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Toxic Liver Injury

THE METABOLISM OF DIMETHYLNITROSAMINE

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In a previous paper (Barnes & Magee, 1954) dimethylnitrosamine was shown to cause acute centrilobular necrosis of the liver in the rat, mouse, guinea pig, rabbit and dog, when given in doses of the order of 25 mg./kg. body weight. No gross abnormality was found in organs or tissues other than the liver. In long-term feeding experiments, when dimethylnitrosamine was added to the diet of rats at a level of 50 parts per million, a high incidence of malignant hepatic tumours occurred, with no evidence of primary tumours elsewhere. In similar experiments with rabbits no tumours were produced (Magee & Barnes, 1956).

It appears that dimethylnitrosamine is a highly specific liver poison, and the present paper describes experiments which were performed in the hope of throwing some light on the mechanism of this specificity. A polarographic method for the estimation of dimethylnitrosamine in tissues and body fluids has been developed in this Laboratory (Heath & Jarvis, 1955) and this method was used to study the distribution of the compound in the animal body after administration, and its excretion.

METHODS

The animals used were albino rats of the Porton strain, mice of the C. 57 Chester Beatty strain and commercially obtained albino mice, and cross-bred rabbits. The rats and mice were maintained on M.R.C. diet 41 (Bruce & Parkes, 1949) and the rabbits on M.R.C. diet 18 (Bruce, 1947). Dimethylnitrosamine, when given orally, was diluted with water and administered by a metal cannula attached to a syringe. For parenteral administration dilution was with 0·9% NaCl. Intravenous injection was by tail vein in rats and by ear vein in rabbits, both procedures being without anaesthesia, unless otherwise stated. The compound was estimated in tissues and body fluids by the polarographic method of Heath & Jarvis (1955). In determinations of the total body content of dimethylnitrosamine in rats the animals were killed by intraperitoneal injection of Na pentobarbital (Veterinary Nembutal, Abbott) and immediately frozen by immersion in liquid N_2. The rats were then transferred to a bucket and fragmented by percussion with a heavy brass ram-rod. The fragments were disintegrated further in 2·5% (w/v) sulphosalicylic acid by means of an Atomix blender (Measuring and Scientific Equipment Ltd., London, S.W. 1), and the resulting tissue suspension was made up to a known volume and centrifuged. Dimethylnitrosamine was estimated in a suitable portion of the supernatant fluid. A similar procedure was used for whole mice except that they were killed by cervical dislocation, and it was found possible to disintegrate them in the blender without preliminary freezing. In experiments on the excretion of dimethylnitrosamine, rats were placed in conventional metabolism cages constructed of metal coated with bakelite. Urine and faeces were collected separately by means of a glass separator. Total hepatectomy was performed on rats by Dr K. K. Cheng, using a two-stage technique (Cheng, 1951). The second stage of the operation on the previously prepared animals was carried out under ether anaesthesia. Control animals of similar weight to that of the hepatectomized rats underwent a sham operation, in which the abdomen was opened and closed. Dimethylnitrosamine was injected intravenously into the hepatectomized and the sham-operated control rats at operation, while they were still under anaesthesia. Total nephrectomy on rats was performed by Dr J. M. Barnes, and again control animals underwent a sham operation, both

procedures being carried out under ether anaesthesia. Blood samples were obtained from an ear vein in rabbits without anaesthesia and from the inferior vena cava, by means of a syringe, in rats immediately after they had been killed with coal gas. Heparin was always used as anticoagulant. Plasma was separated from red cells by centrifuging, and dimethylnitrosamine was determined in known volumes of plasma, red cells and whole blood respectively. Dialysis of 3 ml. samples of rat plasma was carried out in cellophan sacs against 5 l. of 0·9% NaCl, for 22 hr. at 2°, with mechanical stirring.

