Toxic Liver Injury

INHIBITION OF PROTEIN SYNTHESIS IN RAT LIVER BY DIMETHYLNITROSAMINE *IN VIVO*

By P. N. MAGEE
Toxicology Research Unit, Medical Research Council Laboratories, Woodmansterne Road, Carshalton, Surrey

(*Received* 18 *November* 1957)

Dimethylnitrosamine has been shown to cause acute haemorrhagic centrilobular necrosis of the liver in several mammalian species (Barnes & Magee, 1954). It can also produce malignant tumours of the liver in the rat after prolonged administration (Magee & Barnes, 1956). The compound is rapidly metabolized *in vivo*, probably exclusively by the liver (Magee, 1956; Dutton & Heath, 1956). It appears to be uniformly distributed throughout the body water soon after administration, without preferential accumulation in the liver. The metabolism of the compound *in vitro* has been studied (Magee & Vandekar, 1958) and found to occur only in the non-mitochondrial part of the cytoplasm. The metabolism *in vitro* has many features in common with that of the carcinogenic azo dyes and many other foreign compounds. In all these systems the microsome fraction is responsible for the activity. Some histochemical and chemical changes in rat liver during the development of the necrosis have been studied (P. N. Magee, in preparation) in amplification of earlier histological work (Barnes & Magee, 1954). The development of the lesion in the liver after necrotizing doses of dimethylnitrosamine (25–50 mg./kg. body wt.) is regular and reproducible. Three hours after the dose there is no constant microscopical evidence of damage, although there may be a discernible loss of cytoplasmic basophil material. At 6 hr. there is definite loss of cytoplasmic basophilia in the centrilobular zones but the nuclei appear intact. At the same time there is reduction in chemically determined ribonucleic acid but not in deoxyribonucleic acid, and the content of the latter in isolated nuclei is not significantly altered. As the necrosis develops, the parenchymal cell nuclei disintegrate, there is increase of stainable and chemically determined lipid and the hepatic glycogen is greatly reduced. The centrilobular zonal character of these changes must be emphasized since there is evidence of active regeneration in the periportal parenchyma 48 hr. after the dose. It was suggested that the point of attack of dimethylnitrosamine or a metabolite might be on the cytoplasmic basophil material. This material has been shown to correspond to the endoplasmic reticulum as seen in the electron microscope (Porter, 1954), which in turn is believed to correspond with the microsome material isolated by differential centrifuging (Kuff, Hogeboom & Dalton, 1956). In view of these findings the effect of dimethylnitrosamine on protein synthesis in the liver was studied, since the microsomes are believed to play a dominant part in this function (Hultin, 1950; Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1950; Keller, Zamecnik & Loftfield, 1954; Simkin & Work, 1957*a*, *b*). This work is reported in the present paper. A preliminary account has already been given (Magee, 1957).

MATERIALS AND METHODS

Dimethylnitrosamine. This was obtained commercially and purified by distillation (b.p. 151°).

Isotopes. Mixed [^{14}C]amino acids were obtained as a hydrolysate of ^{14}C-labelled *Chlorella* protein (Catch, 1954) with a specific activity of approx. 100 μc/mg. of protein. The hydrolysate was dissolved in 0·9% NaCl before injection. ^{32}P was obtained as a solution of inorganic orthophosphate in sterile phosphate–saline buffer (pH 7·4) and was kindly standardized by Dr H. B. Stoner by the method of Stoner, Threlfall & Green (1952). All isotopically labelled material was supplied by The Radiochemical Centre, Amersham, Bucks.

Animals. Albino rats of the Porton strain were maintained on M.R.C. diet no. 41 (Bruce & Parkes, 1949).

Conduct of animal experiments

Dimethylnitrosamine, suitably diluted with water, was given to the rats by stomach tube. The dose was 50 mg./kg. body wt. Mixed [^{14}C]amino acids were injected into a tail vein without anaesthesia, 0·5 ml. containing approx. 5 μc (50 μg.) being given. Unless otherwise stated, the rats were killed 15 min. after the injection. The ^{32}P solution was injected subcutaneously and the animals were killed 2 hr. later. The details of the experiments are shown in the tables and legends for the figures.

