significant increase in liver-to-body weight ratio found at the highest dose level in both males and females may also have been indicative of a toxic effect of VCM on the liver. This suggestion was not clearly supported, however, by the histological liver changes observed (foci of hyperbasophilic hepatocytes), which were found only in a few rats on the two higher dose levels and which were very slight in degree and not necessarily related to treatment. In addition, the histochemical enzyme studies failed to produce evidence of any effect of VCM on the liver, while the hypertrophy of the rough endoplasmic reticulum found in hepatocytes of top-dose animals without other ultrastructural changes was probably a non-specific reaction not necessarily indicative of a toxic response. The absence of liver damage in the present study may be related to the relatively short duration of the experiment and the short daily exposure of the rats to VCM.

At the highest dose level, a statistically significant depression of the relative weights of the adrenals compared with the controls was seen in males. This decrease was due, at least in part, to the relatively heavy adrenals of the control animals (the adrenal-to-body weight ratio for rats of the strain and age used being on average 0·0138 g/100 g); it did not occur at all in females and was not accompanied by histological changes in the adrenals. The decrease, therefore, was not considered to be an effect of toxicological importance.

Though special attention was paid to the microscopic appearance of the ceruminous glands (Zymbal's glands), which are known to be target organs

of VCM administered by inhalation (Maltoni & Lefemine, 1974), no histopathological changes could be detected at this site.

From this oral toxicity study, the no-toxic-effect level may be placed at 30 mg VCM/kg body weight, but it may be higher, as the effects noticed at the 100- and 300-mg/kg dosage levels were considered of doubtful toxicological significance.

Although daily administration of VCM in oil by gavage seems to be acceptable also for long-term studies, the method has several disadvantages. These include technical problems inherent in the daily intubation of a large number of rats for a prolonged period, the daily exposure of the test animals to a very large amount of VCM for a relatively short time and the difficulty of interpreting the toxicological significance of possible gastric lesions. Observations described in the present report indicate that the feeding of rats on diets containing PVC powder with a high VCM content is a more practical way of administering the test compound orally than is stomach intubation and is, moreover, unencumbered by the above problems. It may be argued that even when rats are provided daily with a fresh portion of feed, the loss of VCM from the diet is considerable (about 80%) over a 24-hr period. This difficulty can be obviated, however, by training the rats to eat their daily portion of diet within a period of 2–4 hr, a procedure which is still a much more realistic way of administering VCM than is stomach intubation. In addition, shortening the feeding period has the advantage of reducing automatically the period during which rats are unavoidably exposed to VCM evaporating from the test diets.

Starting with PVC powder containing 4000 ppm VCM, a maximal level of PVC in the diet of 10% and a (pessimistically) estimated average 50% loss of VCM from the diet before ingestion, VCM can be administered in this way at a maximum dietary level of 200 ppm, which is equivalent to 10 mg VCM/kg body weight/day. This level is very high when compared with the likely oral daily intake by man in Europe, estimated to be less than 0·0017 mg/kg body weight (van Esch & van Logten, 1975), but is relatively low compared with the 300 mg VCM/kg body weight (given daily to rats by gavage), which merely induced effects of questionable toxicological importance. A 2-yr chronic toxicity/carcinogenicity study in rats receiving VCM in either soya-bean oil by gavage (one very high level) or in PVC powder incorporated into the diet (three much lower levels) is currently in progress.

Acknowledgement—The authors thank Dr. H. P. Til for carrying out the haematological and biochemical studies, Mr. M. Th. Spanjers for supervising the 90-day toxicity study and Drs. J. A. Wijsman for performing the fatty-acid analyses.

REFERENCES

Barka, T. & Anderson, P. J. (1963). *Histochemistry. Theory, Practice, and Bibliography*. p. 239. Hoeber Medical Division, Harper & Row, Publishers, Inc., New York.

Basalaev, A. V., Vazin, A. N. & Kochetkov, A. G. (1972). On the pathogenesis of changes developing due to a long-term exposure to the effect of vinyl chloride. *Gig. Truda prof. Zabol.* **16**, 24.

Block, J. B. (1974). Angiosarcoma of the liver following vinyl chloride exposure. *J. Am. med. Ass.* **229**, 53.

Creech, J. L., Jr. & Johnson, M. N. (1974). Angiosarcoma of liver in the manufacture of polyvinyl chloride. *J. occup. Med.* **16**, 150.

Dinman, B. D., Cook, W. A., Whitehouse, W. M., Magnuson, H. J. & Ditcheck, T. (1971). Occupational acroosteolysis. I. An epidemiological study. *Archs envir. Hlth* **22**, 61.

Falk, H., Creech, J. L., Jr., Heath, C. W., Jr., Johnson, M. N. & Key, M. M. (1974). Hepatic disease among workers at a vinyl chloride polymerization plant. *J. Am. med. Ass.* **230**, 59.

Gomori, G. (1939). Microtechnical demonstration of phosphatase in tissue sections. *Proc. Soc. exp. Biol. Med.* **42**, 23.

Hardie, D. W. F. (1964). Chlorocarbons and chlorohydrocarbons. Vinyl chloride. In *Encyclopedia of Chemical Technology*. 2nd Ed., Vol. 5. Edited by R. E. Kirk and D. F. Othmer. p. 171. John Wiley & Sons, New York.

Harris, D. K. & Adams, W. G. F. (1967). Acro-osteolysis occurring in men engaged in the polymerization of vinyl chloride. *Br. med. J.* **3**, 712.

Jaeger, R. J., Reynolds, E. S. Conolly, R. B., Moslen, Mary T., Szabo, S. & Murphy, S. D. (1974). Acute hepatic injury by vinyl chloride in rats pretreated with phenobarbital. *Nature. Lond.* **252**, 724.

Kramer, C. G. & Mutchler, J. E. (1972). The correlation of clinical and environmental measurements for workers exposed to vinyl chloride. *Am. ind. Hyg. Ass. J.* **33**, 19.

Lange, C. E., Jühe, S., Stein, G. u. Veltman, G. (1974). Die sogenannte Vinylchloride-Krankheit–eine berufsbedingte Systemsklerose? *Int. Arch. Arbeitsmed.* **32**, 1.

Lee, F. I. & Harry, D. S. (1974). Angiosarcoma of the liver in a vinyl-chloride worker. *Lancet* **I**, 1316.

Makk, L., Creech, J. L., Whelan, J. G., Jr. & Johnson, M. N. (1974). Liver damage and angiosarcoma in vinyl chloride workers. A systematic detection program. *J. Am. med. Ass.* **230**, 64.

Maltoni, C. & Lefemine, G. (1974). Carcinogenicity bioassays of vinyl chloride. I. Research plan and early results. *Envir. Res.* **7**, 387.

Markowitz, S. S., McDonald, C. J., Fethiere, W. & Kerzner, M. S. (1972). Occupational acroosteolysis. *Archs Derm.* **106**, 219.

Marsteller, H. J., Lelbach, W. K., Müller, R., Jühe, S., Lange, C. E., Rohner, H. G. u. Veltman, G. (1973). Chronisch-toxische Leberschäden bei Arbeitern in der PVC-Produktion. *Dt. med. Wschr.* **98**, 2311.

Monson, R. R., Peters, J. M. & Johnson, M. N. (1974). Proportional mortality among vinyl-chloride workers. *Lancet* **II**, 397.

Randolph, W. F. (1973). Prior-sanctioned polyvinyl chloride resin. *Federal Register* **38**, 12934.

Suciu, I., Drejman, I. & Valaskai, M. (1967). Étude des maladies dues au chlorure de vinyle. *Medna Lav.* **58**, 261.

Torkelson, T. R., Oyen, F. & Rowe, V. K. (1961). The toxicity of vinyl chloride as determined by repeated exposure of laboratory animals. *Am. ind. Hyg. Ass. J.* **22**, 354.

van Esch, G. J. & van Logten, M. J. (1975). Vinyl chloride: A report of a European assessment. *Toxicology* **4**, 1.

Viola, P. L. (1970). Pathology of vinyl chloride. *Medna Lav.* **61**, 174.

Viola, P. L., Bigotti, A. & Caputo, A. (1971). Oncogenic response of rat skin, lungs, and bones to vinyl chloride. *Cancer Res.* **31**, 516.

Wachstein, M. & Meisel, E. (1957). Histochemistry of hepatic phosphatases at a physiologic pH. *Am. J. clin. Path.* **27**, 13.

Fd Cosmet. Toxicol. Vol. 13, pp. 639–647. Pergamon Press 1975. Printed in Great Britain

BIOLOGICAL TESTING OF FOOD GROWN IN THE TRANSKEI

I. F. H. PURCHASE*, R. C. TUSTIN and S. J. VAN RENSBURG

National Research Institute for Nutritional Diseases, SA Medical Research Council, PO Box 70, Tygerlei, Cape Province, South Africa

(*Received 11 April 1975*)

Abstract—Oesophageal cancer is the commonest form of cancer occurring in the Transkei, South Africa. In an attempt to identify carcinogens contributing to this high incidence, food collected from gardens belonging to families in which a member had developed oesophageal cancer was fed to BD IX rats from conception until death. One group was fed a diet consisting of maize, beans and a salt mixture while the second group received a full Transkei diet consisting of the same ingredients with the addition of *imifino* (*Solanum nigrum* and *Sonchus oleraceus*). The latter group developed severe liver lesions (characterized by bile-duct proliferation and hyperplastic nodules) and epithelial-cell dysplasia of the oesophagus. This group also suffered from an increased incidence of tumours of various types. It proved impossible to breed successfully from rats on the full Transkei diet. The liver lesions were exacerbated by supplying distillate, collected when the diets were dried, to the rats as drinking-water. The effect of the distillate was probably not due to the presence of nitrosamines. The possibility that the full Transkei diet was producing these effects indirectly by the interaction of nitrites and secondary amines in the stomach was tested by the addition of sodium nitrite to the diets but this did not induce liver lesions. These results suggest the diet collected from the Transkei contains toxic factors which produce liver and oesophageal lesions and an increased incidence of cancer in rats. These same factors may also contribute to the high incidence of oesophageal and liver cancer occurring in man in the Transkei.

INTRODUCTION

Before 1950 oesophageal cancer was considered to be a very rare disease among the Bantu of South Africa (Oettlé, 1964), but since then reports have indicated an increase in the incidence of the disease (Burrell, 1957; Higginson, 1951). This increase, although occurring over much of Southern Africa, was particularly evident in the eastern Cape Province (Burrell, 1957), which includes the Transkei where there was a fivefold increase in hospital cases over a 15-yr period (Rose, 1965). As a result, oesophageal cancer is now the commonest neoplasm in the Transkei and elsewhere in Southern Africa (Oettlé, 1964).

Further evidence for an increase in oesophageal cancer and for the present high incidence is provided by the records of mine hospitals, which serve a population comprising mainly short-term migrant labourers originating from many different areas of Southern Africa. There was an increase in the number of cases observed between 1937 and 1963 (Oettlé, 1964) and 13% of all cancers reported during the period from 1964–1968 were oesophageal cancer (Robertson, Harington & Bradshaw, 1971). A further noteworthy finding from the latter survey is that the crude incidence rate of oesophageal cancer (16·2/100,000/yr) was much higher in the Bantu miners from the Transkei than from other areas in Southern Africa and that liver cancer, with a crude incidence rate of 11·6/100,000/yr, was also relatively common in the Transkei Bantu.

A feature of great significance concerning oesophageal cancer in Southern Africa is its distribution, which is characterized by variations in incidence differing by a factor of as much as 200 (Oettlé, 1964). Variations may also occur between different localities within individual areas of high incidence, as was observed in the Transkei (Burrell, 1969; Rose, 1965). Recent figures show that the age-standardized rate for males in the Transkei varies from 10/100,000/yr to over 130/100,000/yr (Rose, 1965 & 1973).

Burrell (1957) noticed this marked variation in the incidence and distribution of the disease in the Transkei at an early stage in his investigations and arranged for a geological survey of this part of South Africa to be undertaken (Marais & Drewes, 1962). This survey revealed that the incidence was greater in people living in areas where the soil was derived from Beaufort sediments than where it was derived from dolomite. This was particularly evident in the district of Butterworth, which has the highest incidence of oesophageal cancer in the Transkei; here only 18% of cases reported in the survey occurred in people living on dolomitic soils which cover 45% of the surface area of the district.

In a study of the gardens in this district, it was found that those belonging to families in which one or more members had suffered or were suffering from oesophageal cancer were less productive than were the gardens of tumour-free families (Burrell, Roach & Shadwell, 1966). It was also noticed that many of the plants grown in the gardens of cancer sufferers showed signs of mineral deficiencies, which were shown by means of diagnostic leaf injections to be due to deficiencies of molybdenum, iron, copper and

*Present address: Central Toxicology Laboratory, Imperial Chemical Industries Limited, Alderley Park, Nr Macclesfield, Cheshire, SK10 4TJ, England

zinc. Because of the clearly marked distinction between these two types of garden, the deficient gardens were termed 'cancer gardens'. A deficiency of trace elements, particularly of molybdenum, can influence the metabolism of plants and it was suggested that the high nitrate content of molybdenum-deficient plants and the interference with reduction of nitrite, which is dependent on trace elements, could lead to the production of nitrosamines in the plant (Burrell *et al.* 1966). This observation was followed by an assay of crops and plants from this area for nitrosamine content. One of the first plants assayed was the fruit of *Solanum incanum*, which is used by the local population to curdle milk, and this was found to contain small amounts of dimethylnitrosamine (du Plessis, Nunn & Roach, 1969).

Apart from these studies there have been few direct attempts to identify the aetiological agents that may contribute to the high incidence of the disease. One recent epidemiological approach suggested that fermented beverages may be implicated (Cook, 1971). One disadvantage of this type of approach is, of course, that it can only show whether there is or is not an association between the cancer incidence and the occurrence of a particular agent. No direct proof can be forthcoming. The problem may be further complicated by the wide variation in social and dietary habits between different ethnic groups in different countries and by the fact that a cancer may be caused by more than one agent.

In this paper we describe, as an alternative approach, a more direct method to determine whether any carcinogen(s) were being ingested during a certain period of time by the exposed population in a high-incidence area for oesophageal cancer in the Transkei, which has a population of 1·7 million and an area of 16,440 square miles. A preliminary report of part of this work has already been presented (Purchase & Joubert, 1971). Before this work was undertaken the following points were considered:

(a) Restriction of the search for the aetiological agent to a small geographical area would increase the possibility of determining that a single carcinogen or combination of carcinogens was operating (Purchase, 1968).

(b) Available evidence suggested that the occurrence of oesophageal cancer was related to the soil ('cancer gardens') and that the edible plants growing in this soil could be the intermediate vehicle by which the carcinogenic influence was transmitted to the population (Burrell *et al.* 1966).

(c) As the nature of the carcinogen was unknown, a test relatively non-specific in terms of chemical properties was required. A biological test complied with this requirement and the BD IX strain of rat, which is susceptible to experimentally induced oesophageal carcinogenesis but does not under normal conditions develop this form of cancer (H. Druckrey, personal communication 1967), was considered to be an ideal test animal.

(d) It was acknowledged that extrapolation of results from animal tests to the human situation is difficult.

(e) It was recognized that the induction period of most forms of human neoplasia probably spans many years, that the habits of people change and that the specific carcinogen(s) may no longer have been present in the food of the exposed population at the time of our experiment. However, the local habit of a fairly stable population living in a small locality and eating predominantly traditional, home-grown food increased the chances that the carcinogen was still present in that restricted environment.

EXPERIMENTAL

Animals and maintenance. Black rats of the BD IX strain (Druckrey, 1971), which had been inbred for over 100 brother–sister generations, were obtained from Professor H. Druckrey, Freiburg, West Germany. They were housed two to a cage for the first 6 months of the experiment (until May 1968) and thereafter singly in an air-conditioned room maintained at 24°C, with a minimum relative humidity of 50%, illuminated with fluorescent lights for 12 hr/day and situated at the Frere Hospital, East London, approximately 80 miles from the Kentani district near Butterworth in the Transkei, where the food was collected.

Food samples. As far as possible, food was collected from 'cancer gardens'. Attempts to buy food directly from the families cultivating these gardens were unsuccessful, mainly because they were loth to part with any personal belonging which might become the means by which an evil spell could be cast on them. It is the custom in the area to take maize to local millers for milling into meal. With the co-operation of one of the millers it was thus possible to collect food originating from 'cancer gardens'. Maize and beans collected in this way were stored in metal storage bins, insect infestation being controlled by the locally common technique of placing a few ml of carbon disulphide in an open container on top of the food.

During the 1968/69 growing season, crops were poor because of low and irregular rainfall, which occurs predominantly during the warmer months of the year (October–April). Difficulty was encountered in the collection of sufficient food from 'cancer gardens' and some was therefore obtained also from neighbours of the families mentioned above. All samples were obtained, however, in the Kentani district, which has one of the highest incidence rates of oesophageal cancer in the Transkei, the rates standardized for world population and age being 94/100,000/yr for males and 48/100,000/yr for females (E.F. Rose, personal communication 1972).

Maize, the staple diet in the area, and locally produced beans formed the diet of most of the rats in the experiment. The maize was a mixture of yellow and white varieties and the beans were brown Haricot beans. In addition, various green vegetables or *imifino* were collected and fed to some of the rats. This *imifino* is used by the local population to augment the diet and comprises numerous species of plants, many of them growing in the wild state. In the experiments, however, two plants, *umsobo* (*Solanum nigrum*) and *irwabe* (*Sonchus oleraceus*), were used exclusively.

These were collected fresh when they were available to the local population (from September to May) and were stored at 4°C until used, maximum storage being 2 days. Samples of each batch of maize used were analysed for aflatoxin and sterigmatocystin content.

Diet preparation. The animals were fed one of the following three basic diets, with numbers in parentheses indicating parts by weight:

Diet A (Transkei maize and beans diet) consisted of maize (64), beans (32) and salt mixture* (4). The maize and beans were mixed in the correct proportion and milled in a hammer mill. The resultant meal was cooked with water as a stiff porridge in an iron pot for 20 min by a method typical of that used in the Transkei. As the porridge was inconvenient for feeding to the rats, it was dried in a forced draught oven at ±50°C and remilled to a fine meal before use. The salt mixture was added after remilling.

Diet B (full Transkei diet) consisted of maize, beans and salt mixture as in A (85) and a 1:1 mixture of *imifino* and maize (15). Leaves, and fruit when present, were picked from the fresh *imifino* and mixed with an equal weight of maize meal. The whole mixture was cooked in water for 20 min and dried and milled as described above.

Diet C (supplemented full Transkei diet) was a mixture of diet B (74), milk powder (25) and vitamin mixture (1). The vitamin mixture contained Vitamin K (5·0 mg), thiamine hydrochloride (11·2 mg), riboflavine (25·0 mg), Ca pantothenate (60·0 mg), vitamin B_{12} (0·15 mg), biotin (2·0 mg), pyridoxine HCl (12·1 mg), inositol (5·0 g), choline chloride (5·0 g), ascorbic acid (0·5 g), α-tocopherol (250 μg), vitamin A acetate (15,000 IU), vitamin D_3 (5000 IU) and dextrin to 100 g.

In one experiment (no. 2) water evaporated and collected during the drying of the food in the forced-draught oven was given to the rats as drinking-water. This distillate was collected through a double-coil water-cooled condenser to which a suction pump was attached to increase recovery. It was estimated that 70–75% of the water was actually recovered. The water collected from the preparation of all diets was pooled and stored at 4°C until used.

Animal experiments

The general plan of these experiments is summarized in Table 1.

Experiment 1. This was planned as a multi-generation study. Eight male and 14 female rats originating from Germany were given diet B for 8 days before mating. Between matings the males were kept on the standard rat ration fed in our laboratories. The females received diet C from day 15 of gestation until the offspring were weaned when 3–4 wk old. From weaning, 21 males and 19 females from the first mating were fed diet B (group 1), while those derived from the second and third matings (24 males and 16 females) were placed on diet A (group 2). Feeding of these diets was continued until the rats died. A third group (1A) consisted of 16 males and 20 females bred from locally produced BD IX rats, which had received diet C throughout gestation. They were given the same diet as their parents for 307 days during which attempts were made to breed from them, and subsequently were placed on diet B until day 781, when the 16 survivors were killed.

Experiment 2. This was started approximately 1 yr after the commencement of experiment 1. The young of locally bred BD IX parents, which had received diet C during gestation, were divided into two groups. One of these (group 1B), comprising 11 males and eight females, received diet B plus the distillate as drinking-water, while group 2B, comprising nine males and ten females, received diet A plus the distillate as drinking-water. One sample of distillate was taken for nitrosamine analysis by gas chromatography and polarography. Surviving rats (one in group 1B and two in group 2B) were killed on day 829. The animals in groups 1 and 2 (experiment 1) served as controls for this experiment.

Experiment 3. Weanling male BD IX rats, bred in our laboratory in Pretoria and flown to the laboratory in East London, were divided into four groups of ten rats. These were fed either a diet consisting of 85% (by weight) standard rat ration and 15% *imifino*/maize mixture prepared as described above (group 3), standard rat ration containing 0·5% sodium nitrite (group 4), diet B containing 0·5% sodium nitrite (group 5) or diet A containing 0·5% sodium nitrite (group 6).

Pathological examinations

Due to unforeseen circumstances, all surviving rats had to be killed on 28 June 1971. The animals killed, and those that died during the experiments were autopsied and the oesophagus, stomach, liver, spleen, kidney, pancreas, heart, lung and any other macroscopically abnormal organ or tissue were placed in 10% buffered formalin. Sections 5 μm thick were cut for histological examination after appropriate preparation and were stained with haematoxylin and eosin and, where necessary, with Masson's trichrome, Gomori's reticulin, periodic acid–Schiff and other special stains.

RESULTS

Experiment 1

The patterns of mortality in the animals in groups 1, 1A and 2 are given in Fig. 1. The rats in group 1, receiving diet B (the full Transkei diet), survived for a shorter time (mean 610 days) than did those on diet A, the Transkei maize and beans diet (group 2, mean 691 days). During the late spring and winter of 1968 (from about day 200 to day 350) the rats in groups 1 and 2 were unthrifty, with dull coats and scabby skin lesions. The following year during the same season these clinical signs were more advanced and, in addition, alopecia, haemorrhages into the eye, xerophthalmia and even blindness occurred in some of them. The rats in group 1 were more severely affected than those in group 2. The basic hepatic

*The salt mixture contained (in g) $CaCO_3$ (610), K_2HPO_4 (696), $CaHPO_4$ (688), $Na_2HPO_4.12H_2O$ (358); $MgSO_4.7H_2O$ (246), NaCl (234), Ca lactate (154), Fe citrate (59·8), KI (1·6), $MnSO_4.2H_2O$ (0·615), $CuSO_4.5H_2O$ (0·563) and $ZnCl_2$ (0·523).

Table 1. *Experimental protocols and survival data for experiments 1, 2 and 3*

Experiment no.	Rat strain	Maternal diet (gestation to weaning)	Group no.	No. of rats/group M	No. of rats/group F	Diet	Drinking-water	Duration of feeding* (days)	Survival time† (days) Time of First death	Time of Last death	Mean
1	ex Germany	C‡	1‡	21	19	B	Standard	980	135	980	610
			2‡	24	16	A	Standard	1140	100	1140	687
	BD IX (local)	C	1A	16	20	C/B§	Standard	307/474§	171	781‖ (16)	—
2	BD IX (local)	C	1B	11	8	B	Distillate	829	213	829‖ (1)	331
			2B	9	10	A	Distillate	829	436	829‖ (2)	694
3	BD IX (Pretoria)	R	3	10	0	R + *imifino*	Standard	474	—	474‖ (10)	—
			4	10	0	R + $NaNO_2$	Standard	474	469	474‖ (9)	—
			5	10	0	B + $NaNO_2$	Standard	474	137	474‖ (1)	180¶
			6	10	0	A + $NaNO_2$	Standard	378	130	378	214

A = Transkei maize and beans diet B = Full Transkei diet C = Supplemented full Transkei diet R = Standard rat ration

*Time from birth to last death or the killing of survivors (i.e. duration of test feeding plus 21 days).

†The detailed mortality patterns for groups 1, 2 and 1A and for groups 1B and 2B are shown in Figs 1 and 2, respectively.

‡Before each mating, males and females were fed diet B for 8 days, males being given the standard ration at other times; rats in group no. 1 were derived from the first mating and those in group no. 2 from the second and third matings.

§Diets C and B fed in succession for 307 and 474 days, respectively.