RESULTS

Metabolism of dimethylnitrosamine

Recovery of dimethylnitrosamine from the whole animal. Twelve mice weighing 20 g. were given 1 mg. of dimethylnitrosamine (purified by distillation, b.p. 151°) orally. Three were killed immediately and analysed for dimethylnitrosamine. The remaining nine animals were placed in glass beakers so that all excreta could be collected and were killed in groups of 3 at 0·5, 1 and 4 hr. after injection. Immediately after death each animal, together with any excreta produced during the experimental period, was analysed. As shown in Table 1, good recovery of the total dose was obtained at zero time, and the amounts of dimethylnitrosamine recovered fell off quite uniformly with time, until at 4 hr. none was recovered.

In a second experiment, 4 rats were given orally 50 mg. of dimethylnitrosamine/kg. body wt. One was killed immediately by intraperitoneal Nembutal and the remainder at 4, 8 and 24 hr. after injection. Again good recovery was obtained at zero time, the amount of the compound recovered fell off with time, and none was found at 24 hr. (Table 1).

Excretion of dimethylnitrosamine. Two rats were each given 10 mg. of dimethylnitrosamine orally (50 mg./kg. body wt.) and were placed in the same metabolism cage. Urine and faeces were collected at known times after injection and analysed for dimethylnitrosamine. In a similar experiment two pairs of rats were given 100 mg. of dimethylnitrosamine (500 mg./kg. body wt.) intravenously, and the combined urine from each pair was analysed after 24 hr. The results are shown in Table 2.

These results suggested either that the compound was metabolized by the animal or that it became combined with a tissue constituent in such a way that it could no longer be extracted with sulphosalicylic acid. The latter possibility was not easily

Table 1. *Recovery of dimethylnitrosamine from the whole animal*

Rats and mice received 50 mg. of dimethylnitrosamine/kg. body wt. orally. In all cases, except the rat killed 24 hr. after administration, excreta produced during the experimental period were included with the whole animal for analysis.

Mice				Rats		
		Dimethylnitrosamine recovered, as percentage of injected dose				
No. of animals	Time after injection (min.)	Mean	Range	No. of animals	Time after injection (hr.)	Dimethylnitrosamine recovered, as percentage of injected dose
3	0	97·1	96·0–99·5	1	0	94
3	30	65·4	57·5–70·0	1	4	57
3	60	33·6	31·2–36·0	1	8	34
3	240	0	—	1	24	0

Table 2. *Excretion of dimethylnitrosamine*

In Expt. *A*, one pair of rats received 50 mg. of dimethylnitrosamine/kg. body wt. orally, providing a total of 20 mg. for excretion. In Expt. *B*, two pairs of rats received 500 mg. of dimethylnitrosamine/kg. body wt. intravenously, providing 200 mg. for excretion from each pair.

Expt. *A*			Expt. *B*		
	Dimethylnitrosamine recovered (μg.)			Dimethylnitrosamine recovered (from urine only)	
Time after injection (hr.)	Urine	Faeces	Time after injection (hr.)	First pair (mg.)	Second pair (mg.)
3·5	96	No sample	24	9·45	21·87
4·5	30	No sample	—	—	—
23	208	Nil	—	—	—
24	Nil	Nil	—	—	—
Total	334	0	—	—	—
Percentage of total injected dose	1·7	0	Percentage of total injected dose	4·7	11

Table 3. *Recovery of dimethylnitrosamine from rats which had undergone various experimental procedures*

All animals received 50 mg. of dimethylnitrosamine/kg. body wt. intravenously.

Experimental procedure	Time after injection of dimethylnitrosamine (hr.)	Percentage of injected dose recovered from whole animal	
		Experimental	Control
Total hepatectomy. Controls sham-operated. Dimethylnitrosamine injected at operation	4	99	75
	6	98	73
Total nephrectomy 24 hr. before injection of dimethylnitrosamine. Control sham-operated	4	71	62
Total nephrectomy 7 hr. before injection of dimethylnitrosamine. Control sham-operated	19	4	2
Dimethylnitrosamine 50 mg./kg. body wt. orally 24 hr. before injection of a second dose intravenously. Two normal rats as controls	6	74	35 25