Preparation of tissues

Whole tissues. The rats were killed by a blow on the head followed by bleeding from the neck vessels. Samples of liver and other tissues were removed as quickly as possible

and plunged into liquid N_2. The frozen tissues were then rapidly weighed and disintegrated in water with an all-glass homogenizer (Potter & Elvehjem, 1936) in a cold room at 2°.

Blood. The rats were anaesthetized with sodium pentobarbital (Veterinary Nembutal-Abbot) and the thorax was opened and the great vessels severed. Blood was collected with a Pasteur pipette from the thoracic cavity and transferred to a plastic centrifuge tube containing heparin, and plasma was obtained by centrifuging.

Cell fractionation. The livers were not frozen, but immersed in ice-cold 0·25 M-sucrose. They were then blotted rapidly, weighed and disintegrated in about 10 vols. of 0·25 M-sucrose with a modified homogenizer in which a Perspex pestle rotated at 1500 rev./min. in a smooth-glass test tube with a clearance of 0·25 mm. The tube and pestle were cooled on ice before use and the disintegration took less than 1 min. The suspensions were fractionated by differential centrifuging, essentially according to Schneider (1948), in a refrigerated centrifuge at 0° (Measuring and Scientific Equipment Ltd., Angle 13). Nuclei and cell debris were sedimented at 600 *g* for 10 min. and washed twice with cold 0·25 M-sucrose. The pooled supernatant and washings were then centrifuged at 8500 *g* to bring down the mitochondrial fraction, which was also washed twice with sucrose. The washings were added to the supernatant, from which the microsomal fraction was sedimented at 20 000 *g* for 2 hr. The supernatant, constituting the cell-sap fraction, was removed, and the microsomal pellet was not washed. All operations were carried out at 0°.

Analytical methods

Glycogen. This was determined by the method of Good, Kramer & Somogyi (1933), the glucose being estimated according to Nelson (1944).

Phosphorus. This was determined by a modification of the method of Fiske & Subbarow (1925). Separation of the phosphorus-containing fractions of liver into acid-soluble, lipid and residual phosphorus was done by the procedure of Davidson, Frazer & Hutchison (1951), and partially purified ribonucleic acid (RNA) was prepared according to Davidson & Smellie (1952). Plasma inorganic phosphorus was precipitated by the method of Stoner *et al.* (1952).

Free amino acids in liver. These were extracted by the procedure of Awapara (1948). Their concentration was measured titrimetrically, after trapping in baryta the CO_2 released by ninhydrin, according to Van Slyke, MacFadyen & Hamilton (1941).

Determinations of radioactivity. Radioactivity of tissue proteins was determined according to Simkin & Work (1957*a*). The dry protein powders were assayed at 'infinite thickness' on polythene planchets (Popják, 1950). For the determination of the radioactivity of the free amino acids, the CO_2 released by ninhydrin from a fraction of the extract was trapped in NaOH. A suitable amount of Na_2CO_3 was added so that enough carrier $BaCO_3$ was produced on the addition of excess of $BaCl_2$ to permit assay at 'infinite thickness'. The $BaCO_3$ was collected on weighed sintered-glass disks by filtering the slurry with gentle suction, and was washed with water, ethanol and ether. After drying, the disks were weighed again to give the weight of $BaCO_3$ (about 200 mg.) and assayed for radioactivity. The CO_2 released by ninhydrin from another fraction of the free amino acid extract was determined titrimetrically and the weight of $BaCO_3$ corresponding to this CO_2 was calculated. The specific radioactivity of the free amino acids is expressed as counts/min. of the sample of $BaCO_3$ on the disk multiplied by the wt. of $BaCO_3$ on the disk and divided by the wt. of $BaCO_3$ derived from the free amino acids. Radioactivity of material containing ^{32}P was determined on the solutions of the blue phosphomolybdic acid complex obtained in the Fiske & Subbarow (1925) procedure. In all measurements of radioactivity enough counts were taken to reduce the counting error below 2%. Solid samples were assayed with an end-window counter (G.E.C. EHM25) and liquids with an M6 counter (20th Century Electronics Ltd.).