‖Day on which survivors (no. in parenthesis) were killed.

¶The one survivor killed on day 474 was excluded from this calculation.

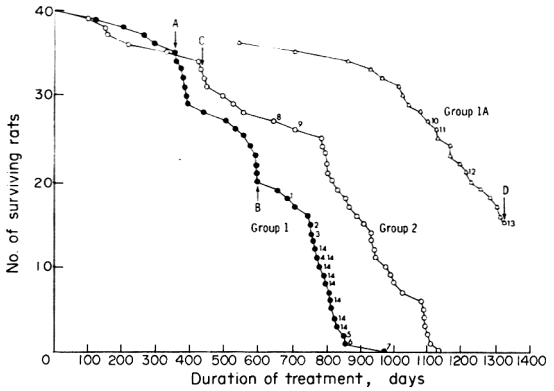

Fig. 1. Time of death of individual rats in group 1 (diet B), group 2 (diet A) and group 1A (diet C for 307 days). Arrows indicate (A) first sign of pigment deposition in groups 1 and 2. (B) first sign of marked bile-duct proliferation in group 1. (C) first sign of bile-duct adenoma in group 2 and (D) killing of survivors. The rats marked with figures had the following tumours: (1) anaplastic sarcoma, abdomen; (2) haemangioendothelioma, abdomen; (3) fibrosarcoma, bladder; (4) bronchial carcinoma; (5) adenosarcoma, subcutaneous; (6) fibrosarcoma, rectum; (7) anaplastic sarcoma, subcutaneous; (8) reticulum-cell sarcoma, stomach; (9) adenoma, adrenal; (10) plasmacytoma, liver and kidney; (11) fibrosarcoma, subcutaneous; (12) adenoma, subcutaneous; (13) adenoma, adrenal in one rat; (14) hyperplastic nodules in the liver.

lesions observed in individuals in groups 1 and 2 also differed.

A meaningful average survival time for the animals in group 1A could not be calculated because those surviving to day 781 had to be killed. Until then, the mortality pattern in these rats, which received diet C (full Transkei diet supplemented with milk powder and vitamins) for 307 days followed by diet B for the remainder of the period, was similar to that of the other two groups in this experiment (Fig. 1), although the feeding did not run concurrently.

The mean body weights of group 1 rats were lower than those of group 2 up to wk 15, slightly higher from wk 20 to wk 80 and slightly lower thereafter.

In none of the food samples analysed was aflatoxin or sterigmatocystin found.

Group 1 pathology. Marked macroscopic evidence of liver damage was noted for the first time in the rat that died on day 751, and the livers of most animals dying later showed similar changes. Macroscopically the livers were yellow, with roughened surfaces. These changes were associated with ascites. Histologically, however, lesions were apparent in the livers of rats dying at an earlier stage and the liver changes in all the animals in this group were classified into three categories according to their severity, which could, in most cases, be correlated with the length of treatment.

The first four rats that died had few significant liver lesions (category 1). The liver of the fifth rat that died (on day 359) (Fig. 1, arrow A) and those of seven of the next 13 rats that died showed the presence of small foci of proliferating cells in the bile-duct epithelium (category 2). These, although distributed throughout the liver lobule, tended to be more prevalent in the periportal areas and consisted of relatively compact groups of cells, some of which were arranged in a circular pattern with or without a primitive lumen, while others had a more definite ductular configuration. These cells were considered to be similar to 'oval cells' or proliferating bile-duct cells (Grisham & Hartroft, 1961). Hepatocellular pleomorphism was present, particularly in one case. In addition, cells laden with pigment were present in the livers of many of these animals and in those dying later. These cells were mainly present either in the centre of lobules or in association with portal tracts of proliferating bile-duct cells. The pigment was yellowish-brown in sections stained with haematoxylin and eosin, brown with periodic acid–Schiff and Masson's trichrome stains, and was positive for fat in frozen sections stained with Oil Red O and for iron after staining with Prussian blue, but was negative with Turnbull's blue. It was considered to be of a ceroid type.

Liver lesions classified in the third category were first encountered in the rat that died on day 600 (Fig. 1, arrow B) and were seen in varying and usually progressive degrees in 17 of the 23 animals dying later. The milder of these lesions resembled those in category 2, but were larger and more frequent, while the most severe lesions seen in seven of the 17 consisted primarily of an extensive intralobular proliferation of bile-duct epithelial cells, which in many parts had replaced up to some 60–70% of parenchymal elements (Figs 3 & 4). These hyperplastic cells were gathered in parts into relatively compact masses supported by delicate connective-tissue fibres, in parts into structures resembling primitive bile ducts with or without lumina and in other areas into focal accumulations of well-developed ducts frequently exhibiting cystic dilation of the lumina (Fig. 5). The latter change was, however, not as common as the former two. In eight of the livers showing the most severe changes and in one of the others in category 3, hyperplastic nodules of hepatocytes were present (Fig. 6). These varied in size and, in four of the livers particularly, the nodules were numerous and the cells comprising them appeared primitive.

In the livers of many rats in this group, as well as in those of animals in other groups, evidence of hepatocellular degeneration was present, notably fatty infiltration, particularly in the centrilobular areas, single-cell necrosis and, in a few cases, even more extensive necrosis. These more acute degenerative changes were considered non-specific and, in at least some of the cases, were related to a pneumonia which was present in many of the animals.

While no evidence of frank neoplasia occurred in the livers, various neoplasms were encountered in other organs in seven rats in this group (Fig. 1). The number of tumours seen in this group was greater than that seen in group 2 and the difference was statistically significant when tested by a modification of the life table technique, which takes into account differences in mortality pattern (Peto, 1974). There was no evidence that tumours were restricted to certain litters, as the seven tumours in group 1 occurred in five of the seven litters that made up the group. Additional lesions were nephrocalcinosis in many animals, deposition of ceroid pigment in the lungs and spleens of a few, and pericarditis, epicarditis and abscesses in various organs of single rats.

Group 2 pathology. The liver lesions in these rats were less severe than those in the preceding group. Macroscopically visible liver lesions were seen in only four rats (all of which died after more than 920 days) and ascites occurred in two of these. Histologically, deposition of ceroid pigment within the cytoplasm of cells in the centrilobular areas of the liver was first observed after about the same time as those in group 1 (see arrow A, Fig. 1). The most outstanding lesion was again a proliferation of bile-duct epithelial cells (Figs 7 & 8). It was first encountered to a mild degree in the animal that died on day 436 (Fig. 1, arrow C), and was present in 29 of the remaining 30 rats; in general, the longer the animal survived the larger and more frequent were the lesions, although all were much less severe than the advanced lesions in group 1. In contrast to the relatively widespread hyperplasia of this cell type in the liver lobules of rats in category 3 in the former group, the change in these animals was circumscribed and, with few exceptions, was confined to the periportal regions. Another distinction was that the proliferating cells were all arranged in a well-defined ductular pattern, the more advanced lesion resembling a small bile-duct adenoma. Only in very rare instances did a duct or the ducts in a group show cystic dilation. Some contained inspissated bile. Two neoplasms, a reticulum-cell sarcoma of the stomach and an adenoma of one adrenal cortex, were observed in the animals dying on days 649 and 725, respectively (Fig. 1). Several of the rats suffered from lesions such as myocardial fibrosis and acanthoma of the stomach. The incidence of other changes in the lungs, livers and kidneys of these animals was similar to that in the preceding group.

Group 1A pathology. In this group, foci of bile-duct cell hyperplasia within the liver lobules were first seen in the liver of the rat that died on day 422. This lesion was present in 28 of the 32 animals surviving beyond this point and, although it was never as extensive or as aggressive as those in category 3 in group 1, it resembled the change in this group more than that in group 2 and it was unfortunate that the rats surviving to day 781 had to be killed. The hyperplastic change was not confined to any particular area in the liver lobule and the involved cells tended for the most part to be arranged into well-defined ducts occurring singly or in relatively small groups. In many of the animals surviving beyond day 557, the odd focus of these primitive intralobular bile ducts had become cystic and some of these small cysts were visible macroscopically. Tumours were encountered in four of the rats (Fig. 1).

This group of animals was the second generation of a planned multigeneration study, the experiment having been commenced during their gestation and approximately 17 months after that of group 1. However, although the animals were mated regularly during the initial period of 307 days when their diet was supplemented, their breeding performance was extremely poor. Very few of the females conceived and none of the offspring was reared.

Oesophageal lesions in experiment 1. Detailed oesophageal examinations revealed mild, subtle changes which appeared to follow a temporal sequence and which differed in the two main groups. Although exceptions occurred, there was a predominant pattern. At 100–300 days, there was ballooning degeneration and nuclear pycnosis in the oesophageal basal epithelial cells of group 1 animals; in group 2 animals there was vacuolation, particularly of the nuclei in these cells. At 300–500 days, the epithelium was thinner in group 1 than in group 2 rats and there were occasional areas of mild dysplasia, interspersed with cells with pycnotic nuclei; pycnosis of the basal epithelial cell nuclei was frequent and at times severe in group 2 animals and cytoplasmic ballooning degeneration was common. Between 500 and 700 days, group 1 animals showed some thickening of the epithelium with disarrangement of basal-cell replication indicated by mild hyperchromasia, anaplasia and dysplasia; nuclear pycnosis was still common. The epithelium in group 2 animals was thin and the basal cells were sparse. Most animals in group 1 died by day 900. From day 700 to termination, the epithelium of some rats was thickened with hyperkeratosis, parakeratosis and dysplasia and there was basal-cell hyperplasia with foci of cellular infiltration in the lamina propria. Two rats had small papillomas in the distal third of the oesophagus, causing obstruction and moderate distension of the proximal portion. The epithelia of group 2 rats showed some thickening but the changes were milder than those seen in group 1. Very few alterations were noted before day 550 in group 1A. After this time the changes seen in groups 1 and 2, such as parakeratosis and hyperkeratosis, were seen. In the 16 survivors killed on day 781 all the changes, including anaplasia and dysplasia, were common.

Experiment 2

The mean survival times (Table 1) of the animals in groups 1B and 2B (331 and 694 days, respectively) were much shorter than those in Experiment 1, in which no animals received distillate. Many of the rats in group 2B, however, died from pneumonia during one relatively short period (Fig. 2).

Group 1B pathology. The autopsies on the rats in group 1B revealed macroscopic signs of liver damage similar to those observed in group 1. Microscopically, too, the hepatic lesions were similar, except that the intralobular bile-duct epithelial-cell hyperplasia was generally more aggressive and the lesions were more advanced, those of all the rats resembling the changes

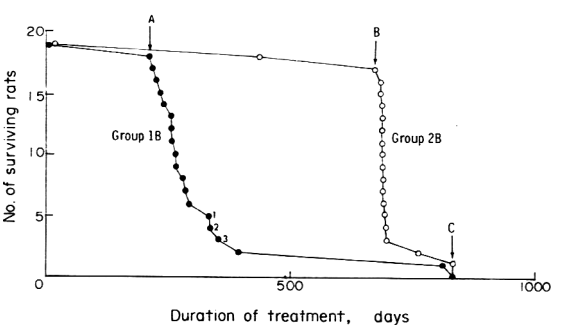

Fig. 2. Times of death of individual rats in group 1B (diet B plus distillate) and group 2B (diet A plus distillate). Arrows indicate (A) first sign of bile-duct hyperplasia in group 1B, (B) first sign of bile-duct hyperplasia in group 2B and (C) killing of survivors. Rats numbered 1, 2 and 3 had hyperplastic liver nodules.

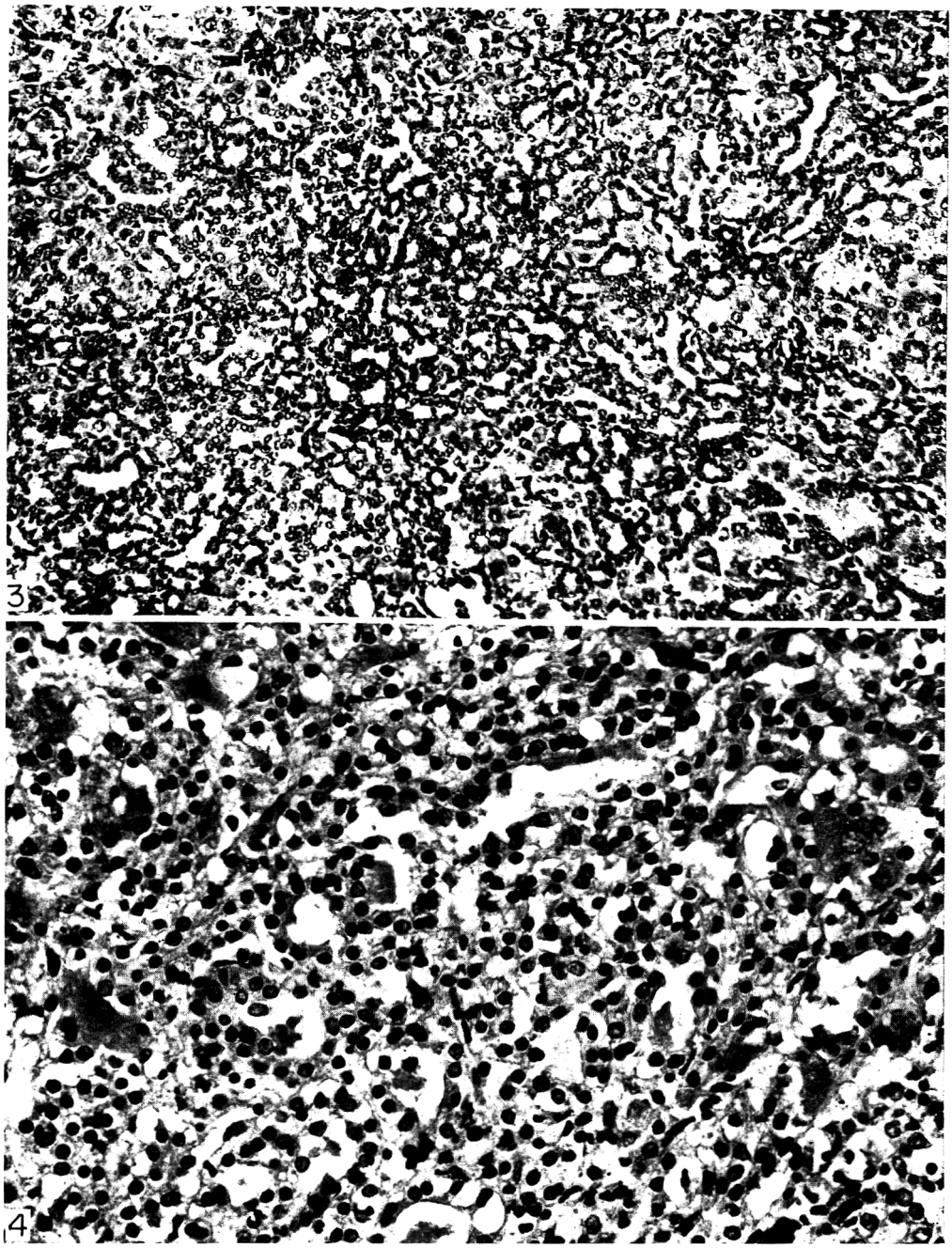

Fig. 3. Liver lesion in a rat dying after 799 days on diet B, showing extensive bile-duct proliferation. Haematoxylin and eosin × 50.

Fig. 4. Liver lesion in a rat fed for 774 days on diet B, with proliferation of bile-duct cells, many of which are arranged around a lumen. Haematoxylin and eosin × 125.

Facing p. 644

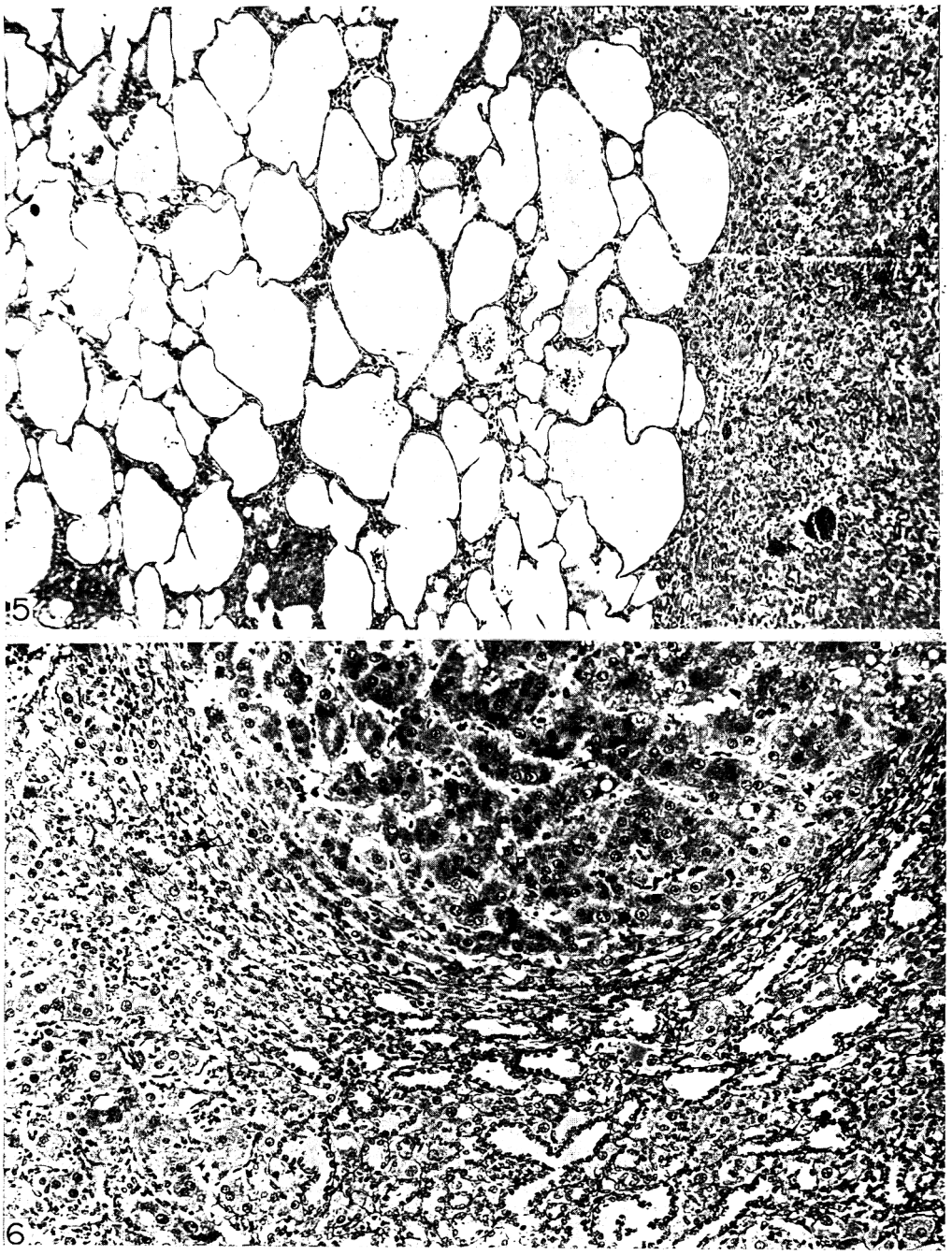

Fig. 5. Cystic dilation of bile ducts in a rat fed for 831 days on diet B. Haematoxylin and eosin ×25.

Fig. 6. The edge of a hyperplastic nodule compressing abnormal live rparenchyma in the rat fed diet B for 799 days. Reticulin × 50.

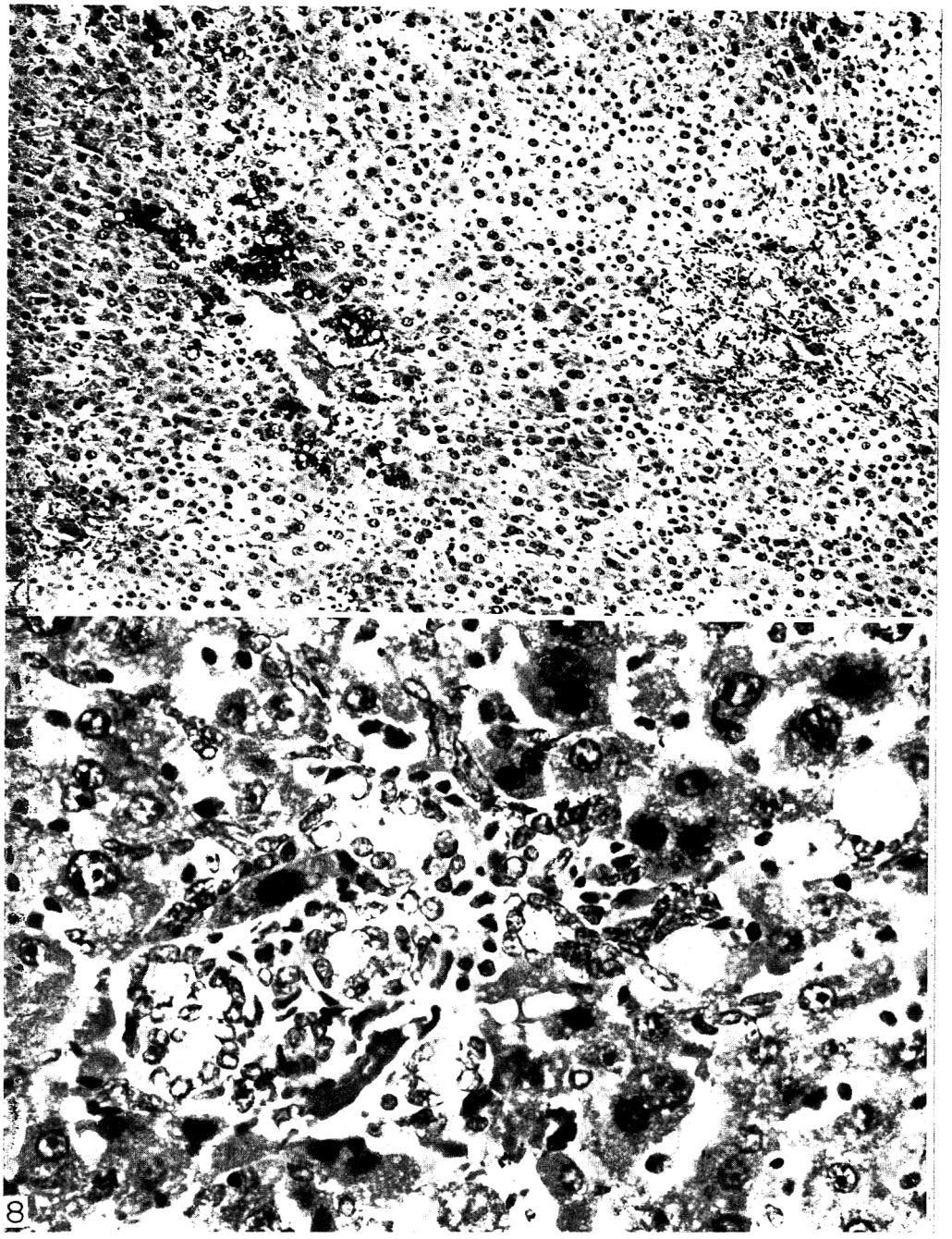

Fig. 7. Liver from a rat on diet A for 747 days, showing dark-staining pigmented cells in the centrilobular area and proliferating bile ducts in the immediate periportal area. Haematoxylin and eosin × 50.

Fig. 8. A small area of bile-duct proliferation in the liver of a rat fed on diet A for 649 days. Haematoxylin and eosin × 160.

