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Research Section

ENHANCING EFFECTS OF PHENOBARBITONE AND BUTYLATED HYDROXYTOLUENE ON 2-ACETYLAMINOFLUORENE-INDUCED HEPATIC TUMORIGENESIS IN THE RAT

C. PERAINO, R. J. M. FRY, E. STAFFELDT and J. P. CHRISTOPHER*

Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois 60439, USA

(*Received 29 August 1976*)

Abstract—A comparison has been made between the enhancing effects of 0·05% dietary phenobarbitone and of 0·5% dietary butylated hydroxytoluene (BHT) on hepatic tumorigenesis in rats previously fed 2-acetylaminofluorene for a brief period. Prolonged feeding of the BHT diet produced a significant degree of enhancement which, however, was both lower in magnitude and delayed, in comparison with the enhancement produced by the phenobarbitone diet. Daily phenobarbitone injections stimulated persistent liver enlargement and a transient fourfold increase in DNA synthesis over a 5-day period. Similar treatment with BHT produced less pronounced liver enlargement after a delay of 1 day and did not stimulate DNA synthesis as did phenobarbitone. The results suggest that the dissimilar tumorigenic-enhancing abilities of BHT and phenobarbitone may result from differences in the effects of these agents on biochemical processes related to liver growth.

INTRODUCTION

Studies in this laboratory have shown that dietary phenobarbitone enhances hepatic tumorigenesis in rats previously fed 2-acetylaminofluorene (AAF) for a brief period (Peraino, Fry & Staffeldt, 1971; Peraino, Fry, Staffeldt & Kisieleski, 1973). Additional use of this sequential treatment protocol showed that DDT—which resembles phenobarbitone in its effects on liver metabolism and morphology (Peraino, Fry, Staffeldt & Christopher, 1975; Schulte-Hermann, 1974)—was as effective as phenobarbitone in enhancing AAF-induced hepatic tumorigenesis (Peraino *et al.* 1975).

Butylated hydroxytoluene (BHT), an antioxidant commonly used as a food additive, exerts stimulatory effects on liver growth that are qualitatively similar to those produced by phenobarbitone and DDT (Schulte-Hermann, 1974). In addition, BHT reduced the incidence of AAF-induced hepatic tumorigenesis when fed simultaneously with the carcinogen (Ulland, Weisburger, Yamamoto & Weisburger, 1973). This protective effect resembles that produced by feeding phenobarbitone simultaneously with AAF (Peraino *et al.* 1971). Both BHT and phenobarbitone evidently stimulate metabolic pathways leading to AAF detoxification, thereby reducing the proportion of the carcinogen dose available for activation (Grantham, Weisburger & Weisburger, 1973; Matsushima, Grantham, Weisburger & Weisburger, 1972; Mushlin & Peraino, 1974). Several other studies, reviewed by Wattenberg, Loub, Lam & Speier (1976), have shown that BHT reduces the tumour incidence from a variety of carcinogens in a number of target tissues when BHT and the carcinogen are administered concurrently. Similar protective effects of phenobarbitone with different carcinogens and in different tissues have also been observed (Grube, Peraino & Fry, 1975; Ishidate, Watanabe & Odashima, 1967; Kunz, Schaude & Thomas, 1969).

The similarity of the effects of BHT and phenobarbitone suggested that BHT would show tumorigenic enhancing activity in rat liver when tested according to the sequential treatment protocol that had revealed such activity for phenobarbitone and DDT. The present study, therefore, compares the effects of dietary BHT and phenobarbitone on liver tumorigenesis in rats previously fed AAF.

EXPERIMENTAL

Tumour incidence study. Male Sprague–Dawley rats (22 days old) were fed a diet containing 0·02% AAF for 18 days, and then were fed the diet without AAF for 7 days. The rats were divided into three groups, each containing 120 rats, which were then given a control diet, the control diet plus 0·05% phenobarbitone or the control diet plus 0·5% BHT for up to 407 days. On the basis of food-consumption measurements, the average daily intake was estimated as 20–50 mg/kg for phenobarbitone and 200–500 mg/kg for BHT. No signs of sedation or toxicity appeared in any of the rats throughout the experiment.

Rats selected randomly from each group were killed at eight intervals and examined for liver tumours as described previously (Peraino *et al.* 1975). The data from pairs of adjacent examinations were

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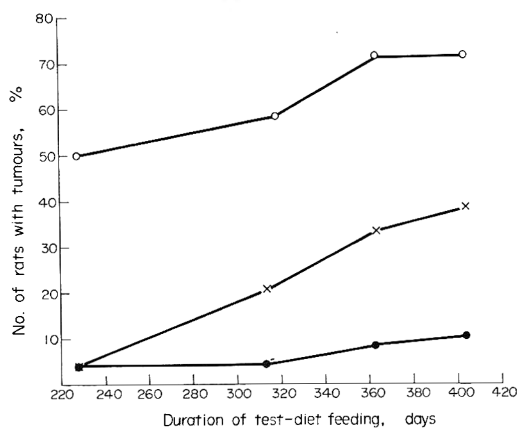

Fig. 1. Development of liver adenomas or carcinomas in rats (20–24 for each point) fed 0·02% dietary AAF for 18 days followed, after 7 days, by control diet (●), 0·05% phenobarbitone (O) or 0·5% BHT (×).

pooled and plotted at the four mean intervals between the original killings. Additional experimental details concerning rats, diets, husbandry and histological preparations have been described elsewhere (Peraino *et al.* 1971).

Acute exposure study. The experimental conditions were similar to those reported previously (Peraino *et al.* 1975). Weanling rats were fed the control diet for 25 days. Beginning on day 26 and continuing for 5 days the rats were given single daily ip injections (between 07.00 and 09.00 hr) of BHT (500 mg/kg) dissolved in cotton-seed oil. Control rats received equivalent volumes of cotton-seed oil. Liver DNA synthesis and relative liver weights were measured daily during this period by procedures described previously (Peraino *et al.* 1975). The phenobarbitone data shown in Fig. 2 were taken from the earlier study (Peraino *et al.* 1975), in which phenobarbitone sodium was injected in distilled water at a dosage of 83 mg/kg.

RESULTS

Tumour incidence study

Deaths (23% in each group) occurred randomly throughout the experiment and showed no apparent relationship to the experimental treatments. Examination of the lungs at autopsy implicated pneumonia as the major cause of these deaths.

The observed hepatic lesions were classified as indicated previously (Peraino *et al.* 1975). Only those lesions classified as adenomas or carcinomas were used to compile the tumour data described below. The ratio of adenomas to carcinomas (approximately 3:1) did not differ significantly among the three groups.

Figure 1 compares the development of hepatic tumours in rats fed AAF followed by a control diet or one containing phenobarbitone or BHT. In agreement with our earlier observations (Peraino *et al.* 1971, 1973 & 1975), phenobarbitone markedly enhanced tumorigenesis in rats previously fed AAF. In rats fed BHT, tumour incidence began to increase much later than in the phenobarbitone group and then rose more rapidly than in the control group during the remainder of the experiment.

Table 1 compares the total tumour incidences in the three groups and shows that the overall tumour incidence in the BHT group was significantly greater than that in the control group but significantly less than that in the phenobarbitone group.

Acute exposure study

Both liver weight and liver DNA synthesis increased in rats injected daily with phenobarbitone over a 5-day period. The increase in DNA synthesis was transient, rising progressively to four times the control values during the first 3 days of treatment and returning to control levels by day 5, despite continued phenobarbitone administration. However, the liver weights in these rats remained high at day 5. In rats given BHT under the same conditions as those for phenobarbitone, although at a dose level six times higher, liver weight also increased, but the response was less pronounced, and did not begin until day 2 (Fig. 2). BHT also did not cause the transient stimulation of DNA synthesis previously observed during acute phenobarbitone treatment, but instead produced an initial drop in synthesis on day 1 followed by a return to pretreatment levels by day 5. Autoradiographic measurements of hepatocyte labelling in these animals (data not shown) indicated a stimulatory effect of BHT on hepatocyte proliferation by day 5.

DISCUSSION

The results of the present study indicate that the prolonged feeding of BHT significantly enhances hepatic tumorigenesis in rats previously fed AAF for a brief period. Recent studies have shown that BHT also increases the tumorigenic effect of diethylnitrosamine (DENA) in lung and forestomach in male mice (Clapp, Satterfield & Klima, 1975; Clapp, Tyndall, Cumming & Otten, 1974). In these experiments BHT at a dietary level of 0·75% and DENA were administered concurrently, and the enhancing effect of BHT

Table 1. *Hepatic tumour incidence in rats continuously fed phenobarbitone or BHT for 407 days starting 7 days after an 18-day period of AAF feeding*

Treatment after AAF feeding	No. of rats	No. of rats with tumours	Percentage of rats with tumours	Total no. of tumours	Average no. tumours/rat
Control diet	92	6	7	12	0·13
0·05% Phenobarbitone	93	58	62*	112	1·20
0·5% BHT	93	24	26*	31	0·33

*The values for rats fed phenobarbitone or BHT were significantly ($P < 0{\cdot}001$) higher than control values, but the value for rats fed BHT was significantly ($P < 0{\cdot}001$) lower than that for rats fed phenobarbitone.

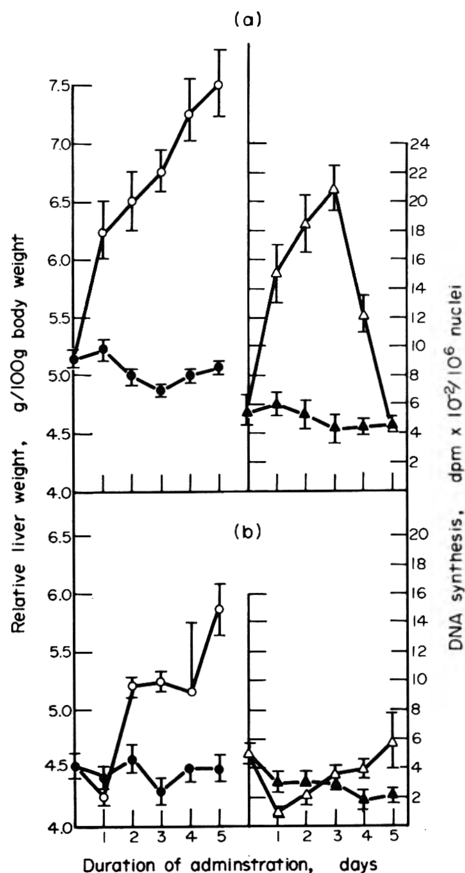

Fig. 2. Relative liver weight (O) and liver DNA synthesis (△) in rats injected with (a) phenobarbitone and (b) BHT, and the corresponding values for injection vehicles above (●, ▲). Each point represents the mean ± SEM for a group of five rats.

was attributed to its induction of microsomal enzymes catalysing the conversion of DENA to an active carcinogen (Clapp *et al.* 1974). The sequential treatment protocol used in the present study, however, decreases the likelihood that this mechanism is the basis for the enhancement of AAF tumorigenesis by BHT. Earlier studies with AAF and phenobarbitone suggested that in the sequential protocol the metabolic fate of the carcinogen is not altered by the subsequently administered enhancing agent (Mushlin & Peraino, 1974). Moreover, as noted above, the pattern of microsomal enzyme induction produced by BHT and phenobarbitone favours AAF inactivation and a reduction in tumorigenesis.

Although both phenobarbitone and BHT enhanced tumorigenesis, BHT was much less efficient, and the BHT effect did not become apparent during the first 200 days of feeding—a period during which phenobarbitone, fed at a tenfold lower level, produced marked enhancement (Fig. 1). The differences in the characteristics of the enhancement produced by BHT and phenobarbitone are also reflected in the relative responses of liver size and DNA synthesis to acute administration of the two agents (Fig. 2). These observations raise the possibility that differences in the effects of BHT and phenobarbitone on biochemical processes associated with liver growth may underlie the observed differences in the tumorigenic enhancing abilities of these agents.

Further studies of the comparative effects of BHT and phenobarbitone on liver tumorigenesis and biochemical responses should help to identify molecular changes specifically related to various components of the enhancement process. Such identification is essential, both for an understanding of the mechanism of tumorigenesis and for the development of relatively rapid methods for estimating the tumorigenic risk associated with the ingestion of food additives and environmental contaminants.

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ON THE AETIOLOGY OF GASTRIC CANCER: MUTAGENICITY OF FOOD EXTRACTS AFTER INCUBATION WITH NITRITE

H. MARQUARDT, F. RUFINO and J. H. WEISBURGER

Naylor Dana Institute for Disease Prevention, Valhalla, New York 10595, USA

(*Received 4 September 1976*)

Abstract—Treatment of certain foods, such as fish, beans and borscht which are typical of areas with a high incidence of gastric cancer, with 5000 ppm nitrite resulted in the formation of one or more mutagenic principles, as indicated by a *Salmonella typhimurium* test. *S. typhimurium* strain TA 1535 was the most sensitive of several indicator organisms tested. Formation of mutagenic material was highest at pH 3 with 5000 ppm nitrite, but significant amounts were also produced with 1000 ppm nitrite. More acidic as well as alkaline media led to a loss of the mutagenic activity over a 24-hr period. Ascorbic acid prevented the formation of the mutagenic material in nitrite-treated fish. Nitrite treatment of hot dogs, pork, beef, barley and sour milk produced no detectable mutagenic material.

INTRODUCTION

The well-established organ-specific carcinogenicity of nitrosamines and nitrosamides in animals (Magee, 1971; Magee & Barnes, 1956) is possibly of relevance to the development of human cancer, since *in vivo* nitrosation of certain dietary amines and amides under the acidic conditions of the stomach could lead to the formation of these agents in human gastric juices, which have a pH range of 1–4 (Druckrey, 1975; Endo, Takahashi, Kinoshita & Baba, 1974; Sander, 1970 & 1971; Schoental & Bensted, 1969; Sugimura & Kawachi, 1973). Nitrite is used as a food preservative to inhibit *Clostridium botulinum*, and is also secreted, sometimes in substantial quantities, in human saliva (Tannenbaum, Sinskey, Weisman & Bishop, 1974). Nitrate, especially that in starchy foods and in vegetables, is another source, being converted to nitrite when such foods are stored at room temperature (Weisburger & Raineri, 1975a). The nitrosatable products (amines and amides) also occur in our diet (Endo, Takahashi, Kinoshita, Utsunomiya & Baba, 1975).

In a search for the aetiological factors responsible for gastric cancer, we examined extracts of nitrite-treated foods for their mutagenic activity in different strains of *Salmonella typhimurium* (Ames, McCann & Yamasaki, 1973). Since the stomach is the most likely site of cancers induced by this nitrosation reaction of alkylamide-type substrates, we investigated food consumed predominantly in regions with a high stomach-cancer incidence. While fish is a key component of the diet in Japan (the country with the highest stomach-cancer rate in the world), pork, beans, barley, sour milk and borscht are eaten mostly in Poland and other Eastern European countries where the stomach-cancer rate is also high. Hot dogs and beef, components of the diet in America (a low-risk area for gastric cancer), were also investigated.

EXPERIMENTAL

Food materials. The foods were purchased from local stores in Westchester County, except for Japanese fish (Sanma Hiraki) which was imported deep-frozen and purchased from a dealer in Flushing, New York.

Preparation of the extracts. Samples of about 10 g of each food were minced and homogenized in a Waring blender. Except for the dose-response study, 5000 ppm $NaNO_2$ and 20,000 ppm NaCl both in 1 ml water were added to 5-g aliquots of food; in control mixtures, 1 ml distilled water was added instead of $NaNO_2$. Each mixture was incubated in the dark for 1 hr at 25°C and pH 3. The reaction was stopped by addition of 5000 ppm ammonium sulphamate in 1 ml water. Thereafter, the mixture (c. 8 ml) was cooled to 4°C and extracted twice with 10 ml ice-cold hexane to remove the lipids. The remainder was then extracted four times with 15 ml ice-cold ether, the combined ether extracts were filtered by suction through Whatman no. 40 filter paper, and the filter was washed with 15 ml ether. The ether extract was dried with anhydrous Na_2SO_4 and reduced under a stream of nitrogen to about 0·1 ml.

Mutagenesis assay. A mutagenesis assay with *S. typhimurium* was performed following the procedure described by Dr. B. N. Ames, Berkeley, California, who also generously provided the different tester strains (McCann, Choi, Yamasaki & Ames, 1975). The tests were performed by incubation for 48 hr at 37°C with and without the addition of a 9000-**g** liver supernatant (S-9 fraction) isolated from Aroclor-treated male CDF rats (Ames *et al.* 1973). *N*-Methyl-*N*-nitro-*N*-nitrosoguanidine (20 μg/plate) and picrolonic acid (250 μg/plate), neither of which requires metabolic activation, were used as positive controls with TA 100 and 1535 and with TA 98, 1537 and 1538, respectively. 2-Aminofluorene (250 μg/plate), which requires metabolic activation, was used as a positive control with

the S-9 fraction and TA 1538. Each assay was performed in duplicate, each food product was tested in two independent experiments, and the values presented are the means ± SD.

Effect of pH. In preliminary experiments, the incubations were performed at pH 1, 2, 3 and 4. The highest numbers of revertant colonies were seen at pH 3 and therefore this pH was used as a standard condition for all subsequent tests in which various types of foods were exposed to nitrite.

Level of nitrite. To determine the effect of different levels of sodium nitrite, concentrations of 1000, 2000, 3000, 4000, 5000 and 20,000 ppm of the compound were added to homogenates of 5 g raw fish. The mixture was incubated and processed as described above.

Stability of mutagenic material. The residue from an ether extract containing mutagen derived from nitrite-treated fish was taken up in 1 ml water. This solution was adjusted to pH 1, 3, 7 or 10 and allowed to stand at 25°C for 24 hr. The residual mutagenic activity was then evaluated.

Dose-response study on mutagenic material. Increasing amounts of the aqueous residue from the final ether extract from nitrite-treated foods obtained as described above were applied to plates containing the tester strains. The number of revertant colonies was determined.

RESULTS

With the indicator organism *S. typhimurium*, incubation at pH 3 appeared to be optimal for the production of a mutagenic principle (Table 1). In preliminary experiments it was noted that incubation for 1 hr was optimal.

A dose-response study involving increasing amounts of nitrite suggested that maximum activity was observed in the range 5000–20,000 ppm sodium nitrite (Table 2). Thereafter, 5000 ppm nitrite was used as the standard amount in this experimental series.

Extracts of nitrite-treated fish contained a powerful mutagen which was best revealed by the use of tester strains, such as *S. typhimurium* strain TA 1535, susceptible to mutation through base-pair substitution (Table 3). Other strains of *S. typhimurium* were less sensitive. Control samples of fish without nitrite did not contain appreciable mutagenic material. The treatment of homogenates of sour milk, hot dog, beef and pork with nitrite failed to yield measurable mutagenic activity with *S. typhimurium* strain TA 1535. In this system, extracts of borscht and beans gave a moderate increase in mutagenic activity (Table 3).

Table 1. *Mutagenic effects of 10 μl extract of nitrite-treated raw fish in* S. typhimurium *TA 1535*

Incubation at pH	Number of his$^+$ revertant colonies*
1	42 ± 0
2	65 ± 14·8
3	252 ± 29·0
4	125 ± 8·4

*The number of spontaneous revertants was 7 ± 2·8.

Table 2. *Effect of amount of nitrite on the production in fish of material mutagenic to* S. typhimurium *TA 1535*

Concn of sodium nitrite (ppm)	Number of his$^+$ revertant colonies*
1000	42 ± 7·8
2000	60 ± 0·7
3000	158 ± 19·8
4000	173 ± 5·7
5000	297 ± 8·5
20,000	311 ± 14·9

*The number of spontaneous revertants was 12 ± 1·4.

Of the positive controls, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine produced an average of 1535 revertant colonies/plate with TA 1535 and 100, picrolonic acid produced 654, 130 and 930 with TA 98, 1537 and 1538, respectively, and 2-aminofluorene produced 185 with TA 1538.

With the nitrosated foodstuffs that gave rise to a mutagen, there was a dose–activity relationship with respect to the amount of the extract applied to the plate (Table 4). A study on the stability of the mutagenic principle as a function of pH showed that the material was most stable at pH 3 (57% of the original activity remaining after 24 hr) but disappeared substantially at pH 1 (7% remaining), 7 (12% remaining) or 10 (4% remaining). Incubation for 1 hr at 25°C at pH 3 developed 252 revertants/plate with 10 μl extract, while incubation at 60°C at pH 3 reduced the revertants developed to 124, and heating to 100°C destroyed the mutagenic activity. In addition, incubation with 28,000 ppm ascorbic acid (twice the molar equivalent of nitrite) completely prevented the formation of the mutagenic material.

DISCUSSION

We have presented data bearing on the concept that gastric cancer in man might stem from the *in vivo* nitrosation of an as yet unknown substrate with the production of an alkylnitrosoureido type of compound. Sugimura & Kawachi (1973) and Druckrey (1975) showed these to be powerful and specific gastric carcinogens in a variety of species, leading to cancer in the glandular stomach like that seen in man. Previous studies in our laboratories have provided evidence that in high-risk populations, substantial amounts of nitrite occurred in foods through deliberate addition or by reduction of nitrite (Weisburger & Raineri, 1975a,b).

The current series of experiments was started in order to obtain information on the nature of the substrate nitrosated, and an assessment of mutagenicity seemed to be a useful tool for enquiring into the nature of a possibly carcinogenic principle. The results described justify this approach. Nitrosation of foods, such as fish, consumed in countries where the incidence of gastric cancer is high, yielded mutagenically-active material, whereas similar treatment of foods such as meats, consumed largely in countries where the gastric-cancer incidence is low, failed to yield a measurable amount of mutagenic activity, perhaps because of the reaction of nitrite with myoglobin

Table 3. *Mutagenic effects of nitrite-treated food extracts in* S. typhimurium

				Number of his⁺ revertant colonies			
				Without S-9		With S-9*	
Food	Nitrite treatment	*S. typhimurium* strain TA-	Dose/plate µl	With extract	Without extract†	With extract	Without extract†
Fish	+	1535	10 st	297 ± 8·5	12 ± 1·4	290 ± 25·0	8 ± 3·5
		1535	10 pa	252 ± 29·0	14 ± 2·8	235 ± 5·7	8 ± 2·8
		100	10 pa	320 ± 42·0	80 ± 2·8	311 ± 14·9	88 ± 8·5
		1537	10 pa	7 ± 5·7	4 ± 2·8	7 ± 2·8	4 ± 2·8
		1538	10 pa	14 ± 4·2	8 ± 5·0	11 ± 12·7	8 ± 5·7
		98	10 pa	30 ± 5·7	17 ± 3·5	20 ± 7·1	24 ± 5·7
	−	1535	10 st	3 ± 2·1	1 ± 0·7		
		1535	10 pa	18 ± 0·7	14 ± 2·8		
		100	10 pa	134 ± 11·3	80 ± 2·8		
		1537	10 pa	4 ± 2·8	4 ± 1·4		
		1538	10 pa	12 ± 2·8	8 ± 5·0		
		98	10 pa	20 ± 2·8	11 ± 3·5		
Hot dog‡	+	1535	10 st	6 ± 0	6 ± 0·7	3 ± 2·1	8 ± 3·5
		1535	10 pa	16 ± 5·7	14 ± 2·8	24 ± 9·9	15 ± 7·1
		100	10 pa	138 ± 19·8	98 ± 8·5	170 ± 31·0	91 ± 26·0
		98	10 pa	26 ± 7·1	11 ± 1·4	32 ± 1·4	12 ± 4·2
	−	1535	10 st	4 ± 2·4	6 ± 2·1	6 ± 1·4	7 ± 4·2
		1535	10 pa	15 ± 1·4	14 ± 2·8	9 ± 4·2	15 ± 7·1
		100	10 pa	114 ± 8·5	98 ± 8·5	142 ± 14·0	91 ± 26·0
		98	10 pa	16 ± 2·8	11 ± 1·4	13 ± 5·7	12 ± 4·2
Beef	+	1535	10 st	15 ± 5·7	8 ± 3·5	9 ± 2·1	7 ± 0·7
Beef + salt	−	1535	10 st	9 ± 0·7	8 ± 3·5	9 ± 2·1	7 ± 0·7
Pork	+	1535	10 st	5 ± 2·1	6 ± 0·7	5 ± 1·4	8 ± 3·5
	−	1535	10 st	4 ± 1·4	6 ± 0·7	3 ± 7·1	8 ± 3·5
Beans	+	1535	10 st	26 ± 5·0	12 ± 1·4	27 ± 9·2	8 ± 3·5
			25 st	27 ± 7·8		153 ± 19·8	
	−	1535	10 st	9 ± 1·4	12 ± 1·4	11 ± 0·7	8 ± 3·5
Barley	+	1535	10 st	6 ± 1·4	12 ± 1·4	3 ± 0·7	8 ± 3·5
	−	1535	10 st	6 ± 0·7	12 ± 1·4	5 ± 0·7	8 ± 3·5
Borscht	+	1535	10 st	23 ± 7·1	12 ± 1·4	29 ± 4·2	8 ± 3·5
			25 st	65 ± 0		93 ± 7·1	
	−	1535	10 st	9 ± 1·4	12 ± 1·4	12 ± 0·7	8 ± 3·5
Sour milk	+	1535	10 st	15 ± 0	12 ± 1·4	18 ± 3·5	8 ± 3·5
	−	1535	10 st	9 ± 1·4	12 ± 1·4	6 ± 0·7	8 ± 3·5

st = Spot test pa = Plate incorporation assay
*The 9000-g supernatant derived from the liver of Aroclor-induced male CDF rats; 400 µg protein/plate.
†Spontaneous reversions.
‡Hot dog contained pork and beef.
Values are means ± SD for duplicate assays in two independent experiments.

(Druckrey, Steinhoff, Benthner, Schnieder & Klärner, 1963). This latter negative result is also an important finding in view of the fact that nitrite in small amounts is currently used for several purposes, including protection against botulism, in these meat products (Binkerd & Kolari, 1975). Long-term feeding of nitrite plus meat to rats has not provided evidence of a carcinogenic effect, perhaps in agreement with the failure to yield a mutagen in our tests (van Logten, den Tonkelaar, Kroes, Berkvens & van Esch, 1972).

Endo *et al.* (1975) have nitrosated methylguanidine under simulated gastric conditions and have noted the formation of a mutagenic principle identified as nitrosocyanamide. This interesting finding requires extension and verification, for it seems that the older literature on the presence of methylguanidine in foods may be at least partially incorrect (Dr. H. Endo, personal communication 1976). Also, nitrosocyanamide, while highly mutagenic, induced mainly forestomach tumours in rats, and thus does not exhibit the specificity for inducing human-like cancer of the glandular stomach such as is induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or *N'*-acetylmethylnitrosourea (Druckrey, 1975).

The fact that the mutagenic material was shown to be present in fish and also to a lesser extent in beans and borscht without metabolic activation suggests that the mutagen is direct-acting. Thus, it may well have a structure corresponding to that of an alkylnitrosamide, a possibility supported by the demonstrations of the varying stability of the material with pH and temperature. In addition, however, the activity in beans and borscht was increased when an

Table 4. *Mutagenic effects of extract of nitrite-treated food in* S. typhimurium *TA 1535 plate incorporation assay as a function of dose/plate*

Extract	Dose/plate (μl)	Number of his$^+$ revertant colonies
Fish*	1	70 ± 12·7
	5	184 ± 19·8
	10	252 ± 29·0
	20	212 ± 5·7
	40	—‡
Beans†	1	26 ± 1·4
	10	59 ± 5·7
	25	73 ± 0
Borscht†	1	26 ± 7·1
	10	29 ± 3·5
	25	65 ± 0

*The number of spontaneous revertants was 8 ± 2·8.
†The number of spontaneous revertants was 6 ± 1·4.
‡Lethal dose.

activating enzyme system from rat liver was used, suggesting that types of mutagens other than alkylnitrosamides were also produced.

The purification and identification of the mutagens in the nitrite-treated foods, especially in fish, are currently being investigated, and animal experiments have been started to examine whether the mutagen demonstrated in nitrosated fish is also carcinogenic.

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ABSORPTION AND METABOLISM OF CHLORINATED FATTY ACIDS AND TRIGLYCERIDES IN RATS

H. M. Cunningham and G. A. Lawrence

Food Directorate, Health Protection Branch, Health and Welfare Canada, Tunney's Pasture, Ottawa, Canada

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Abstract—Oleic, linoleic and linolenic acids and their triglycerides were chlorinated with $^{36}Cl_2$ and stable chlorine, before being given orally along with [^{3}H]oleic acid and corn oil to one of six groups of 100-g Wistar rats. Absorption, tissue distribution and excretion were determined over 24 hr. Chlorination of oleic acid or triolein reduced absorption in the digestive tract and deposition in most tissues of the body except the heart, compared with values for oleic acid itself. Chlorinated linoleic and linolenic acids and their triglycerides showed an even greater decrease in absorption and an exceptionally large reduction in deposition in all tissues. Bile collected for 3 days from four additional rats intubated with ^{36}Cl-chlorinated oleic acid and [^{3}H]oleic acid showed that negligible amounts of both labels were excreted by this route.

INTRODUCTION

Chlorinated lipids resulting from the bleaching of flour with chlorine have been found to be toxic to growing rats (Cunningham, Lawrence & Tryphonas, 1977). Radioisotope studies have shown that the chlorination of oleic acid reduces its absorption by about 10–15% and that the chlorination of flour lipids results in a further 15–20% decrease in absorption (Cunningham & Lawrence, 1977). However, these studies indicated that much less of the chlorinated flour lipids were deposited in the tissues than would be expected from the amount that is apparently absorbed and it appeared that variations in the type of lipid chlorinated could be responsible for differences in metabolism.

The present studies were designed to determine how chlorination affects the absorption and metabolism of oleic, linoleic and linolenic acids and their triglycerides. The amount of labelled lipids recycled through the bile was also determined in order to establish figures for 'true absorption'.

EXPERIMENTAL

Materials. Chlorine-36 was purchased as 3·25 N-H^{36}Cl with a specific activity of 4·4 mCi/g from Atomic Energy of Canada Limited, Commercial Products, Ottawa. Oleic acid ($C_{18}H_{34}O_2$), linoleic acid ($C_{18}H_{32}O_2$) and linolenic acid ($C_{18}H_{30}O_2$) and their triglycerides, triolein, trilinolein and trilinolenin (all 99·0% pure) were obtained from the Sigma Chemical Company, St. Louis, Mo. The fatty acids and triglycerides in 0·5-g aliquots were chlorinated in the dark by techniques described previously for ^{36}Cl-chlorination of oleic acid (Cunningham & Lawrence, 1976). Each preparation, containing 15 μCi ^{36}Cl and 75 mg carrier chlorine, was then exposed to an excess of chlorine gas for 30 min to ensure 100% saturation. Surplus chlorine was removed on a rotary evaporator, 150 μCi [^{3}H]-oleic acid in 5 ml corn oil was added, and the preparation was diluted to 10 ml with corn oil.

Animals and administration. Twenty-four 100-g male Wistar rats were allocated according to body weight to six equal groups. Each rat was given by intubation 1·0 ml of one of the six chlorinated lipid preparations and placed in a metabolism cage equipped for the separate collection of urine and faeces. After 24 hr the rats were anaesthetized with ether, blood samples were taken by heart puncture and the animals were killed by exsanguination. The liver, kidneys, heart, omentum, brain, intestinal contents, faeces and urine were frozen and kept for analysis.

Bile cannulae were surgically introduced into four additional male Wistar rats weighing 425–585 g. Each received by intubation 1·0 ml of the chlorinated oleic acid preparation and the bile was collected for 72 hr.

Analytical methods. Samples of tissue, intestinal contents, excreta and bile were analysed in duplicate for total lipids by Folch extraction (Folch, Lees & Sloane Stanley, 1957). The lipids from the Folch extraction were weighed and counted for ^{3}H and ^{36}Cl (Cunningham & Lawrence, 1976) and ^{3}H and ^{36}Cl were also determined in samples of urine and the aqueous phase of the Folch extraction of blood and bile.

RESULTS

Table 1 shows that 24 hr after dosing most of the unabsorbed radioactive lipid was in the faeces, only a small percentage remaining in the intestine. All chlorinated fatty acids and triglycerides were less efficiently absorbed than the [^{3}H]oleic acid given simultaneously, and the amount of unabsorbed chlorinated lipid had no significant effect on the absorption of oleic acid. Chlorinated oleic acid was better absorbed than chlorinated linoleic or chlorinated linolenic acids, while chlorinated triolein and trilinolenin were more completely absorbed than chlorinated trilinolein.