investigated, since procedures adequate to degrade the main tissue components would also cause chemical breakdown of dimethylnitrosamine. Alkaline hydrolysis of the acid-extracted tissue residue was the method of choice, since dimethylnitrosamine is more resistant to alkali than to acid. Liver from a rat killed 4 hr. after an oral dose of 50 mg./kg. body wt. was extracted with sulphosalicylic acid in the usual way, and the residue was re-extracted until the washings no longer contained detectable dimethylnitrosamine. This residue was then suspended in 50 ml. of water, to which was added 50 g. of KOH, and the mixture was refluxed for 2·5 hr. The resulting digest was analysed. A similar experiment was done with two mice, which were killed 45 min. after receiving 50 mg. of dimethylnitrosamine/kg. body wt. by intraperitoneal injection. In this experiment the livers and also the rest of the bodies were acid-extracted separately and the residue was refluxed with KOH for 4 hr. Small quantities of dimethylnitrosamine were recovered from the acid extracts, but none from the alkaline digests.

In a control experiment duplicate samples containing 50 μg. of dimethylnitrosamine were refluxed with KOH of the same concentration for 4 hr., after which 39 and 38 μg. respectively were recovered. Since as little as 0·25 μg. of dimethylnitrosamine/ml. of sample can be detected by the polarographic method, under the conditions of the experiment not more than 10 μg. could have been present in a combination resistant to acid extraction. These results suggested that the greater part, if not all, of the injected dose was metabolized, apart from the small amount excreted in the urine.

Experiments to investigate the site of metabolism of dimethylnitrosamine. Two rats which had undergone total hepatectomy were given 50 mg. of dimethylnitrosamine/kg. body wt. intravenously, and two other animals received the same dose after sham operations. The hepatectomized rats were killed at 4 and 6 hr. respectively after injection of dimethylnitrosamine, and the controls were killed at the same times. The total body content of the compound in the four animals was determined. As shown in Table 3 the amount of the compound recovered from the hepatectomized animals suggested that none had been metabolized, as it was well within the range of error of the method. The control animals, on the other hand, showed substantial loss in spite of the immediately preceding anaesthetic and abdominal incision. The effect of previous damage to the liver was investigated by giving a rat 50 mg. of dimethylnitrosamine/kg. body wt. orally, a dose sufficient to produce extensive necrosis (Barnes & Magee, 1954), and then, 24 hr. later, 50 mg./kg. intravenously. Two control animals were given the same dose intravenously, and all three were killed 6 hr. later. The percentage recoveries shown in Table 3 indicate that previous damage to the liver causes considerable reduction in the rate at which dimethylnitrosamine disappears from the body. The effect of total nephrectomy was then tried. One

Table 4. *Effect of SKF 525A on the metabolism of dimethylnitrosamine by the mouse*

The animals received an oral dose of SKF 525A (mg./kg. body wt.) as indicated, followed by intraperitoneal injection of 50 mg. of dimethylnitrosamine/kg. body wt. 45 min. later, and were killed after a further 90 min.

Dose of SKF 525A	Recovery of dimethylnitrosamine from whole mouse (% of injected dose)
100	19
100	17
—	9
—	11
200	48
—	0
400	58
—	0

nephrectomized and one control animal were given 50 mg. of dimethylnitrosamine/kg. body wt. intravenously 7 hr. after the operation and killed 19 hr. after the injection. The other nephrectomized animal and its control received the same dose 24 hr. after operation, and were killed 4 hr. later. It will be seen from Table 3 that total nephrectomy reduces the rate of disappearance of dimethylnitrosamine only slightly, and that virtually none was recovered 24 hr. after administration. These experiments strongly suggested that the liver is the most important, perhaps the only, site of metabolism of dimethylnitrosamine.

Effect of 2-diethylaminoethyl diphenylpropylacetate hydrochloride (SKF 525A) on the metabolism of dimethylnitrosamine by the whole animal. Two mice were given 100 mg. of SKF 525A/kg. body wt. in water orally and 40 min. later 50 mg. of dimethylnitrosamine/kg. body wt. intraperitoneally. Two control mice received the same treatment except that water replaced the SKF 525A. In another experiment two mice received 200 and 400 mg. of SKF 525A/kg. body wt. respectively, followed by the same dose of dimethylnitrosamine as before. The amounts recovered from the whole mice are shown in Table 4.