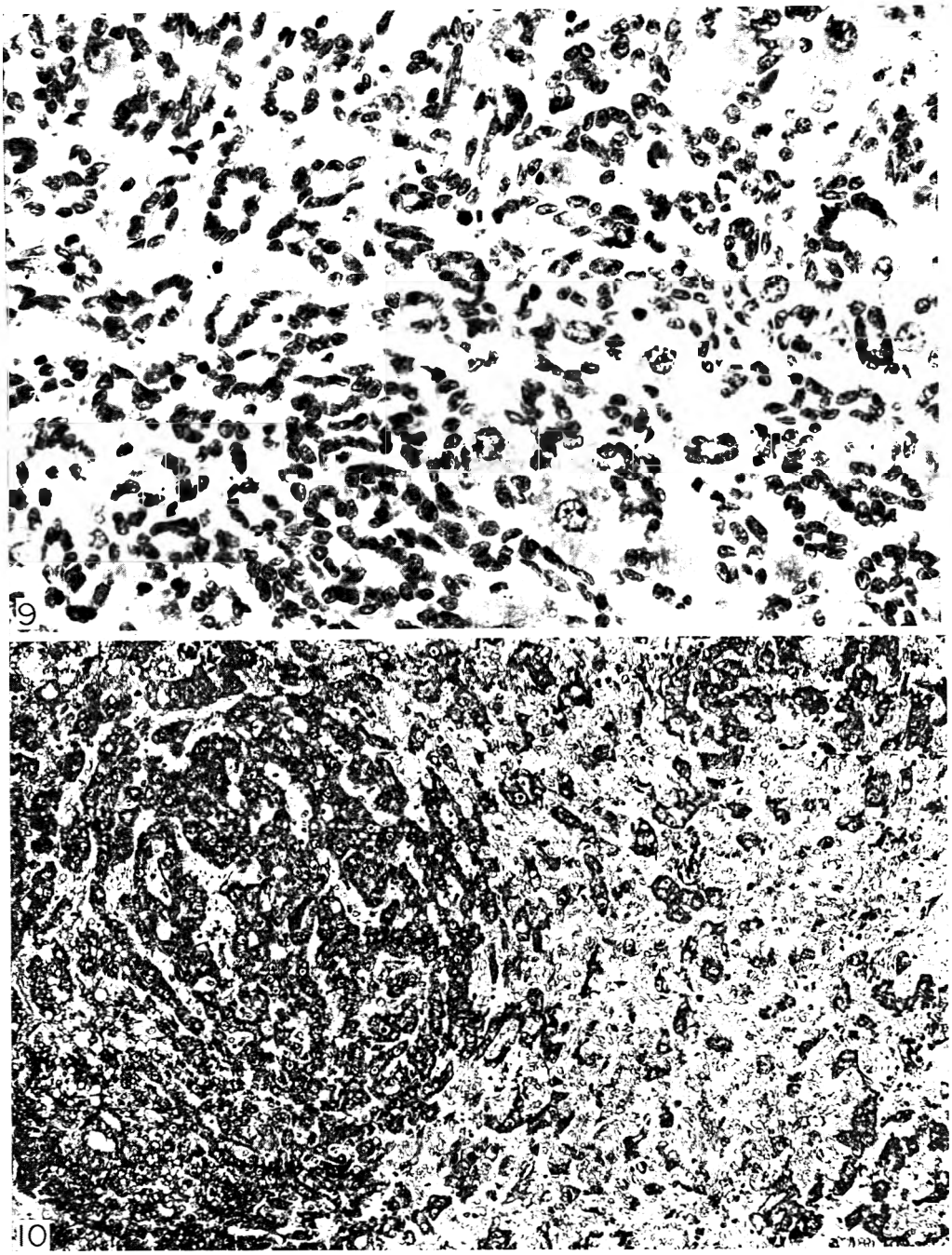

Fig. 9. Liver from a rat in group 1B that died after 283 days on diet B and distillate, showing aggressive bile-duct proliferation. Haematoxylin and eosin × 125.

Fig. 10. Liver of a rat in group 1B that died after 353 days on diet B and distillate, showing extensive bile-duct proliferation and a focal hyperplastic area. Haematoxylin and eosin × 50.

in category 3 of group 1 (Figs 9 & 10). No hepatic or extrahepatic neoplasms were encountered, but nodules of hepatocellular hyperplasia were seen in four rats (Fig. 2). Only one of the 19 animals in this group survived to day 829, when it was killed. Changes observed in other organs were not regarded as being of any significance.

Group 2B pathology. Only two animals in this group had macroscopic signs of liver disease. Histologically, intralobular and particularly periportal proliferation of bile ducts occurred in the livers of 13 of the 19 rats, and although the appearance of the individual liver lesions resembled those of group 2, they were less extensive than the latter and were considerably smaller and of more benign appearance than those of group 1B. From the nature of these lesions it is apparent that none of the animals in group 2B had died from hepatic failure or had even suffered to any extent as a result of damage to this organ. Two of the animals survived to day 829 and had to be killed. Deposition of ceroid pigment was a common finding. No extrahepatic lesions considered to be of significance to the experiment were observed.

Oesophageal lesions in experiment 2. Few animals survived beyond 400 days. Up to this time the lesions already seen in groups 1 and 2, particularly the tendency to anaplasia, were well advanced in group 1B. The lesions in group 2B were less severe.

Distillate analyses. The sample of distillate analysed for nitrosamine content by gas chromatography was found to contain no traces of low-molecular-weight nitrosamines (J. R. Nunn, personal communication 1970). Similarly, polarography failed to reveal the presence of nitrosamines, although there was some evidence of a low concentration (0·35 ppm) of alkyl nitrate in the distillate (C. L. Walters, personal communication 1970).

Experiment 3

The mortality pattern in this experiment is indicated in Table 1. Although, again, surviving rats had to be killed on day 474, this only affected 19 of the 20 rats in groups 3 and 4, and one in group 5, the remainder in groups 5 and 6 having died spontaneously before this time. With the exception of a sweat-gland adenoma in one animal in group 3, no lesions of possible relevance to this experiment were observed in the animals. No oesophageal lesions of the type seen in the previous experiments were seen.

DISCUSSION

The prospective end point of these experiments was the induction of oesophageal carcinoma in the rats. This was not achieved, although a considerable degree of epithelial-cell atypia and dysplasia of the oesophagus was encountered in the rats in experiments 1 and 2. This lesion, although mild, was much more marked than any seen in rats of this strain maintained on a standard laboratory diet or in the rats in experiment 3. It may have been associated with the syndrome in which lesions of the skin and eyes occurred, although no seasonal variation in the oesophageal lesion corresponding to the seasonal nature of the clinical syndrome was seen. Thus it seems more likely that it was caused by toxic factors in the diet.

The most dramatic lesions in these rats were the liver lesions that occurred in groups 1 and 1B. The earliest was a proliferation of cells resembling primitive bile-duct epithelial cells within the liver lobule. This was accompanied by mild degenerative changes in the hepatocytes and culminated in the development of nodules of hepatocellular hyperplasia in eight animals in group 1 and four in group 1B. These lesions are considered to be caused by the full Transkei diet, because the only liver lesions seen in group 2 animals, receiving Transkei maize and beans, were a much milder bile-duct proliferation and a deposition of ceroid. It seems unlikely, therefore, that the advanced liver lesions were due to a dietary deficiency, a view confirmed by the absence of any protective effect by protein and vitamin supplementation in group 1A. The latter finding also suggests that the lesions were produced by toxin(s) in the full Transkei diet. The presence of toxins would explain the poor breeding performance of group 1A rats and the higher mortality in group 1. The incidence of tumours was significantly higher in group 1 than in group 2, a difference accentuated when adjustment for mortality was introduced. The incidence in the three groups receiving the full Transkei diet (12 tumours in 95 rats) was considerably higher than that in those receiving Transkei maize and beans (two tumours in 59 animals), despite the longer survival times in the latter groups. There is no strictly comparable tumour-incidence data for BD IX rats, although experiments with this strain using other diets have yielded a higher total tumour incidence but a lower incidence of malignant tumours than was recorded in group 1 (S. J. van Rensburg and I. F. H. Purchase, unpublished observations 1973). The higher incidence of tumours in group 1 was not associated with marked differences in body weight between the two groups and is thus unlikely to have been due to a lower food intake by group 2. Neither was there evidence that the higher tumour incidence was related to any increase in susceptibility in individual litters. It appears, therefore, that the higher tumour yield in group 1 represents a real increase in the tumour incidence.

In animals receiving Transkei diets there were three distinct toxic responses. The mildest was the bile-duct proliferation and ceroid deposition in the liver and the slight oesophageal changes in group 2. More extensive bile-duct proliferation with subsequent development of hepatocellular hyperplastic nodules represented a more severe intoxication. Finally the increased tumour yield suggested a third toxic effect. The two latter effects were associated with more severe oesophageal lesions in group 1 and 1B. Increasing doses of the same toxin may have been involved in the production of these responses. However, as group 1 was receiving the same dietary ingredients, with the addition of *imifino*, as group 2, it seems more likely that at least two toxins were present. One toxin, producing the mild oesophageal and bile-duct lesions, could have been acting in both groups. A second toxin, associated with the incorporation of *imifino* into the diet (and possibly enhanced by the mild toxin mentioned above), produced the more severe oesophageal and liver lesions and may also be the car-

cinogen in the full Transkei diet. The exact nature of the toxin was not determined, although no aflatoxin or sterigmatocystin, both hepatocarcinogenic myocotoxins (Newberne & Butler, 1969; Purchase & van der Watt, 1970), were detected in the diet. Other mycotoxins or, as was suggested by Burrell *et al.* (1966), nitrosamines or toxins unique to *imifino* could be responsible.

The liver lesions observed in this experiment are not unique. Several carcinogens are known to produce an 'oval' cell or bile-duct proliferation which progresses to neoplasia; among these are 3'-methyl-4-dimethylaminoazobenzene. 4'-fluoro-4-dimethylaminoazobenzene (Price, Harman, Miller & Miller, 1952), 2-acetylaminofluorene and ethionine (Farber, 1956). The presence of 'oval' cells is not unique to carcinogens, as 2-aminochrysene and trichloroethylene induce a similar response (Farber, 1956). The progression to regenerative or hyperplastic nodules may, however, indicate that the response is to a carcinogen.

The suggestion by Burrell and his colleagues that nitrosamines resulting from metabolic abnormalities induced in plants by molybdenum deficiency could be responsible for the high incidence of cancer, led to the second series of experiments. Nitrosamines, particularly those with a low molecular weight, are volatile and can form azeotropes with water. The process by which the diets were dried, with temperatures of 50° in a forced-draught oven, could have reduced drastically the nitrosamine levels in the diets. The use of the distillate as the drinking-water of the rats accentuated the lethal and hepatotoxic effects of the full Transkei diet but not of the maize and beans diet. This effect was unfortunately complicated by simultaneous mortality in group 1 (between 750 and 850 days, i.e. 20 November 1969 to 7 March 1970) and group 1B receiving distillate (between 217 and 290 days, i.e. 24 October 1969 to 5 January 1970). There is therefore a possibility of a seasonal effect acting through the diet but unconnected with the distillate. Analysis of a single sample of the distillate by gas chromatography and polarography failed to detect nitrosamines. This observation is not considered unequivocal because it was made on a single sample which had to be stored for some time in transit before analysis. A low concentration of alkyl nitrate was detected, however, by polarography.

The reason for the enhancement of the liver lesions by the distillate is not clear. The possibility that it could be due to the interaction in the stomach of nitrite or nitrate from the distillate with secondary amines from the food (Sander, Schweinsberg & Menz, 1968) was investigated in the third experiment. In experiments 1 and 2, slight bile-duct cell hyperplasia was seen in group 1 and pronounced hyperplasia in group 1B by 124 and 208 days, respectively. The rats in groups 2 and 2B developed the earliest bile-duct lesions at 436 and 683 days respectively. The absence of the slightest bile-duct proliferation in any of the rats receiving 0·5% sodium nitrite in the diet suggests that interaction with amines did not occur. Animals on Transkei diets were, however, more susceptible to the toxic effects of sodium nitrite than animals on normal rat ration. In another experiment, where 0·1% sodium nitrite was fed to BD IX rats for 267 days (when the experiment had to be terminated), there was no effect on mortality or on the development of liver lesions.

Oesophageal carcinoma was not produced in BD IX rats by the Transkei diets, but a carcinogen, which produced a variety of tumours and possibly a progressive liver lesion, was certainly present in the food. This type of observation is difficult to extrapolate to man, even though the diet and the dietary preparation technique were similar to those used in the Transkei. Some carcinogens have varying organ specificity, which depends on the route of administration and the species of animal used. Thus β-naphthylamine is a bladder carcinogen in man and in the dog, although it produces only liver tumours in rats (Hueper, 1961). The significance of the findings in this experiment may be that the carcinogen present in the food is responsible for oesophageal cancer in man and a variety of tumours and liver disease in rats. Alternatively, the liver lesions may reflect the presence of a hepatotoxic carcinogen which is also active in man, producing the high level of liver cancer in the population. A third alternative is that the carcinogen in the diet was responsible in the rat for the liver lesions and the tumour response, while in man it is responsible for both liver and oesophageal cancer. Unfortunately no detailed epidemiology capable of identifying a geographical coincidence of these two tumours in the Transkei is available. In as far as the whole of Southern Africa is concerned, liver and oesophageal cancer do not usually occur in high incidence in the same area (Robertson *et al.* 1971). If a single carcinogen is responsible for both cancers, other factors may be operating, which influence the site of the cancer.

The precise nature of the carcinogen and the factors that contribute to the effects of Transkei diets on rats are undergoing further investigation.

Acknowledgements—We thank Professor J. J. Theron for his support during the setting up of this experiment. Dr. E. F. Rose for her continued interest and help and Mr. H. J. B. Joubert for his continuous and enthusiastic technical assistance.

REFERENCES

Burrell, R. J. W. (1957). Oesophageal cancer in the Bantu. *S. Afr. med. J.* **31**, 401.

Burrel, R. J. W. (1969). Distribution maps of esophageal cancer among Bantu in the Transkei. *J. natn. Cancer Inst.* **43**, 877.

Burrell, R. J. W., Roach, W. A. & Shadwell, A. (1966). Esophageal cancer in the Bantu of the Transkei associated with mineral deficiency in garden plants. *J. natn. Cancer Inst.* **36**, 201.

Cook, Paula (1971). Cancer of the oesophagus in Africa. A summary and evaluation of the evidence for the frequency of occurrence, and a preliminary indication of the possible association with the consumption of alcoholic drinks made from maize. *Br. J. Cancer* **25**, 853.

Druckrey, H. (1971). Genotypes and phenotypes of ten inbred strains of BD-rats. *Arneimittel-Forsch.* **21**, 1274.

du Plessis, L. S., Nunn, J. R. & Roach, W. A. (1969). Carcinogen in a Transkeian Bantu food additive. *Nature, Lond.* **222**, 1198.

Farber, E. (1956). Similarities in the sequence of early histological changes induced in the liver of the rat by ethionine, 2-acetylaminofluorine, 3'-methyl-4-dimethylaminoazobenzene. *Cancer Res.* **16**, 142.

Grisham, J. W. & Hartroft, W. S. (1961). Morphological identification by electron microscopy of 'oval cells' in experimental hepatic degeneration. *Lab. Invest.* **10**, 317.

Higginson, J. (1951). Malignant neoplastic disease in the South African Bantu. *Cancer, N.Y.* **4**, 1224.

Hueper, W. C. (1961). Environmental carcinogenesis and cancers. *Cancer Res.* **21**, 842.

Marais, J. A. H. & Drewes, E. F. R. (1962). The relationship between solid geology and oesophageal cancer distribution in the Transkei. *Ann. geol. Surv. S. Afr.* **1**, 105.

Newberne, P. M. & Butler, W. H. (1969). Acute and chronic effects of aflatoxin on the liver of domestic and laboratory animals: A review. *Cancer Res.* **29**, 236.

Oettlé, A. G. (1964). Cancer in Africa, especially in regions south of the Sahara. *J. natn. Cancer Inst.* **33**, 383.

Peto, R. (1974). Guidelines on the analysis of tumour rates and death rates in experimental animals. *Br. J. Cancer* **29**, 101.

Price, J. M., Harman, J. W., Miller, E. C. & Miller, J. A. (1952). Progressive microscopic alterations in the livers of rats fed the hepatic carcinogens 3'-methyl-4-dimethylaminoazobenzene and 4'-fluoro-4-dimethylaminoazobenzene. *Cancer Res.* **12**, 192.

Purchase, I. F. H. (1968). Mycotoxins. In *Cancer in Africa.* Edited by P. Clifford, C. H. Linsell and G. L. Timms. p. 327. East African Publishing House, Nairobi.

Purchase, I. F. H. & Joubert, H. J. B. (1971). Biological screening as a laboratory aid in determining cancer aetiology. In *Mycotoxins in Human Health.* Edited by I. F. H. Purchase. p. 291. Macmillan, London.

Purchase, I. F. H. & van der Watt, J. J. (1970). Carcinogenicity of sterigmatocystin. *Fd Cosmet. Toxicol.* **8**, 289.

Robertson, M. A., Harington, J. S. & Bradshaw, Evelyn (1971). The cancer pattern in African gold miners. *Br. J. Cancer* **25**, 395.

Rose, E. F. (1965). Interim report on the survey of cancer of the oesophagus in the Transkei. *S. Afr. med. J.* **39**, 1098.

Rose, Elizabeth F. (1973). Esophageal cancer in the Transkei: 1955–69. *J. natn. Cancer Inst.* **51**, 7.

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.

Fd Cosmet. Toxicol. Vol. 13, pp. 649–651. Pergamon Press 1975. Printed in Great Britain

A STUDY OF KINETIC PARAMETERS FOR THE USE OF SERUM ORNITHINE CARBAMOYLTRANSFERASE AS AN INDEX OF LIVER DAMAGE

R. B. DROTMAN

The Procter and Gamble Company, Ivorydale Technical Center, 5299 Spring Grove Avenue, Cincinnati, Ohio 45217, USA

(*Received 1 April 1975*)

Abstract—A simple radiometric assay for ornithine carbamoyltransferase (EC 2.1.3.3) in serum is described in detail. The method utilizes $^{14}CO_2$ released from [^{14}C-ureido]-L-citrulline by arsenolysis as a measure of enzyme activity. The $^{14}CO_2$ is entrapped in base and measured directly by liquid scintillation spectrometry. Ornithine carbamoyltransferase in serum was found to be stable for at least 2 wk when stored frozen. Significant inhibition of enzyme activity by endogenous compounds was not apparent. Linear relationships were demonstrated between $^{14}CO_2$ formation and the length (time) of incubation and also between $^{14}CO_2$ formation and enzyme concentration. The assay was found to be a relatively sensitive method for detecting liver damage.

INTRODUCTION

Ornithine carbamoyltransferase (EC 2.1.3.3; OCT) is located primarily in the liver (Jones, Anderson, Anderson & Hodes, 1961) and the appearance of substantial amounts of OCT in serum indicates hepatic dysfunction or damage (Tegeris, Smalley, Earl & Curtis, 1969). Because of its localization in the liver and its potential in identifying the liver as the damaged organ, this enzyme has recently received attention as an index of liver damage in toxicological studies (Korsrud, Grice, Kuiper, Goodman, Knipfel & McLaughlan, 1973; Tegeris *et al.* 1969). Despite the fact that OCT in serum has been used to detect liver damage, there is no study describing the kinetic parameters of this enzyme in serum, to make toxicologists using this assay aware of the limitations within which they are working.

OCT activity may be measured directly by following the appearance of citrulline (reaction 1, Fig. 1), or indirectly by arsenolysis (reaction 2, Fig. 1) in which the enzyme catalyses the reverse reaction of citrulline to NH_3, CO_2 and ornithine (Krebs, Eggleston & Knivett, 1955). OCT measurement is most sensitive when $^{14}CO_2$ is the index of enzyme activity, and in this study the arsenolysis reaction was used to determine OCT activity in serum. The method of assay is similar to that described by Korsrud *et al.* (1973), in which trapped $^{14}CO_2$ is counted directly by liquid scintillation instead of being precipitated with $Ba(OH)_2$ before ^{14}C measurement (Reichard & Reichard, 1958).

It is the purpose of this paper to describe in detail the technique used for this assay and to describe the kinetics of OCT in serum from rats that were subjected to liver damage by CCl_4.

EXPERIMENTAL

Animals and dosing. In order to obtain sera containing appreciable levels of OCT, rats were injected ip with CCl_4, a treatment known to produce liver damage and elevate OCT levels (Tegeris *et al.* 1969). For most of the experiments reported here, groups of five male Cox CD albino rats were given a single injection of 0·5 ml CCl_4/kg body weight in corn oil. Following injection, the rats were allowed food (Purina Rat Chow) and water *ad lib.* except in the 12 hr prior to blood withdrawal. The animals were anaesthetized and exsanguinated from the vena cava 24 hr after injection. Sera were pooled and used immediately or frozen. Five groups each of four rats were used to correlate the sensitivity of the OCT assay to the degree of liver damage. The serum samples from these animals were not pooled.

OCT assay procedure. [^{14}C-Ureido]-L-citrulline with a specific activity of 0·1 mCi/mmol was obtained from Amersham-Searle Corp., Mass. It contained [^{14}C]urea as a contaminant, corrections for which were made by determining the urea content with urease or by removing the [^{14}C]urea by the method of Hall, Johnson & Cohen (1960). The final specific radioactivity of citrulline in all cases was 0·0227 μCi/μmol (50,000 dpm/μmol). The final citrulline concentration in all flasks was 10 μmol/ml in 0·1 M-sodium arsenate buffer (pH 6·7). The reaction product, $^{14}CO_2$, was trapped in 1·5 M-NaOH and counted in a mixture of 0·01% 1,4-bis-(2-(4-methyl-5-phenyloxazolyl)benzene) and 0·8% 2,5-diphenyloxazole in toluene–ethanol (1:1, v/v) to which Cab-O-Sil (34 g/litre) was added.

Reactions were carried out in duplicate in 25-ml reaction flasks (Kontes Glass Co., Illinois, no. 882300) which were capped with a one-hole rubber septum

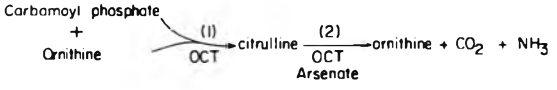

Fig. 1. Reactions catalysed by ornithine carbamoyltransferase.

stopper (Kontes Glass Co., no. 882310). In the hole, a propylene cup, 1 × 1·5 cm, was mounted by a 5·8 cm support rod (Kontes Glass Co., no 882310). Figure 2 is a photograph of the assembled assay unit. To prepare the centerwell-stopper units the support rod was inserted into the one-holed serum stopper so that the top of the centerwell was approximately 1·5 cm below the rubber stopper. The completed centerwell-stopper may be conveniently set in a rack designed for holding 85 × 15 mm serological tubes. Once the appropriate number of centerwell-stopper units was assembled, the centerwells were filled with 0·2 ml of 1·5 N-NaOH. This was easily accomplished from a 1·0 ml syringe fitted with a 20-gauge (1·5 in.) needle. During the filling of the centerwell, it was critical that no NaOH was allowed to come into contact with the outer portion of the cup; if this did occur, that centerwell had to be discarded. An alternative trapping solution, hyamine hydroxide (McDonald, Speeg & Campbell, 1972), may be preferable because of its higher viscosity.

After the centerwells had been prepared, the flasks were charged with 2·0 ml citrulline–arsenate buffer and the reactions were started with 0·4 ml serum (final flask volume, 2·4 ml). The entire aliquot of serum was added at 30–60 sec intervals to begin the reaction and the flask was swirled to ensure good initial mixing. The flask was then placed on a flat surface and the centerwell-stopper unit was carefully inserted to avoid spilling the NaOH. Once the rubber stopper was in place the upraised margin of the stopper was turned down so that a good seal was maintained (Fig. 2). The flask was incubated in a gently shaking waterbath at 30°C.

After 24 hr, the shaker was turned off and the enzyme reaction was stopped by injecting 1 ml 5N-H_2SO_4 through the serum stopper from a syringe fitted with a 20-gauge (1·5 in.) needle. After the needle had entered the flask, the syringe was tilted slightly so that the acid did not touch the centerwell. The flasks were shaken for an additional hour to volatilize the $^{14}CO_2$ following injection of the acid. Previous

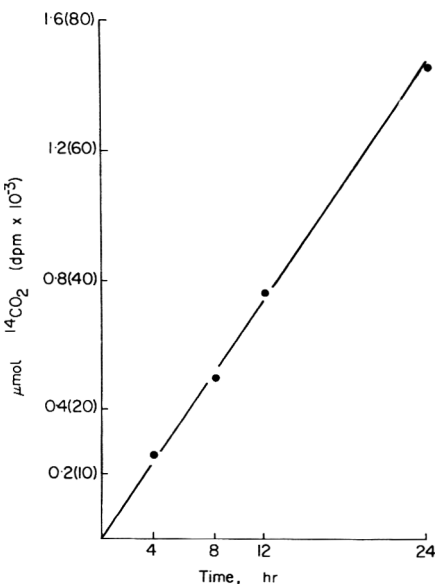

Fig. 3. Reaction rate of OCT.