The distribution of ^{3}H from [^{3}H]oleic acid in the tissue lipids was not significantly affected by the type of chlorinated lipid accompanying it and in most tissues higher levels of oleic acid were deposited than of the chlorinated lipids. The heart was the only tissue in which the levels of ^{36}Cl deposited in tissue lipids was significantly higher than that of ^{3}H. In this case

Table 1. *^{3}H and ^{36}Cl activity in the lipids of tissues and excreta and in the urine and blood water of rats 24 hr after intubation with 15 μCi [^{3}H]oleic acid and 50 mg (1·5 μCi) of a fully chlorinated fatty acid or fatty acid triglyceride*

		Uptake of radioactivity (% of dose)* in rats fed [^{3}H]oleic acid and ^{36}Cl-chlorinated...					
Sample	Isotope	Oleic acid	Linoleic acid	Linolenic acid	Triolein	Trilinolein	Trilinolenin
Lipids							
Faeces	^{3}H	6·87^{abc}	11·31^{ab}	5·68^{abc}	10·90^{abc}	15·33^{ab}	9·75^{abc}
	^{36}Cl	23·80^{aC}	54·26^{A}	49·70^{A}	28·54^{aC}	45·71^{B}	30·03^{aC}
Intestinal contents	^{3}H	0·13^{a}	0·35	0·12^{a}	0·74	0·30	0·60
	^{36}Cl	0·47^{A}	3·09	0·92	2·53	4·60	3·02
Total unabsorbed	^{3}H	7·00^{abcd}	11·66^{abc}	5·80^{abcd}	11·64^{abc}	15·63^{abc}	10·35^{abc}
	^{36}Cl	24·27^{abD}	57·35^{A}	50·62^{A}	31·07^{abC}	50·31^{B}	33·05^{aC}
Liver	^{3}H	1·630^{A}	1·247^{A}	1·390^{A}	1·497^{A}	1·073^{A}	1·194^{A}
	^{36}Cl	1·012^{A}	0·325^{aB}	0·191^{ab}	1·605^{A}	0·343^{a}	0·299^{a}
Kidney	^{3}H	0·322^{A}	0·257^{B}	0·303^{B}	0·281^{B}	0·239^{B}	0·370^{B}
	^{36}Cl	0·163^{aB}	0·033^{ab}	0·027^{ab}	0·162^{aB}	0·037^{ab}	0·066^{ab}
Heart	^{3}H	0·028^{aB}	0·028^{aB}	0·038^{aB}	0·034^{aB}	0·023^{aC}	0·035^{aB}
	^{36}Cl	0·035^{aB}	0·009^{abC}	0·003^{abc}	0·073^{A}	0·010^{ab}	0·013^{ab}
Brain	^{3}H	0·022^{A}	0·018^{A}	0·019^{A}	0·019^{A}	0·018^{A}	0·018^{A}
	^{36}Cl	0·006^{aB}	0·002^{abc}	0·001^{abc}	0·010^{aC}	0·003^{ac}	0·002^{abc}
Omentum	^{3}H	1·446^{A}	1·051^{B}	1·134^{B}	1·032^{B}	0·771^{B}	1·186^{B}
	^{36}Cl	0·766^{aB}	0·079^{ab}	0·129^{ab}	0·601^{ab}	0·103^{ab}	0·286^{ab}
Blood†	^{3}H	0·046^{B}	0·069^{A}	0·066^{A}	0·065^{A}	0·043^{B}	0·063^{A}
	^{36}Cl	0·030^{B}	0·018^{a}	0·009^{ab}	0·060^{A}	0·021^{a}	0·021^{a}
Water							
Blood	^{3}H	2·774	2·425	2·836	2·795	2·367	2·671
	^{36}Cl	3·903^{A}	1·525^{a}	2·106^{a}	2·811^{A}	1·570^{a}	3·429^{A}
Urine	^{3}H	3·98^{ab}	6·05^{ab}	5·33^{ab}	4·50^{abc}	5·58^{ab}	4·37^{ab}
	^{36}Cl	19·60^{A}	9·03^{ab}	13·44^{ab}	10·95^{aC}	8·72^{a}	17·15^{B}

*Values are the means for groups of four animals. Statistical differences ($P < 0·01$) determined for each tissue are indicated by different superscripts of the same letter (e.g. "A" is significantly greater than "a").
†The total blood volume was calculated as 6·41% of body weight (Altman & Dittmer, 1974).

the concentration of ^{36}Cl from chlorinated triolein significantly exceeded that of ^{3}H from oleic acid, and that of ^{36}Cl from chlorinated oleic acid was slightly higher (not significantly) than that of ^{3}H from oleic acid (Table 1).

In general, a decrease in the absorption of a chlorinated lipid was accompanied by a decrease in its deposition in tissue lipids, but in some cases (e.g. chlorinated linoleic and linolenic acids compared with chlorinated oleic acid) the deposition was much lower than could be accounted for by the amount absorbed. The chlorinated triglycerides of these fatty acids tended to behave similarly. There was generally little difference in the deposition of ^{36}Cl from chlorinated linoleic or linolenic acids but, in the liver and heart lipids, there was significantly more ^{36}Cl deposited from chlorinated linoleic acid (Table 1).

There was no significant differences in water-soluble ^{3}H in the blood of rats receiving the six chlorinated lipid preparations, but the lowest levels of water-soluble ^{36}Cl occurred in the blood of rats given chlorinated linoleic acid and trilinolein. The urine showed a similar pattern in ^{36}Cl content (Table 1).

Figure 1 shows that only very low levels of ^{3}H- and ^{36}Cl-labelled lipids were excreted in the bile of rats given [^{3}H]oleic acid and ^{36}Cl-chlorinated oleic acid, and that there was little difference between the concentrations of the two isotopes. A peak of 0·002% of the dose of ^{36}Cl was reached 4–6 hr after dosing. Total excretion of ^{36}Cl in the bile averaged 0·014 ± 0·0018% of the dose during the first 24 hr, 0·0057 ± 0·007% from 24 to 48 hr and 0·0050 ± 0·0014% from 48 to 72 hr. The concentration of ^{36}Cl in the aqueous fraction of the bile remained higher than that of ^{3}H. A similar difference was observed in the aqueous frac-

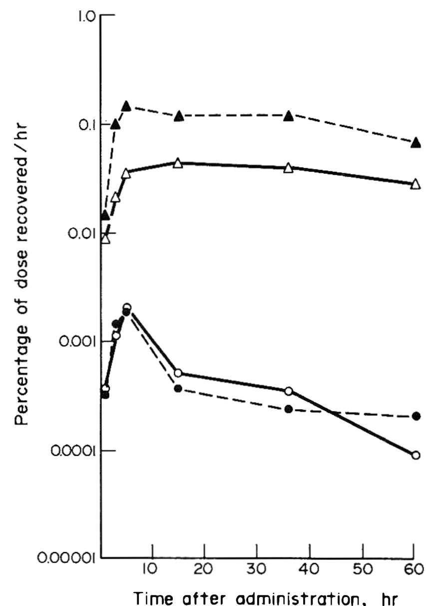

Fig. 1. Distribution of ^{3}H (△, ○) and ^{36}Cl (▲, ●) in bile water (△, ▲) and in bile lipids (○, ●) of four rats after intubation with 50 mg 15%-chlorinated oleic acid containing 15 μCi [^{3}H]oleic and 1·5 μCi ^{36}Cl-chlorinated oleic acid. Values for each collection period are shown at the midpoint in time of that collection interval.

tion of the blood of rats 24 hr after receiving [^{3}H]oleic and ^{36}Cl-chlorinated oleic acid (Table 1).

DISCUSSION

The data show that the chlorination of unsaturated fatty acids reduced their absorption in the digestive tract with respect to that of oleic acid. The observation that recycling of labelled fatty acids through the bile is negligible indicates that the figures for absorption obtained in this study closely represent 'true absorption'. The levels of 93·0% absorption recorded for oleic acid and 75·7% for chlorinated oleic acid agree closely with figures of 91·5 and 72·3%, respectively, obtained in an earlier study (Cunningham & Lawrence, 1976).

It is well known that stearic acid is less digestible than oleic acid (Deuel, 1955) and it was not surprising to find that the addition of chlorine to the double bond of oleic acid reduced absorption. However, it was surprising to find that although further introduction of chlorine, accomplished by chlorinating linoleic acid, resulted in an additional decrease in absorption, still further introduction of chlorine by chlorination of linolenic acid caused no further decrease in absorption. Wheat lipids contain high levels of both oleic and linoleic acids (Hilditch, 1956) and it would appear that the lower absorption of chlorinated wheat-flour lipids compared with that of chlorinated oleic acid observed in earlier work (Cunningham & Lawrence, 1977) could have resulted from the linoleic acid present in the wheat lipids.

The reason why extremely small amounts of the ^{36}Cl from chlorinated linoleic and linolenic acids were deposited in the tissues compared with that from chlorinated oleic acid cannot be explained, even when some allowance is made for the differences in absorption. This was particularly evident when it was found that ^{36}Cl-chlorinated trilinolenin was 2% less well absorbed than ^{36}Cl-chlorinated triolein but resulted in only one third to one fifth as much ^{36}Cl in the tissues. It is also interesting to note that less chlorinated linoleic acid is absorbed than chlorinated oleic acid and results in less chlorinated lipid in the tissues, while recent studies on its LD_{50} (H.M. Cunningham and G. A. Lawrence, unpublished data 1976) show that it is considerably more toxic.

This is the third successive study in which the ^{36}Cl from chlorinated oleic acid was found in the heart, 24 hr after dosing, at a slightly higher concentration than ^{3}H from oleic acid (Cunningham & Lawrence, 1976 & 1977), while in all other tissues analysed it was always lower. The observation that the ^{36}Cl from chlorinated triolein was significantly higher is a further indication that the heart may either tend to retain chlorinated oleic acid or have difficulty in eliminating it. It may be relevant to recall that brominated vegetable oil has been found to result in an accumulation of lipid in heart muscle (Munro, Salem, Goodman & Hasnain, 1971).

The relatively high levels of water-soluble chlorine present in the blood and urine indicate that the rat has the ability to dechlorinate all six of the chlorinated lipids tested. Since any differences in the levels of water-soluble ^{36}Cl would be related to the amounts absorbed and available for metabolism, this indicates that these lipids are dechlorinated at relatively similar rates.

It may be concluded that the chlorination of oleic acid reduces its absorption in the digestive tract and its deposition in most tissues of the body except the heart. Chlorination of linoleic or linolenic acid results in a further decrease in absorption and an exceptionally large reduction in deposition in all tissues.

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ABSORPTION AND DISTRIBUTION STUDIES ON CHLORINATED OLEIC ACID AND EXTRACTS OF CHLORINATED LIPID AND PROTEIN FRACTIONS OF FLOUR IN RATS

H. M. Cunningham and G. A. Lawrence

Food Directorate, Health Protection Branch, Health and Welfare Canada, Tunney's Pasture, Ottawa, Canada

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Abstract—Sixteen 100-g rats were given [^{3}H]oleic and ^{36}Cl-chlorinated oleic acid by intubation and killed at intervals from 4 to 24 hr. Faeces and urine collections were made on those killed at 24 hr. Four additional groups of four 100-g rats were intubated with low- or high-chlorine flour lipids or water extracts of fat-free chlorinated flour or of chlorinated gluten, the chlorine in all preparations being labelled with ^{36}Cl. Distribution studies showed that the peak tissue concentration of chlorinated oleic acid occurred at the same time after ingestion (4–8 hr) as that of oleic acid but that the chlorinated acid was not as completely absorbed. Chlorinated flour lipids were less efficiently absorbed than chlorinated oleic acid and less than one half as much was deposited in the tissues. Chlorinated compounds in water-soluble extracts of flour or gluten were readily absorbed, showed no detectable incorporation into body lipids or proteins and were rapidly excreted in the urine.

INTRODUCTION

Earlier experiments showed that chlorinated cake flour and chlorinated flour lipids reduced growth rate and increased relative liver size in growing rats (Cunningham, Lawrence & Tryphonas, 1977). These experiments also showed that fat-free chlorinated wheat gluten had similar effects and that 95–99% of the chlorine in ether-extracted chlorinated flour or gluten could be extracted with water. Further experiments compared the absorption, distribution and biological half-life of ^{36}Cl-chlorinated oleic acid with [^{3}H]oleic acid in rats over a period of 1–28 days after intubation (Cunningham & Lawrence, 1976). The rapidity with which the level of chlorinated lipids declined in the liver compared to other tissues indicated that the liver was probably the main site of dechlorination, but the peak concentrations of chlorinated lipids in all tissues apparently occurred prior to 24 hr when the first determinations were made.

The present experiments were conducted to study the absorption and distribution of chlorinated and non-chlorinated oleic acid during the first 24 hr after dosing and to compare the absorption and distribution of chlorinated oleic acid with that of chlorinated flour lipids and water extracts of chlorinated flour and chlorinated gluten.

EXPERIMENTAL

Chlorine and $^{36}Cl_2$ were generated for the following preparations by the reaction of HCl or $H^{36}Cl$ and $KMnO_4$ (Holmes, 1941) and chlorination was carried out by techniques described previously for oleic acid (Cunningham & Lawrence, 1976) and flour (Cunningham *et al.* 1977).

[^{3}H]Oleic and ^{36}Cl-chlorinated oleic acids. Using corn oil as a vehicle, 50 μCi [^{3}H]oleic acid (New England Nuclear, Boston, Mass.), 1·5 μCi ^{36}Cl-chlorinated oleic acid and 25 mg carrier oleic acid chlorinated with 3·75 mg chlorine were made up to 1 ml. The ^{36}Cl-chlorinated oleic acid contained 85% dichlorostearic acid, the remainder being more polar compounds (Cunningham & Lawrence, 1976).

Low-chlorine flour lipids. Unbleached pastry flour (7·93% protein, N × 5·7; 1·3% lipids) was chlorinated with $^{36}Cl_2$ using a carrier level of 0·2 g Cl_2/100 g flour. The lipids were extracted for 8 hr with diethyl ether in a Soxhlet apparatus and contained 63·8 mg Cl/g. This was diluted to a concentration of 67 mg (0·66 μCi ^{36}Cl)/ml in corn oil and 25 μCi [^{3}H]oleic acid/ml was added.

High-chlorine flour lipids. The lipids were extracted from unbleached flour and chlorinated with $^{36}Cl_2$ and 0·2 g Cl_2/100 g flour. The lipids contained 132 mg Cl/g and were diluted to 50 mg (1·03 μCi ^{36}Cl)/ml in corn oil and 25 μCi [^{3}H]oleic acid/ml was added.

Water extract of fat-free chlorinated flour. The residue of the low-chlorine flour after ether extraction was shaken with 10 vol water, allowed to stand overnight, shaken and centrifuged. The supernatant was decanted, the residue was washed twice with 10 vol water and the washings were combined, neutralized with NaOH and reduced on a rotary evaporator until the volume was equivalent to 4·0 ml for every 3·0 g of residue. This extracted 95% of the ^{36}Cl, to give an activity of 0·12 μCi/ml.

Water extract of chlorinated gluten. Wheat gluten (Teklad Test Diets, Madison, Wisc.) was extracted for 8 hr with diethyl ether and chlorinated with $^{36}Cl_2$ using a level of 2% Cl_2 as carrier. This was extracted three times with 30 vol water and concentrated in the same manner as the extract of flour residue. The extraction removed 97% of the ^{36}Cl, to give an activity of 0·10 μCi/ml.

Animals. Thirty-two 100-g male Wistar rats (Biobreeding Laboratories of Canada Ltd., Ottawa) were allocated according to body weight into eight groups of four. Each rat in the first four groups received by

intubation 1·0 ml of the preparation containing [^{3}H]oleic and ^{36}Cl-chlorinated oleic acids, and the groups were killed at 4, 8, 16 and 24 hr. Faeces and urine collections were made from the 24-hr group and were included in a comparison with rats given the other preparations. Rats in the remaining four groups received 1 ml low- or high-chlorine flour lipids, or 4 ml water extract of fat-free chlorinated flour or chlorinated gluten preparations. Faeces and urine were collected simultaneously from all rats in a group for 24 hr, at which time they were killed.

Analysis. Samples of blood, liver, kidney, heart, omentum, brain and faeces were analysed in duplicate for total lipids by Folch extraction (Folch, Lees & Sloane Stanley, 1957). The ^{3}H and ^{36}Cl activity of the lipids, proteins and aqueous phase of these extractions were determined as described previously (Cunningham & Lawrence, 1976). The ^{3}H and ^{36}Cl activity was determined directly on samples of urine.

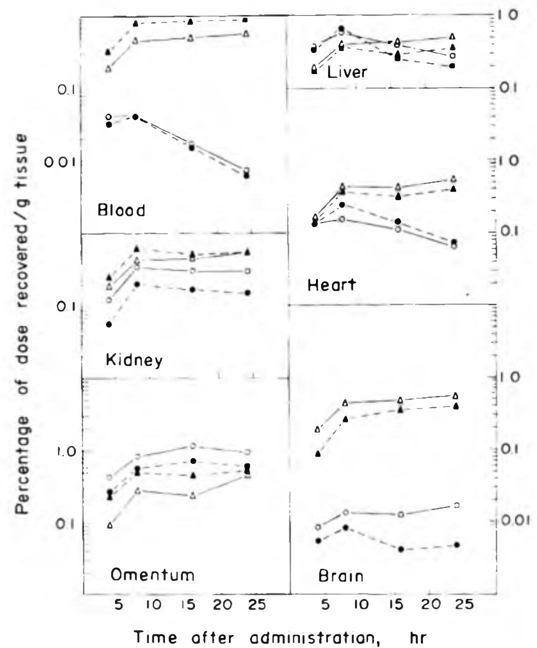

Fig. 1. Distribution of ^{3}H (△, ○) and ^{36}Cl (▲, ●) in tissue water (△, ▲) and tissue lipids (○, ●) in 16 rats killed 4–24 hr after intubation with 50 μCi [^{3}H]oleic acid, 1·5 μCi ^{36}Cl-chlorinated oleic acid and 25 mg oleic acid chlorinated with 3·75 mg Cl_2.

RESULTS

Tritium levels in the tissue water were similar in all tissues, rising quickly from 4 to 8 hr after dosage thereafter increasing only slightly (Fig. 1). The ^{36}Cl in tissue water followed a similar trend to ^{3}H but was lower in the liver, heart and brain and higher in the blood, kidney and omentum.

The levels of ^{3}H- and ^{36}Cl-labelled lipids in tissues varied considerably from one tissue to another, the highest being recorded in the omentum and lowest in the brain. The heart was the only tissue in which the lipid ^{36}Cl exceeded the lipid ^{3}H throughout the first 24 hr after dosing. In most tissues the ^{36}Cl concentration was less than that of ^{3}H and the spread between these widened in the liver and brain as the experiment progressed. The largest initial difference between the concentrations of the two isotopes at 4 hr was observed in the kidney and brain. At 24 hr there was 3·3 times as much ^{3}H lipid/g brain as ^{36}Cl lipid.

The liver accumulated considerably more ^{3}H and ^{36}Cl than any other tissue (Table 1), reaching a maximum of 3·085% of ingested ^{36}Cl at 8 hr compared to only 0·012% in the brain. The ^{36}Cl in liver protein rose from 0·07% of the dose at 4 hr to 0·13% at 8 hr and was 0·11% at 16 and 24 hr. The ^{3}H in liver protein only changed slightly from 0·04% of the dose at 4, 8 and 16 hr to 0·05% at 24 hr. Only traces of these isotopes were detected in the proteins of the other tissues.

Comparison of the 24-hr absorption and distribution of chlorinated oleic acid with that of chlorinated flour lipids (Table 2) indicates that more of the former than of the latter (at both low and high levels of chlorination) was absorbed and deposited in the tissues. The differences in tissue levels were large, the greatest differences being recorded in the heart where the ^{36}Cl deposited from chlorinated flour lipids was

Table 1. *^{3}H and ^{36}Cl activity in the tissue lipids of rats intubated with 50 μCi [^{3}H]oleic acid, 1·5 μCi ^{36}Cl-chlorinated oleic acid and 25 mg oleic acid chlorinated with 3·75 mg Cl_2*

		Uptake of radioactivity (% of dose)* in lipids of tissue at			
Tissue	Isotope	4 hr	8 hr	16 hr	24 hr
Liver	^{3}H	1·817 ± 0·310	2·782 ± 0·247	2·090 ± 0·150	1·511 ± 0·348
	^{36}Cl	1·618 ± 0·367	3·085 ± 0·604	1·469 ± 0·070	1·039 ± 0·137
Kidney	^{3}H	0·126 ± 0·020	0·354 ± 0·030	0·314 ± 0·011	0·311 ± 0·057
	^{36}Cl	0·055 ± 0·012	0·213 ± 0·031	0·176 ± 0·006	0·161 ± 0·027
Heart	^{3}H	0·056 ± 0·005	0·062 ± 0·011	0·044 ± 0·007	0·029 ± 0·004
	^{36}Cl	0·051 ± 0·008	0·097 ± 0·018	0·057 ± 0·008	0·032 ± 0·005
Brain	^{3}H	0·013 ± 0·001	0·020 ± 0·001	0·017 ± 0·002	0·027 ± 0·001
	^{36}Cl	0·008 ± 0·001	0·012 ± 0·001	0·006 ± 0·001	0·007 ± 0·001
Omentum†	^{3}H	0·423 ± 0·319	0·819 ± 0·176	1·163 ± 0·224	0·950 ± 0·344
	^{36}Cl	0·266 ± 0·073	0·561 ± 0·094	0·706 ± 0·112	0·598 ± 0·157
Blood‡	^{3}H	0·259 ± 0·065	0·261 ± 0·042	0·100 ± 0·009	0·043 ± 0·003
	^{36}Cl	0·207 ± 0·051	0·266 ± 0·022	0·089 ± 0·012	0·037 ± 0·003

*Values are means ± SEM for groups of four animals.
†The omentum was not totally recovered and data are given as % of dose/g omentum.
‡The total blood volume was calculated as 6·41% of body weight (Altman & Dittmer, 1974).

Table 2. *^3H and ^{36}Cl activity in the lipids of tissues, blood and faeces of rats 24 hr after intubation with [^3H]oleic acid and three ^{36}Cl-chlorinated lipid preparations*

		Uptake of radioactivity (% of dose)* in lipids of rats fed [^{3}H]oleic acid and ^{36}Cl-chlorinated...		
Sample	Isotope	Oleic acid	Flour lipids (low-chlorine)	Flour lipids (high-chlorine)
Liver	^{3}H	1·511 ± 0·348	1·424 ± 0·079	1·013 ± 0·230
	^{36}Cl	1·039 ± 0·137	0·388 ± 0·021	0·479 ± 0·139
Kidney	^{3}H	0·311 ± 0·057	0·338 ± 0·042	0·258 ± 0·009
	^{36}Cl	0·161 ± 0·027	0·073 ± 0·011	0·058 ± 0·009
Heart	^{3}H	0·029 ± 0·004	0·031 ± 0·002	0·027 ± 0·002
	^{36}Cl	0·032 ± 0·001	0·008 ± 0·001	0·007 ± 0·001
Brain	^{3}H	0·027 ± 0·002	0·019 ± 0·001	0·020 ± 0·001
	^{36}Cl	0·008 ± 0·001	0·002 ± 0·001	0·004 ± 0·001
Omentum†	^{3}H	0·950 ± 0·344	1·468 ± 0·263	0·650 ± 0·037
	^{36}Cl	0·598 ± 0·157	0·383 ± 0·062	0·137 ± 0·022
Blood‡	^{3}H	0·043 ± 0·003	0·035 ± 0·003	0·041 ± 0·005
	^{36}Cl	0·037 ± 0·003	0·012 ± 0·001	0·020 ± 0·005
Faeces§	^{3}H	3·289 ± 1·808	2·946 ± 0·796	6·224 ± 1·956
	^{36}Cl	14·053 ± 1·465	27·371 ± 3·184	34·999 ± 6·489

*Values are mean ± SEM for groups of four animals.
†The omentum was not totally recovered and data are given as % of dose/g omentum.
‡The total blood volume was calculated as 6·41% of body weight (Altman & Dittmer, 1974).
§Collected for 24 hr after dosing.
For details of dose levels see Experimental section.

less than one quarter of that from chlorinated oleic acid.

Earlier work indicated that only 1·1% of chlorinated oleic acid was absorbed on the second day after ingestion and only 0·05% thereafter (Cunningham & Lawrence, 1976). However, in case the excretion of unabsorbed chlorinated flour lipids was slower than that of chlorinated oleic acid, a 48-hr absorption study was conducted, four additional rats being fed each type of chlorinated flour lipids. It was found that 35·6 ± 4·6% of the high-chlorine lipids appeared in the faeces during a 48-hr period and 32·0 ± 2·9% of the low-chlorine lipids were unabsorbed over the same period compared to 35·0 ± 6·5 and 27·4 ± 3·2%, respectively, at 24 hr.

All ^{36}Cl from the water extracts of chlorinated flour or gluten was detected in the aqueous phase of the Folch extraction of the tissues, none being found in the tissue lipids or proteins.

Table 3 shows that the ^{36}Cl in the water of all tissues was lower when chlorinated flour lipids were ingested than when chlorinated oleic acid was ingested but the ^{36}Cl in the aqueous phase of faeces and urine was slightly higher with the chlorinated flour lipids. Only 2% of the water-soluble ^{36}Cl of chlorinated flour or gluten was excreted in the faeces. There was no appreciable difference between these fractions in their distribution in tissue water or urine but the levels of both were considerably higher than when either chlorinated oleic acid or flour lipids were ingested.

Table 3. *^{36}Cl activity in the water of tissues, blood and excreta of rats 24 hr after intubation with chlorinated lipids and water extracts of chlorinated flour and wheat gluten*

	Uptake of ^{36}Cl (% of dose)* in tissue water of rats fed				
	^{36}Cl-chlorinated...			Water extract of ^{36}Cl-chlorinated...	
Sample	Oleic acid	Flour lipids (low-chlorine)	Flour lipids (high-chlorine)	Flour	Gluten
Liver	1·716 ± 0·154	1·360 ± 0·044	1·121 ± 0·151	3·317 ± 0·163	2·901 ± 0·210
Kidney	0·572 ± 0·039	0·409 ± 0·014	0·400 ± 0·052	1·086 ± 0·003	0·975 ± 0·075
Heart	0·169 ± 0·016	0·097 ± 0·002	0·101 ± 0·013	0·258 ± 0·007	0·264 ± 0·008
Brain	0·618 ± 0·053	0·348 ± 0·023	0·345 ± 0·046	0·948 ± 0·013	0·878 ± 0·114
Omentum†	0·553 ± 0·079	0·331 ± 0·016	0·324 ± 0·050	0·841 ± 0·019	0·821 ± 0·074
Blood‡	5·391 ± 0·544	3·408 ± 0·185	3·390 ± 0·410	8·415 ± 0·393	7·587 ± 0·911
Faeces§	1·242 ± 0·324	3·287 ± 0·389	2·012 ± 0·409	2·140 ± 0·444	2·009 ± 0·171
Urine§	17·184 ± 3·042	20·953 ± 1·810	17·877 ± 2·256	37·038 ± 0·951	30·325 ± 3·399

*Values are means ± SEM for groups of four animals.
†The omentum was not totally recovered and data are given as % of dose/g omentum.
‡The total blood volume was calculated as 6·41% of body weight (Altman & Dittmer, 1974).
§Collected for 24 hr after dosing.
For details of dose levels see Experimental section.

DISCUSSION

The levels of ^3H and ^{36}Cl in tissue water and lipids 24 hr after dosing agree closely with the 24-hr data from earlier experiments in which rats were dosed with the same amounts of tritiated and chlorinated oleic acid and killed at intervals from 24 hr to 28 days (Cunningham & Lawrence, 1976). The present studies therefore extend the observations back to 4 hr after dosage at which time none of the tracers had reached peak levels in the tissues. They also show a number of relationships which are the reverse of those observed after 24 hr. Tritium levels in tissue water were lower than ^{36}Cl levels in the blood, kidney and omentum up to 24 hr in the present study but by 14 days were found to be the reverse (Cunningham & Lawrence, 1976). Similarly, ^3H levels in heart lipids were lower than ^{36}Cl levels up to 24 hr but were higher after 24 hr. It is still not known how these changes are related to rates of metabolism and clearance of the labelled lipids, but the data confirm earlier work in showing that the brain has an extraordinary ability to discriminate against the uptake of chlorinated lipids either by metabolizing or selectively eliminating them from the tissue.

One factor that could reduce the toxicity of chlorinated lipids would be lower absorption. The studies on the absorption of chlorinated oleic acid and flour lipids indicated that not only does chlorination reduce absorption of lipids but the chlorination of very unsaturated lipids, such as those in wheat which are high in linoleic acid (Hilditch & Williams, 1964), results in a greater reduction in absorption. Increasing the level of chlorination of wheat flour lipids also appears to result in a slight further reduction in absorption. It has been observed that, on chlorination, oleic acid and flour lipids become more viscous at room temperature and it would appear the chlorination has an effect on absorption somewhat similar to that of hydrogenation.

Another factor that could affect the toxicity of chlorinated lipids is the discrimination against their deposition in the tissues. The deposition of chlorinated flour lipids was several times less than that of chlorinated oleic acid in vital tissues such as the brain, heart, liver and kidney. Uptake of chlorinated flour lipids in adipose tissue of the omentum is also substantially reduced, compared with the deposition of non-chlorinated lipids in the same animal. Deposition of ^{36}Cl from chlorinated oleic acid in omental lipids was 62·9% of that of ^3H from oleic acid, compared with 26·1% for ^{36}Cl from low-chlorine flour lipids and 21·1% from high-chlorine flour lipids (Table 2).

The data show that water-soluble ^{36}Cl-labelled material extracted from either flour or gluten is almost completely absorbed and is distributed throughout the tissues in approximately the same manner as water-soluble ^{36}Cl-labelled compounds resulting from the metabolism of chlorinated lipids. It is well known that chlorine used to bleach flour cleaves peptide bonds, oxidizes thiol groups and forms substitution compounds with amino acids (Kissell, 1971; Tsen & Kulp, 1971), and this results in an increased solubility of flour proteins in water and a decrease in pH due to the formation of HCl. Over 95% of these chlorinated compounds are water soluble, and upon neutralization of the water extract with NaOH the ^{36}Cl-labelled compounds represent a mixture of $Na^{36}Cl$ and ^{36}Cl-substituted amino acids and proteins. Since none of this chlorine is incorporated into proteins or lipids in the body it is evidently excreted rapidly in the urine. Nevertheless, wheat gluten chlorinated at a level of 2% (w/w) showed toxic effects when included at a level of 10% in a rat chow diet (Cunningham *et al.* 1976), and so the chlorinated compounds would appear to have some adverse effect in passing through the body.

It may be concluded that peak tissue concentrations of chlorinated lipids occur at the same time after ingestion (4–8 hr) as those of non-chlorinated lipids but that the chlorinated lipids are not as well absorbed. Chlorinated flour lipids are less efficiently absorbed than chlorinated oleic acid and much smaller amounts are deposited in the tissues. Water-soluble chlorine compounds resulting from the chlorination of fat-free flour or gluten are readily absorbed and rapidly eliminated from the body and show no measureable incorporation into lipid or protein constituents of the body.

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SYNTHESIS AND PROPERTIES OF N^{ϵ}-(DL-2-AMINO-2-CARBOXYETHYL)-L-LYSINE, LYSINOALANINE*

J. C. WOODARD, D. D. SHORT, C. E. STRATTAN and J. H. DUNCAN

Division of Comparative Pathology, Department of Pathology and Department of Pharmacology and Radiology, J. Hillis Miller Health Center, University of Florida and Veterans Administration Hospital, Gainesville, Florida 32610, USA

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Abstract—A method for the organic synthesis of N^{ϵ}-(DL-2-amino-2-carboxyethyl)-L-lysine (lysinoalanine) is described. Intermediates in the reaction sequence were isolated and characterized by thin-layer chromatography and nuclear magnetic resonance spectroscopy. Analytically-pure lysinoalanine was prepared and characterized by infra-red mass spectroscopy. Aqueous solutions of the amino acid were found to be heat sensitive. Degradation occurred at temperatures as low as 40°C, and 70% of the lysinoalanine was destroyed when a 1% solution was heated to 115°C for 16 hr.

INTRODUCTION

Alterations in the nutritional value of food during processing and storage have held the attention of experimental nutritionists for a number of years. For the most part, studies have been concerned with the destruction of nutrients or changes in product acceptability. Although it is well known that chemical alterations occur during food processing, preparation and storage, little attention has been paid to the fact that harmful substances may be synthesized. This problem is complicated further because nutritional feeding studies may not indicate the presence of these potentially harmful substances.

Several investigators have observed that alkali treatment of different proteins may cause chemical changes that lead to the formation of new amino acids such as lanthionine (Horn, Jones & Ringel, 1941), ornithinoalanine (Ziegler, Melchert & Lürken, 1967) and lysinoalanine (Bohak, 1964; Patchornik & Sokolovsky, 1964). Studies in our laboratory have indicated that one of these, lysinoalanine, has a nephrotoxic effect when fed to rats. Renal lesions are limited to the pars recta, and tubular cells in this area have an increased nuclear size and DNA content. In toxicity studies with other substances, the induction of similar nuclear changes has been associated with neoplastic transformation of cells, and such histological alterations should be considered as indicative of possible tumorigenic properties. This importance has been magnified recently because a number of investigators, including Sternberg, Kim & Schwende (1975), have found significant quantities of lysinoalanine in a wide variety of foods.

Since lysinoalanine is not a naturally occurring amino acid but is produced when protein is modified, the development of a method for synthesizing chemically pure lysinoalanine would greatly assist the determination of its importance to human and animal health. Lysinoalanine was first synthesized by Bohak (1964), but yields by this procedure are too low to be useful for animal experimentation. The method of Okuda & Zaki has been used to synthesize both lysinoalanine (Okuda & Zahn, 1965) and ornithinoalanine (Miro, Puente, Anguera & Febrer, 1968). The original method gave adequate yields of lysinoalanine, but, in our hands, the product was contaminated with other substances which could not be separated conveniently. This paper reports additional studies on the synthesis and properties of lysinolanine.

EXPERIMENTAL

Materials and methods. In order to synthesize quantities of lysinoalanine sufficient for experimental purposes, modifications were made to the method used by Okuda & Zahn (1965). At each step in the synthesis the chemical intermediates were isolated and identified by physico–chemical methods, and each step was altered until the desired results were obtained.

Nuclear magnetic resonance (NMR) spectra were obtained by dissolving samples in appropriate solvents. After filtration, the dissolved samples were placed in standard glass NMR tubes (Wilmed Glass Co., Inc., Buena, N.J.). Concentrations were dependent on the limited solubility of the chemical to be analysed. Samples were run on a Varian A60A Analytical Nuclear Magnetic Spectrometer. Spectra were obtained at 0·5 R_f field (mG), at a filter bandwidth of 4, and at room temperature.

Infra-red spectra were obtained by mixing sample with a small quantity of potassium bromide and forming a pellet by pressing in a mould at 7000 psi for 5 min under vacuum. The amount of potassium bromide was adjusted until a pellet of appropriate opacity and integrity was obtained. Samples were run on a Perkin-Elmer 137 Sodium Chloride Spectrometer. The slit width was set at 30, and the 3-min speed was used. The absorbance was varied to obtain the best scale.

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Mass spectra were obtained by preparing volatile derivatives of lysinoalanine by the method of Roach & Gehrke (1969). Analytical gas-chromatographic analysis was performed on a Varian model 1200 gas chromatograph with a stainless-steel (6 ft × 1/8 in.) column packed with 3% Dexsil 300 (Analabs Inc., North Haven, Conn.) under the following conditions: injector 250°C; detector flame ionization 310°C; carrier gas (He) 30 ml/min; column oven 200 to 300°C at 8 deg C/min. Combined gas chromatography–mass spectrometry was performed on a DuPont 21-491 mass spectrometer which was interfaced with the Varian model 1200 gas chromatograph via a stainless-steel single-stage jet separator maintained at 260°C (ion source 275°C; ionizing voltage 70 eV).

For thin-layer chromatography (TLC), 1–5 μg of the sample in water was applied to silica gel G (E.M. Laboratories, Inc., Elmsford, N.Y.) thin-layer plates. Application was always 2 cm from the bottom of the plate, and the solvent, *n*-butanol–glacial acetic acid–water (4:1:1, by vol.), was allowed to migrate 10 cm.

Ion-exchange material was obtained from Bio Rad Laboratories, Richmond, Cal. Ethanol refers to absolute ethanol and water refers to distilled water.