Distribution of dimethylnitrosamine in the body

Recovery of dimethylnitrosamine from tissues and organs. Four rats were given 50 mg. of dimethylnitrosamine/kg. body wt. and were killed by intraperitoneal Nembutal 0·5, 1, 2 and 4 hr. respectively after injection. Various organs, as indicated in Table 5, were removed and analysed individually. The remainder of the carcass was also analysed. From the results shown in Table 5 it was concluded that the compound was distributed throughout the organs and tissues of the body. In another experiment, two rats were given 50 mg./kg. body wt. and the concentration in some organs was compared with that in the blood 4 hr. after injection. The results are also shown in Table 5, where it can be seen that the concentrations are again very similar, including that in the blood. This experiment indicated that the dimethylnitrosamine found in the various organs could not have been due merely to their content of blood.

The distribution between plasma and red cells was studied in 4 rats and 1 rabbit, all of which received 500 mg. of dimethylnitrosamine/kg. body wt. intravenously. Blood was taken from the rats at 2 hr. and from the rabbit at 30 min. and 2 hr. after injection. The concentrations of dimethylnitrosamine in red cells, plasma and whole blood were about equal.

Recovery of dimethylnitrosamine from the circulating blood. A rabbit weighing 1·6 kg. was given 80 mg. of dimethylnitrosamine by intravenous injection. Blood for analysis was withdrawn at intervals from an ear vein. Fig. 1 shows log (concentration of dimethylnitrosamine) plotted against time. There is a rapid initial reduction in concentra-

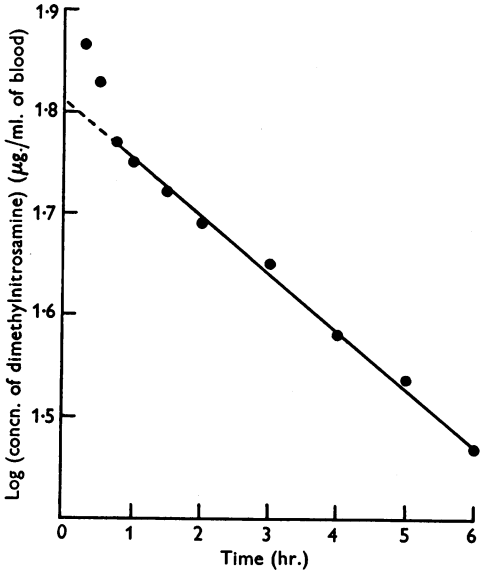

Fig. 1. Fall in concentration of dimethylnitrosamine in the circulating blood of a rabbit weighing 1·6 kg. after the intravenous injection of 80 mg. of the compound.

Table 5. *Distribution of dimethylnitrosamine in tissues and organs*

Rats were given 50 mg. of dimethylnitrosamine/kg. body wt. intravenously.

Expt.	No. of animals	Time after injection (hr.)	Concn. of dimethylnitrosamine in tissues (μg./g. wet wt.)							
			Blood	Liver	Kidney	Spleen	Brain	Heart and lungs	Intestinal tract	Carcass
1	1	0·5	—	78	82	62	39	74	—	62
	1	1	—	35	37	33	32	38	28	28
	1	2	—	33	39	31	34	33	14	22
	1	4	—	35	39	33	40	37	—	25
2	2	4	23	25	28	20	—	26	—	—
			20	20	26	25	—	27	—	—

tion followed by a more gradual and linear fall. On extrapolation to zero time this line cuts the ordinate at a point corresponding to 64 μg. of dimethylnitrosamine/ml. of blood.