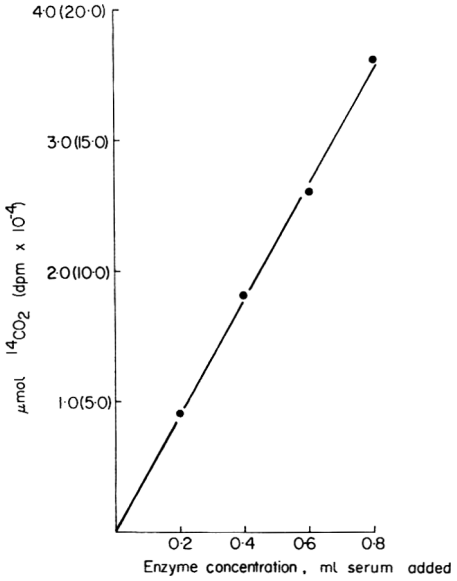

Fig. 4. Effect of varying amounts of enzyme (OCT) on $^{14}CO_2$ generation.

experiments by McDonald *et al.* (1972) and data generated by ourselves (unpublished) indicated that 100% of the $^{14}CO_2$ was quantitatively recovered within 15 min.

The centerwell was then pulled to within 1 cm of the stopper, and the stopper was removed by a slight twisting motion. Condensation was wiped off the centerwell which was separated from the supporting rod with scissors. The entire centerwell was dropped into a scintillation vial and shaken with the scintillation fluid, and the radioactivity was measured by a liquid scintillation spectrometer. A control flask (all components minus serum) was run and the resultant background radioactivity was subtracted from the values obtained for the experimental flasks.

The apparatus and basic procedure described were used to determine the effects of reaction time, enzyme concentration, serum storage and inhibitors on the results obtained and to relate changes in OCT levels to known conditions of liver damage induced by CCl_4.

RESULTS

Measurement and reaction rate

In order to demonstrate the relationship between $^{14}CO_2$ formation and time, enzymic reactions were stopped with acid after 4, 8, 12 and 24 hr and the entrapped $^{14}CO_2$ was measured. The formation of $^{14}CO_2$ from [^{14}C-ureido]-L-citrulline as a function of time (Fig. 3) indicates a linear relationship.

Influence of enzyme concentration

Flasks were prepared as described except that the volume of serum added was 0·2, 0·4, 0·6 or 0·8 ml. Citrulline concentration and specific radioactivity were adjusted so that both remained constant with the different volumes of serum. Figure 4 shows the results of increasing enzyme concentration on $^{14}CO_2$ formation. A linear relationship was again demonstrated over the range studied.

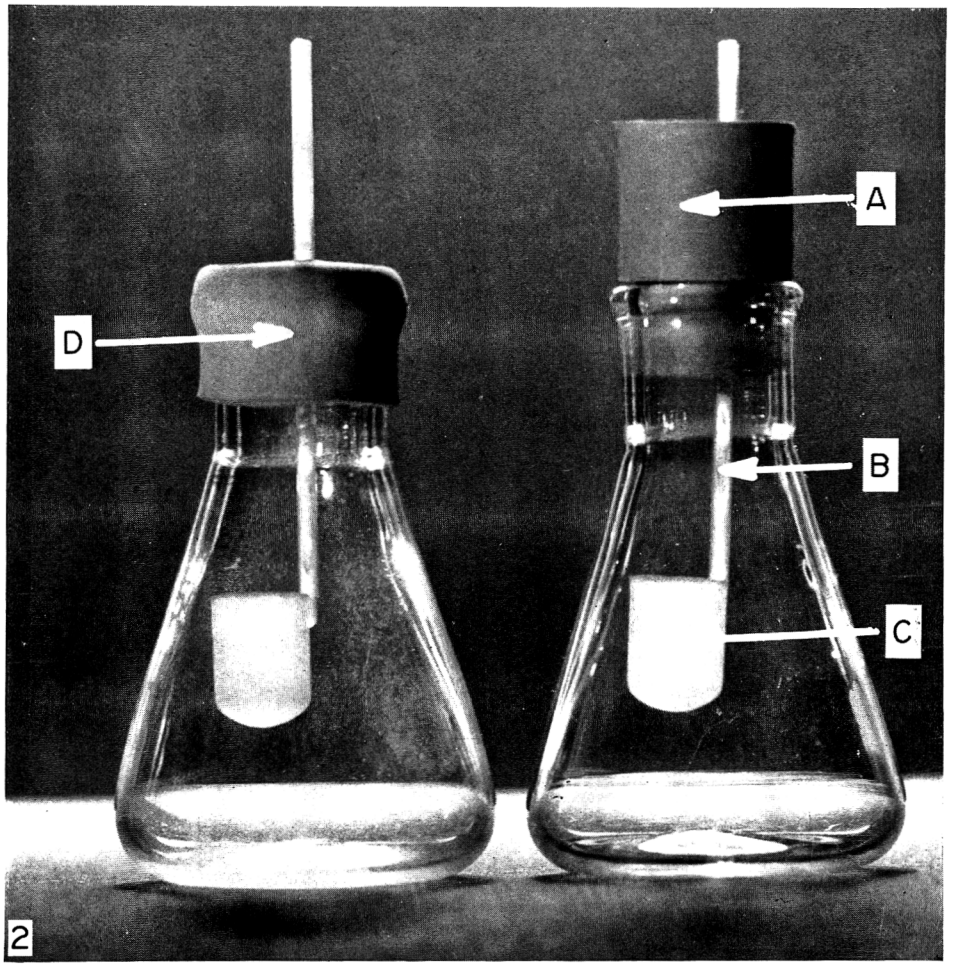

Fig. 2. Assembled OCT assay unit showing (A) upraised margin of rubber septum stopper, (B) supporting rod of polypropylene centerwell, (C) polypropylene centerwell and (D) lowered margin of rubber septum stopper.

Facing p. 650

Stability studies

Since a toxicology laboratory may not be able to perform an assay immediately, it is of value to know whether the enzyme is stable for a reasonable period of time. Serum was stored in a freezer, therefore, at −5°C in 2-ml aliquots and assayed on alternate days over a period of 2 wk. These assays indicated no loss in OCT activity in this period (day 0, 77,000 dpm $^{14}CO_2$/0·4 ml; day 14, 78,200 dpm $^{14}CO_2$/0·4 ml).

Inhibitor studies

Krebs *et al.* (1955) have shown that ornithine and phosphate inhibit the arsenolysis of citrulline. Both of these compounds are found in serum, and since ornithine is also a reaction product, serum was dialysed to determine whether any compound was present in sufficient concentration to inhibit the reaction. Dialysis was performed with a Bio-Fiber 50 membrane (Bio-Rad Laboratories, Richmond, Cal.) with 10 ml serum outside the fibres and the dialysate, double-distilled water, inside the fibres. Dialysis was allowed to proceed for 30 min at a flow rate of 3 ml/min. The assay results obtained were 1·6 μmol $^{14}CO_2$/0·4 ml undialysed serum compared with 1·3 μmol following dialysis.

Sensitivity of the assay for detection of liver damage

The ability of this assay to detect changes in OCT levels in response to liver damage was established by injecting rats ip with CCl_4 in graded doses and assaying the serum for enzyme activity. The doses of CCl_4 used and the serum activity of OCT expressed both as dpm $^{14}CO_2$/0·4 ml serum and as μmol CO_2/litre serum/24 hr are given in Table 1. OCT activity in the serum was evident at all levels of CCl_4 injection.

Table 1. *Detection of CCl_4-induced liver damage by the assay of OCT in rat serum*

Dose of CCl_4 (μl/kg body weight)	$^{14}CO_2$ produced	
	dpm/0·4 ml serum	μmol/24 hr/litre serum
0·0*	104	4 (0·6–6·0)
37·5	650	26 (8·0–62·0)
75·0	1478	59 (10·8–106·0)
150·0	11,275	360 (160·0–460·0)
300·0	33,308	1350 (620·0–3200·0)

*Control animals were given corn oil ip.
Values are means for groups of four animals. Numbers in parenthesis indicate the range.

DISCUSSION

The 24-hr incubation period used for the assay of OCT in the majority of experiments was convenient and the results in Fig. 3 indicate that the slope is linear within this period. Therefore, a 24-hr incubation or one of shorter duration is recommended. Further flexibility may be added to the assay in the selection of the amount of serum used, since Fig. 4 indicates that there is linearity up to 0·8 ml. It appears that the radioactivity liberated is directly proportional to the OCT activity in the serum. For this reason the sensitivity of the assay can be increased by the use of larger serum samples or, conversely, if only a small amount of sample is available it may still be utilized. In addition to the above kinetic parameters, the enzyme is stable during storage for at least 2 wk and no inhibitors are present in serum in high enough concentration to affect the enzyme activity substantially.

For an enzyme that appears in the serum to be useful in the detection of organ damage or disease, it must show significant increases in activity even when the damage is minimal. The data in Table 1 indicate a significant increase of OCT activity with injection of CCl_4 in doses known to produce minimal amounts of liver damage (Grice, Barth, Cornish, Foster & Gray, 1971). Histological examination of liver tissue stained with haematoxylin-eosin showed no distinctive liver damage with 37·5 μl CCl_4/kg body weight, even though there was a sixfold increase in OCT activity in the serum. Histological damage was noted at all higher levels of CCl_4. In addition, there appeared to be a graded response of OCT to increasing levels of CCl_4, more enzyme being lost from the cells and appearing in the serum with higher doses of injected CCl_4. Therefore measurement of OCT activity in the serum is a sensitive index of liver damage and may be of practical use in the toxicology laboratory.

Acknowledgements—Mr. G. T. Lawhorn provided expert technical assistance. Dr. H. E. Black carried out the histological evaluation of the liver. Thanks are due to Messrs. R. Schneider and H. Lampe for radiometric measurements. Dr. N. R. Artman and Dr. H. H. Cornish read the paper and made many valuable suggestions.

REFERENCES

Grice, H. C., Barth, M. L., Cornish, H. H., Foster, G. V. & Gray, R. H. (1971). Correlation between serum enzymes, isozyme patterns and histologically detectable organ damage. *Fd Cosmet. Toxicol.* **9**, 847.

Hall, L. M., Johnson, R. C. & Cohen, P. P. (1960). The presence of carbamyl phosphate synthetase in intestinal mucosa. *Biochim. biophys. Acta* **37**, 144.

Jones, Mary E., Anderson, Ann. D., Anderson, Constance & Hodes, Susan (1961). Citrulline synthesis in rat tissues. *Archs Biochem. Biophys.* **95**, 499.

Korsrud, G. O., Grice, H. C., Kuiper Goodman, T., Knipfel, J. E. & McLaughlan, J. M. (1973). Sensitivity of several serum enzymes for the detection of thioacetamide-, dimethylnitrosamine- and diethanolamine-induced liver damage in rats. *Toxic, appl. Pharmac.* **26**, 299.

Krebs, H. A., Eggleston, L. V. & Knivett, V. A. (1955). Arsenolysis and phosphorolysis of citrulline in mammalian liver. *Biochem. J.* **59**, 185.

McDonald, J. A., Speeg, K. V., Jr. & Campbell, J. W. (1972). Urease: A sensitive and specific radiometric assay. *Enzymologia* **42**, 1.

Reichard, H. & Reichard, P. (1958). Determination of ornithine carbamyl transferase in serum. *J. Lab. clin. Med.* **52**, 709.

Tegeris, A. S., Smalley, H. E., Jr., Earl, F. L. & Curtis, J. M. (1969). Ornithine carbamyl transferase as a liver function test: Comparative studies in the dog, swine and man. *Toxic. appl. Pharmac.* **14**, 54.

Fd Cosmet Toxicol. Vol. 13, pp. 653–654. Pergamon Press 1975. Printed in Great Britain

SHORT PAPER

DIMETHYLNITROSAMINE IN SOUSE AND SIMILAR JELLIED CURED-MEAT PRODUCTS

W. FIDDLER, J. I. FEINBERG, J. W. PENSABENE, A. C. WILLIAMS and C. J. DOOLEY

Eastern Regional Research Center, Philadelphia, Pennsylvania 19118, USA*

(*Received* 16 *April* 1975)

Summary—Dimethylnitrosamine was found in eight of ten samples of commercial souse and similar gelatin-containing cured-meat products at levels ranging from 3 to 63 μg/kg. One of the samples was also found to contain 19 μg nitrosopyrrolidine/kg. There appeared to be no correlation between residual levels of sodium nitrite and the nitrosamine concentration. The nitrosamines were determined quantitatively by gas–liquid chromatography (glc) using an alkali flame ionization detector and the analyses were confirmed by glc–high resolution mass spectrometry.

Introduction

A paucity of information is available on the presence of nitrosamines in the human diet. Accumulation of such data is important so that the contribution of nitrosamines to human health hazards can be assessed properly. Several volatile nitrosamines, particularly dimethylnitrosamine (DMNA) and nitrosopyrrolidine (NPy), have been confirmed by mass spectrometry in a variety of cured-meat products (Crosby, Foreman, Palframan & Sawyer, 1972; Fazio, White, Dusold & Howard, 1973; Fazio, White & Howard, 1971; Panalaks, Iyengar, Donaldson, Miles & Sen, 1974; Sen, 1972; Sen, Donaldson, Iyengar & Panalaks, T. 1973; Wasserman, Fiddler, Doerr, Osman & Dooley, 1972). For the most part, the positive samples occurred in a random fashion and contained μg/kg (ppb) concentrations of nitrosamines.

Prior to the finding of NPy in fried bacon (Crosby *et al.* 1972; Fazio *et al.* 1973; Sen *et al.* 1973), it had been suggested that the pyrolysis of protein and the cooking of protein food might produce free amino acids, such as proline, hydroxyproline and arginine and nitrosatable secondary amines, such as pyrrolidine and piperidine (Lijinsky & Epstein, 1970). Bills, Hildrum, Scanlan & Libbey (1973) and Pensabene, Fiddler, Gates, Fagan & Wasserman (1974) reported that NPy was formed in model systems from the decarboxylation of nitrosoproline at temperatures used for frying. Recently, workers in our laboratory identified nitrosoproline in uncooked bacon (Kushnir, Feinberg, Pensabene, Piotrowski, Fiddler & Wasserman, 1975). In addition, we found NPy in the adipose tissue but not in the lean tissue of fried bacon. As a result, we have suggested that collagen may be responsible for NPy formation in fried bacon (Fiddler, Pensabene, Fagan, Thorne, Piotrowski & Wasserman, 1974). Huxel, Scanlan & Libbey (1974) produced NPy from samples of buffered collagen (from bovine achilles tendon) and $NaNO_2$ at temperatures of 120–195°C in a model system. Collagen, the major constituent of skin, tendon and connective tissues, contains large concentrations of glycine, proline and hydroxyproline and is the most abundant mammalian protein (Bodwell & McClain, 1971). Gelatin is commonly prepared by the alkaline treatment of collagen (Fysh, 1958). Gelatin, therefore, may serve as a source of nitrosatable proline, and the product may convert to NPy under certain conditions.

There are a number of meat products made with gelatin and cured non-skeletal and organ meats. This paper reports a limited survey designed to determine the presence of volatile nitrosamines in such products.

Experimental

Seven samples of souse, two of blood and tongue and one of head cheese, produced by seven different manufacturers, were purchased in local retail outlets. Details of the ingredients and processing and storage conditions of these samples were unknown.

These samples were analysed for six volatile nitrosamines, namely DMNA, methylethylnitrosamine, diethylnitrosamine, nitrosopiperidine, NPy and nitrosomorpholine. Concentrations as low as 0·5 μg/kg could be detected and 3 μg nitrosamine/kg could be confirmed by mass spectrometry. Only DMNA and NPy were confirmed by matching the gas–liquid chromatographic (glc) retention times with those of authentic nitrosamines and by peak-matching the exact masses of the nitrosamine parent ions, m/e 74·0480 and 100·06366, respectively, using perfluorokerosene as an internal standard. The procedures for the isolation, separation, detection and confirmation of volatile nitrosamines, including information regarding recoveries from spiked samples, glc and glc–high resolution mass spectrometric conditions have been published elsewhere (Fiddler *et al.* 1974; Kushnir *et al.* 1975; Pensabene *et al.* 1974).

*Agricultural Research Service, US Department of Agriculture.

Precaution should be exercised in the handling of nitrosamines, since they are potential carcinogens.

Results and Discussion

The results of the nitrosamine survey are shown in Table 1. DMNA was confirmed in six of the seven samples of souse and in both samples of blood and tongue in concentrations ranging from 3 to 63 μg/kg. The presence of NPy (19 μg/kg) in one sample of souse was confirmed by mass spectrometry. The sample of head cheese contained no detectable nitrosamine. Residual nitrite varied in the samples from no detectable amount to 135 μg/kg, but there appeared to be no correlation between residual $NaNO_2$ content and the DMNA concentration found.

Table 1. *Nitrosamines in souse and similar products*

Type	No.	Residual $NaNO_2$ (mg/kg)	Content of nitrosamines* (μg/kg)	
			DMNA	NPy
Souse	1	12	26	19
	2	118	63	ND
	3	39	3	ND
	4	4	5	ND
	5	3	6	ND
	6	27	6	ND
	7	ND	ND	ND
Blood and tongue	1	5	45	ND
	2	135	7	ND
Head cheese	1	15	ND	ND

DMNA = Dimethylnitrosamine ND = None detected
NPy = Nitrosopyrrolidine
*Identity confirmed by mass spectrometry.

In a general method for the preparation of souse, non-skeletal meats previously cured with nitrite or nitrite–nitrate are ground through a 1-in. plate and then mixed with gelatin, broth, vinegar and spices. The mixture is heated to 74°C (165°F), poured into moulds and chilled to solidify (Kramlich, Pearson & Tauber, 1973). This temperature is considerably lower than the 185°C (365°F) optimum temperature reported for the decarboxylation of nitrosoproline to NPy (Pensabene *et al.* 1974).

Lecithin (Möhler & Hallermayer, 1973; Pensabene, Fiddler, Doerr, Lakritz & Wasserman, 1975) and its decomposition products (Pensabene *et al.* 1975) have been shown to form DMNA when reacted with nitrite in model systems. Since non-skeletal tissues may contain higher concentrations of lecithin than does skeletal tissue (Dugan, 1971), the former may be a source of DMNA.

The nitrite available for nitrosamine formation could arise from the initial cooking of the cured non-skeletal meats; the broth resulting from the tenderizing process may be used in the preparation of souse.

Currently our laboratory is investigating the source of DMNA in products containing gelatin. While the jellied products described in this paper constitute only a very small portion of the total production of cured meats in the United States, they may play a more important dietary role elsewhere.

REFERENCES

Bills, D. D, Hildrum, K. I., Scanlan, R. A. & Libbey, L. M. (1973). Potential precursors of *N*-nitrosopyrrolidine in bacon and other fried foods. *J. agric. Fd Chem.* **21**, 876.

Bodwell, C E. & McClain, P. E. (1971). Chemistry of animal tissues, proteins. In *The Science of Meat and Meat Products* 2nd ed. Edited by J. F. Price and B. S. Schweigert. p. 112. W. H. Freeman and Co., San Francisco, Cal.

Crosby, N. T., Foreman, J. K., Palframan, J. F. & Sawyer, R. (1972). Estimation of steam-volatile N-nitrosamines in foods at the 1 μg/kg level. *Nature, Lond.* **238**, 342.

Dugan, L. R., Jr. (1971). Chemistry of animal tissues, fats. In *The Science of Meat and Meat Products*. 2nd ed. Edited by J. F. Price and B. S. Schweigert. p. 142. W. H. Freeman and Co., San Francisco, Cal.

Fazio, T., White, R. H., Dusold, L. R. & Howard, J. W. (1973). Nitrosopyrrolidine in cooked bacon. *J. Ass. off. analyt. Chem.* **56**, 919.

Fazio, T., White, R. H. & Howard, J. W. (1971). Analysis of nitrite- and/or nitrate-processed meats for *N*-nitrosodimethylamine. *J. Ass. off. analyt. Chem.* **54**, 1157.

Fiddler, T., Pensabene, J. W., Fagan, J. C., Thorne, E. J., Piotrowski, E. G. & Wasserman, A. E. (1974). The role of lean and adipose tissue in the formation of nitrosopyrrolidine in fried bacon. *J. Fd Sci.* **39**, 1070.

Fysh, D. (1958). The influence of the mode of preparation on the physical properties of gelatin. In *Recent Advances in Gelatin and Glue Research*. Edited by G. Stainsby. p. 141. Pergamon Press, London.

Huxel, E. T., Scanlan, R. A. & Libbey, L. M. (1974). Formation of N-nitrosopyrrolidine from pyrrolidine ring containing compounds at elevated temperatures. *J. agric. Fd Chem.* **22**, 698.

Kramlich, W. E., Pearson, A. M. & Tauber, F. W. (1973). *Processed Meats*. p. 218. Avi Publishing Co., Inc., Westport, Conn.

Kushnir, I., Feinberg, J. I., Pensabene, J. W., Piotrowski, E. G., Fiddler, W. & Wasserman, A. E. (1975). The isolation and identification of nitrosoproline in uncooked bacon. *J. Fd Sci.* **40**, 427.

Lijinsky, W. & Epstein, S. S. (1970). Nitrosamines as environmental carcinogens. *Nature, Lond.* **225**, 21.

Möhler, K. u. Hallermayer, E. (1973). Bildung von Nitrosaminen aus Lecithin und Nitrit. *Z. Lebensmittelunters. u. -Forsch.* **151**, 52.

Panalaks, T., Iyengar, J. R., Donaldson, B. A., Miles, W. F. & Sen, N. P. (1974). Further survey of cured meat products for volatile N-nitrosamines. *J. Ass. off. analyt. Chem.* **57**, 806.

Pensabene, J. W., Fiddler, W., Doerr, R. C., Lakritz, L. & Wasserman, A. E. (1975). The formation of dimethylnitrosamine from lecithin and its components. *J. agric. Fd Chem.* In press.

Pensabene, J. W., Fiddler, W., Gates, R. A., Fagan, J. C. & Wasserman, A. E. (1974). Effect of frying and other cooking conditions on nitrosopyrrolidine formation in bacon. *J. Fd Sci.* **39**, 314.

Sen, N. P (1972). The evidence for the presence of dimethylnitrosamine in meat products. *Fd Cosmet. Toxicol.* **10**, 219.

Sen, N. P., Donaldson, Barbara, Iyengar, J. R. & Panalaks, T. (1973). Nitrosopyrrolidine and dimethylnitrosamine in bacon. *Nature, Lond.* **241**, 473.

Wasserman, A. E., Fiddler, W., Doerr, R. C., Osman, S. F. & Dooley, C. J. (1972). Dimethylnitrosamine in frankfurters. *Fd Cosmet. Toxicol.* **10**, 681.

Fd Cosmet. Toxicol. Vol. 13, pp. 655–661. Pergamon Press 1975. Printed in Great Britain

Review Section

THE HISTORY AND USE OF NITRATE AND NITRITE IN THE CURING OF MEAT*

E. F. BINKERD and O. E. KOLARI
Armour and Company, Food Research Division, Oak Brook, Illinois 60521, USA

(*Received 3 February 1975*)

The curing of meat is today based in part upon the art as practised through aeons of time and perhaps to a far greater extent upon sound scientific principles developed since about the turn of the century. It is the purpose of this review to describe briefly the early curing of meat, reaching deep into antiquity, to review curing practices from colonial to modern times, to outline the basic studies elucidating the use of nitrite, to discuss the studies leading to current regulations governing the use of nitrate and nitrite and to describe current practice in the industry. This discussion is not intended as a reference work on the actual curing of meat but rather as a survey of the chain of events leading to industry practice in the use of nitrate and nitrite.

The origin of the use of nitrate in the curing of meat is lost in history, but it is certain that the preservation of meat with salt preceded the intentional use of nitrate by many centuries.

The preservation of cooked meats and fish in sesame oil in jars was practised as early as 3000 BC in Mesopotamia. Early Summerians enjoyed dried salted meat and fish as part of their diet. Indeed, salt was considered a dietary essential by the ancient Babylonians. Salt was in common use in the ancient Jewish Kingdom as early as 1600 BC because of its availability from the salt-rich Dead Sea. The technology of sea-salt production was also known by at least 1200 BC by the Chinese, who early made salt from drilled wells (Jensen, 1953 & 1954).

It appears that meat preservation was first practised in the saline deserts of Hither Asia and in coastal areas. Desert salts contained nitrates and borax as impurities. However, the reddening effect of nitrates was not mentioned until late Roman times.