Preparation of the monoethyl ester of acetamidomalonic acid. The preparation of the monoethyl ester was similar to that described by Hellman, Teichmann & Lingens (1958). The diethyl ester of acetamidomalonic acid was obtained from Sigma Chemical Co., St. Louis, Mo., and 21·72 g (0·1 mol) was added to 100 ml ethanol. Reagent-grade potassium hydroxide (6·4 g) was placed in 100 ml ethanol, and the molarity of the solution was determined by titration against a standard acid. Exactly 0·1 mol alcoholic potassium hydroxide was added dropwise to the stirred mixture of diester over 2 hr. The mixture cleared partially, but a hazy solution remained. The mixture was stirred for 16 hr. After cooling to 4°C potassium chloride was precipitated by the dropwise addition of concentrated hydrochloric acid. The solution was filtered when the pH reached 5·0, and the addition of acid was continued until the pH of the solution was 1·5. The chilled solution was filtered, the ethanol was air evaporated, and the resulting powder was suspended in 200 ml chloroform and stirred for 1 hr. The solution was then filtered, and the resulting crystals of the monoethyl ester of acetamidomalonic acid were air dried and stored at 4°C for later use.

Preparation of ethyl 2-acetamidoacrylate ('acrylate'). A modification of the method of Hellman *et al.* (1958) was used. The monoethyl ester of acetamidomalonic acid (9·46 g, 0·05 mol) was mixed with 5 ml (0·06 mol) 37% formaldehyde solution (analytical reagent) in a 100-ml round-bottom flask, and then 1 ml absolute methanol was added to produce a slurry. This mixture was cooled to 4°C in an ice-bath and 5 ml 57% dimethylamine solution (prepared from anhydrous dimethylamine obtained from Eastman Kodak, Rochester, N.Y.) in methanol was added with vigorous stirring. After the solution had cleared, it was removed from the ice bath and stirred at room temperature until no further gas evolution was seen, and then 0·1 g hydroquinone was added.

The reaction vessel, connected to a distillation apparatus, was placed in a water bath at room temperature, and the contents were agitated by a magnetic stirrer. The collecting vessel of the distillation apparatus was placed in a bath of dry ice and acetone, and the pressure was reduced slowly to <1 mm Hg. After the methanol, as well as most of the water, had distilled off, the water bath was replaced by a heating mantle prewarmed to 40°C. When the reaction mixture had virtually ceased to bubble, the temperature of the mantle was raised to 85°C, and vacuum distillation was continued. When the reaction mixture became less viscous and developed an orange colour, the distillation apparatus was removed from the round-bottom flask, and replaced with one that had heating tape wrapped around the vertical distillation column. The temperature of both the mantle and tape were raised to 92°C, and a clean collecting vessel was placed in an ice bath. Vacuum distillation was continued, and the clear acrylate solidified in the cold receiving flask. When distillation was complete, the collecting flask was stoppered and stored at <0°C for 24 hr.

The frozen acrylate was placed on absorbent paper and pressed with a spatula. (Caution must be exercised at this point; gloves must be used as the acrylate can cause a severe skin reaction.) Any oil was absorbed by the paper, and the pressing procedure was repeated using clean paper until no further absorption was observed. The semi-purified crystals were dissolved in petroleum ether with stirring, and more acrylate was added until the solution became cloudy (saturated). Petroleum ether was then added until the solution just cleared. The solution was poured into a flask that could be stoppered tightly and was cooled below 0°C in an explosion-proof freezer. Long needle-like crystals appeared within 30 min. The ether was removed, the crystals were dried under vacuum, and the purification step was repeated. The dry acrylate was stored in a stoppered brown bottle at 0°C.

Purification of N^{α}*-acetyl-L-lysine.* N^{α}-Acetyl-L-lysine (NAL) was obtained from Sigma Chemical Co. It was dissolved in distilled water and placed on a weakly acidic ion-exchange column (2 × 30 cm, Bio Rex "70") that had been converted to the hydrogen form and washed with water. After rinsing the sample into the resin with an additional 10 ml water, the sample was eluted with water. The eluate was concentrated by vacuum evaporation, and NAL was precipitated with ethanol, washed with ethanol, dried under vacuum, and stored at 0°C for later use.

Purification of N^{ϵ}*-(DL-2-acetamido-2-carbethoxyethyl-*N^{α}*-acetyl)-L-lysine (lysinoalanine with blocking groups; LAB).* NAL (2 g) was added with stirring to 20 ml ethanol containing 426 mg sodium hydroxide. After the NAL had dissolved, the solution was chilled in an ice bath and 1·76 g acrylate was slowly added over 60 min. The solution was removed from the ice bath, and the reaction was allowed to continue at room temperature for an additional 6 hr. The sample was then evaporated to dryness in a rotary evaporator at a constant temperature of 40°C. The foam that resulted was dissolved in 20 ml absolute methanol, and 5-ml aliquots were added dropwise with constant mixing to four separate large centrifuge tubes containing 250 ml diethyl ether. This precipitation step was repeated twice after centrifuging, decanting the supernatant and drying the precipitate under vacuum.

Preparation of N$^{\epsilon}$-(DL-*2-acetamido-2-carboxyethyl-N$^{\alpha}$-acetyl*)-L-*lysine*(N$^{\alpha,\epsilon}$-*diacetyllysinoalanine*; *LA*(*Ac*)$_2$). The dry hygroscopic foam (LAB) was dissolved in 100 ml 0·1 N-sodium hydroxide and stirred for 12 hr at room temperature. A weakly acidic ion-exchange resin (Bio Rex "70") in the hydrogen form was added until a pH of 6 was obtained. The solution was filtered to remove the resin, and the resin was washed three times with 50-ml aliquots of water. The filtrate and washings were taken to dryness by rotary evaporation. The resulting white foam was redissolved in 20 ml water and placed on a weakly acidic ion-exchange column (2 × 30 cm, Bio Rex "70") in the hydrogen form. After rinsing the sample into the resin with an additional 10 ml water, the sample was eluted with water and concentrated by rotary evaporation.

The concentrated mixture was placed on a weakly basic ion-exchange column (2 × 30 cm, AG 3X4) in the acetate form, and 10 ml water was used to rinse the solution into the resin. Water was used as the first eluant, and this was continued until no further ninhydrin-positive material was detected in the eluate. After the collection vessel had been changed, elution was continued with 1 N-acetic acid until ninhydrin-positive material was no longer being eluted. The acid fraction was concentrated by rotary evaporation at 40°C and washed several times with 1 N-hydrochloric acid to remove the acetic acid.

Preparation of N$^{\epsilon}$-(DL-*2-amino-2 carboxyethyl*)-L-*lysine* (*lysinoalanine*). The white foam (LA(Ac)$_2$) was dissolved in 250 ml 6 N-hydrochloric acid, and the solution was heated under reflux at 100°C with stirring for 6 hr. The acidic solution was evaporated at 25°C under reduced pressure in a rotary evaporator, and the remaining hydrochloric acid was removed by repeated washings with water. The resulting white foam was redissolved in 10 ml water and placed on an ion retardation-resin column (2 × 30 cm, AG 11A8) in the self-absorb form. After rinsing the sample into the resin with 5 ml water, the column was eluted with water. Collection was continued until ninhydrin-positive material was no longer detected, the aqueous eluate was concentrated to a small volume in a rotary evaporator at 25°C, and the free base of lysinoalanine was precipitated by the addition of ethanol. The precipitate was removed by filtration, air-dried, and redissolved in 100 ml water. The solution was placed on a weakly acidic ion-exchange column (2 × 30 cm, Bio Rex "70") in the hydrogen form. The sample was rinsed into the column with 10 ml water, and the column was washed with water until the eluate was negative by the ninhydrin method. Lysinoalanine was eluted from the column with 100 ml 0·1 N-hydrochloric acid. The acid solution was concentrated by rotary evaporation at 25°C, and the foam was washed repeatedly with water to remove the excess of hydrochloric acid. The lysinoalanine was dissolved in 10 ml water and placed on an AG 11A8 column in the self-absorb form. After rinsing the sample onto the resin and eluting with water, the eluate was concentrated to a small volume by rotary evaporation at 25°C. Lysinoalanine was precipitated from the water with ethanol. The sample was filtered, lyophilized and stored below 0°C.

RESULTS AND DISCUSSION

The method consists of two main reaction steps. The first is the synthesis of ethyl 2-acetamidoacrylate. In the second step, NAL is added across the double bond of the acrylate, and lysinoalanine is produced after removal of the blocking groups. An abbreviated scheme is shown in Figs 1 & 2. At each step it was necessary to separate the desired reaction product from unwanted starting materials and other contaminants by column chromatography.

The monoethyl ester of acetamidomalonic acid was prepared from the diethyl ester. This reaction was found to be time dependent, greater yields being obtained when the reaction mixture was stirred for 14–16 hr. The final product was washed with ice-cold water to remove any diacid that might have formed. Since potassium hydroxide in ethanol frequently produces a yellow colour (due to peroxide), the final

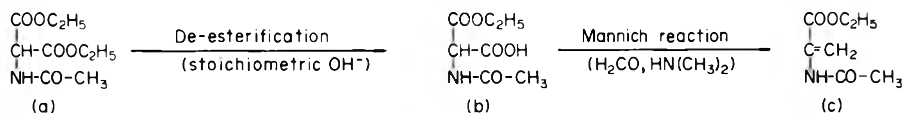

Fig. 1. Synthesis of ethyl 2-acetamidoacrylate (c) from the monoethyl ester (b) of acetamidomalonic acid, which is formed from the diethyl ester (a).

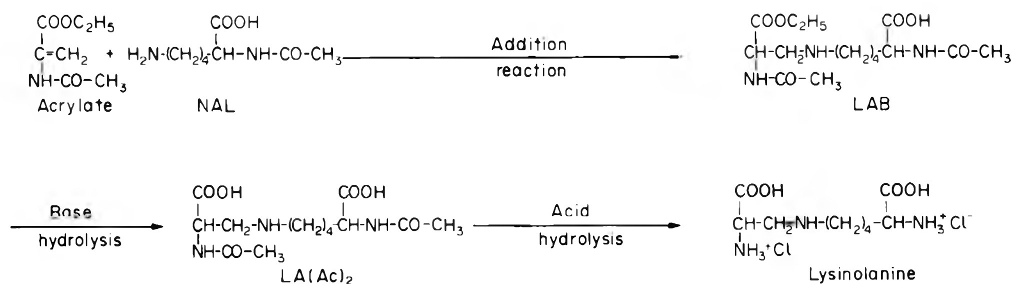

Fig. 2. Synthesis of N^{ϵ}-(DL-2-amino-2-carboxyethyl)-L-lysine (lysinoalanine) from ethyl 2-acetamidoacrylate ('acrylate') and N^{α}-acetyl-L-lysine (NAL), showing intermediates N^{ϵ}-(DL-2-acetamido-2-carbethoxyethyl-N^{α}-acetyl)-L-lysine (LAB) and N^{ϵ}-(DL-2-acetamido-2-carboxyethyl)-N^{α}-acetyl-L-lysine (LA(Ac)$_2$).

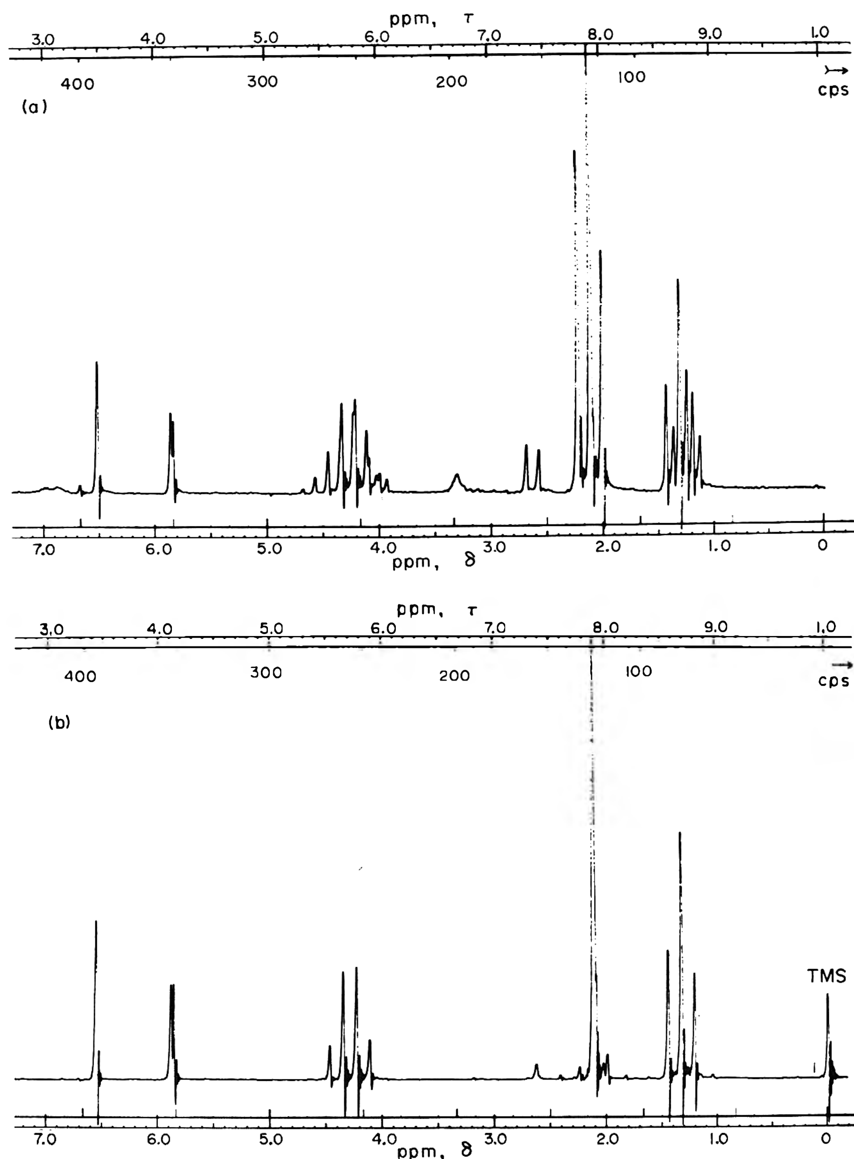

Fig. 3. NMR spectra of (a) freshly distilled and (b) recrystallized ethyl 2-acetamidoacrylate in deuterated chloroform, showing reduction of the multiple signals in the region of the ethyl triplet (1·3δ), acyl methyl group (2·1δ) and ethyl quartet (4·3δ) after purification.

product was washed with chloroform to remove this impurity.

The formation of the acrylate from the monoethyl ester is thought to proceed via the Mannich reaction. The mechanism of this reaction is poorly understood, and the synthesis of acrylate was found to be the most difficult step in the preparation of lysinoalanine. Numerous attempts were made to find a suitable substitute for this chemical in the reaction sequence, but the presence of the acrylamino and ethoxy groups appeared essential to obtain good yields in the step in which acrylate was reacted with NAL. Since the acrylate has a tendency to undergo polymerization, several aspects of its preparation require special attention. All steps should be completed quickly and without interruption. Distillation temperatures should be reached rapidly, and distillation should proceed as quickly as possible. Water must be completely distilled from the reaction mixture before the temperature of the reaction vessel is raised to 92°C. The acrylate that is finally distilled will be clear if of good quality; otherwise, it may be light yellow. Even though the acrylate may be clear, it is contaminated with substances which alter its solubility in petroleum ether. The presence of contaminants can be detected in the NMR spectrum of freshly distilled acrylate (Fig. 3a). By pressing the solidified acrylate onto porous paper with a metal spatula, an oily substance is absorbed by the paper. This process allows the acrylate to crystallize from cold petroleum ether. Acrylate was purified after repeating the pressing procedure and the recrystallization. The purification can be followed by noting the changes in the NMR spectrum after recrystallization (Fig. 3b).

In the next step of the synthetic scheme, NAL is reacted with freshly purified acrylate. Purification of NAL was necessary because the commercial product contained lysine. Separation was conveniently per-

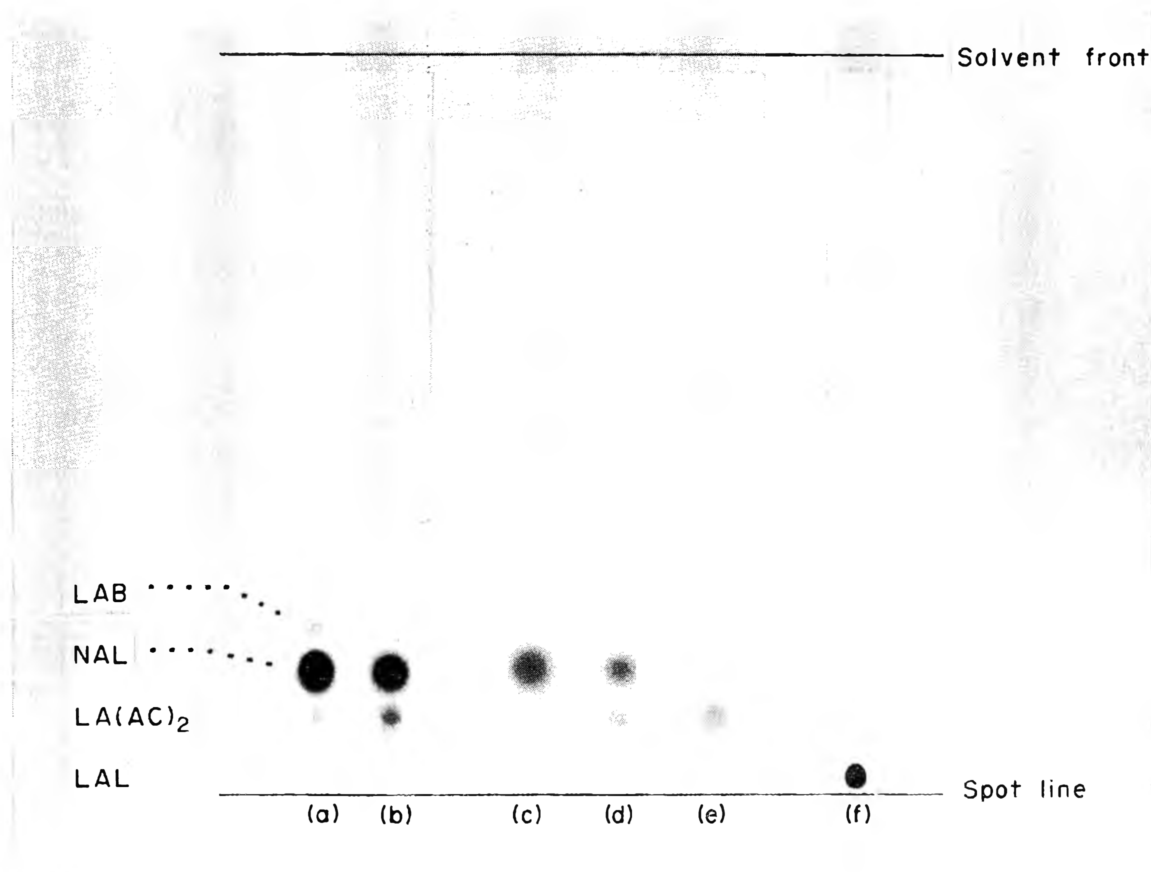

Fig. 4. Thin-layer chromatograms after various steps in the reaction sequence. (a) After reaction of N^{α}-acetyl-L-lysine and ethyl 2-acetamidoacrylate; (b and d) after base hydrolysis of (a); (c) aqueous and (e) acid eluate from column chromatography of (d): (f) after acid hydrolysis of (e) and column chromatography.

formed by passing an aqueous solution through a weakly acidic ion-exchange resin in the hydrogen form. Since NAL is a neutral amino acid, it passes through the column, whereas lysine, a basic amino acid, is held by the resin. Water was not used as a solvent in the reaction because higher yields were obtained when ethanol was used. NAL dissolved in ethanol after the addition of sodium hydroxide. The lower yields with water were probably the result of rearrangements and side reactions that the acrylate could undergo in this medium. Sodium hydroxide was found to be necessary, as the reaction did not proceed when organic bases such as triethylamine and pyridine were used.

The reaction of NAL and acrylate did not produce a single compound as implied by Okuda & Zahn (1965). When the reaction was carried out in ethanol, three ninhydrin-positive spots could be detected by TLC of the final product (Fig. 4a). One spot had an R_F value of 0·19 and was identified as NAL. The other spots had R_F values of 0·11 and 0·24. An NMR spectrum of the reaction mixture before TLC showed that the major component was probably the diacetyl monoethyl ester of lysinoalanine, LAB (Fig. 5a). After recrystallizing the reaction mixture and removing any residual acrylate by ether precipitation, the mixture was treated with sodium hydroxide. Sodium ion was removed by passing the reaction mixture through a weakly acidic ion-exchange resin. TLC showed that the compound with R_F 0·24 was no longer present, and there was a corresponding increase in the amount of the compound with R_F 0·11 (Fig. 4b). These results

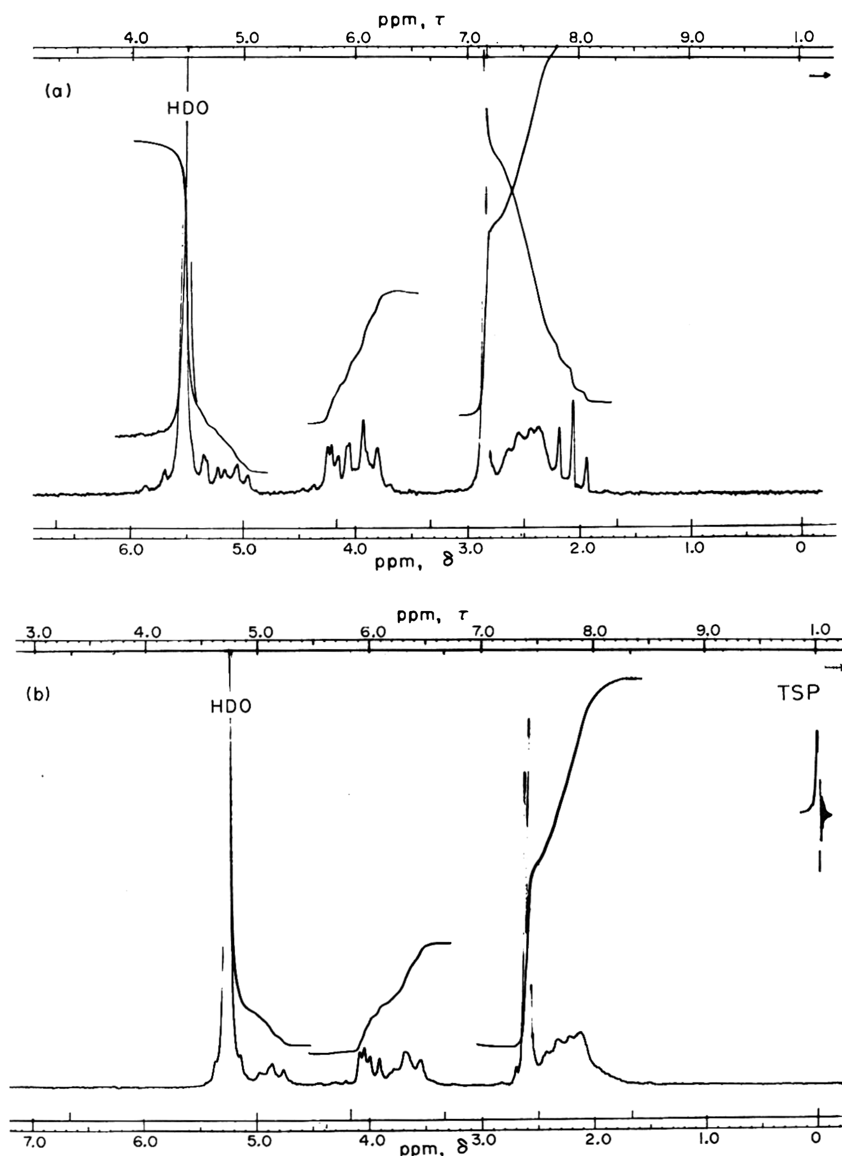

Fig. 5. NMR spectra in deuterium oxide of the reaction products of N^{α}-acetyl-L-lysine and ethyl 2-acetamidoacrylate (a) before and (b) after base hydrolysis and separation showing the large acyl methyl singlet (2·8δ) in both spectra. The presence of an ethyl triplet (2·1δ) in the reaction mixture before hydrolysis indicates that the major component was LAB, whereas after base hydrolysis and chromatographic separation of $LA(Ac)_2$, the ethyl triplet of LAB and the vinyl proton signals (5·9δ and 6·6δ) of the acrylate (cf. Fig. 3) were no longer present.

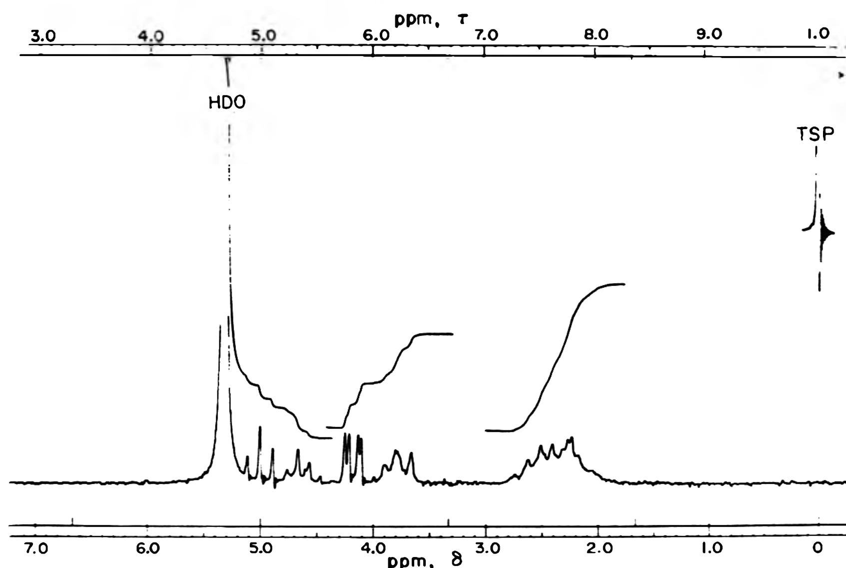

Fig. 6. NMR spectrum of lysinoalanine in deuterium oxide. The absence of an acyl methyl signal (2·8δ) indicates the removal of the final blocking groups, and the complex multiplets remaining arise from the proton interactions of the alkyl chain.

indicated the probable hydrolysis of the ethoxy group to produce diacetyl lysinoalanine. NAL could be separated by passing the mixture through an ion-exchange column containing a weakly basic resin in the acetate form. The NAL passed through the column in the water fraction, and $LA(Ac)_2$ was eluted with weak acid. TLC showed a complete separation of the compounds (Fig. 4c–e), and an NMR spectrum of the acid fraction was consistent with its being diacetyl lysinoalanine (Fig. 5b).

After acid hydrolysis of $LA(Ac)_2$ to remove the acetyl blocking groups, TLC showed the presence of lysinoalanine as well as several degradation products. Crystallization of lysinoalanine hydrochloride from ethanol–water or pyridine–ethanol produced a slightly yellow hygroscopic powder. If the mixture was passed through an ion-retardation resin in the self-absorb form, a non-hygroscopic powder was obtained after crystallization of the free base from ethanol–water. The degradation products were removed by passing the sample through a weakly acidic ion-exchange resin in the hydrogen form. The contaminants passed through the column, and lysinoalanine was eluted with weak acid (Fig. 4e). After forming the free base and recrystallizing, the purified lysinoalanine was characterized by NMR and infra-red spectroscopy (Figs 6 & 7). The tris-(*N*-trifluoroacetyl)-di-(*n*-butyl)ester derivative gave a gas-chromatographic peak (3% Dexsil 300) at Kovats Index 2586 (Kovats, 1965). Combined gas chromatography–mass spectrometry (Fig. 8) of this derivative resulted in a spectrum characterized by a molecular ion at *m/e* 633 and the expected fragment ions (Gelpi, Koenig, Gilbert & Oro, 1969). The derivative also exhibited identical characteristics by gas chromatography and gas chromatography–mass spectrometry to a sample of lysinoalanine supplied through the courtesy of Dr. P. Slump, CIVO, TNO, Zeist, The Netherlands.

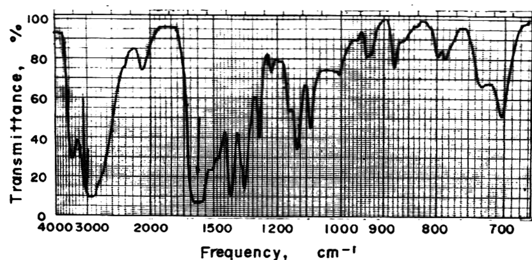

Fig. 7. The infra-red spectrum of lysinoalanine showing, in addition to absorption values characteristic of amino acids, the C–N vibrations between 1200 and 1500 cm^{-1}.

Lysinoalanine was found to be unstable to heat. Slight destruction occurred when aqueous solutions were heated to 40°C, more occurred at 60°C, and as much as 70% destruction occurred at 115°C. After a 1% aqueous solution of lysinoalanine had been heated to 115°C for 16 hr, amino acid analysis showed the presence of chromatographic peaks consistent with the following: lysinoalanine (29·8%), lysine (65·5%), alanine (1·2%), glycine (2·9%) and serine (0·7%). When hydrochloric acid was added to the solution before heating, less destruction was observed. These findings are consistent with the observations of Bohak (1964), who found instability of lysinoalanine monohydrochloride when it was heated to 60°C in ethanol. In the past we have attempted to isolate lysinoalanine from protein hydrolysates using a heated ion-exchange column. After desalting the

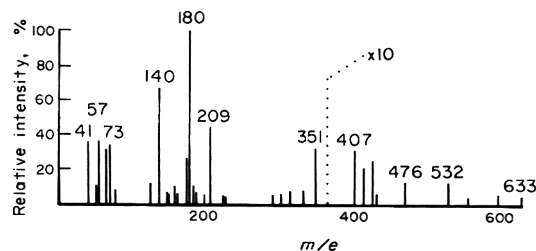

Fig. 8. Mass spectrum of the tris-(*N*-trifluoroacetyl)-di-(*n*-butyl)ester derivative of lysinoalanine.

lysinoalanine fraction, the product was yellow and gave multiple spots on TLC. It is now apparent that the lysinoalanine underwent degradation at 60°C in the citrate buffer at pH 5·28. This information is useful because the standard techniques for amino-acid analysis use heat during protein hydrolysis and use heated chromatography columns. Such methods for measuring lysinoalanine in proteins probably underestimate the true value.

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RENAL TOXICITY OF N^{ϵ}-(DL-2-AMINO-2-CARBOXYETHYL)-L-LYSINE (LYSINOALANINE) IN RATS*

J. C. WOODARD and D. D. SHORT
Division of Comparative Pathology, Department of Pathology, College of Medicine, J. Hillis Miller Health Center, University of Florida, Gainesville, Florida 32610, USA

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Abstract—Organically synthesized lysinoalanine was fed to weanling rats at levels of 0, 0·025, 0·05, 0·1 and 0·3% in a semi-purified diet. Karyomegalic alterations were observed in the renal tubular cells of all animals fed the lysinoalanine diets for 4 wk, and the high dose level also caused tubular necrosis. Renal lesions were observed also after oral or ip administration of 30 mg lysinoalanine for 7 days, and consisted of dilatation of proximal tubules, necrosis of epithelial cells and karyomegalia, limited almost exclusively to the outer medullary stripe. The administration of relatively small quantities of lysinoalanine produced lesions analogous to those seen in rats fed alkali-treated proteins for much longer periods of time.

INTRODUCTION

Previous studies showed that alkali treatment of soya protein altered the protein in such a way that renal lesions were induced in rats (Woodard & Short, 1973). Animals fed diets containing alkali-treated soya protein developed unique histological alterations characterized by cytomegalic changes that were limited to the pars recta, the descending portion of the proximal tubule (Woodard & Alvarez, 1967). Toxicity was induced when edible soya protein was treated with 0·1 N-sodium hydroxide, a treatment found to induce the formation of an unusual amino acid (Bohak, 1964; Patchornik & Sokolovsky, 1964; Woodard & Short, 1973), which has chromatographic properties similar to those of synthesized N^{ϵ}-(DL-2-amino-2-carboxyethyl)-L-lysine, lysinoalanine (Woodard & Short, 1973). Lysinoalanine has been reported in an animal product used as a commercial foaming agent (de Groot & Slump, 1969), and significant quantities are present in a wide variety of foods (Sternberg, Kim & Schwende, 1975).

Studies were conducted to determine the toxicity of synthetic lysinoalanine in order to prove conclusively that the lysinoalanine produced during alkaline soya protein modification was responsible for the renal lesions. Because of the arduous procedure involved in the organic synthesis of this molecule, the amount of material available for study was limited. Nevertheless, results of this study clearly show that lysinoalanine was responsible for the renal lesions previously observed in rats fed alkali-treated soya protein (Woodard & Short, 1973).

EXPERIMENTAL

The synthesis of lysinoalanine is described in an accompanying paper (Woodard, Short, Stratten & Duncan, 1976). The amino acid gave a single peak on a Durrum amino acid analyser (AAA Laboratory, Seattle, Washington) and a single spot on a thin-layer chromatogram developed with butanol–glacial acetic acid–water (4:1:1, by vol). Female weanling albino rats (45–55 g) of the Caesarean-derived (CD) Sprague–Dawley strain were obtained from Charles River Laboratories, Inc., Wilmington, Mass., and were housed singly in air-conditioned animal quarters. Food and water were supplied *ad lib.* Five groups, each of three animals, were fed a semi-purified diet to which lysinoalanine was added to give levels of 0, 0·025, 0·05, 0·1 or 0·3%.

The semi-purified diet comprised the following (g/100 g diet): sucrose 54·5, soya protein (Promine D, Central Soya Co., Chicago; containing 0·1% methionine) 20·0, mineral mixture 5·0, oil (Wesson Oil, Wesson Oil Sales Company, Fullerton, Cal.) 10·0, DL-methionine 0·3, choline bitartrate 0·2 and vitamin mixture 10·0. The mineral mixture was added to the diet in a sucrose carrier and contained the following (g/100 g mixture): dibasic calcium phosphate 43·33, calcium carbonate 7·59, magnesium chloride 19·0, potassium citrate 8·28, potassium chloride 7·88, sodium chloride 3·28, potassium sulphate 0·84, ferric citrate 0·64, potassium iodide 0·016, manganous sulphate 0·38, aluminium potassium sulphate 0·0032, zinc chloride 0·05 and cupric sulphate 0·04. The vitamin mixture was added to the diet in a sucrose carrier and contained the following (mg/100 g diet): thiamine HCl 1·6, pyridoxine HCl 1·6, riboflavin 1·6, calcium D-pantothenate 4·0, nicotin amide 5·0, folic acid 0·5, *meso*-inositol 10·0, DL-α-tocopheryl acetate 1·0, menadione 1·0, vitamin B_{12} 0·0005 and retinol palmitate 2500 IU/100 g diet, ergocalciferol 600 IU/100 g diet.