Where appropriate, experimental results are expressed as the mean ± standard deviation (S.D.) and a statistical comparison of the means is made by Student's *t* test, as modified by Fisher (1934) for small samples.

RESULTS

A preliminary study was made of the incorporation of the mixed [^{14}C]amino acids into the proteins of rat liver and its subcellular fractions at various times after the injection of the tracer (Fig. 1). As expected, the microsomal fraction had the highest activity and the mitochondria the lowest. Incorporation into kidney and spleen proteins was measured in the same animals. The specific radioactivities in kidney were rather higher and in spleen substantially lower than in liver. In the later experiments most of the animals were killed 15 min. after injection of the tracer.

Incorporation of amino acids in rats treated with dimethylnitrosamine was then studied. The specific radioactivities of the liver, kidney and spleen

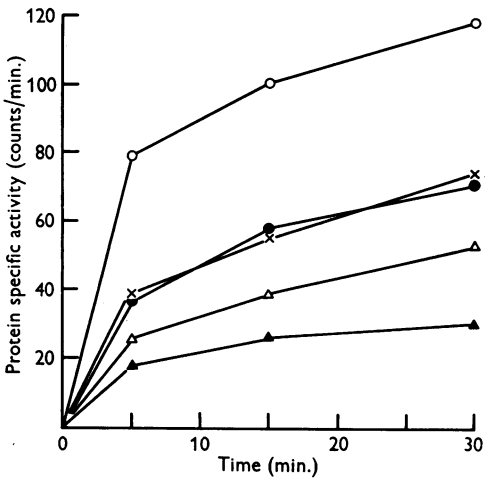

Fig. 1. Uptake of [^{14}C]amino acids into proteins of rat-liver and subcellular fractions. ×, Whole liver; ●, nuclear fraction; △, mitochondrial fraction; ○, microsomal fraction; ▲, cell-sap fraction.

Table 1. *Effect of dimethylnitrosamine on uptake of* [^{14}C]*amino acids into proteins of subcellular fractions of rat liver*

Results are from the same animals as in Fig. 2. Protein specific activities are expressed as counts/min. at infinite thickness under conditions of constant geometry. Number of animals is given in parentheses.

	Protein specific activities (counts/min.)			
		Treated group		
	Untreated group (6)	1 hr. (2)	3 hr. (2)	6 hr. (2)
Original liver suspension	61·5±7·7	62	36	26
Nuclear fraction	60·3±8·9	54	31·5	22
Mitochondrial fraction	26·8±4·8	27	15	11
Microsomal fraction	113·5±23	102	58	38
Cell-sap fraction	42·3±6·2	44	37	20

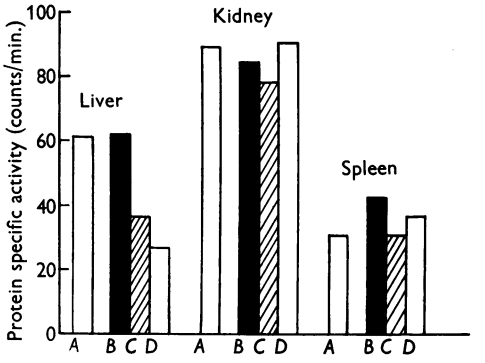

Fig. 2. Effect of dimethylnitrosamine on uptake of [^{14}C]-amino acids into rat proteins. Female rats 200 g. body wt. Pairs were killed 1, 3 and 6 hr. after 50 mg. of dimethylnitrosamine/kg. body wt. was given orally. Six untreated rats were used as controls. All animals received approx. 5 μc of [^{14}C]amino acids intravenously 15 min. before death. *A*, Control; *B*, *C* and *D*, 1, 3 and 6 hr. respectively after treatment with dimethylnitrosamine.

proteins were determined, and the liver suspensions were fractionated into nuclear, mitochondrial, microsomal and cell-sap fractions whose protein specific activities were also determined. The results with whole liver, kidney and spleen are shown in Fig. 2. In the liver there was probably no effect 1 hr. after dimethylnitrosamine, but at 3 and 6 hr. there were marked falls in the specific activities. In the spleen and kidney, however, there was no obvious effect. These findings suggest that dimethylnitrosamine causes an early depression of protein metabolism in the liver, without effect on the other tissues. The protein specific activities of the subcellular fractions of liver are shown in Table 1. The well-known high activity of the microsomal fraction was again observed, and the depression due to dimethylnitrosamine occurs to about the same extent in all the fractions. A similar depression in uptake of labelled amino acids was observed in two rats, 3 hr. after the same dose of dimethylnitrosamine, which were killed 5 min. after intravenous injection of the tracer.