Thus, saltpetre, or 'nitre', was gathered in ancient China and India long before the Christian era. Wall saltpetre ($Ca(NO_3)_2$), found as an efflorescence on the walls of caves and stables, was used by the ancient peoples in the curing of meat. This form of nitrate is formed by nitrifying bacteria (Jensen, 1954). It is reasonable to suggest that desert and wall salts, because of their nitrate contaminants, yielded cured meats that were distinctive not only in colour but also in flavour.

Early Phoenicians from the Eastern edge of the Mediterranean were a seafaring people who traded salt-fish, among other things, as early as 1200 BC (Batterson & Boddie, 1972). In Europe the salt mines of Austria flourished in trade with their neighbours in the early Iron Age (900 BC). The ancient Greeks freely used salt in the preservation of fish prior to 500 BC and "manufactured" salt in "salt gardens" (Jensen, 1954).

The Romans learned the use of salt from the Greeks and cured pork and fish extensively. Salt was apparently in scarce supply, as judged by the etymology of the word 'salary' (*salarium* or allowance of salt). By the time of Homer (900 BC), the curing of meat with salt and by smoking were old practices. The Romans learnt how to pickle various kinds of meat, and the pickle contained other ingredients in addition to salt. They established a trade for these products in the Roman Empire (Jensen, 1954).

Cato (234–149 BC) gave excellent directions for the dry-curing of hams, a process which included an overhaul, rubbing with oil, smoking and again rubbing with a mixture of oil and vinegar. Columella (1st century AD) also gives detailed directions for the dry-curing of pork. Interestingly enough, toasted salt was recommended for dry-curing in a hot climate. One can only speculate on the purpose of the toasting—was it to destroy micro-organisms or to convert some of the nitrate to nitrite (assuming that nitrate was a natural contaminant of the salt)? The use of salt and saltpetre in meat curing was commonplace in mediaeval times, and the effect of saltpetre on colour was recognized. Gradually, sweet pickle and sugar cures evolved as sugar became available, although the use of honey in curing had been practised for a long time.

Edward Smith wrote in 1873: "The oldest and best known preserving agent is salt, with or without saltpetre". He ascribed the preserving action of salt to "its power of attracting moisture, and by thus extracting fluid to harden the tissues". Smith described the common methods of curing as: "Simply rubbing the surface of the meat with salt, or immersing the meat in a strong solution of salt with the addition of saltpetre; and in order that they may be effectual, it is necessary that the meat to be salted should be of

*Presented at the Meeting of the USDA Expert Panel on Nitrites and Nitrosamines, Washington, D.C., 19 June 1974.

a newly killed animal". Smith cautioned: "Meat thus prepared by salt alone loses its colour, but when saltpetre is added the flesh becomes of a reddish colour throughout, provided the action be sufficiently prolonged". The brine solution recommended was "made of 4 lb. of salt and 1/4 lb. of saltpetre in 6 pints of water". The concentration of saltpetre in this formula was a hefty 2·38% or 23,800 ppm. The National Provisioner in its "Handbook" of 1894, gave the following pickle formula for 100 lb of hams packed in regular tierces: 7·66 lb salt, 2 lb sugar and 0·33 lb (3333 ppm, assuming complete absorption) saltpetre and water to make an 80 degree pickle (National Provisioner, 1952).

Salt and saltpetre were commonly used in South America at and before this time in converting dried strips of flesh into a product called "charqui". Amazingly enough, a crude apparatus was described in 1854 for infusing a curing solution throughout the arterial system of a freshly slaughtered bullock (Smith, 1873). The curing solution was described as a preserving material composed of water, saltpetre and salt, with or without flavouring matter. An early reference to saltpetre in curing is in the Farmer's Register of 1835 (Cited from *Red Hot News*, 1968). Saltpetre was claimed to impart to bacon a juiciness and flavour that nothing else could supply. Saltpetre, in the dry-curing method alluded to, was used in "rather stinted" quantities. Actually, about 0·5 lb (5000 ppm) saltpetre was used for each 100 lb meat. A great preservative power was attributed to saltpetre.

In the 19th century, meat curing was an art managed by the rule of thumb. A fledgling packing industry had burgeoned into a major industry, with methods that were antiquated by today's standards. An "expert", who jealously guarded the secrets kept in his little black book or under lock and key, presided over the curing department. But science was on the move. Chemists were starting to penetrate other industries and to apply reason and scientific method to problems that had been viewed with emotion. The first chemists joined the larger packing houses 10 years before the close of the 19th century. "One by one, exact methods of procedure and control were worked out until at the present time spoilage of meats in cure has been reduced to an almost negligible quantity" (Richardson, 1907). Even today, some spoilage of meat can be expected with the ancient long, dry-cure method.

Toward the end of the 19th century, various methods of curing meat were in common use. Dry cure, wet or pickle cures and combinations of the two were in existence. The practice of pumping meats probably started in the early 1890s (National Provisioner, 1952). Before the use of a perforated needle, it was customary to pack saltpetre and salt into an incision in the shank of a ham with a round stick.

Significant changes in meat curing had occurred by the 19th century; wet or pickle curing had become established, rudimentary vascular pumping of curing fluids had emerged, the perforated pumping needle had appeared, saltpetre was firmly entrenched in curing formulae, science and technology and quality control became an integral part of the packing industry, the mechanism of microbial conversion of nitrate to nitrite was understood and the development of cured colour was ascribed to nitrite. The meat-packing industry had arrived and "grown of age".

Various meat-curing mixtures were used in the 1920s. Most mixtures contained salt, sugar and saltpetre. Tomhave reported in 1925 a most interesting recipe for Smithfield hams: "The hams are placed in a large tray of fine salt, then the flesh surface is sprinkled with finely ground saltpetre until they are as white as though covered by a moderate frost, or, to be more exact, use four or six ounces of the powdered saltpetre to each one hundred pounds of green hams". The hams were then packed in salt for 3 days, overhauled, retained in pack for 1 day per pound of ham, washed and partially dried, finely ground pepper was rubbed on the surface, and then they were smoked for 30–40 days.

Tomhave (1925) also suggested a combination dry and wet method of sugar-curing ham, bacon and tongues: dry cure with salt for 8–10 days, then cure 400 lb meat in a pickle made of 5 lb brown sugar and "a small handful of saltpetre", a tablespoon of ginger and 12 lb salt. A 75-degree cover pickle was recommended and, for the handyman, these words of advice were given: "Drop a fresh egg into the pickle; if the egg floats almost submerged, the brine is of the proper strength". Others in this country had often used a potato as a measure of the strength of the pickle.

When nitrite *per se* was first used in curing meat is unknown. Many old recipes for curing stressed the use of a small amount of Sal Prunella to acquire a good colour in the meat. Sal Prunella is prepared from a fused mixture of nitrate and sulphur. Thus, it probably contains some nitrite. The US Dispensatory of 1847 gives directions for preparing Sal Prunella and cites an earlier French pharmacopoeia (Tauber, 1972).

The advent of nitrite in curing was presaged by the classical studies of a handful of scientists. Polenske, in 1891, reported finding nitrite in cured meat and curing pickle. He correctly concluded that the source of nitrite was the bacterial reduction of nitrate. Close on the heels of this discovery, Lehman in 1899 and Kisskalt in 1899, demonstrated that the typical colour of cured meats was due to nitrite and not to nitrate. Haldane (1901) was interested in the colouring matter responsible for the redness of cooked, cured meats. He demonstrated the formation of nitrosohaemoglobin by the addition of nitrite to haemoglobin. Haldane further demonstrated the breakdown of nitrosohaemoglobin to nitrosohaemochromogen and identified the latter as being responsible for the red colour of cooked meat. Hoagland (1908) confirmed Haldane's work by a series of studies with haemoglobin, cured meats and sausages. He explained the reduction of nitrate to nitrite, nitrous acid and nitric oxide (the intermediate) by either bacterial or enzyme action or both. He indicated that the colour of uncooked meats cured with saltpetre was due to nitrosohaemoglobin, and the colour of cooked meats to nitrosohaemochromogen. The colour change during cooking was described as the breakdown of the pigment "into the two constitutents: hematin, the colouring group, and the proteid".

Recognizing the significance of nitrite in curing,

Glage, of Germany, recommended in 1909 the use of partially reduced nitrate (by dry heat) in curing. Shortly after (1913–1915) proprietary curing mixtures containing nitrite were marketed in Germany and Europe and, in 1917, a US patent was issued to Doran on the use of nitrite in curing.

An understanding of the basic chemistry and reactions involved in the colour of cured meat and of the role of micro-organisms in reducing nitrate to nitrite led to studies in this country on the use of nitrite in curing. Up to this time, unsatisfactory or irregular colour development and spoilage of meats during the curing process were unexplainable and plagued the industry. The variability in the bacterial reduction of nitrate to nitrite in pickles was especially noted in new establishments. The adjustment of pH and bacterial 'seeding' of the pickle appeared helpful, although the growth of an undesirable flora in pickle resulted in failure. The increase in nitrite concentration in a nitrate pickle as a result of bacterial reduction is illustrated by the data of Kerr, Marsh, Schroeder & Boyer (1926). The concentration of nitrite increased from 3·6 ppm on the first day to 33 ppm at 15 days and to 887 ppm at 65 days. A solution to this problem of variability in the nitrite content of pickle, with its resultant product variability, was the direct addition of a closely controlled amount of nitrite to either pickle or product.

Permission for the direct use of nitrite by a meat processor, under Federal inspection, was first given on 19 January 1923 by the Bureau of Animal Industry of the United States Department of Agriculture. The first and subsequent series of experiments were closely supervised by Bureau inspectors (Kerr *et al.* 1926).

Authorization of the first experiment in which nitrite was used carried the stipulation that the meat would be destroyed if it contained excessive amounts of nitrites or was otherwise unwholesome or unfit to eat. Kerr *et al.* (1926) had previously determined the nitrite content of 54 samples of various cured meats, which were typical of a nitrate cure, such as ham, corned beef and bacon. Of the samples analysed, 14 exceeded 200 ppm nitrite (average, 485 ppm; range, 208–960 ppm). These data indicated that the nitrite content of meat cured by the processes of the time (nitrate only) yielded extremely variable and, at times, high levels of nitrite in the product.

In the first experiment, 92 pieces of meat weighing 1142 lb were cured in pickle containing approximately 2000 ppm sodium nitrite. A further 94 pieces of bacon weighing 1270 lb were dry-cured with nitrite. The controls consisted of similar products cured with the usual amounts of nitrate. Analysis of the hams indicated an average nitrite content of 42–150 ppm in nitrite-cured hams. The maximum nitrite content of any part of the ham was 200 ppm. Nitrate-cured hams had a maximum nitrite content of 45 ppm nitrite. The flavour and quality of the nitrite-cured meats were equal to those cured by the traditional process, and judges were unable to distinguish meats cured by either method.

Quickly, other experiments were authorized at the original establishment and elsewhere and, on the basis of the first experiment, 200 ppm nitrite was placed as a tentative limit on the nitrite content of the finished meats. Experiments were conducted in a total of 17 establishments ranging in size from the largest to the smallest. All types of products were cured, including items such as shoulders, loins, tongues, hams, bacon and corned and dried beef. The experiments lasted for 2 years and 8 months and the quantity of meat cured was on a commercial scale. Thus, results of these full-scale operations were directly applicable to commercial meat curing.

Experiments in which nitrite was added to sausage demonstrated that the curing period could be materially shortened, an observation similar to that noted with cuts of meat such as ham and bacon. The studies indicated "that 1/4 oz. (156 ppm) or less of sodium nitrite is sufficient to fix the color in 100 lb. of sausage meat". Typical nitrite values in frankfurters, bologna, luncheon meat and minced luncheon meat ranged from 40–150 ppm. On the basis of these studies, Kerr *et al.* (1926) concluded:

> (1) "From one-fourth to 1 oz. of sodium nitrite is sufficient to fix the color in 100 lb, the exact quantity depending on the meat to be cured and process to be employed."
> (2) "Meats cured with sodium nitrite need contain no more nitrites than meats cured with nitrates, and are free from the unconverted nitrates regularly present in nitrate-cured meats."
> (3) A shortening of the customary curing period may be obtained by the use of nitrite.

On the basis of the results obtained in these experiments, the use of sodium nitrite in meat-curing in Federally inspected establishments was formally authorized by the USDA in 1925 (United States Department of Agriculture, 1926). The 19 October authorization by the Bureau of Animal Industry stated:

> "Extended experiments have demonstrated that successful curing may be accomplished by the addition of as small a quantity as one-fourth of an ounce of sodium nitrite to each 100 pounds of meat; therefore, pending further ruling by the Bureau the finished product shall not contain sodium nitrite in excess of 200 parts per million."

Regulations issued in 1970 under the Meat Inspection Regulations, USDA Consumer and Marketing Service (Federal Register 1970, **35**, 15590) permitted the addition of sodium or potassium nitrate at 7 lb to 100 gall. pickle, 3·5 oz to 100 lb meat in dry cure, or 2·75 oz to 100 lb chopped meat. Sodium or potassium nitrite was permitted at 2 lb to 100 gall. pickle at the 10% level of pump, 1 oz to 100 lb meat in dry cure, or 0·25 oz to 100 lb chopped meat and/or meat by-product. The use of nitrites, nitrates or combinations thereof could not result in more than 200 ppm nitrite in the finished product.

The composition of curing formulae used in the meat-packing industry immediately after the BAI order of 1925 permitting the use of nitrite is not precisely known. However, Mohler reported in 1930 that 54% of the packers were using sodium nitrate, 17% sodium nitrite and 30% mixed pump pickles.

In 1936, Mighton, of the Institute of American Meat Packers, determined the amount of nitrate and nitrite in cured meats obtained at retail (Table 1). He was especially interested in the effect of the Federal limitation of 200 ppm nitrite upon the pro-

Table 1. *The nitrate and nitrite contents of cured meats obtained at retail in 1936*

Meat product	No. of samples	Levels (ppm) of Nitrite Average	Nitrite Range	Nitrate Average	Nitrate Range
Smoked hams	6	52	7–145	360	120–610
Picnics	6	54	9–136	160	trace–310
Boiled hams	10	59	11–87	370	30–910
Canned spiced hams	6	17	5–55	700	100–1030
Canned corned beef	6	3	3–5	200	160–270
Pork butts	6	86	31–232	330	100–680
Frankfurters	6	84	55–146	710	570–880
Bologna	6	61	44–86	380	30–790
Raw bacon	6	13	4–22	1400	700–2100
Fried bacon	6	19	4–58	3900	2400–4800

Data from Mighton (1936).

Table 2. *Survey of cured meats in 1937 to determine nitrate and nitrite concentrations*

Meat product	No. of brands	Levels (ppm) of Nitrite Average	Nitrite Range	Nitrate Average	Nitrate Range
Hams	5	80	34–184	600	100–1200
Bacon	5	16	11–29	1200	300–1900
Hams, boiled	5	49	31–63	700	300–1100
Beef, corned	5	75	1–216	1700	500–3000
Bologna	6	100	87–118	210	10–590
Frankfurters	15	54	20–94	815	460–1170
Bologna	5	72	60–114	650	100–1020
Frankfurters	12	69	42–116	815	460–1090
Frankfurters	8	102	13–195	513	100–1090

Data from Lewis (1937).

Table 3. *Nitrite content of cured meat products analysed by USDA MIP Laboratory, Chicago, Ill., in 1970*

Meat product	No. of samples	Nitrite level (ppm) Range	Average
Beef brisket	12	0–10	3
Smoked hams	8	0–120	53
Smoked picnics	8	0–100	46
Pork belly	3	50–100	75
Bacon	6	5–125	43
Pork jowls	3	50–75	67
Beef tongue	7	0–20	8
Meat loaves	16	0–65	19
Frankfurters	21	0–90	21
Bologna	11	0–50	19
Knockwurst	7	0–60	38
Polish sausage	7	0–60	14

Data from American Meat Institute Foundation (1971).

ducts sampled. Mighton concluded: "The most significant thing brought out by this current survey of cured meats is the growing mildness of the cure and the low content of nitrite of soda". A scant 10 years earlier, Kerr *et al.* (1926) had reported on the relatively high level and variability of nitrite in cured products made by the old nitrate process.

Lewis, also of the Institute of American Meat Packers, reported in 1937 an analytical survey of American cured meats as a measure of industry prac-

Table 4. *The nitrite and nitrate content of cured meat products analysed by the AMI Laboratory, Chicago, Ill., in 1970*

Meat product	Levels (ppm) of					
	Nitrite			Nitrate		
	No. of samples	Range	Average	No. of samples	Range	Average
Polish ham	6	10-24	17	6	155–215	173
Ham	16	6–480	54	3	300–700	399
Bacon	22	1–272	60	14	0–310	78
Picnics	4	2·5–22	10	—		—
Bologna	5	43–92	56	1	—	170
Frankfurters	3	0–120	78	—	—	—

Data from American Meat Institute Foundation (1971).

tice and considered how well they came within the BAI order of 1925 permitting the use of nitrite (Table 2). He concluded that the data were typical for commercially cured meats of that era, and that cured meats were well within the regulatory limit of 200 ppm nitrite.

Early in 1970, there was considerable interest in this country and worldwide, especially in Europe, in the amounts of nitrite in various cured-meat products. Because of this interest, data on the residual levels of nitrite were obtained that year by the American Meat Institute Foundation from the USDA Meat Inspection Program Laboratory and the American Meat Institute Laboratory, both located in Chicago, Ill. Data on the nitrite content of various cured-meat products analysed during 1970 by the Chicago MIP laboratory are given in Table 3 (American Meat Institute Foundation, 1971); similar data obtained from the American Meat Institute Laboratory appear in Table 4 (*idem* 1971). Although the data are limited, the results of these two 1970 surveys indicated that the average nitrite content of commercially cured meats was low and was far less than that permitted by Federal regulations.

American Meat Institute Foundation workers (Kolari & Aunan, 1972) again surveyed the amounts of nitrite in selected cured-meat products in 1972 (Tables 5 & 6). In this survey, analytical data for shelf-stable and pasteurized meat products (Table 5) were obtained from a firm in the meat industry. The data in Table 6 were obtained from another firm, data being pooled from five of its plants between December 1971 and June 1972. In these studies, the products were analysed for nitrite 1–2 days after production.

Average nitrite levels reported by industry in 1972 for shelf-stable, pasteurized (Table 5) and other cured-meat products (Table 6) were again far below those permitted by Federal regulations. The amounts of nitrite used in producing the various products were determined from actual processing calculations (Table 5) or from amounts permitted by Federal regulations (Table 6).

Because of the interest in amounts of nitrite and/or nitrate used by meat processors, the ranges of these materials used in 1970 in smoked cured meats were surveyed by the American Meat Institute Foundation (1971) and by the American Meat Institute in 1974

Table 5. *Calculated input, range and average nitrite contents of selected shelf-stable and pasteurized meat products in 1972*

Meat product	Calculated input (ppm)	No. of samples	Nitrite level (ppm)	
			Range	Average
Shelf-stable				
Lunch meat	144	20	13–61	48
	144	30	32–65	47
Vienna sausage				
(packed in meat broth)	124	33	6–16	8
Canned ham	172	34	3–37	11
Pasteurized				
Bulk lunch meat	144	30	13–172	45
	144	33	13–180	28
	144	30	32–102	45
	144	31	14–128	43
Canned ham	172	34	17–130	62
	172	32	26–128	54
	111	34	18–55	34

Data from Kolari & Aunan (1972).

Table 6. *Nitrite concentration in selected cured meat products reported by industry in 1972*

Meat product*	No. of samples	Nitrite levels (ppm)	
		Average	Range
Frankfurters	34	38·2	15–80
Cold cuts (Bolo sausage)	23	35·5	0–76
Bacon	128	95·7	24–170
Smoked ham	56	47·6	16–100
Other smoked meats	18	79·4	19–115
Canned hams	93	55·8	0–160
Miscellaneous	83	48·5	0–178

*Comminuted products manufactured with a theoretical input of 156 ppm nitrite; others prepared with a maximum of 200 ppm nitrite. Data from Kolari & Aunan (1972).

Table 7. *Comparison of the range of input of nitrate and nitrite in 1970* and *1974*

Meat product	Levels of input (ppm)			
	Nitrate		Nitrite	
	1970	1974	1970	1974
Canned refrigerated meats	0–1150	0–558	50–190	104–200
Canned shelf-stable meats	0–1150	0	50–170	77–200
Frankfurters	NA	0	NA	100–156
Other perishable cured meats	0–1140	0–100	78–156	100–200
Fermented sausage	0–1328	0–625	56–156	90–156
Semi-dry sausage	0–1328	0–625	56–156	56–156
Bacon	0–200	0	78–200	120–200
Other pumped primal meats	NA	0	NA	115–200
Country style ham	0–2200	0–2184	0–185	0–156
Other dry cured primal meats	0–2200	0–2184	0–185	0–156
Canned sterile cured meats	0–125	0	88–190	120–200

NA = Not available
Data from the American Meat Institute Foundation (1971) and the American Meat Institute (unpublished data, 1974).

(unpublished data). The data were obtained from the major meat-packing firms and are summarized in Table 7.

In 1970, approximately 50% of the processors surveyed reported that they were not using nitrate in curing shelf-stable meats, such as canned hams. In 1974, all of the manufacturers surveyed had discontinued the use of nitrate in these products. A similar trend was noted for refrigerated canned meats and other perishable cured meats. In 1970, about 33% of the processors surveyed indicated that they had eliminated the use of nitrate in these products, compared with 78% in 1974. By 1974, nitrate was eliminated from cured bacon, other pumped primal meats, canned sterile meats and frankfurters. In addition to the marked decline in the number of surveyed companies using nitrate in the various product categories, a marked decline was also apparent between 1970 and 1974 in the highest amount of nitrate added to a given product by any one company. For example, the highest concentration of nitrate used decreased from 1150 to 558 ppm for canned refrigerated meats, from 1150 to 0 ppm for canned shelf-stable meats, from 1140 to 100 ppm for other perishable meats, and from 1328 to 625 ppm for fermented and semi-dry sausages. On the other hand, only minor differences were noted between 1970 and 1974 in the amounts of nitrite added to the various meat products. The highest levels added were consistent with the amounts permitted under Federal regulations.

REFERENCES

American Meat Institute Foundation (1971). The use of nitrate and nitrite in the meat industry. *Meat Sci. Rev.* **5**, 1.

Batterson, M. & Boddie, W. W. (1972). Salt the Mysterious Necessity, p. 15. Dow Chemical Co., Midland, Mich.

Cato (234–149 BC), *Cato and Varro*. Loeb Classical Library, Harvard University Press. pp. 89, 155.

Columella (1st century AD). *Columella III*. Loeb Classical Library, Harvard University Press. p. 327.

Doran, G. F. (1917). Art of curing meats. U.S. patent 1,212,614.

Glage, M. (1909). Die Konservierung der Roten. Fleischfarbe, p. 27.

Haldane, J. (1901). The red color of salted meat. *J. Hyg., Camb.* **1**, 115.

Hoagland, R. (1908). The action of saltpeter upon the color of meat. United States Department of Agriculture, Bureau of Animal Industry, 25th Ann. Rept. p. 301. Washington. D.C.

Jensen, L. B. (1953). *Man's Foods.* p. 170. The Garrard Press, Champaign, Ill.

Jensen, L. B. (1954). *Microbiology of Meats.* 3rd Ed., p. 6. The Garrard Press, Champaign, Ill.

Kerr, R. H., Marsh, C. T. N., Schroeder, W. F. & Boyer, E. A. (1926). The use of sodium nitrite in curing meat. *J. agric. Res.* **33**, 541.

Kisskalt, K. (1899). Beitrage zur Kenntnis der Ursachen des Rotwerdens des Fleisches beim Kochen, nebst einigen Versuchen über die Wirkung der schwefligen Säure auf die Fleischfarbe. *Arch. Hyg. Bakt.* **35**, 11.

Kolari, O. E. & Aunan, W. J. (1972). The residual levels of nitrite in cured meat products. *Meat Sci. Rev.* **6**, 27.

Lehman, K. B. (1899). Über das Haemorrhodin, ein neues weitverbreitetes Blutfarbstoffderivat. *Sber. phys.-med. Ges. Würzb.* **4**, 57.

Lewis, W. L. (1937). The Use of Nitrite of Soda in Curing Meat. Institute of American Meat Packers, Chicago, Ill.

Mighton, C. J. (1935). An Analytical Survey of Cured Meats. Publn 33, p. 22. Institute of American Meat Packers. Chicago, Ill.

Mohler, J. R. (1930). Incidence and prevention of ham souring. In *Some Observations on Curing.* Edited by W. L. Lewis. p. 29. Institute of American Meat Packers. Chicago, Ill.