Animals were killed after 4 wk, and sections of kidney, liver, pancreas, salivary gland, uterus and gastrointestinal tract were processed by standard methods for light-microscopic examination.

Six additional groups of two rats were fed the semi-purified diet. The first group received daily ip injections of 30 mg lysinoalanine dissolved in 1 ml water

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with the aid of a sonicator. The second group was given daily ip injections of 30 mg L-lysine monohydrochloride. Two other groups were given solutions of 30 mg lysinoalanine or L-lysine monohydrochloride daily by stomach tube. The rats in these four groups received a total of 210 mg amino acid in 7 days and were killed on day 8. Those in the fifth group were intubated with a single dose of 210 mg lysinoalanine dissolved in 0·8 ml 1·105 N-HCl. The sixth, control, group received a single oral dose of 0·8 ml 1·105 N-HCl. Animals in the last two groups were killed 8 days after treatment. Renal tissues from the six groups of animals were processed for light microscopy by standard methods.

RESULTS

All rats receiving lysinoalanine in the diet had renal lesions, but no histological changes were found in the other organs examined. Kidney changes were more severe in animals receiving the highest dietary level of lysinoalanine, but, except for differences in severity, the lesions in all animals were morphologically similar to those described in rats fed alkali-treated protein (Woodard & Alverez, 1967; Woodard & Short, 1973).

These changes were located principally in the outer medullary stripe, the anatomical zone referred to as the inner cortex in older publications (Maunsbach, 1969). In animals fed 0·025% lysinoalanine, morphological changes were limited to this zone, where a marked karyomegalic reaction was observed in the cells of the pars recta (Fig. 1). Cells were more basophilic than normal, but a brush border could be discerned on the luminal surface and there was little evidence of cell necrosis. Tissue sections from animals fed lysinoalanine at a dietary level of 0·05 or 0·1% showed similar morphological changes, except that animals fed 0·1% showed occasional cortical changes including dilated tubules, groups of tubules with regenerative epithelium and evidence of periglomerular sclerosis.

The renal lesions in animals fed 0·3% lysinoalanine were more severe than those observed at the lower dietary levels. Although the most severe changes were observed in the outer medullary stripe, there was dilatation of tubules in the renal papillae and inner medullary stripe. There were also extensive anatomical changes in the cortex (Fig. 2). In the outer medullary stripe, the tubular brush border was not present, and the cytoplasmic morphology was not characteristic of epithelial cells of the pars recta (Fig. 3). Moreover, karyomegalic cells located in the outer medullary stripe in animals receiving the highest dietary lysinoalanine level were not as plentiful as in animals receiving lower dietary levels. Karyomegalic alterations were more prominent in the proximal tubules of the medullary rays, and, in contrast to the lower dosage levels, numerous tubular cells of the cortex showed the karyomegalic reaction. Within the cortex, there were numerous zones where the renal tubular epithelium had regenerated. The glomeruli were hypercellular, Bowman's capsule was thickened, and there was hyperplasia of both the visceral and parietal epithelial layers of Bowman's capsule (Fig. 4).

Animals given 30 mg lysinoalanine orally or ip for 7 days had renal lesions, but no lesions were observed in animals treated similarly with L-lysine monohydrochloride (Figs. 5 & 6). The morphological changes were limited to the outer medullary stripe, and epithelial necrosis was very severe when the amino acid was given parenterally (Figs. 7 & 8). The tubules of the outer medullary stripe were dilated and the lining cells were frequently squamoid. The luminal brush border had been lost in other tubules where the epithelium was cuboidal. Necrotic epithelial cells were observed, and there was an occasional leucocyte within the tubular lumen. There was a moderate degree of karyomegalia, and mitotic figures were frequently observed. Animals receiving 210 mg lysinoalanine as a single oral dose had renal lesions similar to those observed in animals that received the same amount of amino acid divided into seven daily doses. However, necrotic epithelial cells were not as frequent. The tubules were dilated, cells lacked a brush border, and there was a moderate degree of karyomegalia.

DISCUSSION

The lesions observed in rats given lysinoalanine were convincing evidence that this amino acid was responsible for the karyomegalic lesions previously observed in animals fed alkali-treated soya protein (Reyniers, Woodard & Alvarez, 1974; Woodard & Alvarez, 1967; Woodard & Short, 1973). Karyomegalic changes were observed in all rats given lysinoalanine. The lesions were limited to the kidney, and almost exclusively to the pars recta. Since both oral and ip administration of lysinoalanine produced a similar effect, it appears that lysinoalanine *per se*, liberated during digestion of alkali-treated protein, is the toxic principal.

Although the renal proximal tubule is divided into three distinct morphological regions, little is known about the differences in physiological function of these regions. The cells of the pars recta are capable of endocytosis of macromolecules and are active in both the secretion of *p*-aminohippurate and the transport of amino acids (McDowell, 1974). The basic amino acids are thought to be resorbed in this portion of the nephron by a single transport mechanism (Meister, 1973). Since the functions of the renal tubular cells of the pars recta have not been adequately defined, it may be difficult to assess physiological changes in lysinoalanine-induced karyomegalic cells. It can be speculated, however, that this amino acid is reabsorbed in this portion of the tubule, where it exerts its toxic effects and induces karyomegalia. Previous studies (Reyniers *et al.* 1974; Woodard, Short, Alvarez & Reyniers, 1975) on the physio-chemical properties of chromatin in hyperdiploid nuclei are consistent with the view that megalocytes represent 'activated' renal tubular cells, and these cells probably have an altered functional state.

The mode of action of this amino acid as a renal toxin is speculative. Its selectivity for the pars recta is reminiscent of lesions produced by transient anoxia or the action of low levels of mercuric chloride. However, its mode of action must be different because the cells of the pars recta are specifically affected over a large dose range, and karyomegalic nuclei are not associated with anoxia or mercuric chloride toxicity.

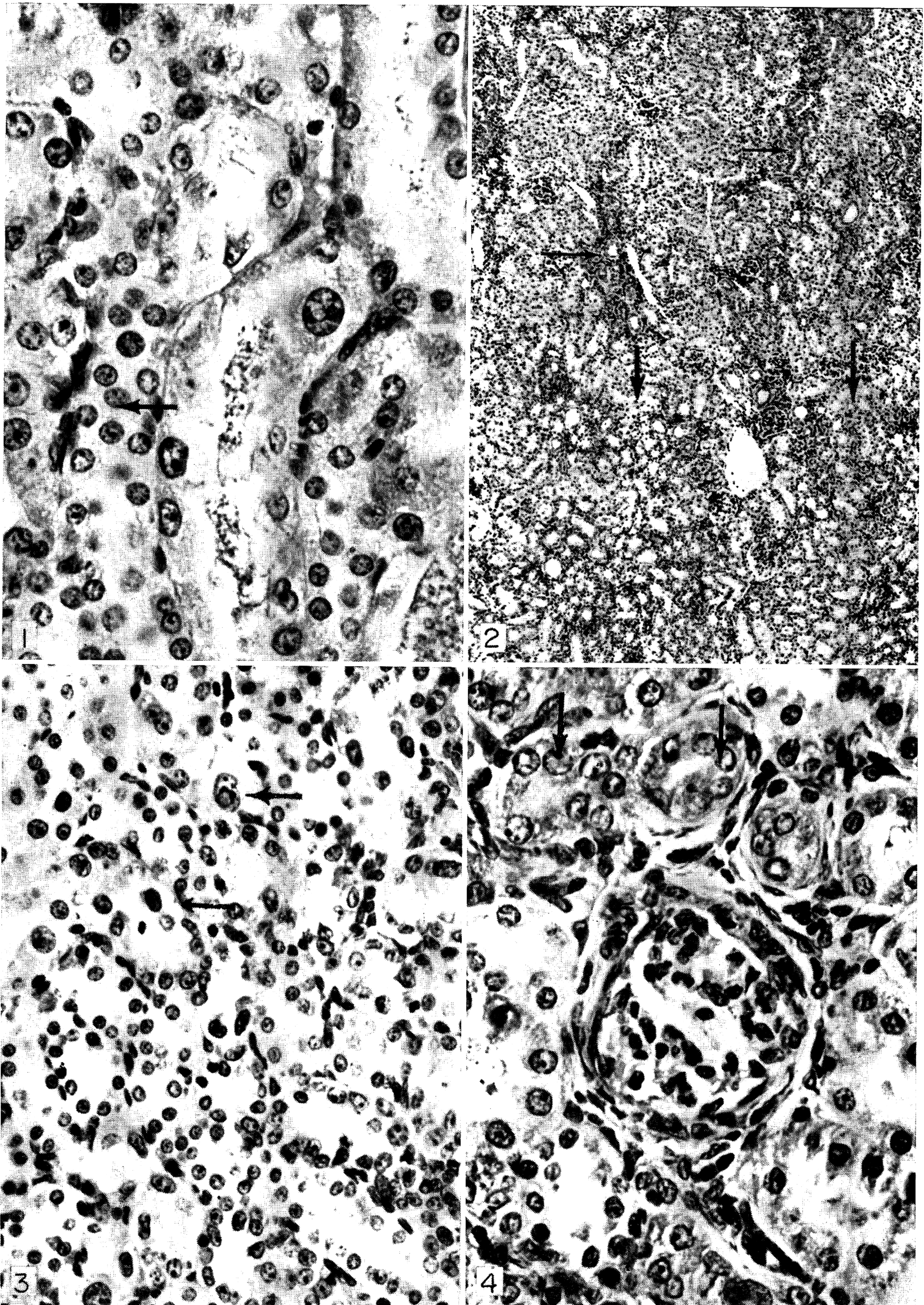

Fig. 1. Outer medullary stripe from a rat fed 0·025% lysinoalanine for 4 wk showing karyomegalic epithelial cells of the pars recta in comparison with the cells of the collecting ducts (↑) which have normal nuclei. Haematoxylin and eosin (H/E) × 344.

Fig. 2. Renal cortex and outer medullary stripe from rat fed 0·3% lysinoalanine for 4 wk showing tubules with regenerative epithelium as dark zones in the cortex (↑). The demarcation between cortex and outer medullary stripe (↑) is unusually prominent because of alterations in the pars recta. H/E × 40.

Fig. 3. Outer medullary stripe from animal shown in Fig. 2 showing karyomegalic nuclei (↑), although many tubules have lost their brush border and do not have enlarged nuclei. H/E × 220.

Fig. 4. Higher magnification of cortex from animal shown in Fig. 2 showing moderate peritubular and periglomerular sclerosis, hyperplasia of cells lining Bowman's capsule, and regenerative tubular epithelium (↑). H/E × 344.

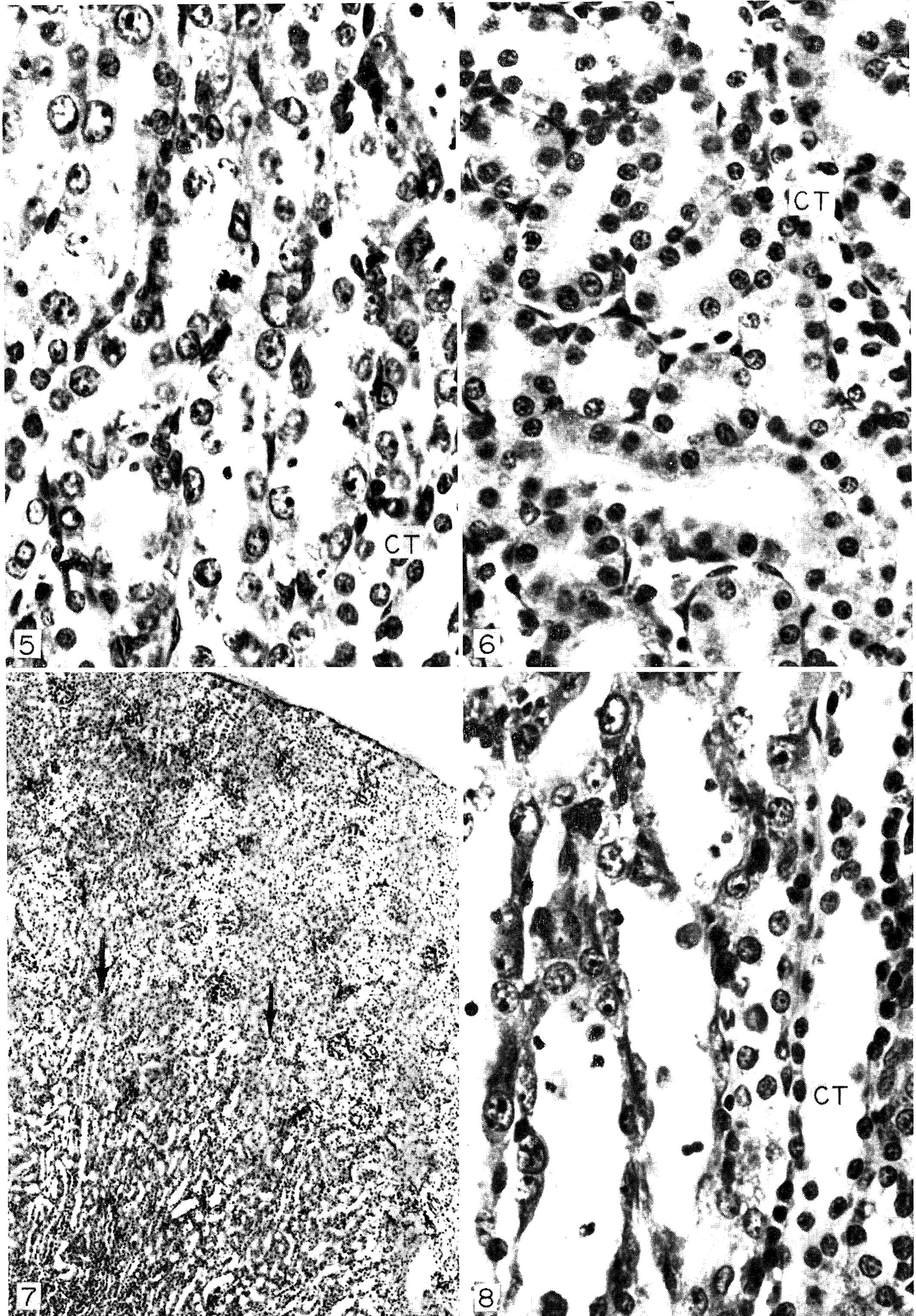

Fig. 5. Outer medullary stripe from a rat given 30 mg lysinoalanine orally for 7 days showing loss of brush border from cells lining the pars recta, cell necrosis, nuclear enlargement, increased mitotic activity and collecting tubule (CT) with normal epithelium. H/E × 344.
Fig. 6. Outer medullary stripe from animal given 30 mg L-lysine monohydrochloride orally for 7 days showing the normal cellular morphology of the pars recta and collecting tubule (CT). H/E × 344.
Fig. 7. Renal cortex and outer medullary stripe of rat given 30 mg lysinoalanine ip for 7 days showing prominent corticomedullary junction (↑) resulting from dilatation of the pars recta. H/E × 45.
Fig. 8. Higher magnification of outer medullary stripe shown in Fig. 7 (cf. Figs. 5 & 6) showing extensive nuclear enlargement and necrotic changes except within collecting tubules (CT). H/E × 344.

It is interesting to note that high dose levels (0·3% of diet) appear to destroy the pars recta before affecting other parts of the nephron. It was calculated that a rat on the 0·3% dietary level ingested 30 mg amino acid/day. When animals were given this amount of lysinoalanine for 1 wk, karyomegalic renal lesions were limited almost exclusively to the outer medulla. However, animals that had eaten equivalent daily amounts of lysinoalanine for 4 wk showed more prominent nuclear alterations in the cortical tubules. A possible explanation for this observation is that karyomegalic lesions develop independently of the necrotic changes. Animals given one or seven doses of lysinoalanine, in total equivalent to the highest dietary level, developed karyomegalic lesions in the outer medulla within 1 wk. Since karyomegalic cells were not prominent in this zone after 4 wk of treatment, it is presumed that necrosis supervened with continued intake of the toxin. The cells of the pars recta regenerated but, probably because they were not functionally mature, failed to resorb lysinoalanine and to redevelop karyomegalic alterations. With the necrosis of the lower segment of the proximal tubule, segments of the proximal tubule in the cortical region probably resorb basic amino acids and thus develop karyomegalia.

It appears that semi-purified diets are more toxic when lysinoalanine is added as the free amino acid than when it is added as an integral part of the protein molecule. Animals fed diets containing 0·025% free amino acid had many more cytomegalic renal tubular cells than animals fed an alkali-treated soya protein of a higher lysinoalanine content (Woodard & Short, 1973). Although the reason for this phenomenon has not been specifically investigated, it could be related to the fact that alkali treatment reduces the digestibility of the protein. Thus, the possibility exists that more lysinoalanine may be absorbed from the intestinal tract when highly digestible proteins of relatively low lysinoalanine content are eaten than when poorly digestible proteins of a higher lysinoalanine content are consumed. Experimental differences in the degree of cytomegalia observed after feeding alkali-treated proteins might be explained, in part, by the availability of the amino acid for absorption.

Many questions remain unanswered regarding the practical importance of lysinoalanine in human or animal health. Many substances (e.g. lead, dimethylgold and pyrrolizidine alkaloids), which induce renal karyomegalia, have been shown to be tumorigenic. Evidence indicates that lysinoalanine is mitogenic for cells of the pars recta, and the possibility that it is carcinogenic or co-carcinogenic must be investigated by animal experimentation.

These studies raise interesting questions concerning the synthesis of new chemical compounds during food processing and indicate the need for thorough investigation of new technological procedures. In the case of lysinoalanine, a model has been established by which renal lesions can be induced by the parenteral administration of relatively small quantities. The lesions are analogous to those seen in rats fed alkali-treated proteins for much longer periods of time. Such a model should prove useful in further evaluations of the biological significance of this unusual amino acid.

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INHALATION OF TALC BABY POWDER BY HAMSTERS

A. P. WEHNER, G. M. ZWICKER and W. C. CANNON
Biology Department, Battelle

C. R. WATSON
Systems Department, Battelle, Pacific Northwest Laboratories, Richland, WA 99352

and

W. W. CARLTON
Department of Microbiology and Pathology, School of Veterinary Science and Medicine, Purdue University, West Lafayette, IN 47907, USA

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Abstract—Groups of 50 male and 50 female Syrian golden hamsters were exposed to talc aerosol for 3, 30 or 150 min/day, 5 days/wk for 30 days, or for 30 or 150 min/day either until they died naturally or for a maximum of 300 days. The mean concentration of the 'respirable' aerosol fraction was approximately 8 μg/litre and the mass mean aerodynamic diameter was 6 μm. Following the exposures, the animals were observed for the remainder of their lifespan. At death, lungs, trachea, larynx, liver, one kidney, stomach, uterus, one ovary, or one testis, and all tissues showing gross lesions were collected for histopathological examination. Deposition of talc particles in the lungs of the exposed animals was demonstrated by X-ray fluorescence and by X-ray diffraction. The exposures to talc aerosol had no effect on body weight, survival or the type, incidence or degree of histopathological change in the exposed groups compared with sham-exposed controls.

INTRODUCTION

Autopsy and radiological findings of talc-induced pneumoconiosis include a heavy accumulation of macrophages, varying degrees of diffuse fibrosis, 'pneumoconiotic nodules', and asbestos bodies in a large percentage of the cases (Worth & Schiller, 1954). The presence of asbestos bodies indicates that the 'talc' to which the patients had been exposed contained appreciable quantities of asbestos and probably various other components, such as quartz.

In recent years, interest has focussed again on the potential health hazard of the dust commonly referred to as talc (Blejer & Arlon, 1973; Chodzikowski, 1975; Henderson, Joslin, Turnbull & Griffiths, 1971; Kleinfeld, Messite, Kooyman & Zaki, 1967; Kleinfeld, Messite & Langer, 1973; Kleinfeld, Messite, Shapiro & Swencicki, 1965; Kleinfeld, Messite & Zaki, 1974; Merliss, 1971; Miller, Tiersfein, Bader, Bader & Selikoff, 1971; Pelfrene & Shubik, 1975; Smith, 1973). It was considered necessary, therefore, to determine the biological effects of talc dust by means of inhalation studies in a suitable animal model under controlled laboratory conditions. Syrian golden hamsters (*Mesocricetus auratus*) were selected because studies in our laboratory have demonstrated the development of pulmonary lesions in these animals following prolonged inhalation of asbestos, cobalt-oxide and nickel-oxide dusts and cigarette smoke. Exposure to chrysotile asbestos aerosol (7 hr/day, 5 days/wk, for 11 months; mean aerosol concn 23 μg/litre) resulted in a 100% incidence of asbestosis in the exposed animals (Wehner, Busch, Olson & Craig, 1974b & 1975a). Exposure to aerosols of cobalt oxide (Wehner *et al.* 1974b) or nickel oxide (Wehner *et al.* 1974b; Wehner, Busch, Olson & Craig, 1975b) caused pneumoconiosis, and cigarette-smoke exposure significantly increased tumour incidence and epithelial lesions of the larynx (Wehner, Busch & Olson, 1974a). The positive response of the Syrian golden hamster to a variety of carcinogens has been reported by a number of investigators (Dontenwill, 1970; Dontenwill & Mohr, 1961; Herrold & Dunham, 1963; Mohr, 1970; Mohr, Wieser & Pielsticker, 1966; Montesano & Saffiotti, 1968 & 1970; Smith, 1974). Montesano, Saffiotti & Shubik (1970) used this species extensively as an animal model for the study of the pathogenesis of lung cancer and found it "markedly susceptible to various respiratory carcinogens and conveniently refractory to chronic pulmonary infections".

EXPERIMENTAL

Experimental design. The hamsters used were of the outbred Ela:ENG strain, from Engle's Laboratory Animals, Inc., Farmersburg, IN, and the experimental design is summarized in Table 1.

Three hundred 4-wk-old hamsters were randomly divided into three groups (1, 2 and 3) each of 50 males and 50 females. These groups were exposed for 30 days to talc aerosol for 3, 30 and 150 min/day, respectively. The mean total aerosol concentration was 37·1 ± 7·4 (SD) μg/litre with a respirable fraction of 9·8 ± 2·4 μg/litre. The sham-exposed control group (group 6) comprised 25 males and 25 females. Two hundred 7-wk-old hamsters were randomly divided

Table 1. *Experimental design for exposure of groups of hamsters to a talc aerosol for various lengths of time*

Group	Exposure regimen*		Calculated cumulative exposure	
	No. of min/day	No. of days	(hr)	(mg hr/m^3)
1	3	30	1·5	12
2	30	30	15	120
3	150	30	75	600
4	30	300	150†	1200†
5	150	300	750†	6000†
6 (control)	150‡	30	75†	—
7 (control)	150‡	300	750†	—

*Animals (50 males and 50 females in groups 1–5 and 25 males and 25 females in groups 6 and 7) were treated on 5 days/wk. Desired respirable fraction of talc aerosol = 8 μg/litre.
†Most of these animals died before completion of 300 exposures.
‡Sham exposures.

into two groups (4 and 5) each of 50 males and 50 females and were exposed to talc aerosol for 30 or 150 min/day, respectively, for 300 days unless they died sooner. Mean total aerosol concentration was 27·4 ± 3·4 μg/litre with a respirable fraction of 8·1 ± 1·0 μg/litre. The sham-exposed controls (group 7) comprised 25 males and 25 females.

Aerosol concentrations and exposure times were based partly on results of simulated infant-exposure experiments conducted by F. D. Pooley (unpublished data, 1973), in which women dusted infant-size dolls with baby powder as they would dust infants, and partly on results of infant-dusting experiments conducted by A. N. Eden (unpublished data, 1971) and were chosen to simulate in young and adult hamsters, as far as was practical, multiples of median infant exposure (MIE) estimated from these data. The MIE was later recalculated using the data of Eden, who determined in a study involving 72 babies ranging from 1 to 24 months of age, that mothers applied talc from one to nine times each day with a median frequency of twice a day, and of R. S. Russell (unpublished data, 1976) who determined the time-weighted average exposure for "one dusting" to be 0·1 mg min/m^3 with a standard error of 0·04 mg min/m^3 in individual tests on 48 mother–infant pairs. Using all frequencies reported by Eden and all the reported "one dusting" exposure values determined by Russell, a computer was used to develop a cumulative frequency distribution of total daily infant exposures by randomly sampling a frequency, randomly sampling an exposure, and multiplying the two numbers together for a total of 1000 cycles. From the resulting cumulative frequency distribution, it was possible to determine that the MIE was 0·058 mg hr/m^3/wk and the upper 95th percentile corresponded to 0·105 mg hr/m^3/wk.

To prevent cross-contamination, the hamsters exposed to talc aerosol and the controls were maintained in separate but identical rooms controlled for temperature (73 ± 2°F) and humidity (45 ± 15%). After completion of the exposures the hamsters were maintained for observation for the remainder of their natural lifespan with the qualification that the experiments were concluded by the killing of all surviving animals when the number of deaths in the group with the most survivors exceeded 90%. Moribund animals were killed by ip injection of sodium pentobarbitone and exsanguination.

The hamsters had free access to Wayne Lab-Blox F6 (Allied Mills, Inc., Chicago, Ill.) and water. Body weights were measured every 2–3 wk during the growing period and every 4 wk thereafter (Figs 1 & 2) and the numbers of deaths were recorded throughout the treatment and observation periods (Figs 3 & 4).

Aerosol exposure system. Each stainless-steel 1500-litre aerosol exposure chamber (Fig. 5) was designed for single-tier exposure of four separate animal cages, which could be inserted and removed independently while the aerosol was being generated. Each 64 × 64 × 15-cm stainless-steel wire-mesh cage held 25 hamsters in individual 12·8 × 12·8-cm compartments, the cages being set in filing-cabinet-like drawers which sealed into the sides of the chamber in either of two positions—fully inserted or fully withdrawn. This arrangement allowed the desired aerosol concentration to be established within the chamber prior to insertion of the drawers with the loaded animal cages. Animals could then be inserted by closing a drawer, with only a relatively small effect on the aerosol concentration within the chamber. This facilitated exposure of animals to given aerosol concentrations for as little as 3 min.

Talc powder. The talc aerosol was generated from Johnson's® Baby Powder, lot 228p, provided by Johnson & Johnson. According to the manufacturers, a multi-step flotation process on Vermont talc is used to provide a high-grade cosmetic talc for the baby powder, lot 228p, a normal production lot, having been prepared as a part of that process. It consists of over 95% (w/w) platey talc with trace quantities of carbonates (magnesite and dolomite) as well as platey chlorite and rutile.

Aerosol generation and characterization. A Wright Dust Feed Mechanism (Wright, 1950) served as the aerosol generator, the talc powder being packed into the cup at a pressure of 263 kg/cm^2. The mechanism was operated with an air flow of approximately 20

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litres/min with an upstream air pressure of 5 lb/in.^{2}, to give a fairly reliable aerosol generation. Air-flow rate through the exposure chamber was 208 litres/min. The concentration of 'respirable' aerosol in the exposure at a sampling rate of 2·5 litres/min. The Casella Type 113A Gravimetric Dust Sampler, which included a horizontal elutriator to remove larger particles from the aerosol prior to collection of the respirable particles on a glass-fibre filter. A sample was taken continuously for the duration of each day's exposure at a sampling rate of 2·5 litres/min. The Whatman GF/A filters from the sampler were weighed before and after sampling to determine the quantity of talc deposited, and the sample volume was measured by the sampler. From these data the respirable fraction of the aerosol concentration was determined.

Total aerosol concentration was measured daily by means of two probes, inserted into opposite quadrants of the chamber. Two probes were used to monitor and ascertain even aerosol distribution within the chamber. Each probe sampled for 60 min concurrent with the Casella sampler at a rate of 2·3 litres/min through a vertically positioned 25-mm-diameter Metricel DM-450 filter paper, which was weighed before and after sampling. Weekly mean aerosol concentrations were computed from the daily data. The mean aerosol concentrations referred to in this paper are the means of the combined weekly means. To monitor the accuracy of the gravimetric data, filter paper samples were subjected periodically to chemical analysis in addition to being weighed. Particle size and particle-size distribution were determined periodically by collecting aerosol samples in an Andersen Cascade Impactor (Andersen, 1966).

Sham exposures consisted of placing the control animals in an identical exposure chamber for the specified periods of time to simulate the stress of handling. Instead of aerosol, filtered room air was drawn through the chamber.

Post-mortem procedures. A detailed autopsy was performed on each animal, and gross findings were recorded. Lungs with trachea and larynx, heart, liver, one kidney, stomach, one ovary and uterus, or one testis, and all tissues showing gross lesions were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 5 μm and stained with haematoxylin and eosin for histopathological examination. For this procedure, the lungs were gently infused through the trachea with formalin, followed by tracheal ligation. All histopathological findings were recorded and coded according to the Systematized Nomenclature of Pathology (Committee on Nomenclature and Classification of Disease, 1969) to facilitate computer analysis of the data.

Qualitative analysis of lung tissue for talc. To identify the particles deposited in the lungs of the exposed hamsters, eight particles of talc were processed for scanning electron microscopy, photographed, and analysed by X-ray fluorescence. Portions of lung tissue from an exposed hamster and from a control animal, and particles removed from the lung of an exposed hamster, were also processed and analysed by X-ray fluorescence and diffraction, and the results were compared with those from the eight talc particles.

RESULTS

Aerosol exposure data

Aerosol data for the 30- and 300-day exposures are summarized in Table 2.

Clinical signs

No clinical signs were observed as a result of the talc exposures. Mean body weights as a function of age and treatment are shown in Figs 1 and 2, in which the 95% confidence intervals have been deleted for clarity. The exposures had no effect on either male or female hamsters as determined by comparison of the 95% confidence intervals of these means.

Number of deaths

There were no significant differences among the survival times of the exposed groups, nor between the exposed groups and the controls (Table 3). However, in all groups the mean survival time of the males was significantly ($P < 0{\cdot}05$) longer than that of the females. The numbers of deaths in the 30- and 300-day exposure groups are shown in Figs 3 and 4, respectively. There was a marked sex-related difference in all groups, which became statistically significant at the age of approximately 12 months, but no treatment-related effect was apparent.

Table 2. *Talc aerosol data for 30- and 300-day exposures*

	Particle size distribution*		
Interval	Upper limit of interval† (μm)	Percentage of total mass in interval	Cumulative percentage mass smaller than upper limit size
	30-day exposures‡		
1	—	34·1	99·9
2	6·7	16·9	65·8
3	4·6	19·5	48·9
4	3·1	21·6	29·4
5	2·0	4·1	7·8
6	1·1	1·2	3·7
7	0·7	1·0	2·5
8	0·5	1·5	1·5
	300-day exposures§		
1	—	32·6	100·0
2	8	26·9	67·5
3	5	20·3	40·6
4	3	9·5	20·3
5	2	3·0	10·8
6	1	2·3	6·8
7	0·7	2·6	4·5
8	0·5	1·9	1·9

*Cumulative 'composite' of 10 (for 30-day exposure) and 53 (for 300-day) Andersen Cascade Impactor samples.
†Particle size expressed as aerodynamic equivalent diameter.
‡Mean aerosol concentrations (μg/litre ± SD), 37·1 ± 7·4 (total) and 9·8 ± 2·4 (respirable fraction); mass median aerodynamic diameter, 4·9 μm; distribution, bimodal with peaks at 0·6 and 2·6 μm.
§Mean aerosol concentrations (μg/litre ± SD), 27·4 ± 3·4 total and 8·1 ± 1·0 (respirable fraction; mass median aerodynamic diameter, 6·0 μm; distribution, bimodal with peaks at 0·6 and 4·0 μm.

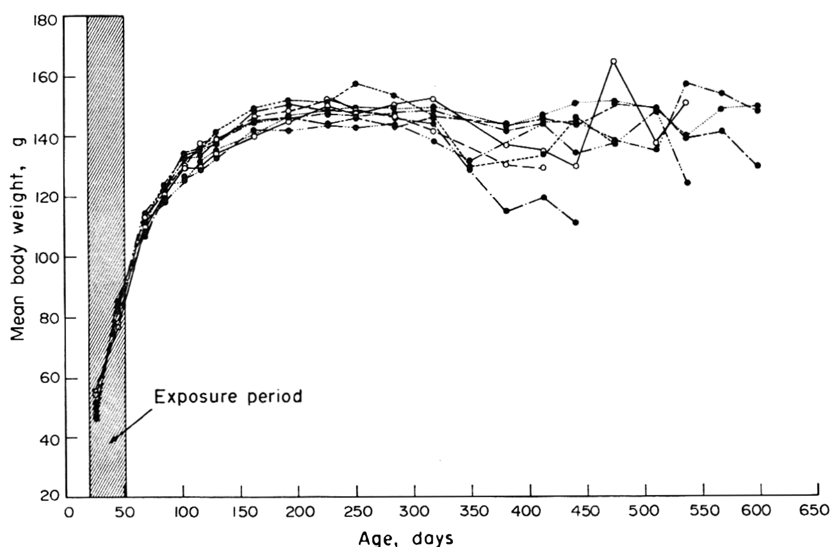


Fig. 1. Mean body weights of hamsters of groups 1 (———, males; —·—, females), 2 (—··—, males; —···—, females) and 3 (·····), males; -----, females), exposed to talc aerosol for 30 days, and of group 6 (——, males; ---, females), the sham-exposed control group.

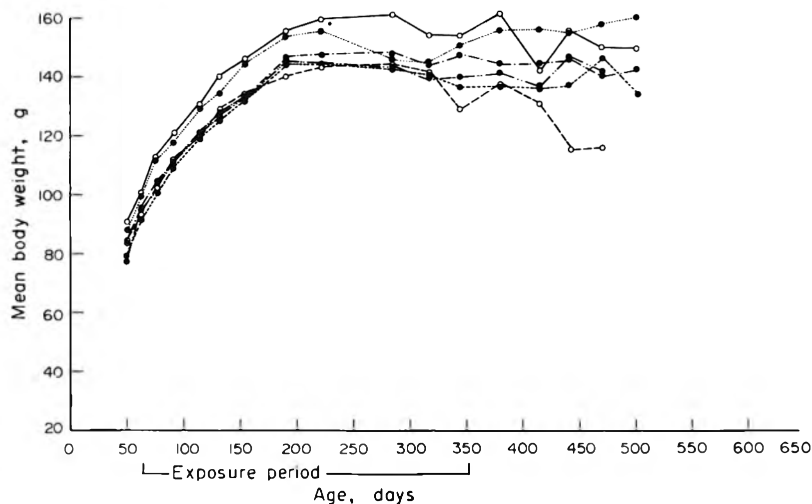


Fig. 2. Mean body weights of hamsters of groups 4 (—·—, males; —··—, females) and 5 (·····, males; -----, females) exposed to talc aerosol for 300 days, and of group 7 (——, males; ---, females), the sham-exposed control group.