Since there is a possibility of vascular disorder in poisoned livers the observed reduction in incorporation of amino acids may have been caused by failure of the tracer to reach the liver cells. The effect of dimethylnitrosamine on the specific activity of the free amino acids in the liver was therefore examined. The specific activities of whole liver and kidney protein and the concentration and specific activities of the free amino acids were determined. There was no significant alteration in the concentration and specific activity of the free amino acids or in the specific activity of the kidney protein, but the liver-protein activity was reduced by about 50 % in the treated animals (Table 2). It seems reasonable to conclude therefore that this depression is caused by a disturbance of the

Table 2. *Effect of dimethylnitrosamine on the incorporation of amino acids into protein and into the free amino acid pool of rat liver* in vivo

Female rats, 200 g. body wt. The treated group received 50 mg. of dimethylnitrosamine/kg. body wt. orally 3 hr. before death. Both groups received approx. 5 μc of [^{14}C]amino acids intravenously 15 min. before death. The specific activities of the proteins and of $BaCO_3$ derived from CO_2 released from the free amino acids by the ninhydrin method are expressed as counts/min. at 'infinite of thickness' under conditions of constant geometry. Five animals were used in each group.

	Untreated group	Treated group
Specific activity of liver protein	84·2 ±3·9	39·8 ±5·2
Specific activity of kidney protein	98·4 ±18·8	93·6 ±12·8
Free amino acid concentration in liver (μg. of amino N/g. wet wt.)	416·6 ±34·6	426·6 ±35·3
Specific activity of free amino acid	2625 ±182	2597 ±164

Table 3. *Effect of dimethylnitrosamine on the incorporation of* ^{32}P *into some phosphorus-containing fractions of rat liver* in vivo

Female rats, 180–200 g. body wt. The treated group received 50 mg. of dimethylnitrosamine/kg. body wt. orally 6 hr. before death, and both groups received 25 μc of ^{32}P/100 g. body wt. subcutaneously 2 hr. before death. The relative specific activities (R.S.A.) were calculated as follows (S.A., specific activity): R.S.A. acid-soluble $P = \frac{\text{S.A. acid-soluble P}}{\text{S.A. plasma inorganic P}}$; R.S.A. lipid $P = \frac{\text{S.A. lipid P} \times 10}{\text{S.A. acid-soluble P}}$, R.S.A. alkali-soluble $P = \frac{\text{S.A. alkali-soluble P} \times 100}{\text{S.A. acid-soluble P}}$. Six animals were used in each group.

	Concentration (mg. of P/100 g. of liver wet wt.)		Relative specific activity	
Constituent	Untreated group	Treated group	Untreated group	Treated group
Acid-soluble phosphorus	102·6 ±5·5	99·3 ±7·6	1·08 ±0·16	1·28 ±0·22
	$0{\cdot}001 > P$		$0{\cdot}1 > P > 0{\cdot}05$	
Lipid phosphorus	103·3 ±6·9	105·3 ±5·2	1·21 ±0·20	1·41 ±0·13
			$0{\cdot}1 > P > 0{\cdot}05$	
Alkali-soluble phosphorus	111·6 ±6·3	89·5 ±3·1	4·18 ±0·29	4·44 ±0·26
	$0{\cdot}001 > P$		$0{\cdot}2 > P > 0{\cdot}1$	

mechanism of incorporation of amino acids rather than by their failure to reach the liver cell.