National Provisioner (1952). The Significant Sixty. Section 2, p. 266. Chicago, Ill.

Polenske, E. (1891). Über den Verlust, welchen das Rinkfleisch und Nahrwert durch das Pokein erleidet, sowie über die Veränderungen salpeterhaltiger Pokellaken. Arb. K. *GesundhAmt.* **7**, 471.

Red Hot News (1968). They just don't make 'em like they used to. *ibid* (Union Carbide Corp., Chicago, Ill.) **30**, no. 2.

Richardson, W. D. (1907). Nitrates in vegetable foods, in cured meats and elsewhere. *J. Am. chem. Soc.* **29**, 1757.

Smith, E. (1873). *Foods.* p. 35. D. Appelton and Co., N.Y.

Tauber, F. W. (1972). Curing of meat and meat products. *Red Hot News* (Union Carbide Corp. Chicago, Ill.) **34**, 279.

Tomhave, W. H. (1925). *Meats and Meat Products.* p. 279. J. B. Lippincott Co., Philadelphia.

United States Department of Agriculture, Bureau of Animal Industry (1926). USDA, BAI Order 211, Amendment 4, 1925, p. 1 (revised).

Fd Cosmet. Toxicol. Vol. 13, p. 663. Pergamon Press 1975. Printed in Great Britain

REVIEWS OF RECENT PUBLICATIONS

Evaluation of Certain Food Additives. Eighteenth Report of the Joint FAO/WHO Expert Committee on Food Additives. Tech. Rep. Ser. Wld Hlth Org. 1974, no. 557. pp. 37. Sw. fr. 5*. (Also issued as *F.A.O. Nutr. Mtg Rep. Ser.* no. 54).

Toxicological Evaluation of Some Food Colours, Enzymes, Flavour Enhancers, Thickening Agents, and Certain Other Food Additives. Joint FAO/WHO Expert Committee on Food Additives. WHO/Food Add./74.6, Geneva, 1975. pp. 204. Sw. fr. 13*. (Also issued as *F.A.O. Nutr. Mtg Rep. Ser.* no 55).

These two publications are the result of a meeting of the Expert Committee held in Rome on 4–13 June 1974.

The first of the reports notes a new decision by the Committee that the acceptable daily intake (ADI) should, as a general rule, include the amounts of a substance naturally present in food, although some exceptions would be permissible. In the light of this change of policy, the ADIs previously allocated for ascorbic acid and benzoic acid will now include the small amounts naturally present, and the position with regard to nitrates, where natural levels contribute significantly to the total intake, will be reviewed in the near future. The Committee decided to use the term "ADI not specified" in place of the previous "ADI not limited" and to provide a detailed definition of this term in a footnote each time it appears. In the case of substances causing only a low incidence of hypersensitivity, or only minor reactions, ADIs would be established, but the Committee hoped that such hazards could be minimized by the appropriate labelling of foods containing such additives.

The Committee also considered the question of specifications, and noted that in many cases it might be possible to lower the limits for arsenic, lead and heavy metals through the application of current technology and good manufacturing practice. Attention was drawn to the possibility that chromium and/or zinc residues may occur in food colourings, in the manufacture of which certain oxidizing and/or reducing agents are employed, and maximum levels of 20 mg/kg for chromium and 200 mg/kg for zinc were recommended, with a limit of 40 mg/kg for other heavy metals. For aromatic amines, the general limit of 0·02% proposed at an earlier meeting was retained until further information on the nature and levels of these contaminants could be obtained.

The additives evaluated fell into the general categories of flavouring substances and enhancers, colourings, food enzymes, thickening agents and a miscellaneous group. In most cases a maximum ADI was recommended, while in some only a temporary acceptance was given and in others no ADI was specified because the Committee considered that the total daily intake resulting from the necessary levels of use and the background levels in food represented no hazard to health. No ADI was allocated for a number of additives because the data provided were inadequate for a satisfactory evaluation. Monographs on the toxicology of most of these additives are included in the second of the publications cited above.

In addition, the Committee prepared new specifications for many of these additives, or revised existing specifications, and specifications were also prepared or revised for a further 22 compounds not considered from the toxicological viewpoint. These will form the subject of a separate publication.

* Obtainable in the United Kingdom through HMSO.

Fd Cosmet. Toxicol. Vol. 13, pp. 665–666. Pergamon Press 1975. Printed in Great Britain

BOOK REVIEWS

Residue Reviews. Residues of Pesticides and Other Contaminants in the Total Environment. Vol. 51. Edited by F. A. Gunther. Springer-Verlag, Berlin, 1974. pp. ix + 189. DM 48·90.

The opening chapters of this volume of *Residue Reviews* are concerned with diazinon (*O,O*-diethyl *O*-(2-isopropyl-4-methy-6-pyrimidinyl) phosphorothioate), a broad-spectrum insecticide effective against a variety of fruit, vegetable and soil pests including locusts, caterpillars, beetles, maggots, flies and aphids. Formulations of diazinon include wettable powders, emulsifiable solutions and aerosol sprays. Procedures for the analysis of these are presented in the first chapter, together with the procedures for the analysis of the technical-grade product, and for the determination of 'pure' diazinon by titration or gas chromatography. In addition, extraction and clean-up procedures for residue analyses are described, as well as various analytical methods relevent to the identification or quantitative estimation of diazinon residues. The estimation of diazinon residues in about 70 agricultural products is reported in the second chapter. The data stem from experiments carried out in different climates and involving different rates of application. It has been estimated that an average of 3 ppm diazinon is found in soil immediately after treatment and the half-life period is 2–4 weeks, the rate of degradation being influenced by the nature of the soil and climatic conditions. As far as the USA is concerned, analysis of total diet samples has led to the conclusion that the maximum possible dietary intake of diazinon by man is in the region of 0·005 μg/kg body weight, an intake some 400 times less than the ADI established by WHO.

Poultry may be exposed to pesticides by ingestion of contaminated feed or through the use of pesticides in poultry houses. Fortunately, the lethal doses—though variable—are generally quite high and are unlikely to be encountered except in cases of accidental contamination. Lower levels of some pesticides can, however, have adverse effects on growth, egg production, egg size, shell thickness, fertility and chick viability. The results of an extensive literature search pertaining to such effects are presented in a chapter entitled "Physiological and biological effects of pesticide residues in poultry". In his conclusion, the author draws attention to the need for continued research, suggesting that a greater uniformity of approach would facilitate comparison of the data obtained. In many cases very little research has been carried out on the physiological effects of metabolites and it is suggested that all residue studies should include major metabolites as well as parent compounds. The author also considers that there is a need for more research on the effects that long-term ingestion of pesticides may have on various biological and physiological functions.

Following the introduction of Guthion (azinphos-methyl) in the USA for use against cotton-infesting insects, the compound has found widespread use against a variety of other insects in field crops such as potatoes, sugar cane, alfalfa, clover and many vegetables. Its broad spectrum of effectiveness, coupled with its relatively low dermal toxicity, lack of phytotoxicity and acceptable rate of degradation has made Guthion the product of choice for many growers, particularly in the USA and Canada. The final chapter of Volume 51 contains a detailed consideration of this insecticide. Toxicological data are included as well as recommended analytical methods for estimating residues in plant and animal tissues and milk, and reference is made to the product's specific use in a wide variety of applications. Details are also given of the tolerances established in the USA and Canada for Guthion residues in crops and food products. The authors conclude that since few instances of resistance or insect tolerance to Guthion have become evident after many years of field application, the chemical should find many more years of effective use in agriculture.

Pollution Criteria for Estuaries. Proceedings of the Conference held at the University of Southampton, July 1973. Edited by P. R. Helliwell and J. Bossanyi. Pentech Press, London, 1975. pp. 326. £10.95.

The adverse effects that may result from changes in the environment are often discussed in general terms. This book, which reports the proceedings of a symposium held in Southampton in 1973, is essentially a practical one, presenting data on pollution by bacteria, chemicals, noise, heat and dust. Consideration of such data is frequently related to the problems—both technical and financial—encountered in controlling the levels of offending agents.

The Solent and adjacent areas feature largely in this volume, but some chapters refer to pollution control in other estuaries such as those of the Clyde, Tay and Forth and Liverpool Bay. Each area has its own special features, depending upon the arrangements for sewage disposal, the extent to which port installations exist, the behaviour of tides and the attractions of the locality as a holiday resort.

The word "criteria" in the title is rather misleading, for throughout the volume the lack of any agreed definition of pollution is seen to create problems for the authorities charged with its control. If adopted criteria were very demanding, the efforts and cost of control would be very high, but the lack of a definite level of attainment leads to differences of opinion between authorities and industry. This is well illustrated in the excellent paper on the medical aspects of pollution presented by Dr. A. McGregor. While he and other participants in the Symposium regard the present situation as reasonably satisfactory, it seems that future pollution control is likely to be an uphill struggle.

For those interested in either aesthetic, technical, medical or economic aspects of control of the environment, this volume cannot fail to provide an increasing

understanding of the many interrelated factors involved. It should be compulsory reading for all students of ecology.

The Normal Microbial Flora of Man. The Society for Applied Bacteriology Symposium Series No. 3. Edited by F. A. Skinner and J. G. Carr. Academic Press. London. 1974. pp. xv + 264. £6.00.

For many years, microbiologists have devoted much of their time, understandably, to the study of bacteria and fungi associated with conditions of disease, and it is only relatively recently that the normal flora of the human body has come under close scrutiny. Many difficulties are encountered in studies of the microflora, not the least being the practical problem of sampling, especially in the case of the skin with its complex surface of crevices, hair follicles and sebaceous glands. In addition there is the difficulty of knowing whether an organism that has been isolated is part of the resident population or has merely been intercepted in transit.

The difficulties involved in the study of the microbial flora are compounded by problems of culture, since many members of the flora are fastidious creatures with exacting nutritional requirements for growth *in vitro*. A further complication is that presented by the atmospheric requirements of bacteria, especially those present in the alimentary tract. For many years, the discrepancy between the numbers of bacteria seen on Gram-stained smears of faeces and the numbers actually cultured led to the belief that faeces contained a large proportion of dead organisms. More recently, however, improvements in anaerobic culture techniques have led to vast increases in viable counts and to the realization that the predominant bacteria in the gut are oxygen-sensitive anaerobes.

In view of these difficulties, it is perhaps surprising that anything more than a qualitative assessment of the types of bacteria present in the normal flora of man is possible, but in most cases much more than that has been achieved, as this volume shows. The book contains papers presented at a symposium held in July 1973 and concentrates mainly on the flora of three sites—the skin, mouth and intestine. Such topics as the effects of soap and disinfectants on the skin flora, the ecology of the flora of skin and hair, the effects of antibacterial agents and other factors on the flora of the mouth and the particular characteristics of the flora of the intestinal tract of children are considered, and there are also chapters on dental plaque, the vaginal flora and the aerial dispersion of organisms from the respiratory tract.

On the whole the contributors to the book have presented their subjects comprehensively but concisely. However, the section on the flora of the gastrointestinal tract may be considered incomplete, in that the influence of various factors, such as race, diet and the presence of pathogens, is discussed without any introductory chapter on the types and numbers of bacteria and yeasts to be found in the normal alimentary tract. In contrast, the chapters on the flora of the mouth present a much more balanced and informative picture, although too much space is perhaps devoted to structural aspects of dental plaque. Nevertheless, this is a book that microbiologists, both in the clinical and research fields, will find a most useful source of reference.

Connective Tissues. Biochemistry and Pathophysiology. Edited by R. Fricke and F. Hartmann. Springer-Verlag. Berlin. 1974. pp. xii + 309. $20.00.

This volume takes the form of an "edited" collection of some 40 papers covering the subjects of lectures delivered at a symposium on connective tissues held in Hannover, probably in 1973 since the Editors' prefatory remarks are dated "Spring 1974". The papers are grouped under the five major headings of structure, synthesis, degradation, immunobiology and pathophysiology and range from general reviews to presentations of on-going laboratory work. Irritatingly, some of the latter are very brief: one example, including references, barely fills a single page. As might be expected from such a heterogeneous authorship, the standard of contributions is variable. Moreover, review papers exhibit a considerable degree of overlap when existing work is surveyed.

Although the copy examined is in 'hardback', the text is presented as an offset litho reproduction of typescript. However, in contrast to the style of some other publishers, at least the same type is used throughout. Even so there is an unusually large crop of typographical errors. The more obvious of these include "plysaccharides" (p. 30), "Egamit" instead of Egami (p. 153) and *Charonias* in place of the correct *Charonia* (p. 153), but there are numerous other examples. Although time has been spent in producing conformity in the text, it is all too apparent that the figures and diagrams are the authors' original versions.

Viewed overall, this is a treatise likely to be accepted most readily by the aficionados of current connective-tissue research. Why it was published as a hardback will remain a mystery.

BOOKS RECEIVED FOR REVIEW

Human Intestinal Flora. Edited by B. S. Drasar and M. J. Hill. Academic Press. London. 1974. pp. xii + 263. £6.50.

Recent Advances in Pathology. No. 9. Edited by C. V. Harrison and K. Weinbren. Churchill Livingstone. Edinburgh. 1975. pp. ix + 279. £9·50.

Foreign Compound Metabolism in Mammals. Vol. 3. Senior Reporter D. E. Hathway. The Chemical Society. London. 1975. pp. xvi + 727. £26.00.

Consumer Health and Product Hazards/Chemicals, Electronic Products, Radiation. Vol. 1 of The Legislation of Product Safety. Edited by S. S. Epstein and R. D. Grundy. The M.I.T. Press. London. 1974. pp. xiii + 342. £7·50.

Chlorinated Insecticides. Vol. II. Biological and Environmental Aspects. By G. T. Brooks. CRC Press. Cleveland, Ohio. 1974. pp. iv + 197. $44.50.

Fd Cosmet. Toxicol. Vol. 13, pp. 667–670. Pergamon Press 1975. Printed in Great Britain

Information Section

ARTICLES OF GENERAL INTEREST

POLYMERS FOR THE SURGEON

The tissue-adhesive and haemostatic properties of 2-cyanoacrylates, which polymerize rapidly in contact with water to form powerful seals, have attracted surgeons for some years. Many studies of the irritancy of these esters and the tissue reactions they induce have been reported (*Cited in F.C.T.* 1973, **11**, 314 & 516).

Cyanoacrylates in skin repair....

Wilkinson (*Archs Derm.* 1972, **106**, 834) has asserted that the important individual characteristics of a cyanoacrylate designed for skin surgery are its cytotoxicity, the speed with which it polymerizes and the heat generated in the process, and the flexibility or brittleness of the resulting film. The tissue reactions induced by methyl 2-cyanoacrylate (MC) have resulted in its replacement largely by the *n*-butyl (NBC) and isobutyl (IBC) esters. The cytotoxicity of these compounds appears to depend on their speed of degradation in the tissue, and this decreases with increase in the length of the alkyl chain. An increase in chain length also increases the speed of polymerization, and this reduces the likelihood of contact of the monomer with adjacent tissues and the consequent risk of irritancy and systemic toxicity. Wetting and spreading, which determine the strength of binding, increase with increasing chain length, but technical problems in connexion with the binding and polymerization processes tend to increase with chains of five carbons or more. The optimal chain length seems to be attained therefore with the butyl esters. NBC and IBC can be applied directly or used in conjunction with gelatin film for haemostatic wound dressings for multiple organ injuries and open donor-tissue sites. The formation of IBC film between wound surfaces is undesirable, since it tends to delay fibroplasia and capillary proliferation across the incision and leads to scarring, but NBC and IBC can be used in conjunction with suturing and can eliminate seepage and graft loss when used to secure skin grafts and hair transplants (Wilkinson *loc. cit.*).

Isoamyl cyanoacrylate (IAC) has been shown to produce greater comfort and less extensive inflammation than IBC when used in the treatment of experimentally induced friction blisters in volunteers (Akers *et al. Archs Derm.* 1973, **107**, 544). The best results were obtained by spreading IAC over the raw blister base. Pentyl cyanoacrylate (PC) was more useful when a partially detached skin fragment was to be joined to the blister base, since its polymerization time (3–5 seconds) permitted wrinkles to be smoothed out before setting occurred.

.... in facial wounds....

Experimental facial wounds produced by incision of the masseter muscle in rats were covered with gauze or terpolymer sponge and sprayed with IBC by Hunsuck *et al.* (*Oral Surg.* 1970, **29**, 305). After 5 days the dressing was removed and the degree of healing was examined. At intervals up to 14 days after this, the animals were killed and the tissue in the vicinity of the wound was sectioned for microscopic examination. Wounds that had been protected by a gauze or sponge dressing sprayed with IBC showed little tissue necrosis, dehydration or secondary infection. There were minimal zones of granulation tissue, and the incision appeared to heal faster than either control wounds packed with saline-moistened gauze or wounds packed with terpolymer sponge and sutured. The downgrowth of epithelium was less in IBC-treated wounds than in the controls. Suturing of the terpolymer sponge into the wound induced faster healing and less inflammation necrosis than the control treatment, but was less effective than the sponge-IBC treatment.

.... and in ophthalmic surgery....

Evaluation of the effects of MC and IBC treatment after various operations carried out on the rabbit eye by Munton (*Expl Eye Res.* 1971, **11**, 1) has shown that the outcome generally depends on the nature of the repair. In conjunctival repair, good closure and healing occurred; by week 12 the regenerated conjunctiva showed normal numbers of goblet cells, there was a slight increase in vascularity at the limbus and the stroma contained few collagen fibres. Resection of the superior rectus muscle resulted in some early muscle necrosis, with amorphous denucleated areas, fibroblasts and giant cells; after 7 days the limbus showed an increase in vascularity and a reduction in the number of goblet cells. At the muscle end, fibroblasts and fibrin, with ghost denucleated fibres, were seen and were tentatively attributed to the heat of polymerization of the adhesive. In corneal Graefe sections, new blood vessels appeared at the limbus, surrounded by chronic inflammatory cells, but at week 19 the wound had healed, with little vascularization. The most variable results appeared after keratoplasty; gross loss of the graft, conical breakdown of the corneal-graft area and staphylomata were seen and vacuolation of the cornea with endothelial metaplasia to polygonal cells occurred.

Perforated keratotomies, 4–6 mm long, in the rabbit eye were experimentally joined by direct application of NBC to the wound area freed from epithelium (Giessmann *et al. Albrecht v. Graefes Arch. klin. exp. Ophthal.* 1972, **183**, 294). Three weeks after treatment only a thin corneal scar was visible. Histological examination of the wound tissue 5 months later

showed a little scar tissue but no sign of vascularization or chronic inflammation.

Spitznas *et al.* (*ibid* 1973, **187**, 89) applied drops of NBC to the rabbit sclera after conjunctival incision and scleral exposure, in order to attach a fragment of lyophilized dura mater as repair material. No major damage resulted, and the slight conjunctival infection observed was no greater than with alternative procedures; histological examination revealed a mild foreign-body reaction with some multinuclear giant cells and formation of a fibrotic capsule round the adhesive. The procedure was applied successfully in man to the repair of retinal detachment and other conditions involving retinal surgery. In a further study by Spitznas *et al.* (*ibid* 1973, **187**, 102) the reaction of choroid, retina and vitreous was noted in rabbits after application of NBC. Acute inflammatory round-cell infiltration appeared during the first few days after intrascleral, suprachoroidal or intraretinal application, but the vitreous showed no reaction to an injection of NBC. A mild protracted foreign-body reaction in surrounding tissue followed treatment of the sclera or suprachoroid while late reactions to NBC in the choroid and retina comprised a loosening and atrophy of the tissue, with circumscribed loss of the nuclear zones of the sensory retina.

In vivo *degradation of cyanoacrylates*

Wade & Leonard (*J. biomed. Mater. Res.* 1972, **6**, 215) treated dogs with sc implants of Ivalon sponge impregnated after insertion with MC labelled with ^{14}C in the C-2 or C-3 position or in the cyano group and determined the activity of various fractions and isolates from the urine collected for 3–4 days after. Urinary urea from animals with the [3-^{14}C]MC-impregnated implants was radioactive, while that from the other rats showed little activity. Treatment of protein-free urine with an anion-exchange resin removed 40% of the total radioactivity, but precipitation with barium hydroxide did not. No activity could be found in distillates from urine acidified with sulphuric acid. Approximately 55% of the urinary metabolites of MC appear to be neutral compounds. The results indicate that random chain scission of MC occurs, with production of formaldehyde, but attempts to discover radioactivity in the form of formic acid were unsuccessful.

Absorbable polymer sutures and other implants

Various polymers have featured in the continuing search for materials ideally suited for use as absorbable sutures. A copolymer of lactide and glycollide (cyclic intermediates derived from lactic and glycollic acids) intended for use as a surgical suture material was embedded by Conn *et al.* (*Am. J. Surg.* 1974, **128**, 19) in the rectus sheath and muscle of rabbits for up to 60 days. Minimal acute inflammatory changes, a small amount of necrotic exudate, minimal giant-cell reaction and a small development of adjacent granulation tissue were apparent after 5 days. This reaction appeared to be no greater than that provoked by the trauma of suturing. No granulation tissue could be seen after 15 days. After 30 days there was no evidence of any necrotic exudate surrounding the suture. Gross inspection of the wound after 60 days failed to distinguish the suture, and microscopic examination showed an absence of giant cells, mononuclear cells and eosinophils. This copolymer (known as polyglactin 910 or Vicryl, from Ethicon, Inc., Somerville, N.J.), therefore appears to be devoid of toxic properties under the conditions of this experiment.

Implantation of blocks of ^{14}C-labelled polylactic acid in the abdominal wall of rats resulted in little distribution of radioactivity in tissues and organs (Brady *et al. J. biomed. Mater. Res.* 1973, **7**, 155). The implant lost about 36·8% of its activity during 168 days. Of this about 29·4% appeared in expired carbon dioxide, 4·6% in the urine and 2·8% in the faeces. Cells that invaded the implant possessed either large double-walled vacuoles or large accumulations of mitochondria and microvesicles. It appears that this type of polylactic acid implant is well tolerated by the host tissue. The material persisted at the implantation site for about 1·5 years, however, in this experiment. While prolonged persistence of an implant might be useful in some circumstances, in others it could delay complete healing, and attention is drawn to the possibility that resorbable implants of this type might be modified by incorporation of other resorbable materials, such as polyglycollic acid, so that they could be 'tailor-made' in respect of both strength and resorbability for a variety of clinical applications.

[P. Cooper—BIBRA]

DEALING WITH ACETALDEHYDE

Acetaldehyde is formed in the body as a metabolite of ethanol and is also an important constituent of tobacco smoke. Previous studies have shown that this aldehyde is retained in the upper respiratory tract after inhalation (*Cited in F.C.T.* 1973, **11**, 922), and that it causes biphasic changes in blood pressure when given by iv injection (*ibid* 1974, **12**, 787). The presence of acetaldehyde produced by microbiological activity in home-brewed lager has caused gastric erosion (*ibid* 1972, **10**, 435).

Metabolism and distribution

The distribution of acetaldehyde in the brains of rats following a single dose of ethanol has been studied by Sippel (*J. Neurochem.* 1974, **23**, 451). An ip injection of 65 mmol ethanol/kg was given 30 minutes before tissue sampling. Brain tissue, excluding the pons–medulla, cerebellum, olfactory lobes and lateral parts of the cerebrum, was taken in the frozen state, treated with thiourea to prevent non-enzymic oxidation of ethanol, and assayed for both acetaldehyde and ethanol by gas-liquid chromatography. Cerebral blood contained 100–360 nmol acetaldehyde/ml, but there was no significant acetaldehyde concentration in the brain tissue when the cerebral blood concentration lay below 250 nmol/ml. The lactate/pyruvate ratio in these tissues gave no indication of ischaemia, so that the absence of acetaldehyde could

not be explained on that basis. Dialysis experiments revealed no binding of acetaldehyde to blood macromolecules, so that there was no physical obstacle to entry into the brain parenchyma. It is suggested that cerebral capillary walls may contain aldehyde dehydrogenase activity, which could prevent access of acetaldehyde to the brain until the enzyme system had become saturated. This hypothesis is supported by the fact that, in the rat, dopa decarboxylase and monoamine oxidase occur in the walls of the cerebral capillaries and bar the entry of L-dopa to the brain tissue.