Table 3. *Mean survival times of groups of hamsters exposed to a talc aerosol and of sham-exposed controls*

Group	Daily aerosol exposure (min)	Actual cumulative exposure (mg hr/m³ ± SD)	Median survival time (days ± SD) Males	Females	M + F	Mean survival time* (days ± SD) Males	Females	M + F
			30-day exposures					
1	3	14·6 ± 3·6	430	370	398	442 ± 15	349 ± 15	396 ± 12
2	30	146 ± 36	398	363	382	415 ± 15	360 ± 13	387 ± 10
3	150	732 ± 180	434	367	388	453 ± 18	372 ± 9	412 ± 11
6	0	0	412	381	393	426 ± 17	372 ± 14	399 ± 8
			300-day exposures					
4	30	1210 ± 150†	482	400	428	491‡ ± 13	380 ± 12	436‡ ± 10
5	150	6060 ± 750†	481	396	428	462† ± 11	405 ± 12	433‡ ± 10
7	0	0	488	354	411	485‡ ± 21	361 ± 16	423 ± 16

*In all groups, the mean survival time for the males was significantly longer ($P < 0·05$) than that for the females.
†Most of these animals died before completion of the 300 exposures (Fig. 5).
‡The survivors of groups 4, 5 and 7 were killed at the age of 20 months. At that time all females were dead and less than 20% of the males were alive. The male mean survival times and consequently the combined mean survival times are therefore artificially low.

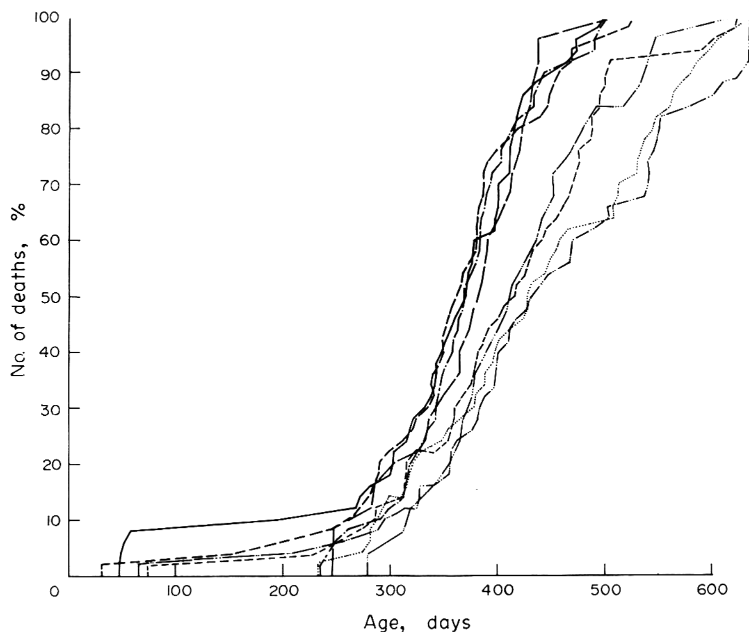


Fig. 3. Death rates in hamsters exposed for 30 days to talc aerosol for 3 min/day (group 1: ·····, males; ——, females), 30 min/day (group 2: ----, males; — — —, females) and 150 min/day (group 3: —··—, males; —·—, females) and in the sham-exposed controls (group 6: —···—, males; ——, females).

Qualitative analysis of lung tissue for talc

Figure 6 shows a photomicrograph of one of the eight talc particles processed. Analysis by X-ray fluorescence at 0–5 keV and at 5–10 keV showed that the particle was composed of magnesium, silicon and traces of iron. Figure 7 is a photomicrograph of lung tissue from a control hamster. One of many talc particles found in the lungs of exposed hamsters is shown in Fig. 8, its identity having been confirmed by X-ray analysis.

Summary of type and incidence of histopathological findings

The type, incidence and severity of the observed lesions indicated no trend toward a dose-response relationship and showed no significant differences between the exposed groups and the controls.

Larynx and trachea. Changes found in the larynx in both control and exposed hamsters included calcification of the mucosal tissue, calcification of the muscles of the larynx, dilation of the glands of the

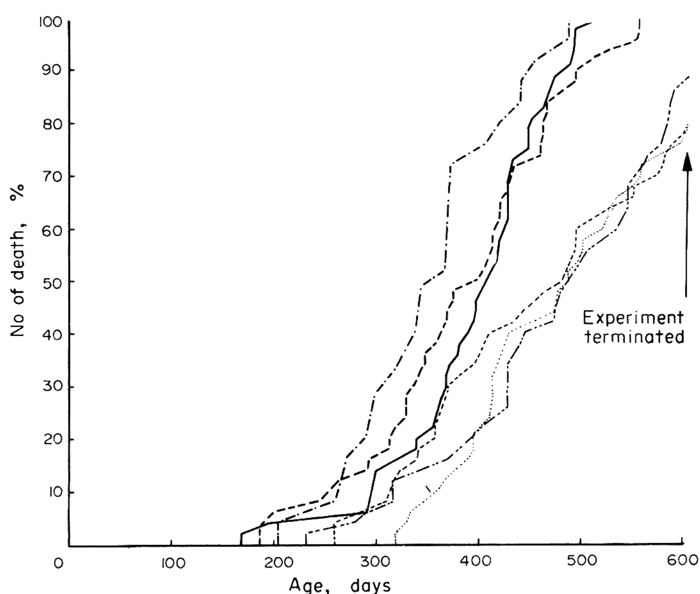


Fig. 4. Death rates in hamsters exposed for 300 days to talc aerosol for 30 min/day (group 4: ·····, males; ——, females) and 150 min/day (group 5: ----, males; — — —, females) and in the sham-exposed controls (group 7: — — — —, males; —·— females).

Table 4. *Summary of incidence of common pulmonary changes observed in hamsters exposed to a talc aerosol*

Pulmonary change	No. of hamsters examined....	1 M	1 F	2 M	2 F	3 M	3 F	4 M	4 F	5 M	5 F	6 M	6 F	7 M	7 F	Total affected	Incidence (%)
		49	47	50	49	48	50	49	50	48	48	25	25	25	25	588	
Alveolar emphysema		16	8	17	5	14	5	4	5	6	8	3	3	3	3	100	17·0
Interstitial pneumonia		23	25	29	24	22	24	18	26	21	30	14	14	12	8	290	49·0
Calcification		21	5	16	5	9	7	3	9	7	12	5	2	3	4	101	17·2
Alveolar hyperplasia		10	5	5	5	11	6	14	11	15	9	7	2	5	0	105	17·9
Alveolar histiocytosis		4	1	5	3	2	4	5	9	6	6	2	2	4	1	54	9·2

Column groups 1–7: No. of animals affected in group*

*See Table 1 for the identification of groups 1–7.

mucosa and focal inflammation and hyperplasia of the mucosa. The most common change was focal mucosal calcification, the incidence of which was not a function of exposure time; for example, the combined data for male and female hamsters showed the highest incidence in group 3 (150 min/day for 30 days) and the lowest in group 5, which received the longest exposure (150 min/day for 300 days). The incidence of other laryngeal changes were similarly unrelated to treatment. Tracheal changes found in both control and exposed hamsters included focal mucosal calcification, mucosal-gland dilation and focal mucosal inflammation. The most common alteration was focal calcification of the mucosa. No treatment effects were evident in any of the alterations of the trachea; the highest incidence (combined male and female data) of focal mucosal calcification occurred in group 1, which had received the shortest exposure (3 min/day for 30 days), and the incidences in the other treatment groups did not differ much from the incidence of the combined sham-exposed control groups.

Lungs. Pulmonary changes (Table 4) in all groups included interstitial pneumonia, focal alveolar and bronchiolar calcification, focal alveolar emphysema, focal alveolar hyperplasia, focal alveolar histiocytosis and pulmonary vasculitis. Interstitial pneumonia was the most common lesion, the incidence being highest in group 2 and lowest in group 4 and in the sham-exposed controls. The other pulmonary changes also varied among the treatment groups without a consistent incidence pattern that could be related to level or duration of talc exposure. Alveolar and bronchiolar calcification and focal alveolar emphysema, for example, were found most frequently in group 1 and least frequently in group 4 of the treated animals.

Table 5. *Incidence of focal alveolar cell hyperplasia*

Exposure (min/day)	Incidence (%) of lesion in groups exposed for 30 days*	300 days*
0	18·0 (group 6)	10·0 (group 7)
3	15·6 (group 1)	—
30	10·1 (group 2)	25·2 (group 4)
150	17·4 (group 3)	25·0 (group 5)

*See Table 1 for the identification of groups 1–7.

The incidence of focal alveolar cell hyperplasia seemed to reflect an effect of treatment, as the highest incidences were found in groups 4 and 5 (Table 5). However, a two-way weighted analysis of variance of the arcsin transformation of the percentage incidence demonstrated that no significant pattern of incidence of focal alveolar cell hyperplasia was associated with the number of exposure days or with the number of exposure minutes per day.

Histological examination, with the aid of a Nomarski system, of a lung section from a randomly selected hamster of group 5 revealed foci of macrophages containing material resembling talc (Fig. 9). The hamster was killed 3·5 months after completion of the 300 days of exposure during which it had received a cumulative exposure of 6000 mg hr/m^3. In a lung section from a randomly selected group 3 hamster, which died during the 30 days of exposure after a cumulative exposure of 420 mg hr/m^3, no talc was observed.

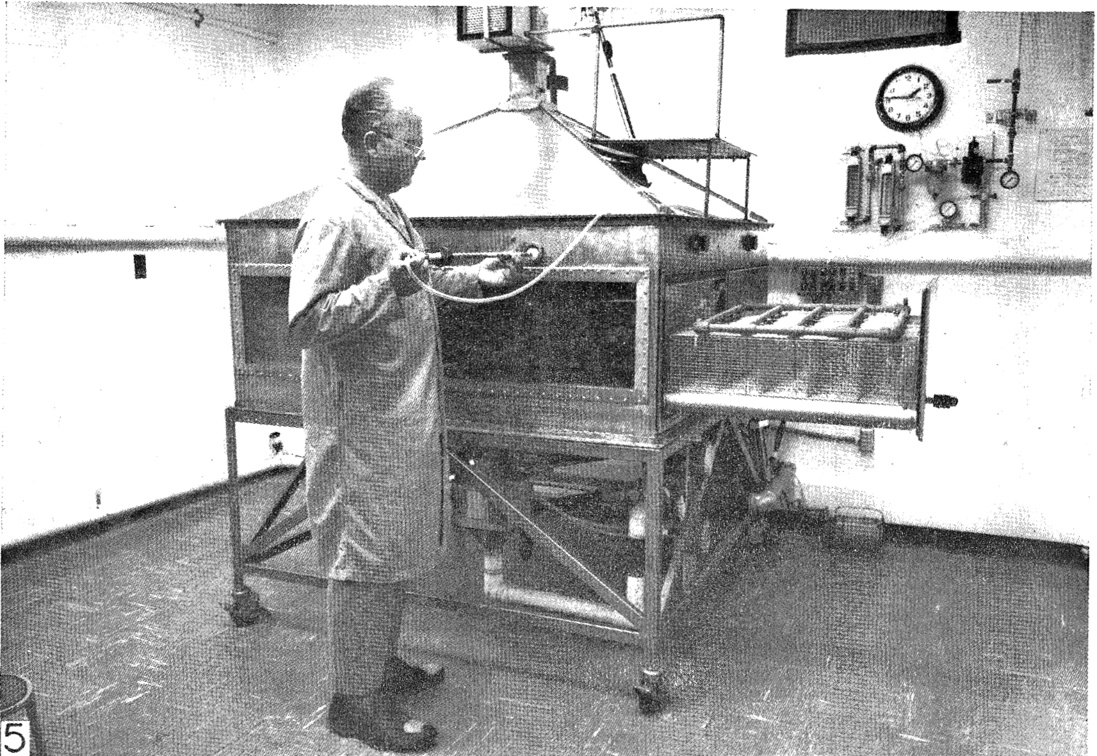
Heart. Common changes in the heart included valvular endocarditis, atrial thrombosis, calcification of the coronary vessels and myocardium and focal myocarditis. The incidence of these alterations did not appear to be related to treatment as no consistent pattern of incidences emerged and the incidence was similar for most of the groups.

Other organs. The incidences of hepatic amyloidosis and 'hepatitis' and of renal amyloidosis and 'nephrosis' were high and were similar for the several treatment groups and for the sham-exposed controls. Changes observed in the stomach included calcification of the mucosal tissues and of the tunica muscularis. No pattern was present to suggest that these alterations were related to the talc exposures. Atrophy of the germinal epithelium was common among the treatment groups, but no pattern consistent with a treatment effect was found. Few changes were found in the uterus, and treatment did not have any apparent effect on the incidence of any of these.

Tumours. Only a few neoplasms (Table 6) were found. They were of several different histological types and their incidence was not related to treatment. No primary neoplasms were found in the respiratory system.

DISCUSSION

The calcification observed in several organs, including the mucosa of the larynx and trachea, alveolar



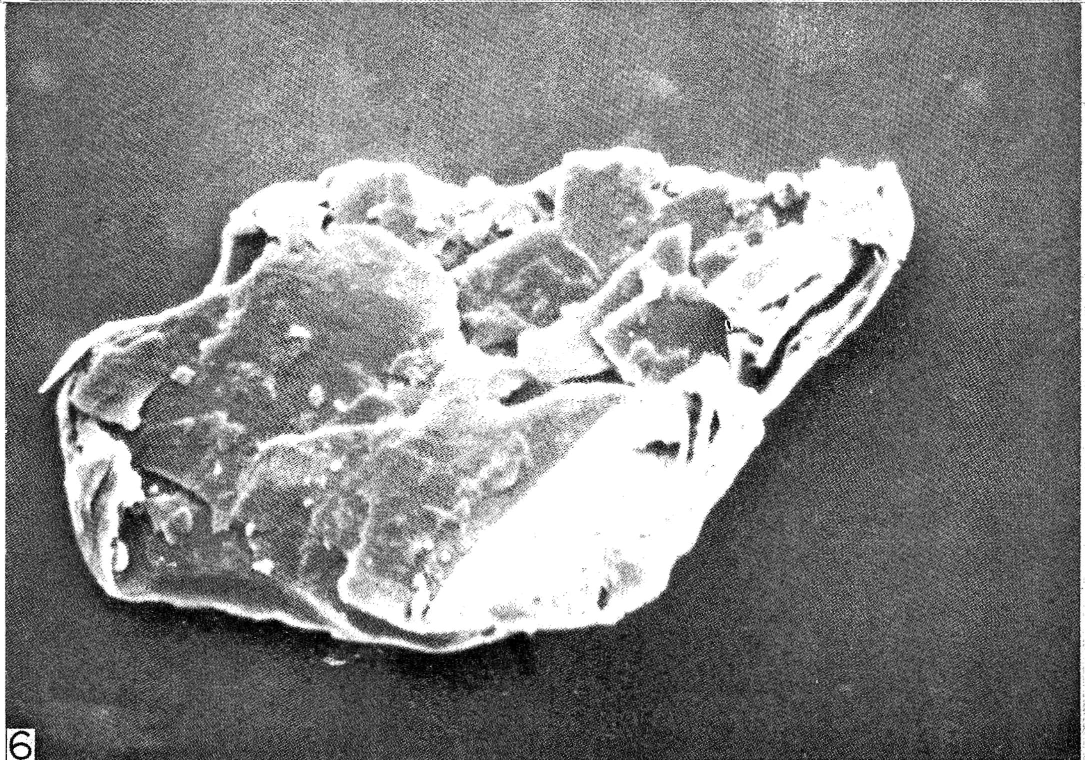


Fig. 5. Aerosol exposure system.

Fig. 6. Scanning electron micrograph of a talc particle. × 1800.

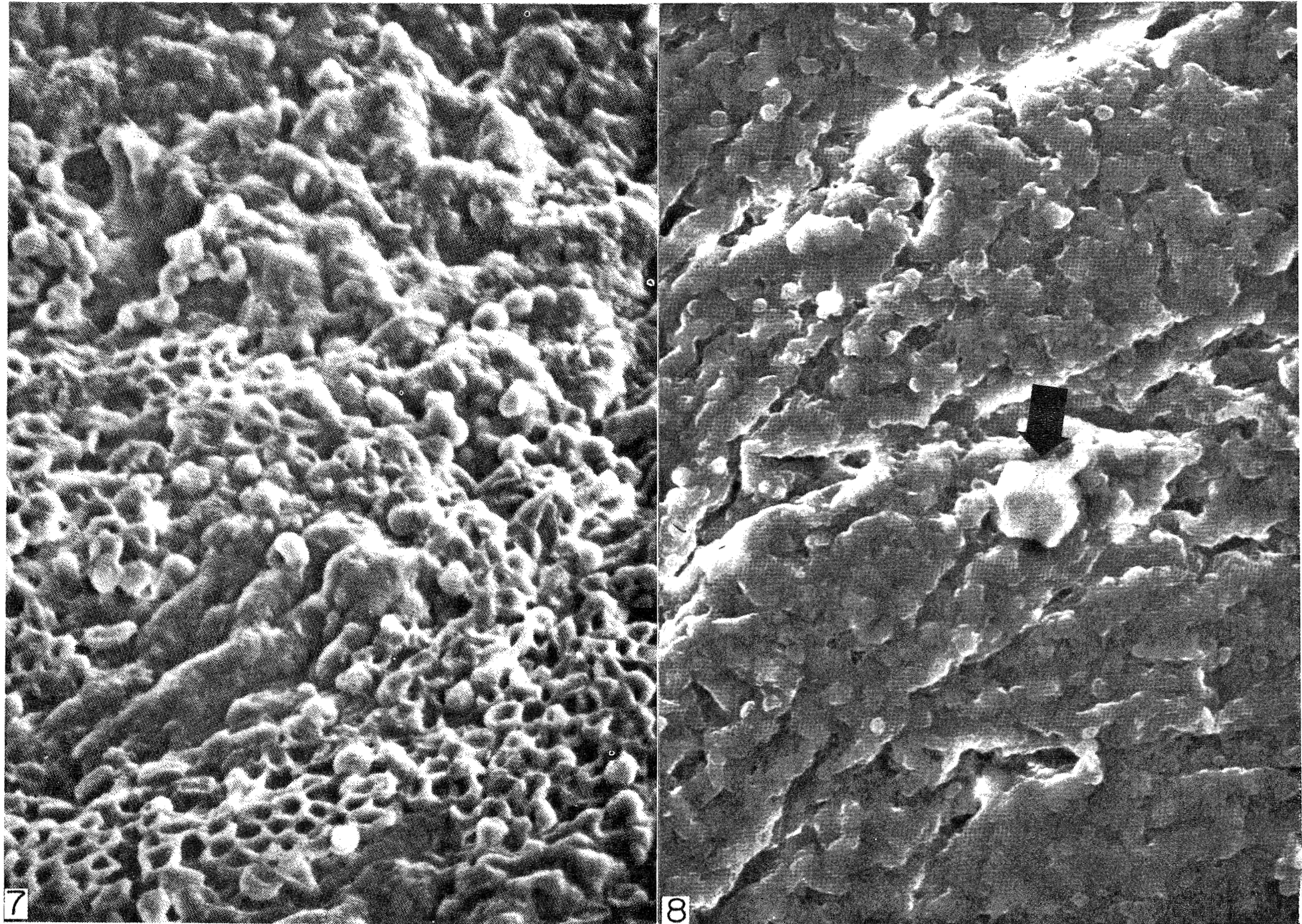


Fig. 7. Scanning electron micrograph of lung tissue. × 1400.

Fig. 8. Scanning electron micrograph of talc particle on lung tissue. × 650.

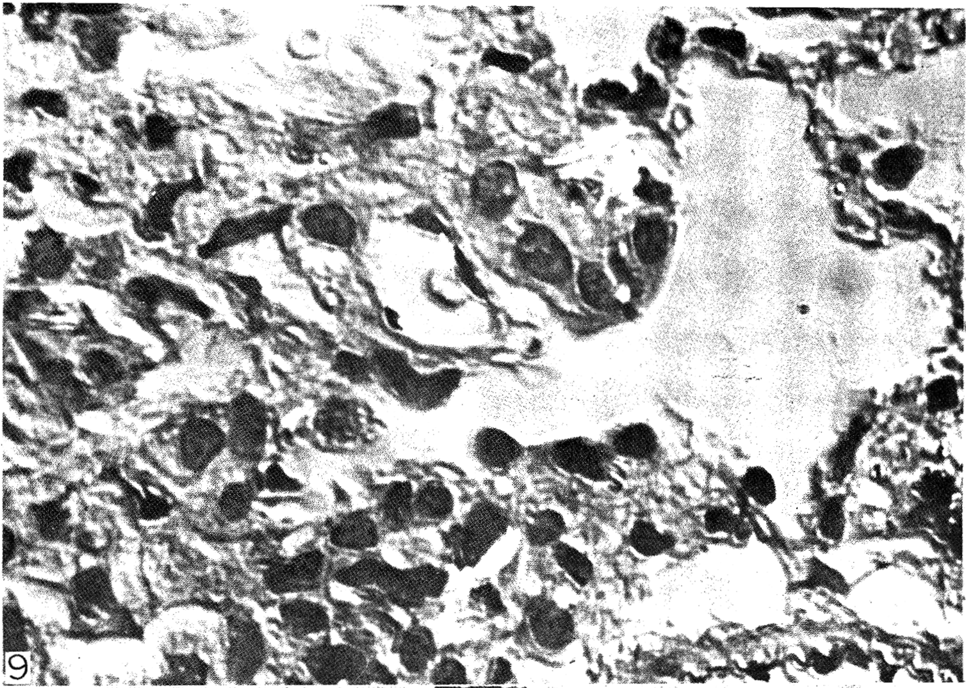


Fig. 9. Photomicrograph of section of hamster lung in Nomarski illumination, showing a cluster of alveolar macrophages containing particles believed to be talc. The male hamster was killed 3·3 months after completion of the 300-day exposure period, during which it had received a cumulative exposure of 6000 mg hr/m³. Haematoxylin and eosin × 870.
Fig. 10. Transmission electron micrograph of talc particles. × 8000.

Table 6. *Summary of incidence of spontaneous neoplasms found in control and talc-exposed hamsters*

Site and neoplasm	No. of affected males and females (as specified)* in groups†					Total affected
	1(M)	3(F)	5(F)	6(F)	7(M)	
Adrenal gland						
Adenoma		1			1	2
Phaeochromocytoma		1				1
Uterus						
Leiomyoma			1			1
Unknown site of origin						
Carcinoma					1	1
Lung						
Carcinoma, metastatic	1				1	2
Thorax						
Rhabdomyosarcoma				1		1
Bone						
Osteosarcoma					1	1
Lymph node						
Malignant lymphoma					1	1
Liver						
Osteosarcoma			1			1
Cholangiocarcinoma	1					1
Malignant lymphoma, metastatic					1	1

*No neoplasms occurred in either sex in groups 2 and 4, in the males of groups 3, 5 and 6 or in the females of groups 1 and 7.
†See Table 1 for the identification of groups 1–7.

and bronchiolar walls, myocardium, coronary vessels and the gastric mucosa and muscularis, is a change commonly associated with ageing. Also, the renal and hepatic changes are common findings in old hamsters (Fortner, 1957). The 'nephrosis' could have altered the serum calcium–phosphorus balance to a marked degree and contributed to the soft-tissue calcification.

Some of the cardiac and pulmonary changes were apparently interrelated. It appears likely that valvular endocarditis was contributory to the atrial thrombosis and, by extension, responsible for the inflammatory lesions of the pulmonary vasculature, as well as contributory to the development of interstitial pneumonia. Thrombosis of the chambers of the heart of ageing hamsters has been described by Fortner (1957) and Chesterman (1972).

Amyloidosis is a common change in the ageing hamster and especially common is renal amyloidosis (Fortner, 1957). The stimulation to the immune system by persistent infection, as seen in the heart valves and lungs, could have contributed to the development of the hepatic and renal amyloidosis.

The incidences of the tissue changes associated with ageing varied greatly among the treatment groups and sham controls, and no consistent pattern was discerned to indicate that treatment increased or accentuated these changes.

The hamsters received cumulative exposures ranging from about 15 to more than 6000 mg hr/m^3 (Table 4). Estimates based on results of a pulmonary deposition study with neutron-activated talc show that 0·05–6 μg talc, depending on the length of exposure, was deposited in the hamster lungs at each exposure (Wehner, 1976). Estimates based on infant-dusting experiments show, according to J. N. Sivertson (personal communication, 1976), that the weekly hamster exposures, expressed in mg hr/m^3, exceeded the average of the weekly infant exposures by some 30 to 1700 times (Table 7).

Unknown quantities of talc also passed through the gastro-intestinal tract of the exposed hamsters. This talc comprised quantities deposited on the ciliated surface of the respiratory tract and subsequently brought up by the mucociliary clearance mechanism and swallowed, talc licked from the pelt and swallowed and talc deposited on the feed during exposure. However, there was no evidence of talc-induced lesions in the gastro-intestinal tract. A gavage study with neutron-activated talc is in progress to determine to what degree talc is absorbed during passage through the gastro-intestinal tract.

It is possible that markedly higher exposure levels would have caused talcosis eventually. Quick clearance of the talc particles from the lung could be at least partly responsible for the absence of talc-induced lesions, but this possibility can only be confirmed by

Table 7. *Comparative levels of exposure to talc aerosols in different groups of hamsters*

Hamster group*	Calculated weekly exposure (mg hr/m^3)	Weekly hamster exposure expressed as multiples of weekly infant exposure†	
		50th percentile	Upper 95th percentile
1	2	30	19
2	20	300	190
3	100	1700	950
4	20	300	190
5	100	1700	950

*See Table 1 for the identification of groups 1–7.
†J. N. Sivertson (personal communication 1976).

determining the fate of the talc particles in the hamster. Early efforts to determine pulmonary deposition and clearance using atomic absorption spectrophotometry were unsuccessful because the relatively high and varying tissue background levels of the elements to be analysed interfered with the measurements. A study is now in progress using neutron-activated talc to determine pulmonary deposition and clearance.

The most plausible explanation for the absence of talc-induced lesions in our hamsters appears to lie in the nature of the talc. It was cosmetic-grade talc (Fig. 10), in which no asbestos fibres were found. Industrial-grade talc, by comparison, contains varying and appreciable quantities of other minerals, including asbestos which is a proven fibrogen and carcinogen. Our results, therefore, should not be interpreted as being in disagreement with the findings in workers engaged in such industries as talc mining and milling, rubber-tyre and cable manufacture and battery-plate casting, in which (prior to the initiation of protective measures) the workers were chronically exposed to atmospheric concentrations of talc-mine dust or industrial-grade talc averaging up to several hundred million particles/ft^3 (Kleinfeld *et al.* 1967). Some of the controversy and confusion about talc can be attributed to the loose application of the term 'talc' for widely varying mixtures of components of different pathogenicity.

We recommend, therefore, that (a) the dust mixtures now commonly referred to as talc should be analysed and graded uniformly according to the type and quantity of their components, (b) an appropriate nomenclature should reflect the type or quality of the dust mixture in question, the term 'talc' being reserved for powder consisting of at least 95% (w/w) platey talc and no asbestos with different names (e.g. talcoid) being assigned to the powder categories of lesser purity, and (c) rather than having one threshold limit value (2×10^7 particles/ft^3 at present) for the dust mixtures now collectively referred to as 'talc', different threshold limits should reflect the differences between the adverse biological effects of cosmetic-grade talc and those of different talcoid mixtures containing varying degrees of asbestos and quartz.

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EVALUATION OF THE CORNEAL IRRITANCY OF TEST SHAMPOOS AND DETERGENTS IN VARIOUS ANIMAL SPECIES

L. L. GERSHBEIN

Neurophysiological Laboratories of Northwest Institute for Medical Research, 5656 West Addison Street, Chicago, Illinois 60634,

and

J. E. McDONALD

Department of Ophthalmology, Loyola University Stritch School of Medicine, Maywood, Illinois 60153, USA

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Abstract—Four commercial shampoos, two cationic detergents (2% Roccal and 2% Isothan Q-15), a nonionic agent (20% Neutronyx 600) and a 1:1 (v/v) mixture of the last two products were instilled into the eyes of several animal species and the corneal changes were followed. To evaluate corneal change, the area, intensity and vascularity of the opacity were rated and scored. In a few instances, the eyes were rinsed with water after the test agent had been in contact with the cornea for a short period: in these cases, the initial contact of the agent with the cornea was shown to be critical. Corneal sensitivity was highest in the rabbit, hamster and mouse, intermediate in the rat and guinea-pig and possibly lowest in the dog, cat, rhesus monkey and chicken.

INTRODUCTION

A procedure widely applied in the evaluation of ophthalmic irritancy is that of Draize, Woodard & Calvery (1944). With minor modifications, it is essentially the method proposed by Friedenwald, Hughes & Hermann (1944), using the rabbit eye. This approach was applied to the screening of the eye irritancy of a number of agents (Hughes, 1947 & 1948) and was recommended by the US FDA (1964) for the grading of eye irritancy. However, there is a lack of agreement in the findings of intra- and interlaboratory surveys of various agents using the Draize rating scheme (Marzulli & Ruggles, 1973; Russell & Hoch, 1962; Weil & Scala, 1971; Weltman, Sparber & Jurtshuk, 1965). Burton (1972) deduced a procedure that was based on the measurement of changes in corneal thickness on instillation of agents into the eye and demonstrated that it yielded results that could be analysed statistically and did not suffer from the variability and bias posed by the Draize method. Corneal thickness has been investigated by others in relation to alkali (Shapiro, 1956) and to such agents as 1,8,9-triacetoxyanthracene (Easty & Mathalone, 1969) and the use of the slit-lamp microscope in following ophthalmic changes has been discussed by several workers (Baldwin, McDonald & Beasley, 1973; McDonald, Baldwin & Beasley, 1973).

The present study was undertaken with a view to elaborating a unique scoring system for corneal changes apart from the grading of the relatively 'recoverable' conjunctival irritation. Complete scoring of all eye changes, as in the Draize procedure, was avoided. As few data relating to other animals are available, the test agents were instilled also into the eyes of other species and the changes were compared with those in the albino rabbit.

EXPERIMENTAL

Test materials. The shampoo formulations tested, SP-1 to SP-4, were four commercial products of pH 8·5–9·3, SP-4 being a Castille shampoo of pH 9·3. In addition, two cationic detergents, 2% Roccal (alkyldimethylbenzylammonium chloride, pH 6·4) and 2% Isothan Q-15 (laurylisoquinolinium bromide, pH 4·3), the nonionic agent, 20% Neutronyx 600 (an aromatic polyglycol ester condensate, pH 6·1) and a mixture of 1:1 (v/v) 20% Neutronyx 600–2% Isothan Q-15 were screened.

Animals. Albino rabbits of either sex weighing 2–2·5 kg were the main test animals. In the species studies, young adult guinea-pigs and rats, both albino, Swiss mice, Syrian hamsters, rhesus monkeys, chickens, dogs and cats were tested. All the animals were given the usual laboratory diets and water *ad lib.*, and except for the smaller species were caged singly.

Instillation of test agents. Each rabbit was immobilized in an injection box, the eyelids were held apart and 0·10 ml of test agent was delivered to the centre of the cornea by means of a syringe. After 15 sec, the hold on the lids was relaxed. Conjunctival alterations were noted at periods of 1, 24, 48 and 72 hr and daily thereafter, until any signs of irritancy had cleared. Corneal changes were followed by means of a slit-lamp on days 1, 4, 7, 15 and 30 after treatment or until clearing had occurred; the scheduling was flexible and depended on the extent of corneal involvement. A similar but somewhat modified procedure was used for other species. Owing to difficulties in restraint and to circumvent the use of anaesthesia, the lids were not held apart for the full 15-sec period in the case of the monkey, but good contact with the cornea was achieved. For the smaller species,

Table 1. *Corneal findings in various animal species following instillation of the eyes with test shampoos and detergents*

Product	Rabbits		Guinea-pigs		Rats		Mice		Hamsters		Dogs		Cats		Monkeys		Chickens	
	No. of lesions*	Mean score (%)†	No. of lesions*	Mean score (%)†	No. of lesions*	Mean score (%)†	No. of lesions*	Mean score (%)†	No. of lesions*	Mean score (%)†	No. of lesions*	Mean score (%)†	No. of lesions*	Mean score (%)†	No. of lesions*	Mean score (%)†	No. of lesions*	Mean score (%)†
SP-1	11 (11)	37·5 ± 6·9	0 (16)	—	5 (15)	11·4 ± 3·9	11 (20)	53·7 ± 12·3	9 (12)	25·9 ± 10·3	1 (10)	17	1‖ (10)	13‖	0 (3)		0 (4)	
SP-2	9 (12)	35·7 ± 13·3	0 (15)	—	0 (17)		1 (20)	8	5 (12)	26·6 ± 8·0	0 (10)		0 (10)		0 (3)		0 (4)	
SP-3	11 (10)	44·4 ± 7·7	2 (15)	3; 3	1 (18)	8	5 (20)	46·0 ± 16·2	7 (11)	59·0 ± 37·4	2 (10)	17; 17	0 (10)	—	0 (3)	—	0 (4)	
SP-4	11 (11)	37·7 ± 4·5	0 (17)	—	2 (17)	13; 34‡	12 (20)	74·4 ± 9·9	7 (11)	84·0 ± 17·4	1 (10)	11§	0 (10)		0 (6)	—		
Roccal (2%)	9 (10)	62·1 ± 7·4	6 (19)	15·3 ± 4·4	10 (14)	23·5 ± 3·5	12 (12)	45·7 ± 10·1	12 (13)	54·6 ± 10·6	4 (13)	6·7 ± 3·5	1‖ (10)	4‖	—			
Isothan Q-15 (2%)	7 (11)	59·2 ± 6·3	6 (18)	19·0 ± 5·0	6 (15)	8·7 ± 2·2	12 (12)	86·2 ± 7·3	12 (13)	57·1 ± 9·1	2 (11)	8; 17	0 (10)		1 (3)	25		
Neutronyx 600 (20%)	10 (10)	34·4 ± 5·9	14 (15)	41·4 ± 7·1	8 (15)	30·8 ± 8·3	11 (12)	70·7 ± 8·8										
Isothan–Neutronyx¶	10 (10)	74·8 ± 6·4	17 (18)	63·4 ± 5·5	16 (16)	29·7 ± 6·3	12 (12)	87·1 ± 6·8									1‖ (4)	4‖

*With total no. of eyes examined given in parenthesis.