The effect of dimethylnitrosamine on the incorporation of ^{32}P into some phosphorus-containing fractions of the liver was then studied. The concentrations and specific radioactivities of the plasma inorganic phosphorus, and liver acid-soluble, lipid and alkali-soluble phosphorus fractions (Davidson *et al.* 1951) were determined. The concentrations and relative specific activities, calculated as shown, are given in Table 3. There are no significant differences between the treated and the control animals except for the concentration of alkali-soluble phosphorus, where there is a significant fall. The heterogeneous nature of the alkali-soluble phosphorus fraction has been emphasized by Davidson & Smellie (1952). Since it contains phosphorus compounds in low concentration but with high specific activity, it seemed possible that these might mask a change in the specific activity of the RNA of the fraction. The incorporation of ^{32}P into partially purified RNA prepared according to Davidson & Smellie (1952) was therefore studied. Ten rats were given ^{32}P, five having had prior treatment with dimethylnitrosamine, the experimental conditions being the same as before. The specific activities of the RNA phosphorus and the total acid-soluble phosphorus were determined and the relative specific activity of the RNA phosphorus was calculated with reference to acid-soluble phosphorus. The results were: dimethylnitrosamine-treated rats, mean $2{\cdot}32 \pm 0{\cdot}15$; control rats, mean $2{\cdot}81 \pm 0{\cdot}31$. The t test gave $0{\cdot}02 > P > 0{\cdot}01$ for the difference between the means, indicating that the reduction in the incorporation of ^{32}P into the RNA fraction is significant.

In view of the marked reduction in incorporation of amino acids into liver protein observed 3 hr. after dimethylnitrosamine (Table 2), determinations of hepatic glycogen were made at the same time, since this constituent has been known for many years to be very sensitive to liver poisons (Drill, 1952). Eighteen rats were used, nine having received dimethylnitrosamine (50 mg./kg. body wt. orally) 3 hr. before death, and the remainder were untreated. Because of the marked diurnal variation in liver glycogen all the animals were killed at the same time of day (12.00 noon). The results were: dimethylnitrosamine-treated rats, mean $4{\cdot}11 \pm 0{\cdot}90$ g./100 g. wet wt.; control rats, mean $4{\cdot}43 \pm 0{\cdot}58$ g./100 g. wet wt. The t test gave $0{\cdot}4 > P > 0{\cdot}3$, indicating no significant difference.

DISCUSSION

The significance of biochemical changes in toxic-liver necrosis is difficult to assess because they may simply indicate death or impending death of cells. The earlier a change can be detected after giving the poison the more likely is it to give a clue to the fundamental biochemical lesion. In the present work the most striking early effect of dimethylnitrosamine was the reduction in incorporation of amino acids into liver protein. Incorporation was reduced by about 50% 3 hr. after a necrotizing dose of dimethylnitrosamine, and this percentage may indicate a greater reduction in the cells which will ultimately die, since the necrosis is zonal.

Current views on protein biosynthesis have been discussed by Borsook (1956) and by Simkin & Work (1957*b*). The necessary factors appear to be free amino acids, an energy source, probably

adenosine triphosphate, and enzyme systems for the activation of the amino acids and for their linkage together in the correct order to form specific proteins. In rat liver the activating enzyme occurs in the cell-sap fraction and the most active incorporation occurs in the microsome protein. Several variants of the template hypothesis have been advanced to explain the replication of specific protein molecules, and RNA, in particular, has been suggested as a possible template. Inhibition of protein synthesis could occur by interference with any of these factors. Obviously the observed effect of dimethylnitrosamine could be explained if the injected [^{14}C]amino acids were not reaching the sites of incorporation in the liver cell and this could be the case if the blood supply to the liver was impaired. This possibility is emphasized by the ischaemic theory of centrilobular necrosis (Himsworth, 1947) and by the finding of Henriques, Henriques & Neuberger (1955) that incorporation of amino acids into liver protein is dependent on blood flow. However, the liver blood flow has been shown to be increased during the development of necrosis due to dimethylnitrosamine (Stoner, 1956). This, together with the finding of normal labelling of the free amino acid pool in the liver (Table 2), makes a failure of access to the pool very unlikely. There is no significant alteration in the concentration of adenosine mono-, di- or tri-phosphate 3 hr. after the same necrotizing dose of dimethylnitrosamine (C. J. Threlfall, personal communication) and there is no marked fall in liver temperature until 24 hr. (Stoner, 1956), suggesting that energy production is not impaired. It appears therefore that the lesion is in the enzymic mechanisms of incorporation. The reduction in uptake of ^{32}P into partially purified RNA is not unexpected in view of the widely found relationship between RNA and protein synthesis (e.g. Brachet, 1955).