Kesäniemi (*Biochem. Pharmac.* 1974, **23**, 1157) has described modifications in the metabolism and effects of ethanol and acetaldehyde during pregnancy in rats. After an injection of 1·2 g ethanol/kg, the ethanol concentration in peripheral blood was similar in pregnant and non-pregnant animals, but the acetaldehyde concentration was higher in pregnant ones. When the livers of pregnant and non-pregnant rats were freeze-clamped 30 minutes after injection of 1·2 g ethanol/kg, the liver content of ethanol and of acetaldehyde was of the same order in both groups, and there were no significant differences in the activities of the respective dehydrogenases determined *in vitro*. Similarly, pregnancy had no significant effect on the lactate/pyruvate ratio of the liver, although levels of both pyruvate and lactate were higher in the livers of pregnant than in those of non-pregnant rats prior to ethanol treatment. The high acetaldehyde content of peripheral blood during pregnancy, allied to the comparable rate of ethanol oxidation in pregnant and non-pregnant rats, suggests a reduction in the extrahepatic metabolism of acetaldehyde during the former condition. Since the foetus is unable to eliminate acetaldehyde acquired from the maternal blood supply, it may be exposed to both ethanol and acetaldehyde and their toxic effects.

The metabolism of acetaldehyde by the myocardium has been investigated by Forsyth *et al.* (*Proc. Soc. exp. Biol. Med.* 1973, **144**, 498) in the isolated rat heart. The organ was perfused with [1,2-^{14}C]acetaldehyde or sodium [1-^{14}C]acetate and the resultant $^{14}CO_2$ was measured. Pretreatment with disulfiram to inactivate aldehyde dehydrogenase prevented CO_2 production from perfused acetaldehyde, a finding which indicates that a myocardial aldehyde dehydrogenase is responsible for the reaction. Oxidation of acetate to CO_2 was more rapid than oxidation of acetaldehyde, suggesting that the dehydrogenation of acetaldehyde may be the rate-limiting factor in its metabolism in heart tissue.

Effects on the circulation and the liver

The systemic, pulmonary and regional circulatory effects of acetaldehyde have been studied in anaesthetized dogs by McCloy *et al.* (*Cardiovasc. Res.* 1974, **8**, 216). After a single iv injection of 16 mg acetaldehyde/kg, there was an increase in systemic and pulmonary arterial pressures, myocardial contractility, cardiac output, systemic venous return and heart rate, but a decrease in left atrial pressure. Intra-arterial injection of 0·1–0·5 mg acetaldehyde/kg was followed by dose-related increases in perfusion pressure of the brachial and renal arteries without any significant change in systemic arterial pressure, and a biphasic change (increase followed by a decrease) in arterial perfusion pressure. After injection of a 4 mg/kg dose, peripheral perfusion pressure rose without any secondary reduction, while coronary and hepatic perfusion pressures declined. These findings indicate that acetaldehyde has a direct vasoconstrictor effect on peripheral arterial vascular beds and a vasodilator effect on those of the coronary and hepatic arteries. The biphasic change in myocardial contractile force following intra-arterial injection of acetaldehyde reflects the overcoming of the direct negative inotropic effect of acetaldehyde on the myocardium by an initially greater positive inotropic action of released catecholamines. Adrenergic blockade with phenoxybenzamine or propranolol greatly reduced the cardiovascular effects of acetaldehyde, supporting the view that most of these effects are due to its action on the release of catecholamines.

Mitochondrial oxygen consumption in the rat liver is reduced by acetaldehyde (Cederbaum *et al. Archs Biochem. Biophys.* 1974, **161**, 26). Incubation of mitochondrial suspensions with acetaldehyde in concentrations of 0·6, 1·5 or 4·5 mM removed 11, 7 and 3% of the aldehyde, respectively, in 10 minutes. When glutamate, β-hydroxybutyrate or α-ketoglutarate was the substrate, mitochondrial oxygen consumption was substantially inhibited by acetaldehyde concentrations as low as 1–3 mM, whereas with succinate or ascorbate much higher concentrations were necessary for significant inhibition. Observations that acetaldehyde inhibited respiratory control, oxidative phosphorylation and ATP–^{32}P exchange reaction indicated that it is an inhibitor of energy production by the mitochondria, and decreases in substrate-supported and ATP-supported calcium ion uptake, in calcium ion-stimulated oxygen uptake and in ATPase activity all pointed to inhibitory effects on energy utilization. Acetaldehyde markedly inhibited malate–aspartate, α-glycerophosphate and fatty acid shuttles for the transfer of reducing equivalents, apparently by interference with NAD^+-dependent state-3 respiration and anion entry and efflux. Since ethanol in concentrations of 6–80 mM failed to have any significant effect on oxygen consumption, anion uptake or energy production and utilization by the mitochondria, the evidence points to the implication of acetaldehyde in some of the adverse metabolic effects associated with chronic ethanol consumption.

Protection against acetaldehyde

Sulphydryl compounds and ascorbic acid afford some protection against the cardiomyopathy and other toxic effects of acetaldehyde. Sprince *et al.* (*Fedn Proc. Fedn Am. Socs exp. Biol.* 1972, **31**, 574 abs) have described experiments in which rats were given thiamine hydrochloride (0·3 mmol/kg), L-cysteine base (2 mmol/kg) or both agents together in the same doses, by oral intubation 30 minutes before an oral dose of 12 mmol acetaldehyde/kg. The percentages of animals anaesthetized within 10 minutes among the control rats and these three treated groups were 66, 43, 26 and 13% respectively, and the percentages of survivors at 24 hours were 12, 43, 61 and 75% respectively. Thus, both thiamine and L-cysteine have some protective effect against acetaldehyde and the effects appeared to be additive.

Further experiments by the same authors (*ibid* 1974, **33**, no. 3, Part 1), involving the treatment of rats with L-cysteine, reduced glutathione, L-ascorbic acid or DL-homocysteine, the oxidized forms of these or DL-thioctic acid prior to acetaldehyde administration, demonstrated that sulphydryl compounds and ascorbic acid could protect rats against the toxicity of the aldehyde. Compared with a 10% survival in control rats pretreated with saline, survival was 100% in rats pretreated with a combination of L-cysteine, thiamine and L-ascorbic acid.

In another study (*idem*, *Agents & Actions* 1974, **4**, 125), L-cysteine base, thiamine hydrochloride or L-2-methylthiazolidine-4-carboxylic acid was given orally to rats in a dose of 2 mmol/kg 30–45 minutes before a dose of 18 mmol acetaldehyde/kg. The survival rate at 24–72 hours was 80, 90 and 75%, respectively, for the three groups, compared with a control value of 10%. In another group, a combination of L-cysteine and thiamine (each at 2 mmol/kg) offered complete protection (100% survival). The oral LD_{50} of each of these protective agents was determined and the ratios of protective to toxic doses were considered to be sufficiently high to render these compounds acceptable candidates for further study as possible agents against chronic ethanol or acetaldehyde intoxication.

[P. Cooper—BIBRA]

Fd Cosmet. Toxicol. Vol. 13, pp 671–677 Pergamon Press 1975. Printed in Great Britain

TOXICOLOGY: ABSTRACTS AND COMMENTS

COLOURING MATTERS

2934. No teratogenic effects from amaranth or Ponceau 4R

Larsson, K. S. (1975). A teratologic study with the dyes amaranth and Ponceau 4R in mice. *Toxicology* **4**, 75.

There has been much controversy over the alleged embryotoxicity of amaranth, but recent work has been reassuring on this score (*Cited in F.C.T.* 1975, **13**, 473). An FDA *ad hoc* advisory committee recently concluded that the colouring did not have any embryotoxic effects when given orally, although a limit of 2 mg/kg/day on its consumption was considered desirable pending further elucidation of its metabolic fate (*Food Chemical News* 1975, **16** (43), 40). Ponceau 4R has not been studied before for reproductive effects, but allegations of weak carcinogenicity (Andrianova, *Vop. Pitan.* 1970, **29** (5), 61) have now been refuted by BIBRA work (unpublished data). The study cited above provides further negative evidence in favour of amaranth and also demonstrates a lack of teratogenicity in Ponceau 4R.

Mice were given amaranth or Ponceau 4R by gavage at dose levels of 7·5, 30 or 100 mg/kg on days 0–7 or 6–18 of pregnancy, and the foetuses were removed on day 18 for detailed examination. No effect could be discerned in any of the groups with regard to the numbers of litters or implantation sites, the frequency of resorptions or foetal deaths, the types and incidence of gross malformations and skeletal and internal malformations or the weight of live foetuses.

[The negative findings from amaranth administration on days 0–7 of pregnancy are particularly significant in view of a previous report of increased resorptions and foetal deaths in rats treated on days 0–19 (Collins, McLaughlin & Gray, *Fd Cosmet. Toxicol.* 1972, **10**, 619). This seems to confirm the suspicion that such effects were artefacts resulting from abnormally low control values. The spectre of amaranth embryotoxicity now seems to have been laid firmly to rest.]

AGRICULTURAL CHEMICALS

2935. Ethylene dibromide residues in apples

Dumas, T. & Bond, E. J. (1975). Bromide residues in apples fumigated with ethylene dibromide. *J. agric. Fd Chem.* **23**, 95.

Squamous-cell carcinomas of the forestomach have been reported in rats and mice given repeated doses of ethylene dibromide (EDB) by stomach tube (*Cited in F.C.T.* 1975, **13**, 144). It would thus appear desirable for residues of this fumigant on agricultural products to be kept as low as possible, and the position with regard to apples has now been investigated.

When three varieties of apples were fumigated with 8–24 mg EDB/litre for 4 hr at 13°C, and subsequently maintained at this temperature, EDB residues in the pulp and skin had declined to below 0·1 ppm in less than 13 days. When similar treatment was given at 20 or 25°, negligible residue levels were attained in only 4–5 days. However, when apples were cold-stored at 4° after being treated and stored for 2 days at 13°, residue levels of 0·1 ppm were attained only after 3–4 wk. Levels of 308 ppm EDB were found in the apple skin immediately after fumigation with 12 mg/litre at 13°, but these had declined to 26 ppm after 4 days. Conversely, levels of 25–30 ppm found in the seeds immediately after treatment did not decline during 13 days of storage at 13°. Inorganic bromide residues determined 7–21 days after fumigation were in the range of 2·0–4·8 ppm, depending on the initial exposure levels and the temperature. As the highest concentration of EDB (24 mg/litre) used in the study was double that found to be effective in practice, these results were regarded as fairly reassuring.

2936. Lindane and liver enzymes

Herbst, M., Guénard, J., Köllmer, H. u. Stötzer, H. (1974). Zur Enzyminduktion in der Leber nach oraler Lindanapplikation. Funktionelle und morphologische Untersuchungen an Ratten. *Arch. Tox.* **32**, 115.

Induction of liver-enzyme activity by chlorinated hydrocarbon insecticides is a well-known phenomenon. The paper cited above is concerned with enzyme induction in the liver of rats given an arachis oil solution of γ-benzene hexachloride (γ-BHC; lindane) at 5 and 20 mg/kg for 1, 2, 4 or 8 consecutive days.

Spontaneous movement and exploration by the animals was not affected by the treatment. There was no alteration in liver weight or in the liver activity of glucose-6-phosphatase, phosphorylase, cytochrome oxidase or adenosine triphosphatase. The glycogen content of the livers of treated animals that were fasted for 24 hr was slightly reduced in comparison with control values, but this effect was not evident when the animals continued to feed *ad lib.* During lindane treatment, urinary ascorbic acid excretion increased markedly, and serum ascorbate levels were

doubled. Electron-microscopy of liver sections from treated rats showed an increase in the smooth endoplasmic reticulum, most marked in the hepatocytes of the intermediary zone, with depletion of glycogen in individual hepatocytes. Pentobarbitone sleeping time in lindane-treated animals was decreased by an amount depending on the magnitude of the dose of the pesticide and the number of doses given, but the reduction was evident even after a single dose of 5 mg/kg. There were no morphological or histochemical changes that could be correlated with the dose levels of lindane or that appeared to be of toxicological significance. The non-specific enzyme-inducing effect of lindane indicated by the increased metabolism of pentobarbitone was therefore interpreted as a benign adaptive change in liver function.

2937. Tetrachlorophenol tested on the rat foetus

Schwetz, B. A., Keeler, P. A. & Gehring, P. J. (1974). Effect of purified and commercial grade tetrachlorophenol on rat embryonal and fetal development. *Toxic. appl. Pharmac.* **28**, 146.

Last month we referred to the effects of pentachlorophenol on foetal development in the rat (*Cited in F.C.T.* 1975, **13**, 585). The same group has carried out a similar study on tetrachlorophenol (TCP), which is used as a fungicide and bactericide, generally for the preservation of wood and similar materials. Commercially available preparations may contain small quantities of non-phenolic contaminants, notably polychlorinated dibenzo-*p*-dioxins. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin has been shown to be highly foetotoxic and teratogenic (*ibid* 1972, **10**, 110), but has not been identified as a tetrachlorophenol contaminant and, indeed, the chemical reaction involved would make its formation therein unlikely. In the experiments described in this paper, two samples of TCP containing this particular dioxin at a level below 0·05 ppm (the limit of sensitivity of the analytical method) were tested in rats. The main contaminants in the commercial-grade sample were hexa-, hepta- and octachlorodibenzo-*p*-dioxin and hexa-, hepta- and octachlorodibenzofurans.

Groups of rats were given purified (99·6%) TCP or commerical-grade (73%) TCP containing 27% pentachlorophenol in corn oil by gavage for 10 days, on days 6–15 of gestation, in doses of 10 or 30 mg/kg/day (the latter being the maximum level tolerated). Maternal body weight was unaffected by treatment, and the dams showed no signs of toxicity from either sample of TCP. No effects were observed on foetal resorptions, foetal body weight or foetal length (crown to rump). No foetal anomalies appeared apart from an increased incidence of delay in the ossification of the skull, an increase which was significant both in foetuses (26% incidence compared with 8% in controls) and litters (50% incidence *v.* 19% in controls) in the group given the higher dose of commercial TCP, and in foetuses (17% incidence) but not in litters in the group given the same dose level of purified TCP. At 10 mg/kg/day neither sample produced this effect. There was an increased incidence of subcutaneous oedema in foetuses and litters from dams given the lower dose of pure or commercial TCP, but since this did not appear after treatment with the higher dose level of either sample, it may have been a chance effect.

Thus, the non-phenolic compounds present in commercial-grade TCP did not contribute significantly to the material's effect on the developing rat foetus, and no teratogenic response was observed.

THE CHEMICAL ENVIRONMENT

2938. Chromium cleared in American cement dermatitis

Perone, V. B., Moffitt, A. E., Jr., Possick, P. A., Key, M. M., Danzinger, S. J. & Gellin, G. A. (1974). The chromium, cobalt, and nickel contents of American cement and their relationship to cement dermatitis. *Am. ind. Hyg. Ass. J.* **35**, 301.

Cement dermatitis is a widely recognized problem and it has generally been assumed that metals in cement, such as chromium, nickel and cobalt, are the agents responsible for the allergic reactions (*Cited in F.C.T.* 1969, **7**, 91). In the study now reported, a group of American workers investigated 42 samples of American Portland Cement.

The concentrations of total chromium and hexavalent chromium (Cr^{VI}), total cobalt and total nickel in cement washings and of total chromium in untreated cement were determined by spectrophotometry. The levels of total chromium ranged from 5 to 124 μg/g raw cement with an average of 29·5 μg/g. Water-soluble chromium in the filtrates represented on average only some 10% of the total chromium in raw cement, and in some cases filtrate levels were below 0·5 μg/g. Only 18 of the 42 filtrates contained measurable amounts of Cr^{VI}, and none contained measurable nickel or cobalt (i.e. levels of 0·5 μg/g or above). The chromium content of cement was not linked to geographical source, as the lowest and highest values were found in cements from the same state.

Patch tests were then carried out on 95 cement workers, more than 50% of whom had been in the industry for more than 15 yr. The group included 25 workers with a history of cutaneous reactions to cement, and at the time of testing 15 were found to have mild dermatitis of the hands. A further 20 showed more active eczema, with vesicles, erythema and fissures at various stages. All 95 men were patch-tested with 0·25% potassium dichromate, 2·0% cobalt nitrate, 5·0% nickel sulphate, 10·0% sodium arsenate, a filtrate containing 4·7 μg Cr^{VI}/g, a filtrate containing no Cr^{IV}, nickel or cobalt, and finally distilled water as a control. No worker reacted to nickel, cobalt, distilled water or the filtrate free from Cr^{VI}, nickel and cobalt. Three workers reacted to the arsenate (a response regarded as due to irritancy rather than to

sensitization) and only one to potassium dichromate or the filtrate containing Cr^{VI}. These findings of a low incidence of sensitivity to chromium and no apparent sensitivity to nickel and cobalt are surprising in view of reports from European workers (Wilkinson & Cronin, *Br. J. Derm.* 1972, **87**, 400; G. Hovking, Thesis, University of Bergen, Norway, 1970). The presence of some unknown factor is apparently ruled out by the lack of reaction to the two filtrates.

The authors concede that 1–2% of additional reactions might have been found by examining patches at 72 as well as 48 hr. However, they feel that these results indicate that chromium, nickel and cobalt are not aetiological factors in cement dermatitis in the USA. They suggest that further information should be obtained by patch-testing workers from other areas of the country.

2939. Leukaemia from benzene

Aksoy, M., Erdem, Ş. & Dinçol, G. (1974). Leukemia in shoe-workers exposed chronically to benzene. *Blood* **44**, 837.

Although leukaemia is now generally recognized as a possible effect of excessive occupational exposure to benzene, there have been only a few quantitative studies of its incidence among workers so exposed (*Cited in F.C.T.* 1971, **9**, 750; *ibid* 1972, **10**, 270). The authors cited above have recently described 26 cases of acute leukaemia, as well as six cases of Hodgkin's disease, among Istanbul workers (*ibid* 1975, **13**, 479) and have now provided further details of the leukaemia cases.

The 26 leukaemia patients were identified during the period 1967–1973 among the 28,500 workers employed in the shoe, slipper and handbag industry, in which benzene is used as a solvent in adhesives. The shops in which they were employed were small and poorly ventilated, and benzene concentrations were found to reach maxima of 210–650 ppm during use of the adhesive. Duration of exposure to benzene varied from 1 to 15 yr, with a mean of 9·7 yr, and the average age at time of diagnosis was 34·2 yr (range 16–58 yr). Acute myeloblastic leukaemia was seen in 14 of the 26, preleukaemia in four, acute erythroleukaemia in three, acute lymphoblastic leukaemia in three, acute monocytic leukaemia in one and acute promyelocytic leukaemia in one. Fever was present in all the cases, and with one exception there were also severe haemorrhagic manifestations, accompanied in three patients by splenomegaly. The overall incidence of leukaemia was equivalent to 13/100,000 over the 7 yr, or 19·7/100,000 over the last 3 yr of the study, compared with an incidence in the general population of only 6/100,000.

2940. Further reassurance on bis-chloromethyl ether formation

Tou, J. C. & Kallos, G. J. (1974). Study of aqueous HCl and formaldehyde mixtures for formation of bis (chloromethyl) ether. *Am. ind. Hyg. Ass. J.* **35**, 419.

It was recently reported (*Cited in F.C.T.* 1975, **13**, 401) that the suggested formation of hazardous concentrations of bis-chloromethyl ether (BCME) in working environments by interaction of formaldehyde (HCHO) and hydrogen chloride (HCl) in moist air was not a practical possibility. However, the same group has demonstrated that very small quantities of BCME are formed when high concentrations (500–3000 ppm) of both of these reactants are present in moist air and are likely also to be formed when the two reactants co-exist in the condensed phase. While BCME is degraded rapidly in aqueous solution, it is very stable in humid air, with a half-life of more than 25 hr, and it was speculated, therefore, that if the rate of escape of BCME from an aqueous solution of HCHO and HCl exceeds the rate of its subsequent hydrolysis, there could be a build-up of BCME in the gaseous phase above the solution.

To investigate this possibility, HCHO and HCl solutions were prepared in a range of concentrations between 2 and 2000 ppm. Equal volumes of the corresponding concentrations were then mixed and stirred in closed reactors at ambient temperature for 18 hr, after which both the aqueous solutions and the air in the reactors were analysed for BCME, the former by mass spectrometry and the latter by gas chromatography–mass spectrometry. The detection limits for BCME by these methods were 9 ppb ($b = 10^9$) in the aqueous phase and 1 ppb in the gas phase above the reaction mixture, but no BCME was detected in any of the test solutions or air samples. Moreover, kinetic studies on the hydrolysis of BCME suggested that even levels much lower than the detectable 9 ppb would have been unlikely to be reached in the aqueous solution.

2941. Liver damage from dimethylformamide in the gerbil

Llewellyn, G. C., Hastings, W. S., Kimbrough, T. D., Rea, F. W. & O'Rear, C. E. (1974). The effects of dimethylformamide on female mongolian gerbils, *Meriones unguiculatus*. *Bull. env. contam. & Toxicol. (U.S.)* **11**, 467.

Dimethylformamide (DMF) is of fairly low acute toxicity, and on prolonged administration to rats the predominant finding at high dose levels is liver damage (*Cited in F.C.T.* 1966, **4**, 547; *ibid* 1973, **11**, 339). An increase in leucocytes and certain changes in blood and urine chemistry have also been produced by repeated ip injection (*ibid* 1972, **10**, 599) and acute pancreatitis has resulted in man from occupational exposure (*ibid* 1975, **13**, 289).

Because toxic effects produced in the Mongolian gerbil following administration of aflatoxin dissolved in DMF were attributed to the latter substance (Dunkin & Llewellyn, *Va J. Sci.* 1971, **22**, 96), a study was undertaken to determine the toxicity of DMF in the female of this species. LD_{50} values by the ip, sc and intragastric routes were found to be in the 3–4 g/kg range, and total doses of this order also caused death when DMF was given in the drinking-water at a concentration of 6·6% for 3 days or 3·4% for 6 days. Death was generally preceded by a marked loss in weight and was accompanied by renal congestion and signs of toxic hepatitis, including zones of diffuse necrosis, hyperchromatic nuclei, abnormal numbers of mitotic figures, giant nuclei, haemosiderin and accumulation of Kupffer cells. A very limited

number of necrotic foci in the liver and no consistent weight loss were seen in animals dying after ingesting drinking-water containing 1·7% DMF for up to 80 days, and necrotic foci were also found in the 25% of the animals that died within 200 days in the group drinking a 1% solution. However, in gerbils given 1% DMF for only 30 days there were no apparent liver, kidney or weight changes.

2942. A no-effect level for dioxane

Kociba, R. J., McCollister, S. B., Park, C., Torkelson, T. R. & Gehring, P. J. (1974). 1,4-Dioxane. I. Results of a 2-year ingestion study in rats. *Toxic. appl. Pharmac.* **30**, 275.

Torkelson, T. R., Leong, B. K. J., Kociba, R. J., Richter, W. A. & Gehring, P. J. (1974) 1,4-Dioxane. II. Results of a 2-year inhalation study in rats. *Toxic. appl. Pharmac.* **30**, 287.

Rats given 1,4-dioxane at concentrations of 0·75–1·8% in their drinking-water for 13 months developed nasal carcinomas, and liver carcinomas were also seen with concentrations of 1·4% or more (*Cited in F.C.T.* 1971, **9**, 157). Damage to the kidney tubules has also resulted from oral administration of dioxane (*ibid* 1971, **9**, 278). More recently, a dose-related increase in hepatic tumours has been described in rats given water containing 0·75–1·8% dioxane for 16 months (Argus *et al. Eur. J. Cancer* 1973, **9**, 237). The present studies were aimed at discovering the long-term effects of lower dose levels, administered by both the oral and inhalation routes.

The first of the two papers cited above describes the effects in rats of 2-yr administration of drinking-water containing 0·01, 0·1 or 1·0% dioxane, equivalent in the males to daily dose levels of 9·6, 94 and 1015 mg/kg and in the females to 19·0, 148 and 1599 mg/kg, respectively. The highest level decreased the survival rate in the early part of the study, reduced body-weight gain and water consumption, increased liver weight, and produced varying degrees of hepatocellular and renal tubular degenerative changes, accompanied by some regenerative activity. Three nasal and ten hepatocellular carcinomas seen at this level were considered to be related to the high dose of dioxane administered. At the 0·1% level, there was similar evidence of degenerative and regenerative changes in the liver and kidney, but no indications of a treatment-related increase in tumour incidence or other adverse effects were detected. At the lowest level, no untoward effects of any kind could be discerned.