†The mean score (± SEM) applies to those eyes displaying corneal lesions and is calculated on the basis of the day-7 readings or the most extreme ratings. In rabbits, lesions of greater severity generally persisted for 30–90 days, but recovery was more rapid with many of the other species. The single lesion noted in the monkey 48 hr after treatment was absent on day 4.

‡Findings on day 3; the respective scores were 0 and 8% on day 13.

§Scoring on day 3; the value was 6% on day 6 and the four lesions elicited by 2% Roccal had disappeared by day 30.

‖Score 24 hr after treatment; lesions were absent 15 hr later.

¶Mixture of 20% Neutronyx 600–2% Isothan Q-15, 1:1 (v/v).

the volume of test agent was 0·05 ml. In any single series of animals, right and left eyes, alternately, were treated with the test agent, 0·90% NaCl being instilled into the other eye. The eyes of the smaller animal species were inspected only for corneal lesions and, with adequate practice, opacities could be evaluated in the trans-illuminated eyes without recourse to the slit-lamp.

Before the instillation of any test agent, the eyes were examined closely. No 5% fluorescein solution was instilled to detect stainable areas in the eyes, as has been advocated by others (Burton, 1972), since many artefacts are possible, and furthermore, no other fluids should be introduced before the test agent. Recovered eyes were used only in the preliminary screening of other agents for ophthalmic irritancy, not in the actual testing. With a few of the formulations, the effect of rinsing was ascertained. The method involved gently rinsing the rabbit eye with a total of 20 ml water at 30°C, delivered by syringe 4 sec after instillation of the test product, and then releasing the hold on the lids.

Rating of eye alterations

Conjunctiva. The extent of conjunctival redness (R), secretion (S), chemosis or swelling (C) and epilation (E) was determined visually, the maximal ratings being 4, 4, 3 and 2, respectively. Epilation, which was included as a change, can stem from the secretion as such, from the agent directly or from a combination of both. Its effect becomes more significant after the first day following treatment. Where a percentage scoring was required for the data, the relation $(R + S + C + E) \times 7{\cdot}7$ could be used, the last factor being obtained by dividing 100 by the maximal or overall extreme score of 13.

Cornea. For the rating of a corneal lesion, the slit-lamp served as an aid in the determination of the area of opacification (I), its intensity (II) and the vascularity (III), the maximal values being 8 (or 2/quadrant), 4 and 16, respectively. Interrelating these by (area × intensity + vascularity), the maximal values yield a figure of 48, so that for conversion to a percentage, multiplication by the factor 100/48 or 2·1 was required. The calculation was likewise applicable to more than one lesion in the cornea. Thus, for two lesions, the first of I, 2; II, 3 and the second of I, 4; II, 1, with a vascularity rating of 10, the score would be: $[(2 \times 3) + (4 \times 1) + 10] \times 2{\cdot}1$, or 42%.

Although photographs of eyes after treatment with test agents have been advanced by some workers for the rating of changes (Marzulli & Ruggles, 1973), such records are less meaningful than slit-lamp observations, especially when eyes display extreme chemosis. The following protocol summaries illustrate the derivation of scores for several corneal opacities:

M3-LE (Castille shampoo, SP-4; 15-day readings). Generalized severe opacification of the cornea occurred and was more dense centrally and below than peripherally. No details of the anterior chamber could be discerned. There was marked thickening in the dense area. Ratings: I, 8; II, 3·5; III, 6. *Score*: 71%.

M14-LE (2% Roccal; 15-day readings). Dense opacification of the entire cornea was noted. Centrally, this lesion was caused by corneal oedema and stromal thickening. Gross defects occurred in the epithelium in this area. A completely vascularized pannus extended all the way round to just outside the pupillary space. Calcium deposits were possibly present in the superficial layer of the central area. Ratings: I, 8; II, 3·5; III, 12. *Score*: 84%.

M16-RE (SP-2; 15-day readings). A very dense complete opacification was present, the centre of which was white and corresponded to epithelial and marked stromal oedema and perhaps to cellular infiltration. The periphery was so densely engorged with blood vessels that the individual constituents could not be discerned. The white area may have been due to perforation of the cornea. Ratings: I, 8; II, 4; III, 12. *Score*: 92%.

M25-RE (2% Isothan Q-15; water-rinsed series; 30-day readings). A phlectenule-like lesion was noted at '4 o'clock' carrying with it a vascular supply from the limbus. Superior to this lesion were small superficial scars with no blood vessel reactions or signs of activity. Ratings: I, 1; II, 3; III, 3. *Score*: 13%.

RESULTS AND DISCUSSION

Corneal findings for the four shampoos and four detergent solutions instilled into the eyes of the rabbit, guinea-pig, rat and mouse and for the shampoo formulations and 2% Roccal and 2% Isothan Q-15 tested in the hamster, dog and cat are summarized in Table 1. In these species at least ten eyes were used for each test agent, and in addition, groups of three monkey eyes were used for the screening of SP-1, SP-2, SP-3 and 2% Isothan Q-15 and six for SP-4, and four chicken eyes in each case for the tests on SP-1, SP-2, SP-3 and the Neutronyx 600–Isothan Q-15 mixture (Table 1).

The albino rabbit proved a very sensitive species for the evaluation of ophthalmic irritancy, its corneal lesions compared with those of the other larger species being generally more persistent and clearing with greater difficulty and only after protracted periods. In a few instances, corneal changes continued beyond month 6. The lesions in the other species were of shorter duration, several that were apparent on day 1 after treatment being absent by days 3–6. Corneal opacities engendered in the guinea-pig and rat by 2% Roccal or 2% Isothan Q-15, however, cleared only by day 25, and generally, corneal recovery was slower with the detergent solutions than with the shampoos.

Conjunctival activity of the various test agents appeared to parallel the corneal irritancy. Again, the rabbit displayed the highest sensitivity among the larger species examined. Changes generally persisted until days 11–17 after instillation of the shampoos into the rabbit eyes but were somewhat less involved with SP-2. With the detergent mixtures, clearing did not occur until well after days 11–15. Epilation was especially prominent in the rabbit eyes treated with 2% Roccal. With the shampoos, 2% Roccal and 2% Isothan Q-15, conjunctival recovery was complete within 48 hr in cats and, except with 2% Isothan Q-15, by 24 hr in the monkeys. With 2% Isothan Q-15, two of the three eyes were clear after 1 hr, but the remaining one, which also displayed a corneal opacity, recovered only by day 4. The dog conjunctiva was somewhat more sensitive to these agents, residual conjunctival irritancy persisting until 48–72 hr.

A decrease in the concentration of a severe ophthalmic irritant would be expected to lead to a lower degree of eye involvement. When six rabbit eyes were

instilled with 0·25% Roccal, five corneal lesions with a mean score of 20·4 ± 4·0% were observed during the first 3 days and were absent by day 6, while with the 2% mixture, lesions occurred in nine of a total of ten rabbit eyes (mean score, 62·1 ± 7·4%; Table 1) and continued beyond month 1. The conjunctival irritancy was also lower with the dilute than with the more concentrated solution, with complete clearing by day 11 in the former case.

The effect of rinsing the rabbit eyes with water was studied in conjunction with four test agents, SP-4 (five eyes, four lesions of score 36·1 ± 14·9%), 20% Neutronyx 600 (five eyes. four lesions of score 24·5 ± 8·8%), 2% Isothan Q-15 (four eyes, three lesions of score 11·0 ± 3·8%) and the Neutronyx–Isothan mixture (three eyes, two lesions of scores 12 and 19%). The conjunctiva cleared by days 4–5 except for one eye in each of the SP-4 and Neutronyx series in which irritation continued beyond day 7. Thus, the initial contact of the agent with the cornea, for however short a period, is critical even though it is followed by rinsing with water.

The vascularity of the corneal lesions was followed closely, especially in the larger species. the ratings of this parameter yielding valuable information on the activity, duration and general dissipation of opacity. The study was also aided by the serial sectioning of fixed eyes from the various species and examination of the anatomical changes engendered by the test for mutations.

On the basis of the findings with the shampoos (Table 1), the following order of decreasing corneal irritancy may be inferred: rabbit, hamster, mouse, rat, guinea-pig, dog, cat, monkey, chicken. It will be recalled, however, that for the last two species, fewer eyes were screened, and SP-4 was not investigated in the chicken. The scheme for the detergents was similar, but the mouse and hamster corneas appeared to display a higher irritancy than the rabbit eye, with the sensitivity of the guinea-pig and rat corneas being somewhat lower. In the remaining species the results, based on few test agents and only a small number of eyes, suggested that these were the least sensitive. A few opacities produced by a test product in these least sensitive animals would be indicative of a high order of corneal irritancy in the rabbit. The use of several species is therefore of value in pinpointing further the activity of test formulations, and in this connexion, animals such as the albino rat and Swiss mouse lend themselves as effective adjuncts to the rabbit in screening for corneal irritancy.

The albino rabbit has been used to delineate or classify eye irritants and, presumably, has been accorded a predictive quality with regard to human usage, but great caution must be exercised in extrapolating the rabbit-eye findings to human experience. The corneal data for the other animals have been deduced from the standpoint of interspecies comparisons and may not be very meaningful at present in the sense of their application to man. Moreover, irritancy to the rabbit eye might profitably be explored in relation to such factors as age, sex, diet and, especially, strain. Burton (1972) pointed out that the rabbit strain might figure prominently in the discrepancies observed in the interlaboratory evaluation of irritants by the Draize method. The use of pigmented rabbit and rodent eyes in corneal studies would be timely; preliminary data from this laboratory indicate that the corneas of pigmented mice, such as field mice, appear to be more resistant to some of the test agents than are those of the Swiss mice.

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SERUM ALKALINE PHOSPHATASE AND MICROSOMAL ENZYMES IN THE BEAGLE DOG

R. T. KEEFE, P. M. NEWBERNE and T. MYERS*

Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

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Abstract—The association between increased serum alkaline phosphatase activity and increased hepatic microsomal-enzyme activity in the beagle has been investigated. Low doses of a combination of ethanol (633 μl/kg/day), propylene glycol (672 μl/kg/day) and chloroform (30 μl/kg/day) raised alkaline phosphatase activities in both serum and liver without concurrently increasing three microsomal enzymes (amidopyrine *N*-demethylase, *p*-nitroanisole *O*-demethylase and benzo[*a*]pyrene hydroxylase). Studies with enzyme inhibitors suggested that the increases in hepatic and serum alkaline-phosphatase levels were predominantly due to enzyme of the hepatic type.

INTRODUCTION

The activity of serum alkaline phosphatase (EC 3.1.3.1; SAP) in the beagle is readily increased by a number of chemicals (Hoffmann & Dorner, 1975). This elevation may occur with a concomitant increase in hepatic microsomal drug-metabolizing enzymes and proliferation of the smooth endoplasmic reticulum (SER). Conning & Litchfield (1971) reported that phenobarbitone administration caused increases in SAP and microsomal-enzyme activities as well as SER proliferation. Furthermore, increases in these parameters could be diminished by inhibiting protein synthesis (Litchfield & Conning, 1972). El-Aaser, Reid & Stevenson (1972) demonstrated that dieldrin and phenobarbitone increased SAP activity and caused liver hypertrophy; Dorner, Hoffmann & Long (1974) reported that prednisolone, a corticosteroid, enhanced SAP and hepatic alkaline phosphatase (HAP) activities. Tettenborn, Luckhaus & Voigt (1973) showed increased levels of SAP and HAP with phenobarbitone, phenylbutazone or clotrimazole treatments, which also caused proliferation of hepatic SER. Leeling, Hartnagel, Bare, Fonesca, Kraus & Kowalski (1975) observed increases in SAP, HAP and cytochrome *P*-450 in beagles treated with a benzoquinoliznyl derivative (TR2379).

Since increased SAP activity in the beagle is considered by some to be indicative of a pathological condition, it was necessary to eliminate cholestasis from the differential diagnosis by monitoring bilirubin levels, bromsulphthalein retention and other indices of liver function. Although cholestatic conditions greatly increase SAP (Kaplan & Righetti, 1970; Righetti & Kaplan, 1971 & 1972), hepatotoxic agents disturb other parameters of liver function well before they affect SAP (Litchfield & Garland, 1972 & 1974). Bone, kidney and intestinal pathology must also be ruled out.

When a mixture containing a number of chemicals capable of inducing microsomal activity increased SAP activity in beagles without increasing serum glutamic–pyruvic or glutamic–oxalacetic transaminase, we were prompted to investigate both the capacity of the individual components to elicit this type of response at the low levels used and the source of the increase in SAP. Obviously, the effects of drugs administered in such a mixture could be potentiated by the activity of the vehicle itself, as shown by Gillette & Jollow (1974), for example.

EXPERIMENTAL

The animals used were all pure-bred beagles supplied by Ridgelan Farms, Michigan. Four young adult females (body weight 8·5–11·5 kg) were treated orally, twice daily on 5 days/wk, with a dose equivalent to 3 ml/kg of a solution containing medium invert syrup (80%, w/v), sodium benzoate (0·1%, w/v), propylene glycol (11·2%, v/v), ethanol (10·55%, v/v) and chloroform (0·50%, v/v). Thus each animal received daily 633 μl ethanol/kg body weight, 672 μl propylene glycol/kg and 30 μl chloroform/kg. SAP activity was determined weekly (Sigma Chemical Company, 1971) in these and in four untreated control beagles. After treatment for 3 wk, a treated and a control animal were killed by exsanguination, and their livers were quickly removed for determination of microsomal-enzyme activity and other parameters, as well as for histopathology. The six remaining dogs were biopsied by laparotomy. Amidopyrine *N*-demethylase, benzo[*a*]pyrene hydroxylase (McLean & McLean, 1966), *p*-nitroanisole *O*-demethylase (Kinoshita, Frawley & DuBois, 1966), alkaline-phosphatase activity alone and in the presence of 5 mM-L-homoarginine or L-phenylalanine (Ghosh & Fishman, 1966; Lin & Fishman, 1972), protein (Lowry, Rosebrough, Farr & Randall, 1951) and DNA and RNA (Prasad, DuMouchelle, Koniuch & Oberleas, 1972) were determined, and samples of liver tissues were prepared for microscopic study. Alkaline phosphatase inhibitors were used to assess the intestinal contribution to the increase in SAP (Marcuson & Tomlinson, 1972). Results are reported as the means ± SEM.

*Present address: Director of Pharmacology and Toxicology, Vick Chemical Company, 1 Bradford Road, Mount Vernon, NY 10553, USA.

RESULTS AND DISCUSSION

SAP activities in the four treated dogs increased at least threefold within 3 wk. The degree of SAP inhibition by L-phenylalanine (22·3 ± 4·9%) and L-homoarginine (65·1 ± 15·5%) was not significantly different from that in the four controls (18·5 ± 1·3% and 63·9 ± 8·8%, respectively).

The HAP activity was more than three times higher in the treated group than in the control group. Inhibition by L-phenylalanine was 19·8 ± 11·3% in the treated, and 25·8 ± 5·8% in the control groups, whereas inhibition by L-homoarginine was 60·2 ± 38·4% and 57·8 ± 24·8% in the treated and control groups, respectively.

Table 1 shows the results of the microsomal-enzyme estimations. Treatment caused a decrease in *p*-nitroanisole *O*-demethylase but no changes in the other two enzymes. The relative concentrations of protein and RNA did not differ between treated and control groups, and histopathology revealed no evidence of cholestasis or hepatic necrosis. No bone, kidney or intestinal abnormalities appeared in the autopsy specimens.

Both HAP and SAP activities were increased in the test beagles, suggesting that the increased SAP activity originated in the liver, a suggestion supported by the results of other workers, mentioned above, who used other chemicals to raise SAP. Our results show a constant inhibition by both L-homoarginine, an inhibitor of non-intestinal alkaline phosphatase and L-phenylalanine, an inhibitor of intestinal alkaline phosphatase.

The hepatic enzyme is the predominant form of SAP activity in control animals (Nagode, Koestner & Steinmeyer, 1969; our own unpublished data). Thus the similar inhibition patterns seen in treated animals and in the controls and the similarity between hepatic and serum inhibition patterns suggest that the hepatic enzyme was responsible for the increase in SAP. The investigation is being continued to elucidate the mechanism of this rise.

Unlike other investigators, we were unable to show an increase in any of three hepatic microsomal enzymes. Litchfield & Conning (1972), for example, reported a tenfold increase in amidopyrine *N*-demethylase in two male beagles treated with phenobarbitone. Our results suggest that *N*-demethylated or hydroxylated drugs will be metabolized in the same manner as they would be with an inert vehicle, although other enzymes not evaluated in this study could be altered by our vehicle. The more generalized effects of the vehicle on the microsomal system are currently being investigated. Our results so far imply that SAP activity can be raised in the beagle without producing evidence of pathology.

In summary, we have shown that a mixture of low levels of commonly ingested chemicals that are reported to increase microsomal enzymes will stimulate SAP and HAP in beagles without concurrently raising amidopyrine *N*-demethylase, *p*-nitroanisole *O*-demethylase or benzo[*a*]pyrene hydroxylase. Our evidence suggests a non-intestinal (probably liver) source of the increased SAP activity. However, studies in progress indicate that the increase in SAP may originate in the small intestine, with subsequent conversion of the enzyme to the hepatic type and its appearance in the serum as such.

Table. 1. *Selected hepatic microsomal-enzyme activities in beagles treated with ethanol, propylene glycol and chloroform and in untreated controls*

Enzyme	Level of enzyme activity*	
	Treated	Control
APDM	4·90 ± 2·09 (3)	3·81 ± 1·67 (3)
PNA	7·95 ± 0·64 (4)	13·72 ± 2·40 (4)
BPOH	1·00 ± 0·12 (4)	1·18 ± 0·19 (4)

APDM = Amidopyrine *N*-demethylase
PNA = *p*-Nitroanisole *O*-demethylase
BPOH = Benzo[*a*]pyrene hydroxylase
*Values are means ± SEM for the numbers of dogs indicated in parentheses, and are expressed in μg aminoantipyrine/hr/mg protein × 10^2 for APDM, in μg *p*-nitrophenol/hr/mg protein × 10 for PNA and in quinine units/mg protein × 10 for BPOH.

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SHORT PAPERS

INHIBITORY EFFECT OF BUTYLATED HYDROXYTOLUENE (BHT) ON INTESTINAL CARCINOGENESIS IN RATS BY AZOXYMETHANE

E. K. WEISBURGER and R. P. EVARTS
Carcinogen Metabolism and Toxicology Branch, National Cancer Institute, Bethesda, Maryland 20014,
and
M. L. WENK
Microbiological Associates, Inc., Bethesda, Maryland 20016, USA

(*Received 3 September 1976*)

Summary—Administration of BHT in the diet to rats concurrently receiving a weekly sc injection of the carcinogen azoxymethane inhibited the development of intestinal tumours. When BHT was fed after a course of treatment with azoxymethane, the number of animals with tumours was not affected but the number of tumours/animal increased slightly.

Introduction

It has been reported that antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have an inhibitory effect on the action of certain chemical carcinogens, including some aromatic amine derivatives (Ulland, Weisburger, Yamamoto & Weisburger, 1973) and polycyclic aromatic hydrocarbons (Wattenberg, 1972 & 1973). The underlying mechanism in most cases appears to be the alteration of the microsomal systems that metabolize the carcinogen, leading to decreased binding of the carcinogen or its metabolites to DNA (Goodman, Trosko & Yager, 1976; Grantham, Weisburger & Weisburger, 1973; Speier & Wattenberg, 1975).

The organ sites affected by the carcinogens mentioned are mainly the liver, breast, forestomach and lung. It was decided to study the effect of BHT on the carcinogenicity of azoxymethane (AOM), a compound which leads primarily to intestinal and secondarily to ear-duct tumours in rats (Ward, Yamamoto, Benjamin, Brown & Weisburger, 1974).

Experimental

Male F344 rats aged 4 wk obtained from the Frederick Cancer Research Center (Maryland) were housed in groups of three in polycarbonate cages (13 × 12 × 6·5 in.) with corncob bedding, maintained at 22 ± 1°C and subjected to a 12-hr light–dark cycle. The basal diet of Wayne laboratory meal and water were available freely.

Azoxymethane (AOM; Ash-Stevens, Inc., Michigan) was injected sc in one weekly dose of 7·4 mg/kg in physiological saline solution for 10 wk (Ward *et al.* 1974). BHT (Gallard-Schlesinger Chemical Manufacturers, New York) at a level of 6600 ppm was mixed with the basal laboratory meal in a Patterson-Kelley V blender. Diets were prepared weekly and stored in a cold room (5°C) until used.

The schedule for administration of the BHT-containing diet and AOM and the number of animals in each group are given in Table 1. Starting at 6 wk of age, group I received AOM, group II, which had been given BHT in the diet for 2 wk before AOM administration began, then received BHT and AOM concurrently, and group III had AOM for 10 wk followed by BHT in the diet for 12 wk. Group IV had BHT in the diet and group V served as controls. In Table 1, wk 0 denotes when AOM administration began (6 wk of age) or the equivalent life time in the other groups. The experiments were terminated at wk 40.

Animals were weighed weekly and examined carefully for signs of gastro-intestinal tumours. Moribund animals or those showing signs of tumours were killed. A careful necropsy was performed on each animal including opening the gastro-intestinal tract and examination for tumours. The ear ducts were also routinely examined. All gross lesions noted and liver, kidneys, colon and duodenum were fixed in 10% buffered formalin. Tissues were sectioned and stained with haematoxylin and eosin. Diagnoses were based on the criteria outlined by Ward (1974). For statistical analysis of data, chi-square and Student's *t*-test were used.

Results

The control group (V) had 100% survival throughout the 40-wk experimental period (Table 1). Groups II and IV, receiving BHT with or without AOM, had up to 12% dead by wk 20 but, since one of these

Table 1. *Administration schedule, body weights and survival of rats fed AOM and/or BHT*

Group	AOM administered on wk	BHT administered on wk	Body weight and survival at wk				
			0	10	20	30	40
I	0–9	—	129 ± 12 (100)	306 ± 17* (92)	392 ± 17*** (92)	428 ± 30** (88)	442 ± 30* (44)
II	0–9	0†–9	125 ± 11 (100)	276 ± 23 (96)	381 ± 20 (94)	430 ± 27 (92)	457 ± 30 (72)
III	0–9	9–20	133 ± 12 (100)	306 ± 23* (100)	370 ± 33* (88)	421 ± 46*** (84)	454 ± 52 (36)
IV	—	0†–9	117 ± 13* (100)	288 ± 26* (96)	387 ± 24*** (88)	447 ± 27 (88)	471 ± 30 (88)
V	—	—	130 ± 13 (100)	332 ± 24 (100)	408 ± 26 (100)	452 ± 28 (100)	475 ± 32 (100)

† Administration began 2 wk prior to wk 0.
Values represent means ± SD for the number of surviving animals (percentage shown in parenthesis), and those marked with asterisks differ significantly from the control values: $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

Table 2. *Distribution of tumours in rats fed AOM and/or BHT*

Group	No. of rats	No. of rats with tumours	Numbers of tumours in									
			Gastro-intestinal canal						Ear duct			
			Mucinous adeno carcinoma	Papillary adeno carcinoma	Adeno carcinoma	Adenoma	Total	No. of gastro-intestinal tumours/tumour-bearing animal	Squamous-cell carcinoma	Acinar sebaceous carcinoma	Fibro-sarcoma	Total
I	23	20 (87%)	10	13	2	3	28	1·4	10	2	0	12 (52%)
II	50	29 (58%)*	15	11	2	1	29	1·0	11	0	1	12 (24%)*
III	25	24 (96%)	18	17	5	0	40	1·7	10	1	0	11 (44%)
IV	25	2 (8%)	0	0	2	0	2	1·0	0	0	0	0 (0%)
V	25	0 (0%)	0	0	0	0	0	0	0	0	0	0 (0%)

The values marked with an asterisk differ significantly ($P < 0{\cdot}05$) from the corresponding values for group I.

groups received BHT only, the result was probably due to the high level of BHT. After wk 30 most of the animals that died, or were killed because of weight loss or anal bleeding, had gastro-intestinal tumours.

Although the food consumption was comparable in all groups, the AOM-treated groups showed somewhat decreased body-weight gain compared with controls. Later losses in body weight were probably due to tumour formation. BHT treatment alone also led to some depression in body weight, but the animals recovered when returned to the control diet.

Tumour incidence in the various groups (Table 2) showed that, as expected, AOM led to a high tumour incidence. Concurrent treatment with BHT appeared to lower the yield of both intestinal and ear-duct tumours. On the other hand, administration of BHT after the course of AOM treatment did not affect the incidence of animals with intestinal tumours but tended to increase the average number of tumours/animal and the distribution in the tract.

Discussion

In this study we used the same level of BHT as in a previous study with Charles River CD rats, a Sprague–Dawley-derived type. In retrospect, this level was somewhat toxic to the F344 rats and it might have been advisable to use a slightly lower dietary level, as the greater tolerance of Sprague–Dawley-derived rats to toxic levels of various xenobiotic compounds has been demonstrated (Reuber & Glover, 1970; Weisburger, Ulland, Schueler, Weisburger & Harris, 1975). The BHT had an inhibitory effect on tumour formation similar to its effect when given with some other carcinogens. In addition, in line with the action of phenobarbitone, which is also an inhibitor of certain carcinogens, treatment with the inhibitor after the carcinogen enhanced the incidence of tumours (Peraino, Fry & Staffeldt, 1971; Peraino, Fry, Staffeldt & Kisieleski, 1973; Weisburger, Madison, Ward, Viguera & Weisburger, 1975).

The mechanism of the inhibitory action of BHT on AOM carcinogenesis has not been elucidated. Previous studies indicated that BHT initiated a change in the excretion pattern and a decrease in the binding of the carcinogen or a metabolite to DNA (Grantham *et al.* 1973). More detailed work has shown that some changes in *P*-450 levels result from feeding the related antioxidant BHA (Speier & Wattenberg, 1975). BHT is an inducer of enzymes and by itself leads to liver enlargement due to the increase in enzyme synthesis (Gilbert, Martin, Gangolli, Abraham & Golberg, 1969; Scully, Bray & Nievel, 1976). However, BHT did not affect DNA repair in Chinese hamster cells *in vitro*, an indication that it acts at the anti-initiation phase (Goodman *et al.* 1976). Of all antioxidants used, BHT was also the most efficient in reducing carcinogen-induced chromosomal breaks in blood leucocyte cultures (Shamberger, Baughman, Kalchert, Willis & Hoffman, 1973).

Further metabolic work should be performed with labelled AOM in order to define the means by which BHT exerts its protective effect.

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NITRATE, NITRITE, DIMETHYLNITROSAMINE AND *N*-NITROSOPYRROLIDINE IN SOME CHINESE FOOD PRODUCTS

Y. Y. FONG and W. C. CHAN

Departments of Biochemistry and Pathology, University of Hong Kong, Hong Kong

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Summary—Over 60 samples of commonly consumed Chinese food products, including dried shrimps, shrimp sauce and paste, oyster sauce, fish sauce, Chinese sausages and dried squid, were analysed for the presence of dimethylnitrosamine and *N*-nitrosopyrrolidine. Low levels of dimethylnitrosamine (1–15 ppb*) and *N*-nitrosopyrrolidine (2–37 ppb) were detected in such foods and were confirmed in several samples by gas chromatography–mass spectrometry. No correlation was found between the amounts of residual nitrate or nitrite present and the levels of nitrosamines found in the food.

Introduction

Dimethylnitrosamine (DMNA) was first reported to be toxic and carcinogenic in animal species by Barnes & Magee in 1954, but a long time elapsed before it was identified in some foods intended for human consumption (Ender, Havre, Helgebostad, Koppang, Madsen & Ceh, 1964; Sakshaug, Sognen, Hansen & Koppang, 1965). Since then numerous reports have appeared on the occurrence of *N*-nitrosamines in foods, mostly cured meat products and smoked fish (Fazio, Damico, Howard, White & Watts, 1971; Sen, 1972; Sen, Donaldson, Iyengar & Panalaks, 1973; Wasserman, Fiddler, Doerr, Osman & Dooley, 1972). We have reported the presence of DMNA in Chinese marine salt fish and have attempted to explain the mechanism of its formation therein (Fong & Chan, 1973a,b & 1976). We thought, therefore, that a study of other commonly consumed Chinese food products might be worthwhile. The present communication reports the detection and confirmation of DMNA and *N*-nitrosopyrrolidine (NPyr) in 61 samples of commonly consumed Chinese foods and the determination of the levels of nitrate and nitrite present.

Experimental

Materials. The samples tested were traditional Chinese foods and sauces and were purchased from local stores. They included dried shrimps (13 samples), shrimp sauce (eight samples), shrimp paste (six samples), fish sauce (ten samples), oyster sauce (eight samples), Chinese sausage (ten samples) and dried squid (six samples). In each experiment, the weight of sample used was 300 g. Nitrosamine extraction was carried out according to Sen, Smith, Schwinghamer & Howsam (1970). Briefly, the sample (if solid) was chopped into small pieces and extracted with methylene chloride at alkaline pH using a blender. After removal of the methylene chloride by evaporation, the nitrosamines were isolated by stream distillation. The distillate was passed through a combined ion-exchange polyamide column and the combined eluate was extracted three times with methylene chloride, dried over anhydrous sodium sulphate and concentrated to 100–300 μl using a Kuderna Danish evaporator.

Analytical procedures. Quantitative measurements for DMNA and NPyr were carried out with a Varian Aerograph 1864-4 gas chromatograph, using a 10 ft × 1/8 in. stainless-steel column packed with 10% Carbowax 20 M on high-performance grade Chromosorb W/AW-DMCS (80–100 mesh), with a nitrogen flow of 25 ml/min and column, injection and detector temperatures of 95, 150 and 190°C respectively (range 10, attenuation 1). The presence of DMNA and NPyr was confirmed by combined gas chromatography–mass spectrometry (GC–MS) using the same column and gas chromatograph interfaced with a Hitachi Perkin–Elmer RMS-4 mass spectrometer. Helium carrier gas was used at a flow rate of 25 ml/min and hydrogen- and air-flow conditions were 40 and 350 ml/min, respectively. The column eluate was split approximately 1:9, 90% passing into the mass spectrometer via an inlet line heated at 200°C. The mass spectrum was obtained at an ionizing voltage of 70 eV.

Nitrate and nitrite determinations. Nitrate and nitrite determinations were carried out on all the samples studied. The method used was that of Follett & Ratcliff (1963).

Results

Recovery studies were carried out prior to the survey and recoveries of DMNA and NPyr added to 100-g samples at levels of 10 and 20 ppb were in

*b = 10^9 throughout this paper.

Table 1. *Nitrate, nitrite, DMNA and NPyr levels in various Chinese food products*

Sample type and no.	Levels of			
	Nitrate (ppm*)	Nitrite (ppm*)	DMNA (ppb†)	NPyr (ppb†)
Dried shrimps				
1	15	13	N	30
2	16	11	4	24‡
3	17	20	4	37‡
4	10	12	2	N
5	9	34	5	25
6	16	13	N	20‡
7	26	7	7	11
8	8	8	10‡	13
9	3	5	N	17‡
10	11	9	N	N
11	15	6	N	10
12	23	12	6	10
13	N	12	N	N
Shrimp sauce				
1	32	8	2	9
2	32	10	10	7
3	64	7	8‡	2
4	29	8	N	10‡
5	49	7	N	N
6	9	10	N	N
7	20	5	2	2
8	15	11	N	N
Shrimp paste				
1	16	12	N	N
2	15	7	11‡	15‡
3	15	12	6	3
4	12	6	N	N
5	10	5	N	N
6	8	6	N	N
Fish sauce				
1	14	N	1	N
2	10	N	N	N
3	10	N	N	N
4	15	N	2	N
5	9	N	N	N
6	26	N	N	N
7	15	N	1	2
8	12	N	N	N
9	15	N	N	N
10	12	N	N	2
Oyster sauce				
1	79	23	N	N
2	85	28	2	N
3	38	27	N	N
4	67	20	N	N
5	35	20	N	N
6	34	34	N	N
7	21	20	N	N
8	62	32	N	N
Chinese sausage				
1	12	4	N	N
2	8	N	3	N
3	15	4	6	N
4	14	4	N	N
5	9	N	6	N
6	2	5	15‡	N
7	25	4	12‡	N
8	10	5	10	N
9	12	4	N	N
10	20	3	N	N
Dried squid				
1	14	10	6	5
2	3	7	8‡	7
3	5	8	N	3
4	4	10	N	N
5	6	8	5	2
6	15	8	2	N

DMNA = Dimethylnitrosamine
NPyr = *N*-Nitrosopyrrolidine
N = Not detected§

*Results expressed as the potassium salt.
†Nitrosamine levels are not corrected for percentage recovery.
‡Identity confirmed by GC–MS.
§Detection limit for nitrosamines was 1 ppb and for nitrate and nitrite 1 ppm.

the ranges of 70–80 and 60–70%, respectively. Table 1 summarizes the results of the study. The average value for nitrate was 20·2 ppm (range 0–85 ppm) and for nitrite 9·2 ppm (0–34 ppm). Of 61 samples analysed, 27 contained detectable amounts of DMNA (1–15 ppb) and 23 contained detectable amounts of NPyr (2–37 ppb). The identity of six samples of DMNA and six of NPyr was confirmed by the GC–MS technique, with isolated DMNA producing strong *m/e* peaks at M^+ (74) and NO^+ (30) and isolated NPyr producing peaks at M^+ (100) and NO^+ (30) as well.

Discussion

The levels of DMNA (1–15 ppb) found in food samples analysed in this study were significantly lower than the levels of 50–300 ppb found in our previous study of Chinese marine salt fish (Fong & Chan, 1973a). There are several possible reasons for this. Many of the food products included in the present study were sauces in fluid or semi-fluid form and therefore the concentration of nitrosatable amines was low. In a previous report (Fong & Chan, 1976), we demonstrated that the amount of nitrosamine produced correlated with the amount of nitrosatable amines present in the food itself. Moreover, it is common practice for the local manufacturer to add benzoate to these sauces to inhibit microbial growth, and this again would cut down the chances of nitrosamine formation (Fong & Chan, 1976). On the other hand, solid food such as dried shrimp and dried squid provide a less favourable environment for bacterial growth than do salt fish, as they can be dried more efficiently. Most of them lose about 70–80% of their original weight during the drying process, compared with about 40–50% in the case of salt fish (McCarthy & Tausz, 1952). It is difficult, therefore, to compare the concentration of nitrosamines present in one sample of food with that in another.