The significance of the reduction in incorporation of amino acids into the liver protein and of ^{32}P into RNA is difficult to assess in relation to the mechanism of cell injury. At this time (3–4 hr. after giving the poison) histological damage is minimal or absent and there is no significant change in the concentration of free amino acids (Table 2) or glycogen. At 6 hr. the concentration and relative specific activities of the acid-soluble and lipid phosphorus are still unchanged (Table 3), as well as water content, sodium, potassium and deoxyribonucleic acid (P. N. Magee, in preparation). Thus although the cells are dying, they still retain much of their normal structural and chemical architecture.

It may be significant that the same subcellular fractions (microsomes + cell sap) are involved in amino acid incorporation *in vitro* (Zamecnik & Keller, 1954; Keller & Zamecnik, 1956) and in the metabolism of dimethylnitrosamine (Magee & Vandekar, 1958). The early inhibition of protein synthesis could be explained by damage to microsome structures either by dimethylnitrosamine itself, perhaps 'activated' in some way, or by a toxic metabolite product in high local concentration close to the site of most active amino acid incorporation. On the other hand, there may be a specific inhibition of protein synthesis, but whether such an inhibition could be expected to kill the cell is not clear since the significance of the dynamic state of the body constituents is still unknown.

The suggestion that some liver toxins may interfere with protein metabolism has been made by several workers. Popper, de la Huerga & Koch-Weser (1954) have advanced a theory of 'conditioned amino acid deficiency' to explain the lesions due to bromobenzene and ethionine. There is evidence that ethionine is incorporated into tissue protein, with the formation of abnormal proteins (Levine & Tarver, 1951; Gross & Tarver, 1955), and Simpson, Farber & Tarver (1950) have shown inhibition of incorporation of labelled amino acids into liver protein shortly after administration of the abnormal amino acid to female rats. Some resemblances between the lesions produced by the carcinogenic azo dyes and dimethylnitrosamine have been discussed previously (Magee & Barnes, 1956). Most work with the azo dyes has been concerned with tumour production by feeding with the compounds, but Orr & Price (1948) have shown that large single doses can produce acute centrilobular necrosis. Miller & Miller (1947) showed that a firmly bound dye–protein complex is formed in the livers of rats, and have suggested that this process may be related to the mechanism of production of the lesions (Miller & Miller, 1953). The early stages of dye-binding in rat liver have been studied in some detail by Hultin (1956*a*, *b*). He found the dye–protein complex as early as 3 hr. after intraperitoneal injection and, at this early stage, the concentration of bound dye was highest in the microsomal fraction. On subfractionation of the microsome material, however, the greatest amount of bound dye was found in fractions with low RNA content, suggesting that the mechanism of dye 'incorporation' was different from the incorporation of amino acids. In more recent work Hultin (1957) has demonstrated that the dye–protein complex can be formed *in vitro* by microsome preparations, but only when the dye is being metabolized. Hultin has concluded that '...the microsomal structures with their vital anabolic functions are particularly exposed to profound disturbances by the carcinogenic azo compounds'. Having regard to the findings of Magee & Vandekar (1958) on the metabolism of dimethylnitrosamine

in vitro, the results of the present work suggest similar conclusions as a working hypothesis for the mechanism of action of this compound.

SUMMARY

1. A study has been made of the turnover of some rat-liver constituents during the early stages of toxic liver necrosis produced by dimethylnitrosamine.

2. Incorporation of [^{14}C]amino acids into liver proteins was markedly reduced at 3 and 6 hr. after a necrotizing dose of dimethylnitrosamine (50 mg./kg. body wt.). This reduction occurred to about the same extent in the different subcellular fractions of liver. Incorporation into kidney and spleen proteins was unimpaired.