The second study involved the exposure of 288 rats of each sex to an average atmospheric concentration of 0·4 mg/litre (111 ppm) for 7 hr/day on 5 days/wk for 2 yr. This level was chosen as being similar to the threshold limit value (TLV), which was 100 ppm when the study was carried out although subsequently it was reduced to 50 ppm. Exposure had no effect on appearance, behaviour, growth or mortality, and minor changes in haematological and clinical-chemistry values, although statistically significant, were well within normal physiological limits and were not considered to indicate any toxic effect. Terminal organ weights and gross and microscopic pathology revealed no treatment-related effects, and tumour incidence was unaffected. In particular, no hepatic or nasal carcinomas were observed. It is calculated that the rats inhaled some 105 mg dioxane/kg/day, a dose level similar to that which produced liver and kidney damage in the first study; they were, however, exposed for only 5 rather than 7 days/wk, providing a smaller overall dose and intermediate recovery periods, and the degree of absorption was probably lower in the lungs than in the gut.

[In addition to providing a basis for defining acceptable levels of exposure to dioxane, these studies are of wider significance, in that they underline the possibility of demonstrating a no-effect level for a carcinogen.]

2943. The rat gut in phenol detoxication

Powell, Gillian M., Miller, J. J., Olavesen, A. H. & Curtis, C. G. (1974). Liver as a major organ of phenol detoxication? *Nature, Lond.* **252**, 234.

This paper presents data in support of the view that the liver is not essential for the detoxication of phenol and that the gastro-intestinal tract plays a major role in providing protection from the potentially toxic effects of phenol. In autoradiographic studies in the rat, the level of isotope in areas corresponding to the liver did not at any time exceed that in the blood following oral or ip administration of ^{14}C-labelled phenol. It is suggested that the [^{14}C]phenol either failed to enter the hepatic cells or, alternatively, had only a transient existence in these cells because of rapid turnover in the liver.

When isolated rat-gut preparations were perfused with fluid containing [^{14}C]phenol either alone or diluted with unlabelled phenol, 50 and 78%, respectively, of the radioactivity was transferred from the mucosal to the serosal circulation in 2 hr. The radioactivity in both mucosal and serosal media was distributed between phenyl sulphate (5%) and phenyl glucuronide (95%). No unchanged [^{14}C]phenol was detected.

The results suggest the gut as the principal site of detoxication of ingested phenol. This possibility was explored *in vivo* by perfusing the rat small intestine *in situ* with media containing [^{14}C]phenol or [^{14}C]phenol/cold phenol mixture and simultaneously sampling the circulating perfusate and blood from the hepatic portal vein at 30-min intervals over 3 hr. In both cases there was a progressive decrease in the amount of radioactivity in the circulating perfusate. Over a 3-hr period, most of the radioactivity was transported from the intestinal lumen and detected in the portal blood. Conjugates of [^{14}C]phenol were found in all the plasma samples but no unchanged labelled phenol was detected. Phenol conjugates appeared in the intestinal perfusate within 30 min but at that time most of the radioactivity was still present as unchanged [^{14}C]phenol. After perfusion for 2 hr, however, free phenol could not be detected and the radioactivity was present as phenyl sulphate (72%) and phenyl glucuronide (28%). Phenol appears, therefore, to be transported in conjugated form from the intestinal lumen, a conclusion in line with the evidently minimal role of the liver in its detoxication following ingestion.

Nevertheless, conjugation with sulphate and glucuronide and subsequent excretion of these conjugates in the urine still occur when phenol is given by the iv route to eliminate direct involvement of the gastro-intestinal tract, and the possibility was explored that in this situation the liver may become a major centre for detoxication. Following iv administration of 50 or 100 mg [^{14}C]phenol/kg to hepatectomized rats, from which 60% of the hepatic tissue and the spleen and gut had been removed, there was no significant difference between these and control rats in the percentage of radioactivity recovered from the urine over a 3-hr period. Urine from both groups contained the phenol conjugates, although the glucuronic acid conjugate was proportionally higher in the urine of the test animals. Thus even without the involvement of the gastro-intestinal tract and the liver, the formation and urinary excretion of phenol detoxication products can still occur.

On the basis of these results, the authors conclude that the intestinal tract acts as a barrier, ensuring that ingested phenol enters the portal blood only in conjugated form. Other potential detoxication sites are therefore not presented with exogenous free phenol. The possible importance of this protective mechanism to man is suggested by an estimation that up to 600 mg phenolic material may be ingested each day in the normal human diet.

2944. Continuing search for sensitizing contaminant in stearyl alcohol

Fisher, A. A. (1974). Contact dermatitis from stearyl alcohol and propylene glycol. *Archs Derm.* **110**, 636.

Shore, R. N. & Shelley, W. B. (1974). Reply. *Archs Derm.* **110**, 636.

The second authors cited above reported last year a severe case of contact dermatitis, which they attributed to some impurity in the commercial stearyl alcohol used as a component of an anti-inflammatory corticosteroid cream (*Cited in F.C.T.* 1975, **13**, 403). This case report prompted the suggestion (Fisher, cited above) that tests should be made of the patient's reaction to oleyl alcohol, a stated impurity in the stearyl alcohol, and to panthenol, the alcohol of pantothenic acid. Fisher (*loc. cit.*) referred to several cases of sensitivity to oleyl alcohol with cross-reaction to stearyl alcohol and to the sensitivity to stearyl alcohol of one of his own patients, who showed cross-reactions to oleyl alcohol. He also drew attention to a report from Germany of a cross-reaction to panthenol shown by a patient sensitive to stearyl alcohol.

Responding to this suggestion, Shore & Shelley (cited above) found that while their patient still gave an intense reaction to commercial stearyl alcohol (30% in petrolatum), no reaction was induced by high-grade (99% pure) stearyl alcohol, oleyl alcohol or panthenol, all again applied in a concentration of 30% in petrolatum.

The identity of the offending impurity in commercial stearyl alcohol remains, therefore, an open question.

2945. Impact of trichloroethane on the cardiovascular system

Herd, P. A., Lipsky, M. & Martin, H. F. (1974). Cardiovascular effects of 1,1,1-trichloroethane. *Archs envir. Hlth* **28**, 227.

1,1,1-Trichloroethane (methylchloroform; TCE) has always been regarded as one of the more innocuous chlorinated solvents in household and industrial use, and recently we reviewed a paper describing a comparative lack of effects in rats exposed to TCE vapour (*Cited in F.C.T.* 1975, **13**, 402). However, the FDA has taken a more serious view of its use in some decongestant aerosol sprays following reports of 21 deaths (mainly through abuse of the preparations), and since 1973 aerosol drug products containing TCE have been subject to regulatory proceedings unless new drug applications have been submitted. It now seems that this wary attitude may have been justified.

The usual mechanism of TCE toxicity is regarded as involving depression of the central nervous system. However, Herd *et al.* (cited above) made the incidental observation that acute exposure of anaesthetized dogs to TCE caused profound cardiovascular depression. In view of its already reported ability to sensitize the heart to arrhythmias (Reinhardt *et al. Toxic. appl. Pharmac.* 1972, **22**, 305) and its increasing use, it was felt that the possible cardiovascular effects of TCE should be explored further.

Nine anaesthetized mongrel dogs were exposed to TCE for 5 min after their normal cardiovascular and pulmonary parameters had been measured during a 30-min control period on mechanical ventilation. After the TCE exposure, the length of time for recovery of blood pressure and heart rate to initial values was measured and found to be about 10 and 45 min, respectively, for most TCE doses tested. A second exposure to TCE followed about 1 hr after the first. The parameters recorded were arterial pressure from a left femoral cannula, left ventricular pressure from cannulation of the left ventricle and cardiac stroke output measured with an electromagnetic flowmeter at the base of the aorta. Arterial pH and blood-gas levels were also monitored during some experiments. In a series of 60 experiments, TCE was administered at hourly intervals, occasionally with drug compounds.

It was found that TCE administration produced a dose-dependent fall in blood pressure 10–15 sec after the start of exposure. This was a biphasic response, the initial decline being due to a marked decrease in total peripheral resistance while both myocardial contractility and cardiac output increased. During the second phase, a reduction in cardiac output and myocardial contractility occurred while total peripheral resistance remained at a roughly constant level. The positive chronotropic and inotropic effects of the initial phase could be abolished by β-receptor blocking induced by propranolol pretreatment, a finding suggestive of a neurohumoral mechanism for the myocardial stimulation occurring in this phase. Continued administration of TCE led to a continued drop in blood pressure, which ultimately resulted in death.

Phenylephrine, an α-agonist, has only minimal effects on the myocardium but produces peripheral vasoconstriction. Its injection during TCE exposure reversed the blood pressure fall, and on cessation of

TCE exposure phenylephrine-treated animals returned to and even exceeded normal blood pressures more quickly than those not treated. No arrhythmias were observed either with TCE on its own or following associated phenylephrine treatment. The mechanism of these peripheral vascular effects is not yet clear and will be the subject of further publications by these authors.

The myocardial changes induced by TCE took much longer to return to normal values. As extracellular calcium ions can affect myocardial contraction dramatically, infusions of calcium gluconate were given during TCE exposure. Contractility increased greatly in the calcium-treated preparation, and blood pressure did not fall. Similar effects of calcium ions on TCE-induced impairment of myocardial contractility were demonstrated in rat papillary-muscle preparations. Again the precise mechanism of TCE inhibition of myocardial contractility is not clear: it has been observed that TCE depresses the respiration of tissue slices from the rat heart and it is possible that this fact may be implicated in its mechanism of action.

Thus acute exposure to TCE may be expected to produce profound changes in cardiovascular function as well as, or perhaps independent of, changes in the central nervous system. The levels of TCE used in this study were higher that those usually encountered, but TCE has a great affinity for lipid and is generally accumulated following repeated exposure to lower doses. It is not known whether lower levels of TCE exposure have an effect on myocardial function that may potentiate other events leading to cardiovascular collapse. No epidemiological data are available on the incidence of cardiovascular accidents in workmen exposed repeatedly to TCE, but the authors of this paper advocate some further investigation into this aspect of TCE toxicity.

2946. The pharmacokinetics of tri-*o*-cresyl phosphate

Sharma, R. P. & Watanabe, P. G. (1974). Time related disposition of tri-*o*-tolyl phosphate (TOTP) and metabolites in chicken. *Pharmac. Res. Commun.* **6**, 475.

The potent neurotoxin tri-*o*-cresyl phosphate (tri-*o*-tolyl phosphate; TOCP) has no anticholinesterase activity itself but is metabolized via its dihydroxymethyl derivative and a monohydroxymethyl intermediate to the active compound, 2-(*o*-cresyl)-4-hydro-1,3,2-benzodioxaphosphoran-2-one (CBDP) (*Cited in F.C.T.* 1963, **1**, 91). The initial hydroxylation takes place in the liver, but cyclization to CBDP is largely catalysed by the albumin fraction of the plasma (Eto *et al. Biochem. Pharmac.* 1967, **16**, 295). The rate and extent of CBDP formation has now been investigated in the chicken. This species was used because its response to TOCP resembles that of man both in clinical symptoms and in the time lapse between exposure and paralysis.

Hens were given ^{32}P-labelled TOCP by crop intubation and then killed after intervals of 1–72 hr. Analyses were carried out on liver and plasma samples from all the birds and on excreta collected from the group killed at 72 hr. In the liver, the total radioactivity increased throughout the sampling period and from 12 hr onwards consisted largely of CBDP, which represented 71 and 74% of the total at 48 and 72 hr respectively. TOCP declined from 70% of the total at 4 hr to 26% at 72 hr, while the hydroxylated intermediates were found at low levels only, the dihydroxymethyl derivative being detected only during the first 24 hr. Plasma levels of radioactivity were consistently lower, and at 24 hr represented only about 5% of those in liver. TOCP accounted for about 80% of this plasma activity, the monohydroxy CBDP intermediate for about 15% and CBDP for the remainder. In the excreta, nearly 99% of the activity consisted of unchanged TOCP, elimination of which was maximal during the first 12 hr, although even by 72 hr only 26·5% of the administered dose had been excreted.

These results indicate that CBDP binds more readily than TOCP with tissue components. The resulting gradual accumulation of this metabolite in the liver and other tissues could account for the delayed neurotoxic effects observed after a single dose of TOCP.

NATURAL PRODUCTS

2947. Congeners and the liver

Siegers, C.-P., Strubelt, O. & Breining, H. (1974). The acute hepatotoxic activity of alcoholic beverages and some of their congeners in guinea pigs. *Pharmacology* **12**, 296.

Recent studies have provided little support for the theory that the congeners in alcoholic beverages, rather than ethanol, are the chief cause of their hepatotoxicity (*Cited in F.C.T.* 1975, **13**, 591), and further negative evidence is now available.

Guinea-pigs were killed 16 hr after a single oral dose of 3·2 or 6·4 g ethanol/kg, given either as a 40% aqueous solution of pure ethanol or in the form of gin, corn-brandy, brandy, vodka, rum or whisky, the dosage volume being 10 or 20 ml/kg. The higher dose of ethanol increased the activities of the serum enzymes, glutamic-oxalacetic transaminase (GOT), glutamic-pyruvic transaminase and glutamic dehydrogenase, while the lower dose increased GOT activity only. The increases in enzyme activity induced by the alcoholic beverages were either of the same order as those induced by pure ethanol or were lower, and no increase in GOT resulted from either gin or corn-brandy. Hepatic triglyceride levels were nearly doubled by both doses of ethanol, whereas the effect of the alcoholic beverages was dose-dependent but did not significantly exceed that of ethanol even at 20 ml/kg. Histologically, the frequency and intensity of vacuolar degeneration of the liver did not differ whether the animals were given pure ethanol or alcoholic beverages.

In another part of this study, guinea-pigs were given a single dose of one of seven congeners dissolved in olive oil in a concentration 10 or 100 times greater than the maximum concentration found in any alcoholic beverage. The doses given were again 10 ml/kg. The top doses were thus equivalent to 50 mg acetaldehyde, 33 mg ethyl formate, 825 mg ethyl acetate, 125 mg methanol, 140 mg *n*-propanol, 335 mg isobutanol and 1200 mg isoamyl alcohol/kg. Even at these high levels none of the congeners increased serum-enzyme activities, determined 16 hr after treatment, and in only three cases was there an increase in liver triglycerides. While both doses of isoamyl alcohol produced triglyceride increases of the same order, the effect of acetaldehyde and methanol was dose-dependent, but even with these compounds the increase was not significantly greater than after ethanol. Solitary hepatic vacuoles were produced by some congeners, but vacuolar degeneration was less marked and less frequent than after the alcoholic beverages.

2948. Another hepatotoxin from a well-known source

Bassir, O. & Emerole, G. O. (1974). Susceptibility of rats to palmotoxins B_0 and G_0. *FEBS Lett.* **40**, 247.

The range of toxic metabolites now known to be produced in addition to aflatoxin by *Aspergillus flavus* includes aspertoxin and flavutoxin (*Cited in F.C.T.* 1975, **13**, 291). Other metabolites, designated palmotoxins B_0 and G_0, have been shown to produce liver lesions and other changes in chick embryos similar to those induced by aflatoxin (Bassir & Adekunle, *W. Afr. J. biol. Chem.* 1969, **12**, 7; *idem, J. Path.* 1970, **102**, 49; *idem, FEBS Lett.* 1970, **10**, 198), but the study now reported is apparently the first to be concerned with their effects in mammals.

When rats were given, from 20 days after birth, daily ip injections of palmotoxin B_0 or G_0 at dose levels of 1·6–6·4 mg/kg/day for 15 days, those treated with palmotoxin B_0 showed consistently lower bodyweight gains than the controls and a reduction in relative liver size. These effects were clearly dose-dependent and were accompanied by peripheral necrosis of the liver cells, a marked increase in serum glutamic–oxalacetic transaminase (GOT) activity and some increase in serum alkaline-phosphatase activity. Similar changes were seen in rats injected with aflatoxin B_1 at a level of 0·5 mg/kg for 15 days, but those treated with palmotoxin G_0 showed only an insignificant loss of weight at the highest dose level.

These findings suggest some similarity between the toxic effects of palmotoxin B_0 and aflatoxin B_1 but no comparable potency in palmotoxin G_0. No deaths occurred during the experiment, however, even in the group given palmotoxin B_0 in doses of 6·4 mg/kg body weight. Increases in serum alkaline-phosphatase and GOT activity are associated with the onset of hepatic necrosis and myocardial infarction, but histological examination of the livers of these rats at termination of treatment showed no marked pathological changes.

PATHOLOGY

2949. Starch in heart surgery

Osborne, M. P., Paneth, M. & Hinson, K. F. W. (1974). Starch granules in the pericardium as a cause of the post-cardiotomy syndrome. *Thorax* **29**, 199.

Starch has been implicated in the aetiology of a number of post-operative syndromes and has even been a cause for concern in extra-corporeal surgery (*Cited in F.C.T.* 1975, **13**, 389 & 485). The authors named above have now described a severe inflammatory reaction following starch contamination during cardiotomy.

A 38-yr-old housewife was admitted to hospital with pain in the posterior left upper chest and was diagnosed as having critical mitral stenosis. After further examination, an operation was performed to replace the mitral valve with a tilting-disc valve prosthesis. During the first 7 days after the operation the patient recovered uneventfully. She then developed a high temperature and productive cough, which improved with antibiotic treatment. Within 48 hr of her discharge from hospital 12 days after the operation, however, she was re-admitted with malaise, abdominal discomfort, nausea and vomiting and, soon after, hypotension developed. The prosthesis was found to be functioning normally.

A post-cardiotomy syndrome with cardiac tamponade was diagnosed, and the aspiration of 250 ml of straw-coloured fluid from the pericardial region produced immediate relief. This pericardial effusion had to be aspirated three times in the following 2 wk, and on the fourth recurrence a second operation was performed. The pericardium was found to be inflamed and covered with a fibrinous exudate, but no discrete lesions were seen. A pericardial drain was inserted and removed 48 hr later. After one further relapse, for which further temporary drainage was inserted, steroid treatment was instituted for several weeks and the patient was subsequently discharged after an uneventful recovery

The fluid removed was sterile. A section of pericardium removed during surgery showed an inflammatory exudate and the presence of clumps of carbohydrate showing the characteristic 'Maltese cross' of starch under polarized light. Intradermal and RAST (radioallergosorbent) tests did not indicate that the patient was sensitive to starch, although at the time of the tests, reactions may have been masked by the steroid treatment she was undergoing.

Starch pericarditis following heart surgery appears not to have been reported previously. Typical starch granulomas were not seen in this patient. Although no immune response was detected on this occasion, there was a non-specific change in serum immunoglobulins. The authors suggest that surgeons should look out for starch as a possible cause of post-cardiotomy syndromes and they express their concern that starch may interact with damaged pericardium to initiate an auto-immune response.

Fd Cosmet. Toxicol. Vol. 13, p. 679. Pergamon Press 1975. Printed in Great Britain

FORTHCOMING PAPERS

It is hoped to publish the following research papers in the next issue of *Food and Cosmetics Toxicology*:

Dose-response study of the carcinogenicity of dietary sodium nitrite and morpholine in rats and hamsters. By R. C. Shank and P. M. Newberne.

Effects of food seasonings on the cell cycle and chromosome morphology of mammalian cells *in vitro* with special reference to turmeric. By C. E. Goodpasture and Frances E. Arrighi.

Cholesterol levels, atherosclerosis and liver morphology in rabbits fed cyclopropenoid fatty acids. By T. L. Ferguson, J. H. Wales, R. O. Sinnhuber and D. J. Lee.

The renal handling of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) in the dog. By J. B. Hook, R. Cardona, J. L. Osborn, M. D. Bailie and P. J. Gehring.

Mirex: A teratogenicity, dominant lethal and tissue distribution study in rats. By K. S. Khera, D. C. Villeneuve, Geraldine Terry, Luz Panopio, Lorena Nash and G. Trivett.

Tissue distribution and excretion of tritiated tetrachlorodibenzo-*p*-dioxin in non-human primates and rats. By J. P. Van Miller, R. J. Marlar and J. R. Allen.

The absence of tetrahydrocannabinol from hops. By Catherine Fenselau, S. Kelly, M. Salmón and S. Billets.

Metabolism and elimination of α-hydroxyethane sulphonate by rats. By W. B. Gibson and F. M. Strong. (Short Paper)

Toxicity studies with δ-aminolaevulinic acid. By G. L. Kennedy, Jr., D. W. Arnold and J. C. Calandra. (Short Paper)

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.

Instructions to Authors

General. Authors from the United Kingdom should send *Original Papers and Reviews* to the Assistant Editor. All other papers and reviews should be sent to the appropriate Regional Editor. All 'Letters to the Editor' should be sent to the Editor and must be signed before they can be considered for publication.

Submission of a paper to the Editor will be held to imply that it reports unpublished original research, that it is not under consideration for publication elsewhere and that if accepted for the Journal, *Food and Cosmetics Toxicology*, it will not be published again, either in English or in any other language, without the consent of the Editor.

Forms of Papers Submitted for Publication. Papers should be headed with the title of the paper, the surnames and initials of the authors (female authors may use one given name) and the names and addresses of the institutions where the work was done. A shortened version of the title not exceeding 45 letters and spaces, to serve as a running title, should be supplied.

In general the text should be subdivided as follows: (a) Summary: brief and self-contained, not exceeding 3% of length of paper (b) Introductory paragraphs (c) Experimental (d) Results, presented as concisely as possible (e) Discussion (if any) and general conclusions (f) Acknowledgements and (g) References. Each subdivision should commence on a separate sheet. Manuscripts should be typewritten on *one side of the paper* and *double spaced.* At least *two copies* should be supplied (one original and one, or preferably two, carbon copies). Papers will be accepted in English, French and German.

References. These should be listed on a separate page, in alphabetical order and arranged as follows: author's name and initials, year, title of the journal [abbreviated according to the rules adopted in the *World List of Scientific Periodicals 1963*, 4th Ed., Butterworth & Co. (Publishers) Ltd. London], volume, first page number:

e.g. Hickman, J. R., McLean, D. L. A. & Ley, F. J. (1964). Rat feeding studies on wheat treated with gamma-radiation. I. Reproduction. *Fd Cosmet. Toxicol.* **2**, 15.

References to books should include the author's name followed by initials, year, title of book, edition, appropriate page number, publisher and place of publication:

e.g. Dow, E. & Moruzzi, G. (1958). *The Physiology and Pathology of the Cerebellum.* 1st Ed., p. 422. The University of Minnesota Press, Minneapolis.

The names of all the authors of papers to be cited should be given when reference is first made in the text. In cases where there are more than two authors subsequent citations should give the first-named author followed by the words *et al.*:

e.g. (McLaughlin, Bidstrup & Konstam, 1963); (McLaughlin *et al.* 1963).

Where more than one paper by the same author(s) has appeared in any one year, the references should be distinguished in the text and the bibliography by the letters, a, b etc. following the citation of the year:

e.g. 1943a, 1943b or (1943a, b).

Footnotes. These as distinct from literature references should be avoided as far as possible. Where they are essential, reference is made by the symbols * † ‡ § ‖ ¶ in that order.

Illustrations and Diagrams. These should be kept to a *minimum* and they should be numbered and marked on the back with the author's name. Legends accompanying illustrations should be typewritten on *separate* sheets. Diagrams and graphs must be drawn in Indian ink on good quality paper or tracing linen. The following standard symbols should be used on line drawings since they are easily available to the printers:

▲ △ ■ □ ● ○ ⊙ × ◇

Photographs and photomicrographs should be submitted unmounted and on glossy paper. When colour plates are to be printed, payment for blockmaking is the responsibility of the author.

Tables and Figures. These should be constructed so as to be intelligible without reference to the text, each table and column being provided with a heading. The same information should not be reproduced in both tables and figures.

Chemical Nomenclature. The fundamental principles of organic and inorganic chemical nomenclature are laid down in the I.U.P.A.C. 1957 Rules (Butterworths Scientific Publications, London, 1958, 1959). These are given in *Handbook for Chemical Society Authors* (1961), pp. 16–163.

Other Nomenclature, Symbols and Abbreviations. In general, authors should follow the recommendations published in the *Handbook for Chemical Society Authors* (1961), p. 164 and in the *I.U.P.A.C. Information Bulletin*, No. 13, p. 64, Appendix B (1961). In the title and summary, abbreviations should be avoided; in the Introduction, Results and Discussion they should be used sparingly.

Page Proofs. These will be sent to the first-named author for correction.

Reprints. Reprints can be ordered on the form accompanying proofs.

Frequency. The Journal will be published bi-monthly.

Printed in Great Britain by A. Wheaton & Co., Exeter