Possibility of binding of dimethylnitrosamine to plasma protein. Rat plasma (3 ml.) was dialysed for 22 hr. at 2° against 5 l. of 0·9 % NaCl to which dimethylnitrosamine had been added to make a concentration of 40 μg./ml. Analysis showed that the final concentrations of dimethylnitrosamine in the fluid in the sac and in the surrounding fluid were 41 and 43 μg./ml. respectively. In another experiment, blood was taken from a rat which had been given 500 mg. of dimethylnitrosamine/kg. body wt. intravenously 2 hr. previously. The plasma was separated and dialysed against 0·9 % NaCl under the same conditions as before. The initial concentration of dimethylnitrosamine found inside the sac was 672 μg./ml. and the final concentration was zero. It was concluded that dimethylnitrosamine is not bound to plasma protein.

DISCUSSION

The results of the experiments on the recovery and excretion of dimethylnitrosamine from the whole animal strongly suggest that the compound is rapidly metabolized. The assumption that satisfactory recovery of dimethylnitrosamine from the tissues can be obtained by extraction with sulphosalicylic acid is based on the work of Heath & Jarvis (1955), who studied the addition of the compound to tissue minces *in vitro* and also recovery from the whole animal immediately after injection. A possible explanation for the progressive decrease in the amount recovered from the whole animal is that it becomes bound to a tissue constituent in such a way as to be no longer extractable with the acid. When mouse liver and remaining carcass were refluxed with strong alkali after thorough extraction with sulphosalicylic acid, no further recovery was obtained. The conditions used would cause saponification of the fat and considerable, if not complete, hydrolysis of the protein, as well as some destruction of the dimethylnitrosamine itself. In the nature of the experiment, conclusions can only be drawn within the limits of sensitivity of the method, and it may be said that probably not more than 1 % of the injected dose can have been bound to tissue constituents. This, of course, would not apply to a form of combination resistant to the alkali treatment. Probably some dimethylnitrosamine is lost through the lungs, but it seems unlikely that the proportion of the total dose excreted in this way can be very large. This assumption is supported by the very high recovery obtained from the rats whose livers had been removed (Table 3).

Dutton & Heath (1956), in this Laboratory, have treated rats and mice with dimethylnitrosamine labelled with ^{14}C and have shown that a large fraction of the radioactivity can be recovered as expired carbon dioxide. This provides conclusive evidence that the compound is, in fact, metabolized.

The almost complete recovery of the injected dose from the totally hepatectomized rats suggests a dominant, if not an exclusive, role for the liver in the metabolism of the compound. Such an experiment is, of course, impossible to control adequately, and it might be that removal of the whole liver could lead to such general depression of the animal that extrahepatic metabolism of dimethylnitrosamine would be reduced to undetectable levels. More dimethylnitrosamine was recovered from the controls to the hepatectomized rats than from normal animals. This slower rate of disappearance of the compound is probably related to the immediately preceding general anaesthetic and abdominal incision. Birnie & Grayson (1952) have shown that general anaesthesia causes reduction in liver temperature and liver blood flow in the rat, and that several hours elapse before normal conditions return. Dr H. B. Stoner (unpublished work) has shown that abdominal incision exacerbates the changes due to anaesthesia. The finding of marked reduction in the rate of disappearance of the poison from rats with previously damaged livers, coupled with the almost negligible effect of removal of the kidneys, is consistent with the idea that the liver is mainly responsible for its metabolic alteration. Support for this conclusion is provided by the work of Dr M. Vandekar (unpublished work) in this Laboratory. He has found that dimethylnitrosamine disappears after addition to rat-liver slices respiring *in vitro*, but not after addition to kidney, spleen or brain slices.

The experiments in which dimethylnitrosamine was measured in different organs and tissues do not indicate any concentration of the compound in the liver which could be held responsible for its toxic action (Table 5). In fact the amounts per unit weight of tissue are remarkably similar in most of the viscera. The lower figures found in the gastrointestinal tract can probably be explained by the effect of the intestinal contents, which might add to the weight of the sample without increasing the amount of dimethylnitrosamine proportionately. This view is supported by the absence of the compound in the faeces. Similar reasoning could be applied to the effect of bone and hair in the carcass. The concentration in the brain is lower than in the other viscera at first, but later reaches the general level (Table 5); this is probably due to the 'blood–brain barrier'. The concentration in the blood was found to be closely similar to that in several of the organs (Table 5) and it is therefore apparent that the blood in a tissue could not account for the amount of the compound found in it. Further conclusions on the distribution in the body can be drawn from the