Comparison of the data in Table 1 suggests that there is no correlation between the levels of nitrosamines and those of nitrate and nitrite. For example, relatively high levels of nitrate (21–85 ppm) and nitrite (20–34 ppm) were found in the eight samples of oyster sauce studied, yet only one sample contained detectable DMNA and then only at a level of 2 ppb. These findings agree well with those of Panalaks, Iyengar & Sen (1973), who found no correlation between the levels of DMNA and those of nitrate and nitrite in commercially processed samples of cured meat products. However, under controlled laboratory conditions we found that DMNA formation in salt fish did in fact increase when the amount of nitrate or nitrite used in the pickling agent was increased (Fong & Chan, 1976).

The results of this study demonstrate that low levels of DMNA and NPyr may be present in some Chinese food products. It is difficult to assess the biological implications of the consumption of trace amounts of nitrosamines by man. However, nitrosamines are potent carcinogens, capable of inducing tumours in a wide range of animal species (Magee and Barnes, 1967) at levels as low as 2 ppm (Terracini, Magee & Barnes, 1967). It is safer, therefore, to adopt the view that no dose of a carcinogen, however small, can be regarded as completely safe.

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EFFECT OF VARIOUS DIETARY COMPONENTS ON ABSORPTION AND TISSUE DISTRIBUTION OF ORALLY ADMINISTERED INORGANIC TIN IN RATS

P. FRITSCH, G. DE SAINT BLANQUAT and R. DERACHE

Groupe de Recherches sur la Toxicologie des Aliments et des Boissons, INSERM, U87, Université Paul Sabatier, Institut de Physiologie, 2 rue François Magendie, 31400—Toulouse, France

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Summary—The absorption of tin from the gastro-intestinal tract and its distribution in the tissues was studied in groups of rats given ^{113}Sn-labelled stannous chloride by gastric intubation together with various other food components, namely sucrose, ascorbic acid and potassium nitrate, given with the tin salt either separately or all together, ethanol (given as a 20% solution), a solution of albumin and an emulsion of sunflower oil and 1% Tween 20. In all groups, 90–99% of the administered radioactivity was excreted in the faeces within 48 hr, at which time faecal excretion and retention in the alimentary tract accounted for 98·7–99·9% of the dose. Only traces of ^{113}Sn were detected in the wide range of organs and tissues examined, irrespective of the other food components administered with the tin salt.

Introduction

Tin is found in various human tissues, particularly the skeleton, muscle, liver, heart, spleen and kidneys (Schroeder, Balassa & Tipton, 1964; Widdowson & Dickerson, 1964) and may be an essential element for man (Nielsen & Sandstead, 1974; Schwarz, 1974).

More interest, however, has been attached to the occurrence of tin in comparatively high concentrations (10–1000 ppm) in certain canned foods, including canned fruit juices and vegetables (Horio, Iwamoto & Komura, 1970). The use of can lacquers completely free of tin has been shown experimentally to be likely to cause problems in connexion with the colour and flavour of canned food. The colour of vegetables such as asparagus, French beans and carrots has been shown to darken (Biston & Guerrier, 1973), and Van Buren & Downing (1969) have suggested that the presence of about 20 ppm tin is in fact essential in certain types of canned product. However, the extent to which tin may dissolve from the can-lining during storage may increase in the presence of certain food constituents, notably nitrates (Farrow, 1970), oxygen (Horio, Iwamoto & Miyazaki, 1972) and amino acids, particularly cystine and cysteine (Gruenwedel & Patnaik, 1973), and as a result the concentrations of tin in canned foods may rise above the permitted level, which is currently set at 250 ppm in many countries, pending further consideration by the Joint FAO/WHO Expert Committee on Food Additives (1975).

Several outbreaks of nausea, vomiting and diarrhoea have been ascribed to tin poisoning (Benoy, Hooper & Schneider, 1971) and similar clinical signs have been observed in cats, dogs and monkeys given high doses of tin experimentally (Cheftel & Truffert, 1971). The latter authors reported the oral LD_{50} for stannous chloride in rats to be 700 mg/kg. Since many investigators have emphasized the high level of faecal excretion of tin derived from orally administered tin salts (Benoy *et al.* 1971; Hiles, 1974), it seemed appropriate to study the possible effects of various other food components on the gastro-intestinal absorption and subsequent systemic distribution of tin. While some earlier studies dealt with a range of tin compounds, including the orthophosphate, oxalate, sulphate, oleate, tartrate and oxides (de Groot, Feron & Til, 1973) or fluoride, citrate and pyrophosphate (Hiles, 1974), we concentrated on stannous chloride, because of its particular importance as a food contaminant.

Experimental

Male Wistar rats, mean body weight 250 g, were divided into eight groups each of eight rats and housed in glass metabolism cages. They were deprived of food for 17 hr prior to intragastric administration of one of eight test mixtures containing a 50-mg/kg dose of stannous chloride labelled with radioactive tin-113 (half-life 122 days; 0·5 μCi/mg tin) either in aqueous solution, alone (group 1) or together with sucrose (5 g/kg body weight; group 2), ascorbic acid (0·5 g/kg; group 3) or potassium nitrate (0·1 g/kg; group 4) or a mixture of all three of these compounds in the same doses (group 5), or in 20% alcoholic solution (2 g ethanol/kg; group 6), in a solution of albumin (2·5 g/kg; group 7) or in a 1:1 (v/v) sunflower

Table 1. *Recovery of tin from gut and excreta of rats following administration of* ^{113}*Sn-labelled stannous chloride with various dietary constituents by gastric intubation*

Sample	Recovery of tin (% of administered radioactivity*) in group†							
	1	2	3	4	5	6	7	8
Alimentary tract								
Stomach	0·27 ± 0·028	0·35 ± 0·023	1·40 ± 0·492	0·36 ± 0·099	0·78 ± 0·326	1·79 ± 0·617	0·007 ± 0·0003	2·79 ± 0·149
Duodenum	0·07 ± 0·006	0·05 ± 0·017	0·28 ± 0·102	0·15 ± 0·127	0·13 ± 0·049	0·52 ± 0·406	0·002 ± 0·0006	0·39 ± 0·290
Ileum	0·26 ± 0·138	0·58 ± 0·195	1·26 ± 0·207	0·24 ± 0·139	1·68 ± 0·663	2·52 ± 0·383	0·003 ± 0·0001	1·88 ± 0·962
Caecum	0·61 ± 0·097	2·28 ± 0·899	2·75 ± 0·311	0·58 ± 0·141	3·08 ± 0·861	2·97 ± 0·713	0·002 ± 0·0005	2·47 ± 1·008
Colon	2·36 ± 0·901	1·77 ± 0·602	1·44 ± 0·383	0·33 ± 0·040	1·82 ± 0·473	2·79 ± 0·817	0·010 ± 0·0003	1·88 ± 0·112
Whole tract	3·57	5·03	7·13	1·66	7·49	10·59	0·024	9·41
Faeces								
0–24 hr	81·61 ± 6·677	45·31 ± 9·012	32·19 ± 3·797	49·69 ± 6·702	41·63 ± 4·307	37·58 ± 2·324	90·40 ± 3·788	35·89 ± 0·761
24–48 hr	13·59 ± 4·156	48·94 ± 8·221	60·44 ± 3·997	48·54 ± 6·683	49·56 ± 2·905	51·69 ± 5·021	9·51 ± 3·797	54·65 ± 4·777
Total	95·20	94·25	92·63	98·23	91·19	89·27	99·91	90·54
Faeces + gut	98·77	99·28	99·76	99·89	98·68	99·86	99·93	99·95
Urine								
0–24 hr	0·07 ± 0·170	0·05 ± 0·026	0·09 ± 0·006	0·03 ± 0·005	0·39 ± 0·024	0·04 ± 0·022	0·04 ± 0·009	0·01 ± 0·006
24–48 hr	0·13 ± 0·057	0·64 ± 0·310	0·12 ± 0·041	0·06 ± 0·021	0·71 ± 0·203	0·10 ± 0·032	0·15 ± 0·098	0·01 ± 0·005
Total	0·20	0·69	0·21	0·09	1·10	0·14	0·19	0·02

*Figures are means or means ± SEM for groups of eight rats.

†Treatments: groups received 50 mg stannous chloride/kg labelled with ^{113}Sn either in aqueous solution, alone (group 1), with sucrose (2), with ascorbic acid (3), with potassium nitrate (4) or with sucrose, ascorbic acid and potassium nitrate (5), or in alcoholic solution (6), albumin solution (7) or sunflower oil–Tween 20 emulsion (8).

oil–1% Tween 20 emulsion (10 ml/kg; group 8). In each case the volume of material intubated was approximately 15 ml/kg body weight.

After intubation, the rats were fasted for a further 6 hr and were then given standard laboratory diet (UAR AO4 Food, Villemoisson-sur-Orge) and water *ad lib.* Urine and faeces were collected separately over the periods 0–24 and 24–48 hr after treatment and at 48 hr the rats were killed by ether anaesthesia and exsanguination by carotid artery catheterization. The gastro-intestinal tract, spleen, kidneys, liver, heart, lungs, gonads, epididymal fat, abdominal muscle, sternum, femur and brain were removed for radioactivity determinations. Total radioactivity of excreta or of whole organs or tissues (Hiles, 1974) was determined using a CG 30 Intertechnique gamma counter, and results were expressed as a percentage of administered radioactivity.

Results and Discussion

Table 1 shows the proportion of the dose of orally administered tin recovered after 48 hr from the various parts of the alimentary tract and their contents in the various groups of rats. The amounts of tin retained in the gut at this stage were generally low, the highest amounts (in total about 10% of the dose) being found in the rats given stannous chloride in alcoholic solution (group 6) or in the oil–Tween 20 emulsion (group 8), and the lowest (about 0.02%) in those given the tin salt in albumin solution (group 7). These figures are reflected in the rate of faecal excretion (Table 1), with the cumulative recovery in the faeces at 48 hr ranging from 90% in group 8 to over 99% in group 7. Thus the total recovery from the alimentary tract and faeces at 48 hr was between 98·7 and 99·9% in all the groups. These findings were comparable with those in previously reported studies (Hiles, 1974; Kutzner & Brod, 1971 & 1974).

The very low levels of radioactivity detected in the 48-hr urine collections (Table 1) were therefore to be expected. Even so, it is possible that the urinary values, ranging from 0·02% in group 8 to 1·10% in group 5 (given stannous chloride with sucrose, ascorbic acid and potassium nitrate), resulted from faecal contamination of the urine. The traces of radioactivity detected in the various tissues, which were selected for study in the light of a report by Yano, Chu & Anger (1973) were equivalent in all cases to less than 0·005% of the dose.

In spite of the apparently negligible absorption of tin in the gastro-intestinal tract, further studies are required to investigate the possibility that the long-term consumption of foods containing appreciable amounts of tin may have nutritional and toxicological effects that could impair the efficiency of the gastro-intestinal mucosa. We intend to start such studies shortly.

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Review Section

BOOK REVIEWS

Experiments and Research with Humans: Values in Conflict. Academy Forum, Third of a Series (Editor B. S. Turvene). National Academy of Sciences, Washington, D.C., 1975. pp. vi + 234. $5.00.

To those who seek a quick and ready answer to the question of when human experimentation may be justified, or to the somewhat different question of when it is legal, this volume will be of little interest.

The book contains an almost verbatim report of the third of the meetings organized by the National Academy of Sciences, under the title 'Academy Forum', to provide an opportunity for subjects of great interest and concern to be debated in public. This particular debate was clearly on a high level, and covered a wide spectrum of situations in which scientific observations on man have been made, including the most controversial ones, such as the use of living tissues from viable and non-viable foetuses.

Naturally, the proceedings were heavily loaded with examples of human experiments drawn from the field of medicine in which, indeed, the diagnosis and treatment of every individual patient constitutes a human experiment. In the realm of drugs and vaccines, there is no alternative to human observation. The Forum participants examined the principle of consent and the special difficulties that arise when consent is not possible owing to the age or medical condition of the experimental subject.

As the President of the NAS commented in his closing remarks, there are dark aspects in the use of man as a guinea-pig. These range from the ferocious brutality often seen in war to experiments in which the risks involved obviously far outweigh any conceivable benefit. Those involved in the debate did not in any way shirk such issues, and the whole of this volume is characterized by its frankness of expression.

One possible point of criticism is that only a sprinkling of 'non-biomedical' individuals participated. The few speakers from the fields of law, philosophy and divinity and those from organizations dealing with 'citizens' rights' presented their views well, but their comments were rather diluted by the weight of medical opinion. Perhaps, in such a debate, this was inevitable, as it is the medical man who most frequently comes face to face with the problems that were under discussion.

This volume can be highly recommended to any readers interested in human values, the methods of preserving them, the risks and sacrifices needed to establish them in practice, and the difficulties posed by questions such as 'is the risk to the few justified in terms of the potential benefit to the many?' Obtainable from the publishing office of the NAS for a mere 5 dollars, this is a document offering excellent value for money.

Neoplastic Development. Vol. 2. By L. Foulds. Academic Press, London, 1975. pp. xiv + 729. £16.80.

The author of this book has devoted most of his professional life to the study of the tissue changes that precede malignancy. A pathologist by trade, he was limited to microscopic observations, but his inventiveness and resourcefulness enabled him to gather data that have provided a valuable insight into the nature of the lesions leading to the emergence of uncontrolled growth.

The basic message of this book, as of the previous volume reviewed some years ago (*Cited in F.C.T.* 1972, **10**, 231), is that cancer is often, but not invariably, preceded by a series of histological changes in the affected or 'target' tissues. The author divides these changes into three principal groups, designated A, B and C. Many group A lesions (designated A_1) are considered to be merely "exposure stigmata", embracing a variety of histological changes commonly associated with the administration of chemical toxins. A further subgroup (A_2) forms a bridge between A_1 and the lesions of group B, which are proliferative but not necessarily cancerous since some of them regress completely. It is difficult to distinguish histologically between lesions of types A and B. Group C lesions are cancerous and are divided into a number of grades according to their degree of malignancy.

The author reviews both experimental and clinical data concerned with the changes that precede the development of cancer in various organs and attempts to classify them into group A or group B lesions. Some of these attempts are more successful than others, largely because some target organs, such as skin and mammary-gland tissue, are easily accessible and as a consequence have been studied intensively along appropriate lines. Nevertheless, despite the many gaps and deficiencies in the available evidence, the applicability of these principles to other target organs is clearly demonstrated in successive chapters of this book.

The picture that emerges from this account is one of great importance to the pathologist engaged in cancer research. In practically every organ one can identify certain lesions that appear to be the precursors of frankly malignant tumours. This is seen most clearly in experimentally induced lesions, but human material provides many instructive examples as well.

One omission from this otherwise excellent account of the developmental stages of neoplasia is the absence of any attempt to distinguish and identify lesions that are clearly the result of repeated trauma and regeneration and that lead, in turn, to precancerous proliferative lesions and ultimately to cancer. The identification of such lesions is an essential step

in any real progress towards understanding the molecular events that lead to cancer.

Clinical Aspects of the Teratogenicity of Drugs. By H. Nishimura and T. Tanimura. American Elsevier Publishing Company, Inc., New York, 1976. pp. xii + 453. Dfl. 135.00.

This book is aimed mainly at the physician who needs to update his knowledge on the possible risks of drugs and other chemicals to pregnant patients.

The first four chapters provide a general introduction to teratology, covering normal human embryonic development, mainly through the medium of a series of photographs, and drug transfer between mother and foetus, and ending with an attempt to explain the mechanisms of teratogenesis at a cellular and molecular level and to identify other critical factors such as dosage regimes and genetic differences. Many readers may find this section a little too general, although the authors have been generous in their provision of references to assist further reading.

Subsequent chapters are concerned mainly with the various categories of chemicals that possess, or are suspected of possessing, teratogenic properties, particular attention being paid to the human foetus. This section accounts for virtually half of the text, and is undoubtedly the most valuable part of the book. Here the coverage is wide and the references are extensive and include many relatively recent papers. Drugs are dealt with first and those considered include antineoplastic and chemotherapeutic agents, hormones, radioisotopes, vaccines, anaesthetics, narcotics and antidepressants. A chapter on environmental chemicals completes this section. Here the areas covered include air pollution, smoking, heavy metals, pesticides, food additives and alcoholic beverages.

The final chapter is concerned with animal testing for teratogens and makes some brief comment on the evaluation of such tests. Most of this chapter is occupied by some 30 pages of tabulated data comparing reported teratological experiments in non-human primates with the corresponding data derived from humans and rodents.

This book succeeds in collating a great deal of information from very scattered sources, and will be of value not only to the practitioner but also to the research worker seeking a guide to the relevant literature.

Pharmacology and Pharmacokinetics. Edited by T. Teorell, R. L. Dedrick and P. G. Condliffe. Plenum Press, New York, 1974. pp. xii + 388. $29.50.

In recent years, rapid advances have been made in the fields of pharmacology and pharmacokinetics. These advances may be exemplified by the appearance of pharmacogenetics as a new and important discipline. The improved understanding of some of the factors that contribute to variations in sensitivity to drugs should allow a more rational approach to chemotherapy, with treatments being adjusted more to the needs of individuals. However, there has been a lag in the application of recent laboratory discoveries to the improvement of treatment regimes, and in view of the increasing need for valid models in this field, a conference was held in 1972 to discuss current concepts and models of drug action. The book under review is a collection of the papers presented at that conference, and the list of participants resembles a 'Who's Who' of pharmacology—a fact that is reflected in the very high quality of the contributions.

The sequence of papers is intelligently organized. Thus, the opening chapters are devoted to an exposition of the present state and limitations of pharmacokinetics, with a forecast of the direction in which some of the more promising lines of research may lead. Successive chapters deal with such problems as the effect of route of administration on drug metabolism and disposition, species differences and scale-up problems in the extrapolation of animal studies to man, and pharmacokinetic studies in cancer chemotherapy. There are also two excellent chapters by B. N. La Du and E. S. Vesell on the importance of genetic factors in unexpected drug effects and in the wide range of sensitivity to drugs seen in normal individuals.

There are some good examples of the derivation and use of mathematical models in experimental studies, and mention is made of the use of computer systems for simulation purposes and in the solution of multicompartment distribution problems. This may be illustrated by the work of R. L. Dedrick and K. B. Bischoff with methotrexate. These workers derived a multicompartment model which fitted the experimental data well for a relatively short period after experimental animals were given a single iv dose of the drug. However, an analysis of data obtained from these animals over a longer period showed that the model did not continue to predict plasma levels of the drug accurately. The authors interpreted the discrepancy as evidence for a further tissue reservoir, which increased in relative importance in the longer term. Clearly one of the hazards that this type of analysis could indicate is accumulation following repeated administration of a compound. In considering the utility of such techniques, however, one author argues the need for a more mathematical approach to be taken in the training of medical students if the potential contribution of pharmacokinetics to medicine is to be realized. One might add that this approach could usefully be extended to the teaching of all subjects with a biological basis.

There are, inevitably, errors and omissions in a volume of this type. Nevertheless, it was surprising to read that "when a metabolic process occurs and the intestinal drug concentration is well below the capacity of the enzyme system [in the intestinal mucosa], gut metabolism is maximal..." Generally however, this book is memorable not so much for its occasional errors as for the excellence of most of the contributions. The nature of the book is such that it is far more likely to be read by the specialist than by the occasional browser, although certain papers are of more general interest and could be of use to many workers involved in biochemical research.

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Information Section

ARTICLES OF GENERAL INTEREST

AMARANTH TERATOLOGY—THE LAST WORD?

Final publication of the outstanding reports on the teratogenicity of amaranth (FD & C Red No. 2), first presented in June 1974, coincided with the exclusion of teratology from the principal issues to be discussed in the formal hearing on the FDA's rejection of this colouring. Concern over the teratogenicity of amaranth stemmed from Russian allegations that it caused foetal damage in rats (Shtenberg & Gavrilenko, *Vop. Pitan.* 1970, **29** (5), 61) but subsequent experiments failed to produce unequivocal results.

In view of this uncertainty an *ad hoc* committee on amaranth, convened by the FDA, instigated a collaborative study involving the FDA laboratories, Industrial Bio-Test Laboratories (IBT) and the National Center for Toxicological Research (NCTR), in an attempt to determine whether reproduceable embryotoxic effects could be demonstrated and to compare the influence of the mode of administration on the teratological response (if any). The relevant reports have now been published as five consecutive papers in volume 1 (1976) of the *Journal of Toxicology and Environmental Health.* Part I (Collins *et al.* p. 851) forms an introduction to the study, part II (*idem*, p. 857) presents FDA data, part III (Keplinger *et al.* p. 863) presents IBT data, part IV (Holson *et al.* p. 867) presents NCTR data and part V (*idem*, p. 875) summarizes and discusses the combined findings.

The same experimental regime was used by each laboratory, and involved the administration of amaranth in a daily dose of 200 mg/kg either by gavage on days 0–19, 6–15 or 7–9 of gestation or from a water-bottle on days 0–20 of gestation. Altogether six control groups were used, reflecting the different methods, vehicles and times of administration of the test material. The experiments differed only in the choice of animal strain, namely Osborne–Mendel at the FDA, Charles River at IBT and both strains at NCTR.

The incidence of skeletal or visceral abnormalities was not significantly higher in treated animals than in the controls in any of the experiments. No evidence was obtained for any embryo- or foetolethal effect in Osborne–Mendel rats fed 200 mg amaranth/kg. While no significant increase in resorptions was seen in these rats, studies with the Charles River strain showed a significant increase in the number of litters with two or more resorptions after dams had been given 200 mg/kg on days 0–19 of gestation. The NCTR study on the latter strain also showed a significant increase in the percentage of resorptions per litter. The suggestion of a foetolethal effect of amaranth in the Charles River strain contrasts with the results of two earlier studies in which rats of this strain were given amaranth by gavage on days 6–15 of gestation (*Cited in F.C.T.* 1975, **13**, 473; Burnett *et al. Toxic appl. Pharmac.* 1974, **29**, 121). Negative results have also been obtained in rabbits, mice and hamsters given up to 1000 mg/kg/day on days 6–18, 6–15 and 6–10 of gestation, respectively.

The increased resorption rate among treated Charles River rats in the studies now under consideration was not corroborated by a significant decrease in the numbers of viable foetuses, as it was in one earlier study in Osborne–Mendel rats (Collins & McLaughlin, *Fd Cosmet. Toxicol.* 1972, **10**, 619). The only other studies indicating possible embryolethal effects after administration of amaranth by gavage to rats were the previously cited Russian studies (Shtenberg & Gavrilenko, *loc. cit.*) and an unpublished FDA study (*Food Chemical News* 1972, **14** (16), 13). However, the Russian work was marred by imprecise details of dye specification and experimental protocol, and the resorption-rate increases in the FDA study were not dose-related (Holson *et al. loc. cit.* part V).

Critical examination of the gavage mode of administration reveals a dearth of data on the physiological and biochemical responses elicited in otherwise normal experimental animals. It is conceivable that homeostatic imbalance resulting from this technique may influence factors that determine the apparent toxicity of the material under investigation, and this might be partly responsible for the inability of many workers to reproduce the embryotoxic effects that have been observed sporadically in the numerous studies with amaranth. Thus, the absence of embryotoxic effects observed in Osborne–Mendel rats by the FDA and NCTR laboratories conflicts with the earlier results (Collins & McLaughlin, *loc. cit.*) in animals given 200 mg amaranth/kg by gavage on days 0–19 of gestation. It is also relevant that a full multigeneration study involving dietary administration of amaranth to Osborne–Mendel rats at the FDA laboratories (Collins *et al. Toxicology* 1975, **3**, 115 & 129) failed to demonstrate a foetotoxic effect.

A comparison between these differing experimental regimes was facilitated by a demonstration (*Cited in F.C.T.* 1977, **15**, 77) that repeated daily doses of amaranth administered by gavage resulted in a cyclic pattern of serum naphthionic acid levels. When the colouring was consumed in the diet, these naphthionic acid levels were constant and much lower than the peak concentrations following gavage. The FDA's multigeneration study involved administration of up to 2400 mg amaranth/kg/day, from which it was inferred that both peak embryonic levels and time-course concentrations for serum naphthionic acid

would have been higher than those in studies involving administration of 200 mg/kg by gavage. This line of reasoning clearly undermines the earlier findings (Collins & McLaughlin, *loc. cit.*)

It is also significant that whereas embryotoxicity is usually indicated by combinations of increased resorption, decreased foetal weight and an increased incidence of skeletal variants, adverse findings in the 17 or more studies on amaranth that have been reported to date have rarely involved more than scattered indications of an increased resorption rate. In the rat, although this finding has not been restricted to a single strain, it has been observed in only two studies, both involving gavage administration. There is need for caution in interpreting statistical findings and biological relevance must be the ultimate arbiter of such matters. Studies on the teratological potential of amaranth have failed to reveal any adverse effects that were both biologically sound and experimentally reproduceable.

[J. J.-P. Drake—BIBRA]

FILLING IN THE ACRYLAMIDE PICTURE

Acrylamide ($CH_2{:}CH \cdot CONH_2$) has been shown to cause peripheral neuropathy in man (*Cited in F.C.T.* 1971, **9**, 912), rats (*ibid* 1974, **12**, 579) and dogs (*ibid* 1975, **13**, 400). It modifies amino-acid incorporation into the spinal cord and peripheral nerves, but not, apparently, into brain or liver tissue (*ibid* 1974, **12**, 579).

Apart from presenting a hazard to those engaged in its manufacture and handling, acrylamide has been shown to be a contaminant of ground-water used for drinking when it has been used as a grouting agent in public works. Igisu *et al.* (*J. Neurol. Neurosurg. Psychiat.* 1975, **38**, 581) have described an incident in Japan in which all five members of a family developed nervous-system disturbances ranging from minor behavioural changes in a child to rhinorrhoea, dizziness, ataxia, hallucinosis and irrational behaviour in adults. The episode was traced to the use of well-water which had become contaminated with 400 ppm acrylamide, together with a trace of dimethylaminopropionitrile, from chemical grouting carried out during sewerage construction in an adjacent street. The severity of the neurotoxicity was proportional to the degree of exposure of the members of the family, the children, who were out at school during the day, being least affected. Mice given water from the affected well developed weakness of the limbs within 2 months.

Edwards (*Chemico-Biol. Interactions* 1976, **12**, 13) gave acrylamide to female rats either in the diet at a level of 200 ppm from mating until parturition or of 400 ppm for the first 20 days of gestation or in a single iv dose of 100 mg/kg on day 9 of gestation. She also measured the concentration of free acrylamide in foetal tissues 1 hour after a single iv dose had been given to pregnant rats on day 14 or 21 of gestation, and found that the placenta presented no effective barrier to the passage of acrylamide from the maternal circulation. Rats given 400 ppm in the diet had foetuses of slightly reduced weight, but this was considered likely to be due to a much reduced maternal food intake. Birth weights in animals exposed to 200 ppm did not differ significantly from those in controls. No external or macroscopic abnormalities were detected in foetuses from any of the treated groups when compared with controls. There is thus no evidence from this study that acrylamide impairs foetal development when given to female rats in doses known to be neurotoxic, although a rapidly reversible neurotoxicity to the foetal system could not be ruled out.

The toxic action of acrylamide on the nervous system was shown to involve the functional block of terminals of muscle-stretching afferent nerves (Sumner, *J. Physiol.* 1975, **246**, 277). A solution of 100 mg acryl amide/ml was injected sc into cats in a daily dose of 10 mg acrylamide/kg, and neuropathy usually became apparent after 20 days as slight hind-limb ataxia. Single afferent fibres derived from the gastrocnemius of these animals were examined after 21–67 days and were shown to conduct impulses at either 72–126 m/sec (group I) or 24–72 m/sec (group II). Of 538 muscle afferents that failed to respond to stretch–receptor stimulation, 99 showed the phenomenon of 'early discharge', indicating that the impulses did not arise from excitation of the muscle spindle, but from muscle action potential. It is argued by Sumner (*loc. cit.*) that the 'early discharge' phenomenon is likely, therefore, to be a physiological artefact that may constitute a pitfall in the examination of the nervous system by initiating synchronous volleys of nerve impulses.

Edwards (*Br. J. ind. Med.* 1975, **32**, 31) also studied the neurotoxicity of several acrylamide analogues fed to rats in the diet. Ethyl crotonate, methyl methacrylate, methylene-bisacrylamide, *N,N*-pentamethyleneacrylamide, *N,N*-bisacrylamidoacetic acid, 3,3-iminodipropionamide and *S*-β-propionamidoglutathione were not neurotoxic, but *N*-hydroxymethyl-, *N*-methyl- and *N,N*-diethylacrylamide produced peripheral neuropathy, which was reversible and indistinguishable from that produced by acrylamide itself. *N*-Hydroxymethylacrylamide fed at 1800 ppm for 1 week and thereafter at 900 ppm induced slight ataxia after 5 weeks. When this intake was supplemented by an ip dose of 50 mg/kg four times during the next 2 weeks, moderate disability was evident after a total of 7 weeks. *N,N*-Diethylacrylamide fed at 800 ppm produced no weakness or ataxia within 10 weeks, but increase of the dietary concentration to 1600 ppm for a further 2 weeks resulted in slight ataxia. At 980 ppm it produced slight ataxia after 8–10 weeks. There was no further deterioration by week 13, but moderate disability resulted from two ip doses given during

week 14. *N*-Methylacrylamide fed at 980 ppm produced slight ataxia after 4–5 weeks and the disability became more marked after 7–8 weeks. The ataxia was again aggravated by two ip doses of the compound, in this case during week 9.

Rats given repeated ip doses of acrylamide in addition to receiving one of the neurotoxic analogues in the diet developed neuropathy earlier than those given acrylamide alone, but this effect was not seen with analogues that were not neurotoxic when administered alone (Edwards, *loc. cit.*). Thus, the effects of acrylamide and its analogues are probably additive. Supplementation of the diet with 5000 IU vitamin A/kg or 52 IU vitamin E/kg did not affect the neuropathy produced by ip injection of 50 mg acrylamide/kg twice weekly or the rate of recovery of the rats after cessation of acrylamide treatment. Acrylamide toxicity was not affected by either DDT or phenobarbitone, both potent inducers of liver enzyme systems.

In a subsequent paper, Edwards (*Biochem. Pharmac.* 1975, **24**, 1277) showed that the neurotoxicity of the three analogues was not due to breakdown to acrylamide itself. When given to rats in a single iv dose, acrylamide and *N*-hydroxymethylacrylamide both distributed throughout the total body water within a few minutes, after which the concentration of both compounds decreased exponentially with a half-life of less than 2 hours. However, a tracer study with *N*-hydroxy[^{14}C]methylacrylamide showed that there was very little breakdown to acrylamide *in vivo*. *N*-Methylacrylamide and *N,N*-dimethylacrylamide were not converted to acrylamide *in vivo*. The reaction rates of acrylamide and *N*-hydroxymethylacrylamide with glutathione *in vitro* were the same and the presence of rat-liver supernatant increased the rates of both reactions, although the analogue did not decompose to acrylamide in the buffer solution.

In hens, 50 mg acrylamide/kg given orally on 3 days/week produced reversible neuropathy after very variable periods. In frogs and goldfish there was no evidence of neuropathy after treatment with sublethal doses of acrylamide, indicating that neither of these animals or their nerve tissue could be used to study the neurotoxic effects of this compound (Edwards, *Br. J. ind. Med.* 1975, **32**, 31).

[P. Cooper—BIBRA]

DISTRIBUTION AND METABOLISM OF CAFFEINE PURSUED

The universal popularity of beverages containing caffeine (1,3,7-trimethylxanthine) has focussed attention on the toxicity and human metabolism of the methylxanthines in general. Since our previous review of this topic (*Cited in F.C.T.* 1972, **10**, 102), several studies have been reported.

Test meals containing 350 ml of a solution of 500 mg caffeine/litre were instilled into the stomachs of six healthy volunteers (Chvasta & Cooke, *Gastroenterology* 1971, **61**, 838). The absorption of caffeine was directly related to its residence time in the stomach, reaching a peak of 16% of the ingested dose after 20 minutes, when about 86% of the test meal had left the stomach. Caffeine increased gastric acid output more than did water. In a further six healthy subjects 100 g glucose/litre was added to the test meals to delay gastric emptying, and the meals were buffered at pH 1–9. The absorption of caffeine was negligible at pH 1, but reached a peak at pH 7, when 21% of the dose was absorbed in 20 minutes.

Male mice were given [^{3}H,^{14}C]caffeine in doses of 5 or 25 mg/kg, and the radioactive metabolites in expired air, urine and faeces were studied (Burg & Stein, *Biochem. Pharmac.* 1972, **21**, 909). Excretion of both ^{3}H and ^{14}C was disproportionately higher after the 25-mg/kg dose than after the lower dose, the amount incorporated into body tissues being approximately constant, irrespective of dose. About 6% of the higher and 3% of the lower dose appeared in the urine as free caffeine (compared with 0·5–1·5% reported in man) but neither 1-methylxanthine nor theobromine was detected. The proportion of 1,7-dimethylxanthine and 7-methylxanthine in mouse urine (11 and 5%) was greater than that in human urine (0·2–0·7 and 0·4–0·9%, respectively). 1-Methyluric acid was present as a minor urinary metabolite (8% of the dose). The total recovery of radioactivity in the urine ranged from 64 to 90%, 80% of this being excreted within 8 hours of administration of the 25-mg/kg dose. Urinary excretion of caffeine in mice fed caffeine since weaning did not differ materially from that in previously untreated mice, but prior feeding did enhance faecal caffeine excretion. Other major urinary metabolites were 3-methylxanthine and 1,3-dimethyluric acid.