curve of disappearance of dimethylnitrosamine from the circulating blood of the rabbit (Fig. 1). The initial rapid fall presumably represents equilibration of the compound in the body as well as loss by metabolism, and the second linear phase further metabolic loss. Both phases, of course, must include the small losses due to excretion. Extrapolation of the second part of the curve to zero time gives a figure of 64 μg. of dimethylnitrosamine/ml. of blood. Since the concentrations in blood and in plasma are about equal, this indicates a concentration of 69 μg./ml. of plasma water, if a water content in plasma of 93 % is assumed (Soberman, 1950). This figure may be regarded as the initial concentration in plasma water if equilibration had been instantaneous, and it may be compared with the concentration that would be expected if the compound was uniformly distributed in the total body water. If a value for total water in the rabbit of 74 % of the body wt. (Soberman, 1950) is taken and again immediate equilibration is assumed, the concentration would be 67 μg./ml. after injection of 80 mg. of dimethylnitrosamine into the rabbit weighing 1·6 kg. This is in excellent agreement with the value found by extrapolation. Similar curves have been reported by Soberman (1950) after the intravenous injection of antipyrine into rabbits. This compound was shown to be rapidly distributed throughout the total body water and, like dimethylnitrosamine, it is metabolized by the animal. It appears, therefore, that dimethylnitrosamine is uniformly distributed throughout the body water and consequently that it must penetrate into the tissue cells. If this is so some explanation is required for the occurrence of selective damage to the liver, since the compound must be relatively innocuous to the other cells. A possible explanation is that a metabolite of dimethylnitrosamine, formed only inside the liver cell, is the toxic substance. This would be present in the liver in higher concentration than elsewhere. Such a metabolic process has been proposed by Miller & Miller (1953) for *p*-dimethylaminoazobenzene in liver carcinogenesis, and also by Nygaard, Eldjarn & Nakken (1954) to explain the specificity of action of thioacetamide on the liver. In a previous paper (Magee & Barnes, 1956) attention has been drawn to similarities in the pathological effects due to *p*-dimethylaminoazobenzene, thioacetamide and dimethylnitrosamine, both in acute and chronic experiments. It is well known that many substances foreign to the body are metabolized only by the liver, and much recent work by Brodie *et al.* (1955) has shown that many of these metabolic transformations, including several demethylation reactions, are inhibited by the compound SKF 525A (2-diethylaminoethyl diphenylpropylacetate hydrochloride) (Cook, Toner & Fellows, 1954). The finding that this compound inhibits the rate of disappearance of dimethylnitrosamine may be significant (Table 4), but the dose required was high and it may be an unspecific effect.

In recent years doubt has been cast on the suitability of the term 'detoxication' when applied to the metabolism of foreign substances. Thus Peters (1952) has developed the idea of a 'lethal synthesis' in which an innocuous substance may be transformed into a toxic one, rather than the reverse. For example, it has been shown that the liver can convert schradan (OMPA) and parathion from weak into strong inhibitors of cholinesterase (Myers, Mendel, Gersmann & Ketelaar, 1952; Dubois, Doull & Coon, 1950; Cheng, 1951). Similar ideas have been advanced by Popper, de la Huerga & Koch-Weser (1954), who suggested that the mechanism of the liver necrosis due to bromobenzene may be a 'conditioned amino acid deficiency' in which the liver is depleted of sulphur-containing amino acids by the excretion of mercapturic acid, and that 'the process of detoxication as such may become detrimental to the liver'.

The goal of any investigation into pathological processes should be the elucidation of the 'biochemical lesion', as defined by Peters (1936). It seems likely that the train of chemical events leading to the death of the liver cell after administration of dimethylnitrosamine is related, in time at least, to the mechanism of metabolism of the compound. Since metabolism of the compound probably starts immediately after injection (Table 2), the biochemical lesion may well occur equally early.