Burg & Werner (*ibid* 1972, **21**, 923) found that after male mice had received a single oral dose of 25 mg [^{3}H,^{14}C]caffeine/kg, radioactivity appeared in the brain, heart, kidneys, liver, lungs, spleen, testes, muscle, plasma and erythrocytes within 5 minutes, becoming generally distributed in proportion to tissue-water content after 1 hour. The maximum half-life of caffeine in these organs was 3 hours. The disposition of a dose of 5 mg/kg was similar, but the half-life was shorter. The major metabolite isolated from the tissues was 1,7-dimethylxanthine, and although little 1-methyluric acid occurred in the tissues, it was present in the urine. The liver contained no detectable quantities of methylxanthine nucleotides. In contrast to these findings in mice, caffeine had a half-life of 11 hours in the plasma of squirrel monkeys given a 5-mg/kg dose (Burg *et al. Toxic. appl. Pharmac.* 1974, **28**, 162). This also contrasted with the half-life of 2·4 hours observed in rhesus monkeys. After repeated oral doses, caffeine was shown to accumulate in the squirrel monkey. Nevertheless, the methylxanthines in the tissues and urine of squirrel monkeys were the same as those in other animal species exam-

ined. The difference probably indicates the absence of an effective catabolic mechanism in the squirrel monkey, rather than a relative inability to excrete caffeine, but the enzymatic defect, if it exists, has not been identified. Thus, the mouse and the rhesus monkey are better animal models than the squirrel monkey for conducting caffeine metabolism studies that may be relevant to man.

In studies by Khanna *et al.* (*ibid* 1972, **23**, 720), male rats received 40 mg [^{3}H]caffeine/kg, and the urine collected over 24 hours was shown to contain 64–67% of the administered radioactivity, although the main urinary metabolite (11·4%) was not identified. *p*-Xanthine constituted 8·8% of the dose, theobromine 5·1% and theophylline 1·2%, and a further unidentified metabolite (1·3%) was associated with traces of 1,3,7-trimethyluric acid and 3-methyluric acid. Unchanged caffeine accounted for 8·8% of the dose. In a further paper (Rao *et al. Experientia* 1973, **29**, 953), the two unidentified metabolites were characterized as 1,3,7-trimethyldihydrouric acid and 3,6,8-trimethylallantoin, respectively. It is suggested that the highly polar methylated allantoins, allantoic acids and urea derivatives may be the metabolites of caffeine reported, but not identified, by earlier investigators.

After ip injection of [1-methyl-^{14}C]caffeine in rats there was rapid penetration of radioactivity into intracellular tissue compartments and accumulation in the cytoplasm (Galli & Spagnuolo, *Pharmac. Res. Commun.* 1975, **7**, 125). However, caffeine and its metabolites did not accumulate in microsomes, mitochondria and synaptosomes. Although the subcellular distribution of total caffeine-derived radioactivity was similar in the liver and brain, caffeine metabolites accumulated in—or were formed in—the particulate fractions of the liver to a greater degree than in those of the brain.

Galli *et al.* (*ibid* 1975, **7**, 217) reported that after an intramuscular injection of 1·4 mg [1-methyl-^{14}C]-caffeine/kg into pregnant rats on day 18 of gestation, radioactivity in the brain and liver of both mother and foetus was similar after 1 hour, but thereafter diminished more rapidly in the maternal than in the foetal brain and more slowly in the maternal than in the foetal liver. Caffeine transferred from maternal to foetal tissues tended to accumulate in foetal brain. The metabolism of caffeine appeared to be similar in both maternal and foetal tissues. These findings suggested that prolonged pharmacological effects are possible during the early stages of the development of the foetal central nervous system, if caffeine is taken by a mother during pregnancy.

[P. Cooper—BIBRA]

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TOXICOLOGY: BIBRA ABSTRACTS AND COMMENTS

AGRICULTURAL CHEMICALS

3123. DDT metabolism

Gingell, R. (1976). Metabolism of ^{14}C-DDT in the mouse and hamster. *Xenobiotica* **6,** 15.

DDT (1,1-bis-(*p*-chlorophenyl)-2,2,2-trichloroethane) has been shown to induce liver tumours in the mouse (Innes *et al. J. natn. Cancer Inst.* 1969, **42,** 1101) but not in the hamster (Agthe *et al. Proc. Soc. exp. Biol. Med.* 1970, **134,** 113), and it is thought that the active tumorigen may be a metabolite of DDT. In the study cited above, several urinary metabolites of DDT were identified in six CF-1 mice and three Syrian golden hamsters of each sex given 25 mg [^{14}C]DDT/kg in olive oil by gavage. Urine from both sexes of mouse and hamster was collected under toluene for 5 days and extracted with hexane before mild alkaline hydrolysis, acidification and extraction with ether. The extracts were chromatographed by thin-layer chromatography, and radioactive metabolites were located by autoradiography of the chromatograms. Radioactive spots were eluted and analysed by gas–liquid chromatography.

Of the administered dose of ^{14}C, male mice excreted 16·1 ± 8·0% and females 14·3 ± 1·8% in the urine in 5 days. Of the ^{14}C in the urine of male mice, 13% was extracted into hexane and 79% into ether. Of the ^{14}C administered, the hexane extract contained 0·5% as DDT, 0·8% as DDE (1,1-bis-(*p*-chlorophenyl)-2,2-dichloroethylene), 0·3% as DDOH (1,1-bis-(*p*-chlorophenyl)-2-ethanol), 0·2% as DDD (1,1-bis-(*p*-chlorophenyl)-2,2-dichloroethane) and 0·1% as DBP (bis-(*p*-chlorophenyl)benzophenone). Most of the ^{14}C excreted was present in the ether extract as DDA (bis-(*p*-chlorophenyl)acetic acid, 7·7%), unidentified DDA conjugates (3·5%), DDA-glycine (2·1%) and DDA-alanine (0·5%). A DDA-serine conjugate was also identified.

Of the administered dose of ^{14}C, male hamsters excreted 13·4 ± 1·7% and females 21·0 ± 3·2% in the urine in 5 days. Of the ^{14}C in male hamster urine, 1·4% was extracted into hexane and 87% into ether. The ether extract contained DDA and its conjugates in similar proportions to those found in mouse urine. In contrast, the hexane extract contained less than 0·3% of the administered dose of ^{14}C as DDT, DDD, DBP and DDOH, and none as DDE.

[The author states that "the results reported here support the suggestion that DDE may be a proximate hepatotumorigenic metabolite formed in mice fed DDT". However, while this work confirms DDE as a urinary metabolite of DDT in mice, 0.8% of a DDT dose of 25 mg/kg being recovered in this form, it provides no direct evidence that DDE is the proximate carcinogen.]

3124. Ethylene thiourea strikes the foetus

Ruddick, J. A. & Khera, K. S. (1975). Pattern of anomalies following single oral doses of ethylenethiourea to pregnant rats. *Teratology* **12,** 277.

Ethylene thiourea (ETU) is a degradation product of the ethylenebis(dithiocarbamate) fungicides. It has been reported to be mutagenic for bacteria (*Cited in F.C.T.* 1975, **13,** 398) and goitrogenic in rats (*ibid* 1973, **11,** 702) and to be capable of inducing foetal malformations in rats and, to a lesser extent, in rabbits (*ibid* 1974, **12,** 282).

The results of giving pregnant rats a dose of 40–240 mg ETU/kg by intubation on 1 day between days 6 and 21 of gestation have now been reported. The rats were killed on day 22 and the numbers of live foetuses and resorptions and the individual litter weights were determined. No maternal toxicity was observed after a dose of 240 mg/kg. No foetal malformations appeared after maternal dosing on days 6–9, but dosing with 240 mg/kg on day 10 was associated with defective external genitalia (in 6% of the offspring), hydronephrosis or kidney hypoplasia (in 20%) and absence or shortness of tail (in 20%). After dosing on day 11, lumbar spina bifida (in 30%), fused ribs and further tail and kidney abnormalities were observed. The foetus was most sensitive on days 12–15, when dosing at this level caused a high rate of anomalies, including hydrocephalus, exencephaly, brachygnathia, hydronephrosis, forelimb micromelia and absence of tail. Doses lower than 240 mg/kg given on these days were associated with fewer and less severe deformities, and a short or absent tail was the only abnormality resulting from a dose of 40 mg/kg. All foetuses exposed to 240 mg ETU/kg on days 17–19 had hydronephrotic or hypoplastic kidneys, but only 42% of those exposed on day 21 had these lesions. Oedema of the head, neck and trunk was seen in all foetuses exposed on days 18–20.

MISCELLANEOUS CONTAMINANTS

3125. Polybrominated biphenyls on the loose

Gutenmann, W. H. & Lisk, D. J. (1975). Tissue storage and excretion in milk of polybrominated biphenyls in ruminants. *J. agric. Fd Chem.* **23,** 1005.

It has been suggested that polybrominated biphenyls (PBBs) may be up to five times as toxic as polychlorinated biphenyls in the environment (*Food Chemical News* 1975, **17** (11), 47), and although no case of human disease from exposure to PBBs

is authenticated, high levels of PBBs have been associated with gross abnormalities in animal reproduction (*ibid* 1975, **17** (5), 27).

In the study cited above, experiments to determine the potential contamination of milk and meat were carried out by feeding 50 ppm PBBs to a lactating cow and a sheep for 15 and 30 days, respectively. The cow excreted 2·22% of the total dose of 17 g PBBs in the milk within 31 days, the half-life during the 15 days following the end of the feeding period being 10·5 days. Production of milk was not affected, but hay consumption decreased. In the sheep, tissue recoveries of PBBs were several-fold higher than in the cow, possible because of better intestinal absorption. In both species, residues were mainly in body fat and liver. Relatively high concentrations of PBBs also occurred in the thyroid of the cow and the muscle of the sheep. Post-mortem examination showed marked glandular hyperplasia of the intrahepatic bile ducts of the cow and the gall bladder of the sheep.

Tolerance limits ranging from 0·3 ppm PBBs in milk and meat to 0·05 ppm in eggs and finished feeds were established by the FDA following the inclusion of a PBB flame retardant instead of magnesium oxide in various animal feeds (*ibid* 1975, **17** (11), 47: *ibid* 1976, **18** (4), 33), a monumental error which led to the slaughter of thousands of cattle, pigs and poultry and the large-scale destruction of eggs and dairy products. Apparently some people in the affected localities of Michigan attributed various minor ailments to exposure to the PBBs, but no conclusions could be reached regarding these suspicions or the possible long-term or delayed effects in man of exposure to PBB-contaminated products. Among people so exposed, however, concentrations up to 2·26 ppm in blood and 174 ppm in adipose tissue have been reported, as well as 92 ppm in human milk (Dunckel, *J. Am. vet. med. Ass.* 1975, **167**, 838).

THE CHEMICAL ENVIRONMENT

3126. Cadmium and chromosomes

Deknudt, Gh. & Léonard, A. (1975). Cytogenetic investigations on leucocytes of workers from a cadmium plant. *Envir. Physiol. Biochem.* **5**, 319.

Cadmium (Cd) has well-recognized toxic effects on the testes and kidneys, is hypertensive, and has been associated with a painful osteomalacia known in Japan as 'itai-itai' (*Cited in F.C.T.* 1972, **10**, 249), a condition apparently characterized additionally by chromosome abnormalities in the leucocytes of peripheral blood (Shiraishi & Yosida, *Proc. Japan. Acad.* 1972, **48**, 248). Cd has also been shown to cause tail abnormalities in the offspring of mice (*Cited in F.C.T.* 1972, **10**, 595).

Studies of workers from a Cd plant have shown that, even with Cd levels that are insufficient to produce osteomalacia, there may be a significant increase in the incidence of chromosomal aberrations. The study group included 23 men exposed occupationally for an average of 12 yr to fumes and dust containing Cd and lead (Pb) but no zinc (Zn), and another 12 exposed in the rolling mill, for an average of 11 yr, mainly to Zn with low concentrations of Pb and Cd. Twelve administrative workers from the same plant were used as controls. Blood samples were taken from each subject and in each case a slide of cultured leucocytes was prepared for the analysis of 200 metaphases. Three of the controls each showed one severe aberration (two chromatid exchanges and one dicentric chromosome). The incidence was markedly higher in the other two groups, the subjects exposed to high levels of Cd and Pb showing a higher number of severe chromosomal anomalies but a lower total number of structural aberrations than the mildly exposed group. The severity of the Cd and Pb exposure of the former group was reflected in higher levels of these metals in the blood samples. The higher incidence of severe abnormalities in the leucocytes of the heavily exposed group may have reflected the absence of Zn exposure, since Zn is known to antagonize the toxic effects of Cd.

3127. Neurological effects of cyanide toxicity

Brierley, J. B., Brown, A. W. & Calverley, J. (1976). Cyanide intoxication in the rat: physiological and neuropathological aspects. *J. Neurol. Neurosurg. Psychiat.* **39**, 129.

Experiments on a range of animal species have demonstrated that intoxication with cyanide compounds can lead to brain damage, the cyanide ion being the common toxic agent. However, it has not been demonstrated that cyanide *per se* exerts a direct effect on the cells of the central nervous system. To resolve this doubt, the authors cited above studied the effects of iv infusion of sodium cyanide in rats.

Since slowing of respiration, leading to apnoea, is the first sign of cyanide overdosing, it was necessary to determine the rate of infusion of a standard sodium cyanide solution that would just avert apnoea in most animals. The solution was then infused, initially at a rate of 0·14 mg/min/kg, into 11 lightly anaesthetized rats and into eight restrained unanaesthetized rats. Electromyogram and electroencephalogram readings were recorded in both experiments, but complete records of cardiovascular and respiratory function were recorded only in the anaesthetized animals.

The white matter of the brain was damaged in six of the 19 rats and the grey matter was affected additionally in one. The treated animals demonstrated the physiological effects of sublethal and lethal cyanide intoxication; hyperventilation was seen in all of them, and concomitant inhibition of aerobic CO_2 production was evident in anaesthetized rats. It was inferred that the low levels of arterial CO_2 probably led to vasoconstriction, and therefore to some restriction in the blood flow in the brain, as well as to a reduced oxygen pressure gradient between blood and tissue.

The distribution of altered neurones in the single

anaesthetized animal was confined to the diencephalon, the neurones of the cerebral cortex, hippocampi and cerebellum being normal. Epileptic seizures preceded the occurrence of apnoea and cardiac arrhythmia and a simultaneous reduction in the mean blood pressure. While cerebral blood flow would have fallen markedly by this time, the results of earlier experiments supported the view that there may have been a simultaneous rise in central venous pressure.

The authors concluded that cyanide damages neurones only as a result of secondary effects on the circulation.

3128. Dioctyl sodium sulphosuccinate doubts

Moffatt, R. E., Kramer, L. L., Lerner, D. & Jones, R. (1975) Studies on dioctyl sodium sulfosuccinate toxicity: Clinical, gross and microscopic pathology in the horse and guinea pig. *Can. J. comp. Med.* **39,** 434.

Dioctyl sodium sulphosuccinate (DSS) is widely regarded as an inert anionic surfactant of low toxicity, but there have been reports of adverse reactions to its topical or internal use in high concentrations in many animal species. Contact skin burns, corneal irritation, gastro-intestinal irritation, diarrhoea and death have been reported. Doses of up to 200 mg/kg are recommended for the treatment of constipation in horses, and, because of the reported adverse effects, the study cited above was designed to determine the changes produced in the horse and guinea-pig by a toxic dose of DSS.

Horses given 0·65 or 1·0 g DSS/kg in water by stomach tube became uneasy within 1 hr and showed a progressive rise in heart and respiration rate within 3 hr, followed by watery diarrhoea, abdominal pain, accumulation of intestinal fluid, cardiac irregularities and death within 14–72 hr. Blood samples from treated horses showed increased haemoglobin, packed cell volume and red cell count, with a moderate reduction in serum chloride. Guinea-pigs given 0·5–0·9 g DSS/kg rapidly grew depressed and inactive, and some died, the majority of deaths being within 4–7 hr. In these animals, muscular weakness progressed to shallow respiration, twitching and convulsions before death. In guinea-pigs given 0·1–0·9 g DSS/kg there was an increase in packed cell volume which correlated with the increase in death rate, and in those given 0·7 or 0·9 g DSS/kg there was an increase in white cell count. The LD_{50} of DSS in this species was about 0·65 g/kg.

In all the animals, dehydration and haemoconcentration were observed *post mortem.* Horses showed organ congestion and alveolar emphysema and oedema. Their livers contained excessive concentrations of bile, and their kidneys exhibited mild proximal tubular degeneration and medullary haemorrhages. Adrenal cortical haemorrhages were also present. Animals surviving 24 hr or longer showed loss of intestinal epithelium. In guinea-pigs dying after 0·5 g DSS/kg or more, necrosis of the gastric epithelium, generalized vascular engorgement and spleens depleted of polymorphonuclear leucocytes were seen. The authors concluded that further studies were needed to establish a safe dose of DSS in horses.

3129. Doubtful carcinogenicity of methotrexate

Bailin, P. L., Tindall, J. P., Roenigk, H. H., Jr. & Hogan, M. D. (1975). Is methotrexate therapy for psoriasis carcinogenic? A modified retrospective-prospective analysis. *J. Am. med. Ass.* **232,** 359.

The incidence of malignant neoplasms and death was studied in a 7-yr follow-up of 205 psoriatic patients treated with methotrexate at two clinics during 1960–1965. Of six patients with cutaneous tumours, some of them multiple, three had received superficial X-ray treatment and two of these had taken inorganic arsenic in addition to methotrexate. Of the other patients, three developed non-fatal internal neoplasms and four died from neoplastic disease. One of the latter, who developed acute myelogenous leukaemia, had received only a single injection of 50 mg methotrexate, which is therefore unlikely to have been implicated. No single type of neoplasm predominated in the series, which suggests that methotrexate was not responsible for the neoplasms observed. On the other hand, there was an eight-fold increase in deaths related to liver diseases, reflecting the suspected hepatotoxicity of this drug.

3130. More evidence for MBK neuropathy

Mallov, J. S. (1976). MBK neuropathy among spray painters. *J. Am. med. Ass.* **235,** 1455.

Recently an epidemic of peripheral neuropathy was described at an Ohio plant producing plastics-coated fabrics (*Cited in F.C.T.* 1976, **14,** 218). This was linked eventually to methyl *n*-butyl ketone (MBK) exposure, although at the time the possibility of a synergistic effect induced by MBK and methyl ethyl ketone could not be discounted. Evidence from animal studies appears to have confirmed the role of MBK in peripheral neuropathy (*ibid* 1976, **14,** 157), and the study cited above further implicates this compound.

Two cases of peripheral neuropathy were reported among spray workers employed in the painting of dam gates on the Ohio river. As a result a further 26 men who had been involved in the work were interviewed and examined. Paint used by the workers was of two formulations. The first and older formulation contained 22% (w/w) methyl isobutyl ketone and 22% methyl isoamyl ketone. These were replaced in the new formulation by MBK at a concentration of 44%. Both formulations contained a plasticizer, tricresyl phosphate, at a concentration of 3·1%, but the long-term use of this had not been associated with any reports of peripheral neuropathy.

Of the individuals examined, three showed clinical evidence of peripheral neuropathy, the signs being similar in many ways to those previously described (*ibid* 1976, **14,** 218) and developing in all three workers within a period of 4 months. All three men had been exposed to both respiratory and skin absorption of MBK and one in particular was more exposed to skin absorption since he never wore gloves and changed his work clothes only once a week. He had also been exposed to lead in the past, although it was considered unlikely that this played any role in the neuropathy. The level of lead in his blood was 55 μg/100 ml, and a range of 10–70 μg/100 ml of blood

is recognized as being compatible with 'safe' industrial exposure.

Animal studies have indicated that a TLV for MBK of 5 ppm would be more suitable than the current limit of 100 ppm (*ibid* 1976, **14,** 218) and the author of this present study recommends the avoidance of MBK-containing materials in paint-spraying operations until adequate industrial hygiene controls are implemented.

3131. Exposure curves for tetrachloroethylene diagnosis

Fernandez, J., Guberan, E. & Caperos, J. (1976). Experimental human exposures to tetrachloroethylene vapor and elimination in breath after inhalation. *Am. ind. Hyg. Ass. J.* **37,** 143.

Tetrachloroethylene, a volatile halogenated hydrocarbon solvent, finds widespread use as an industrial cleansing agent and is known to produce both hepatotoxic and neurotoxic effects (*Cited in F.C.T.* 1970, **8,** 232). Previous studies in man have indicated that the application of serial breath analyses as a diagnostic tool is of great importance in identifying toxic material and in determining the degree of exposure. As a sequel to theoretical studies of the uptake, distribution and elimination of tetrachloroethylene (Guberan & Fernandez, *Br. J. ind. Med.* 1974, **31,** 159), the study cited above provides a preliminary examination of results of human exposures aimed at the establishment of standard exposure curves for the material.

Twenty-four individuals were exposed to tetrachloroethylene vapour at concentrations of 100, 150 or 200 ppm for periods of 1–8 hr. Alveolar air samples taken during the absorption and excretion phases were analysed immediately for tetrachloroethylene by gas chromatography and the urinary excretion rates of trichloroacetic acid and trichloroethanol metabolites were examined in two subjects.

In subjects exposed to concentrations of 100 ppm for a period of 8 hr, the rise in concentration in the alveolar air was rapid during the first 15 min and then slower until the end of exposure, suggesting that the quantity absorbed did not vary proportionately with the duration of exposure. Breath analyses after termination of exposure showed a rapid decrease in the concentration of the compound in alveolar air, in the form of an exponential decay. Exposures of the same duration but to different concentrations of tetrachloroethylene gave rise to decay curves of similar slope with relatively small individual variations, indicating a proportional relationship between the level of exposure and the height of the alveolar desaturation curve.

It appears that decay curves, in which the alveolar concentration is expressed as a fraction of the constant inspired concentration, could provide useful estimates of exposure during the first hours of the post-exposure phase. Neither trichloroethanol (which was not found in the urine) nor trichloroacetic acid was considered to be a reliable quantitative indicator of tetrachloroethylene exposure.

The authors suggest that this method of controlling exposure should be restricted to estimations under conditions of light work and steady exposure, since little is known of the effects on these curves of higher ventilation in heavy work or of a variable inspired concentration.

COSMETICS, TOILETRIES AND HOUSEHOLD PRODUCTS

3132. Aluminium chlorhydrate in the clear

Rieger, K., Gross, J., Hollander, H. u. Weigand, W. (1975). Inhalatorische, histologische und dermatologische Untersuchungen an Aluminiumhydroxydchlorid, einem Wirkstoff in Antiperspirantien. *J. Soc. cosmet. Chem.* **26,** 531.

Antiperspirants rank high in the classes of cosmetics that are liable to cause dermatitis (*FDA Consumer* 1975, **9** (6), 28). One widely used compound, aluminium chlorhydrate (ACH), has been found to be non-irritant to the skin, except in a few isolated individuals, and solutions of it have shown little evidence of toxicity when inhaled as a spray (Martin *et al. Drug Cosmet. Ind.* 1966, **99** (5), 54).

In the study cited above, powdered ACH (85% of particles below 5 μm) was used in inhalation tests in rats, guinea-pigs, rabbits and cats to determine its penetration characteristics. After exposure for 6 hr to 170 mg ACH/m^3, the animals showed no behavioural effects, no changes in body weight or haematological indices and no clinico-chemical abnormalities. Histological examination of animals killed 1–15 days after termination of exposure showed that the inhaled particles were phagocytosed in the lungs and there was a slight transfer of the powder to the peribronchial lymph nodes. Other experiments using a model human lung have indicated that when various commercially available cosmetic sprays are inhaled through the open mouth, only about 1 particle/mm actually reaches the trachea.

In a second experiment, designed to determine whether ACH altered any skin characteristics, two male and two female subjects applied 50% aqueous ACH solution to the axillae daily for 6 months. Biopsy samples of axillary skin and sweat glands at the end of the period showed no pathological changes.

3133. Subacute study on CTAB

Isomaa, B., Reuter, J. & Djupsund, B. M. (1976). The subacute and chronic toxicity of cetyltrimethylammonium bromide (CTAB), a cationic surfactant, in the rat. *Arch. Tox.* **35,** 91.

Surface-active quaternary ammonium salts have been used widely as germicidal agents in cosmetics and patent medicines. Although the dermal toxicity of cetyltrimethylammonium bromide (CTAB; Cetavlon or Cetrimide), the market leader, has been extensively investigated, adequate oral toxicity data have not yet been generated. The paper cited above reports the subacute oral toxicity of CTAB in the rat.

Groups of ten male and ten female rats were given CTAB in their drinking-water at dose levels of 0 (control), 10, 20 or 45 mg/kg/day for 56–60 wk. At the highest dose level, the skeletal growth, as measured by tail length, and the body weights of all animals were significantly lower than those of the controls. The weight differentials persisted throughout the study in males, but only until wk 9 in females. Male rats receiving the highest dose of CTAB exhibited a decreased food conversion up to wk 7, an effect not apparent in the female animals. The only other external sign of toxicity was a wetting of the anterior ventral region in half of the group receiving 45 mg/kg/day.

At autopsy, the stomach and small intestine of all animals were histologically normal. Relative stomach weights were similar in test and control groups. The relative weight of the liver was reduced in males given the highest dose level and that of the caecum was increased in males given the two higher dose levels and in females given the highest dose level. The haematology of the experimental animals at autopsy was similar to that of the controls, and the urine chemistry was normal when studied in ten animals of each group at month 4.

Previous work had indicated that CTAB was not absorbed from the gut (Isomaa, *Fd Cosmet. Toxicol.* 1975, **13,** 231) and that pathological changes at lethal dose levels were restricted to the gastro-intestinal tract. The present authors found no adverse effects on the stomach and intestinal mucosa of the rat at levels up to 45 mg/kg/day. The reduction in the efficiency of food conversion in immature males fed 45 mg CTAB/kg/day supported an earlier observation that CTAB affected absorption and gastric emptying in male rats (Isomaa & Sjöblom, *ibid* 1975, **13,** 517) and suggested that large doses might prevent proper nutrition.

[As chronic toxicity tests in the rat are normally taken to involve at least a 2-yr treatment period the title of this paper seems somewhat misleading.]

BIOCHEMICAL PHARMACOLOGY

3134. Unravelling chloroform's hepatotoxic mechanism

Docks, E. L. & Krishna, G. (1976). The role of glutathione in chloroform-induced hepatotoxicity. *Expl mol. Path.* **24,** 13.

The hepatotoxicity of carbon tetrachloride (CCl_4) is thought to be due to the formation of a free radical, probably $[CCl_3]^{\cdot}$, which causes peroxidative decomposition of the structural lipids of the microsomal endoplasmic reticulum (*Cited in F.C.T.* 1974, **12,** 555). A similar mechanism of toxicity for other halomethanes, including chloroform ($CHCl_3$), was suggested by the finding that injury to the endoplasmic reticulum increased with decreasing effective negative charge on the halogen atoms, an index of free-radical reactivity (*ibid* 1973, **11,** 1149). Moreover, in rats, the hepatotoxicity of $CHCl_3$, like that of CCl_4 (*ibid* 1970, **8,** 88), was greatly enhanced when its metabolism by the microsomal enzymes was stimulated by phenobarbitone (PB) pretreatment (McLean, *Br. J. exp. Path.* 1970, **51,** 317). Such pretreatment also markedly decreased liver glutathione in rats subsequently exposed to 0·5% atmospheric $CHCl_3$ for 2 hr (Brown *et al. Anesthesiology* 1974, **41,** 554), suggesting that the free radical may also react with glutathione. Isopropanol and acetone have also been shown to potentiate $CHCl_3$ hepatotoxicity (*Cited in F.C.T.* 1975, **13,** 588).

The present study revealed a close correlation between liver necrosis and glutathione depletion in rats injected ip first with 80 mg PB/kg and subsequently with $CHCl_3$ in the dose range 0·05–1·0 ml/kg. The lowest dose of $CHCl_3$ was without effect, 0·1 ml/kg decreased liver glutathione by 16% in the PB-treated rats but induced centrilobular necrosis within 24 hr in only one of the six in this group, and 0·2 ml/kg produced a 50% reduction in glutathione, and induced severe necrosis in four of the five rats so treated. Neither liver pathology nor glutathione levels were affected in rats given $CHCl_3$ or PB alone. The development of necrosis could be prevented by administration of either cysteine, a glutathione precursor, or cysteamine, which possesses a thiol group, suggesting that both were acting as nucleophilic agents. Despite the close correlation between glutathione depletion and necrosis, it was found that when glutathione was depleted by pretreatment with diethyl maleate (0·6 ml/kg) the predominant response to 0·2 ml $CHCl_3$/kg was an accumulation of inflammatory cells in the centrilobular area, and necrosis was frequently absent.

Rats pretreated with the enzyme inducers isopropanol, acetone or 3-methylcholanthrene did not show a marked decrease in glutathione after $CHCl_3$ administration, suggesting that the potency of PB may depend on an increase in cytochrome *P*-450 or other specific enzymes which it alone induces. Glutathione depletion in PB-pretreated rats was apparently not due either to glutathione oxidation or to inhibition of glutathione reductase, since there was no decrease in glutathione in kidney, blood or lens or increase of oxidized glutathione in these tissues or in the liver. The haloalkanes CCl_4, CH_2Cl_2, CCl_3Br, $CHBr_3$, CHI_3 and CH_3CCl_3 at dose levels in the range 0·2–1·0 ml/kg failed to produce a marked depletion of glutathione in PB-pretreated rats, and C^2HCl_3 (the deuterium analogue of $CHCl_3$), although active in this respect, was less effective than $CHCl_3$. These findings suggested that glutathione depletion by $CHCl_3$ might be due to the formation of a reactive metabolite other than $[CCl_3]^{\cdot}$, the lower potency of C^2HCl_3 indicating that the hydrogen atom of $CHCl_3$ was involved in some way.

CANCER RESEARCH

3135. Another iodine hypothesis

Stadel, B. V. (1976). Dietary iodine and risk of breast, endometrial, and ovarian cancer. *Lancet* **I**, 890.

It has been suggested that variations in the incidence of large-bowel cancer may be related to dietary differences (*Cited in F.C.T.* 1971, **9**, 900), and the author cited above now suggests that the parallel incidence rates of breast, endometrial and ovarian cancer in different regions of the world are also due to dietary rather than genetic factors. Regional differences in the incidence of breast cancer appear to vary with the prevalence of goitre, and a high incidence of goitre has been observed in women with endometrial cancer. In addition, young women at relatively high risk for endometrial and breast cancer have relatively high levels of oestrogen, although there is no evidence for a hyperoestrogenic state in women at risk for ovarian cancer. Since a deficiency in dietary iodine is associated with goitre (*ibid* 1976, **14**, 356) and since the diets of populations at higher risk for breast, endometrial and ovarian cancers tend to be deficient in iodine, the effects on the endocrine system of a diet relatively low in iodine may be responsible for the increased incidence of these cancers.

TERATOGENESIS

3136. Warfarin under suspicion

Fourie, D. T. & Hay, I. T. (1975). Warfarin as a possible teratogen. *S. Afr. med. J.* **49**, 2081.

The birth of offspring showing various types of defective development has been described after maternal treatment with the indanedione anticoagulant, warfarin (*Cited in F.C.T.* 1972, **10**, 275), and evidence incriminating warfarin in embryopathic conditions is increasing.

The authors of the paper cited above have traced six babies with congenital stippled epiphyses (chondrodysplasia punctata) born to mothers who had been taking warfarin during pregnancy. They report the case of a woman aged 37 who was treated, after a heart-valve replacement, with 5 mg warfarin daily, together with digoxin, frusemide, verapamil and potassium supplements. This treatment was continued during pregnancy, the warfarin being replaced by parenteral heparin only 1 wk before delivery. The woman gave birth to an infant with a hypoplastic upturned nose, mild choanal stenosis, short fingers with dystrophic nails and stippled calcification in the epiphyses of the cuboids, proximal femora, sacrum and vertebral column. This is the fourth such case to be reported in South Africa, although warfarin treatment during pregnancy is relatively uncommon, and warfarin is strongly suspected of being responsible for the malformation observed in this case.

Fd Cosmet. Toxicol. Vol. 15, p. 163. Pergamon Press 1977. Printed in Great Britain

MEETING ANNOUNCEMENTS

BIOLOGICAL OXIDATION OF ORGANIC NITROGEN

The 2nd International Symposium on the Biological Oxidation of Nitrogen in Organic Molecules will be held at Chelsea College, University of London, from 19 to 23 September 1977. Contributions are invited on the occurrence, mechanism and pharmacological and toxicological consequences of the biological oxidation of nitrogen-containing compounds.

Enquiries should be addressed to: Dr. J. W. Gorrod, Chelsea College Annexe, 271 King Street, London, W.6.

PROPOSED PAH MEETING

It is proposed that Battelle's Columbus Laboratories will hold a Second International Symposium on Polynuclear Aromatic Hydrocarbons in September 1977 in Columbus, Ohio. The objectives of the meeting are to bring together scientists working or interested in this field and to provide an informal forum for the exchange of ideas and experimental data.

This 3-day meeting will follow a format similar to that of the First International Symposium, held in October 1975. Dr. P. W. Jones, Associate Manager, Organic and Structural Chemistry Section, and Dr. R. I. Freudenthal, Associate Manager, Pathology/Pharmacology–Toxicology Section, will again be the Co-chairmen responsible for the Chemistry and Analysis and the Biology sessions, respectively. It is envisaged that Chemistry and Analysis sessions will alternate with Biology sessions, and that in addition to approximately 30–40 20-minute presentations there will be a smaller number of 40-minute presentations consisting of overviews or major reports of work.

Questions concerning the meeting should be directed to the appropriate Co-chairman at Battelle, Columbus Laboratories, 505 King Avenue, Columbus, Ohio 43201, USA.

Fd Cosmet. Toxicol. Vol. 15, p. 165. Pergamon Press 1977. Printed in Great Britain

FORTHCOMING PAPERS

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

Phenolic antioxidants and the inhibition of hepatotoxicity from *N,N*-dimethylnitrosamine formed *in situ* in the rat stomach. By B. D. Astill and L. T. Mulligan.

Regeneration of rat liver in the presence of essential oils and their components. By L. L. Gershbein.

Placental and mammary transfer of chlorinated fatty acids in rats. By H. M. Cunningham and G. A. Lawrence.

Effect of benomyl and benomyl hydrolysis products on *Tetrahymena pyriformis*. By P. W. Rankin, J. G. Surak and N. P. Thompson.

Determination of polychlorinated dibenzofurans in tissues of patients with 'Yusho'. By J. Nagayama, Y. Masuda and M. Kuratsune.

The fate of ochratoxin A in rats. By F. C. Chang and F. S. Chu.

Metabolism and toxicity of halogenated carbanilides: Absorption, distribution and excretion of radioactivity from 3,4,4'-trichloro[^{14}C]carbanilide (TCC) and 3-trifluoromethyl-4,4'-dichloro[^{14}C]carbanilide (TFC) in rats. By R. A. Hiles.

Pulmonary deposition, translocation and clearance of inhaled neutron-activated talc in hamsters. By A. P. Wehner, C. L. Wilkerson, W. C. Cannon, R. L. Buschbom and T. M. Tanner.

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