SUMMARY

1. The metabolism, distribution in the body and excretion of dimethylnitrosamine were studied after its administration to rats, mice and rabbits. It has been shown previously that this compound is a selective liver poison in these species, and that it can produce malignant primary liver tumours in the rat.

2. Evidence is presented which suggests that dimethylnitrosamine is rapidly metabolized, and that the liver is the main, probably the only, organ concerned.

3. The compound is distributed uniformly in most tissues of the body, and there is reason to believe that it penetrates into the intracellular space. There is no selective concentration in the liver.

4. SKF 525A (2-diethylaminoethyl diphenylpropylacetate hydrochloride) inhibits the metabolism of dimethylnitrosamine in the mouse, but only when given in large doses.

5. The findings are discussed in relation to the pathogenesis of the acute liver necrosis caused by dimethylnitrosamine.

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The Activity for Chicks of some Vitamin B_{12}-like Compounds

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In the last few years several compounds similar to the cobalamins have been found in nature; in addition to these, new analogues of vitamin B_{12} have been produced by guiding the biosynthetic activities of certain micro-organisms. The preparation, chemical nature and properties of these substances have been reviewed by Kon (1955); they differ from the cobalamins in the nature of the nucleotide part of the molecule. Since many of them have growth-promoting activity for micro-organisms, it was of interest to determine whether or not they were of use to higher animals. The comparatively large amount of material necessary for an animal test limited the number of samples examined to those which could be relatively easily prepared, but we have been able to test on chicks five of the benziminazole series, three purine derivatives, factor B (which has no nucleotide) and factor D (of which the chemical constitution is unknown).

EXPERIMENTAL

Substances tested

Naturally occurring vitamin B_{12}-like compounds. Factors A, B, D and pseudovitamin B_{12} were prepared from calf faeces. Vitamin $B_{12\,III}$, later identified as the 5-hydroxybenziminazole analogue of vitamin B_{12} (Robinson, Miller, McPherson & Folkers, 1955; Friedrich & Bernhauer, 1956), was isolated from sewage by Friedrich & Bernhauer (1953).

Unnatural analogues of vitamin B_{12}. These were prepared by 'shunting' the biosynthesis of vitamin B_{12} by the addition to the medium of the appropriate intermediate (Fantes & O'Callaghan, 1954; Ford, Holdsworth & Kon, 1954). The samples tested were the analogues containing benziminazole, 5 (or 6)-methylbenziminazole, 5:6-dichlorobenziminazole, 2:3-naphthiminazole or 2:6-diaminopurine in the nucleotide.

Preparation for test. All these substances, with the exception of factor D, were purified by repeated ionophoresis and chromatography and contained less than 0·5% of vitamin B_{12}. Factor D and some early samples of factor A were purified by chromatography only. Solutions of the analogues were prepared and standardized by measuring the absorption at 361 mμ.; the $E^{1\%}_{1\,cm.}$ value was assumed to be the same as for cyanocobalamin.

Biological tests

Method and diet. In general, the method of Coates, Harrison & Kon (1951) was followed. Rhode Island Red × Light Sussex chicks were used, bred from hens given a diet low in vitamin B_{12}. Each experimental group consisted of about 10–15 chicks of mixed sexes. The test materials were given either by intramuscular injection twice weekly or were added to the basal diet, which had the following percentage composition: ground maize, 37·8; ground barley, 20; defatted soya grits, 35; dried grass, 3; bone meal, 1·5; limestone flour, 1; arachis oil, 1; salt mixture, 0·7 (NaCl, 0·672 part; $MnSO_4,4H_2O$, 0·028 part). To each 100 g. of diet were added vitamin A (680 i.u.) and vitamin D_3 (64 i.u.) dissolved in the arachis oil, and a vitamin supplement consisting of riboflavin (0·77 mg.), nicotinic acid (5·5 mg.), biotin (0·02 mg.), folic acid (0·08 mg.), thiamine hydrochloride (0·33 mg.), pyridoxine hydrochloride (0·44 mg.) and calcium pantothenate (1·65 mg.).