3. The concentration of free amino acids in the liver was not significantly altered 3 hr. after dimethylnitrosamine, nor was the incorporation of labelled amino acids into the free amino acid pool. The incorporation into the liver proteins in these animals was reduced by about 50%.

4. Incorporation of ^{32}P into phosphorus-containing fractions of rat liver was studied during the period 4–6 hr. after the necrotizing dose of dimethylnitrosamine. There was no significant alteration in the relative specific activities of the acid-soluble phosphorus, the lipid phosphorus and the residual phosphorus (alkali-soluble phosphorus). Incorporation into partially purified ribonucleic acid was significantly reduced during this period.

5. The concentrations of plasma inorganic phosphorus, liver acid-soluble phosphorus and lipid phosphorus were not significantly changed 6 hr. after dimethylnitrosamine. The concentration of residual phosphorus (alkali-soluble phosphorus) was significantly reduced.

6. The concentration of liver glycogen was not significantly altered 3 hr. after the same dose of dimethylnitrosamine.

7. The results are discussed in relation to morphological and metabolic findings published elsewhere. It is suggested that the initial damaging action of dimethylnitrosamine on the liver cell may be on the microsomal structures.

I would like to thank Dr J. M. Barnes for his interest in this work; Drs T. S. Work and J. L. Simkin for advice and discussion, and Mr A. Batchelor for very valuable assistance.

REFERENCES

Awapara, J. (1948). *Arch. Biochem.* **19**, 172.
Barnes, J. M. & Magee, P. N. (1954). *Brit. J. industr. Med.* **11**, 167.
Borsook, H. (1956). *J. cell. comp. Physiol.* **47**, suppl. 1, 35.
Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G. & Lowy, P. H. (1950). *J. biol. Chem.* **187**, 839.
Brachet, J. (1955). *The Nucleic Acids*, vol. 2, p. 475. Ed. by Chargaff, E. & Davidson, J. N. New York: Academic Press Inc.
Bruce, H. M. & Parkes, A. S. (1949). *J. Hyg., Camb.*, **45**, 169.
Catch, J. R. (1954). *Proc. 2nd Radioisotope Conf., Oxford.* London: Butterworths Scientific Publications.
Davidson, J. N., Frazer, S. C. & Hutchison, W. C. (1951). *Biochem. J.* **49**, 311.
Davidson, J. N. & Smellie, R. M. S. (1952). *Biochem. J.* **52**, 594.
Drill, A. (1952). *Pharm. Rev.* **4**, 1.
Dutton, A. H. & Heath, D. F. (1956). *Nature, Lond.*, **178**, 644.
Fisher, R. A. (1934). *Statistical Methods for Research Workers*, 5th ed., pp. 121–158. London: Oliver and Boyd.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Good, C. A., Kramer, H. & Somogyi, M. (1933). *J. biol. Chem.* **100**, 485.
Gross, D. & Tarver, H. (1955). *J. biol. Chem.* **217**, 169.
Henriques, O. B., Henriques, S. B. & Neuberger, A. (1955). *Biochem. J.* **60**, 409.
Himsworth, H. P. (1947). *Lectures on the Liver and its Diseases*: Lowell lectures, Boston, Mass., 1947. Oxford: Blackwell Scientific Publications.
Hultin, T. (1950). *Exp. Cell Res.* **1**, 376.
Hultin, T. (1956*a*). *Exp. Cell Res.* **10**, 71.
Hultin, T. (1956*b*). *Exp. Cell Res.* **10**, 697.
Hultin, T. (1957). *Exp. Cell Res.* **13**, 47.
Keller, E. B. & Zamecnik, P. C. (1956). *J. biol. Chem.* **221**, 45.
Keller, E. B., Zamecnik, P. C. & Loftfield, R. B. (1954). *J. Histochem. Cytochem.* **2**, 378.
Kuff, E. L., Hogeboom, G. H. & Dalton, A. J. (1956). *J. biophys. biochem. Cytol.* **2**, 33.
Levine, M. & Tarver, H. (1951). *J. biol. Chem.* **192**, 835.
Magee, P. N. (1956). *Biochem. J.* **64**, 676.
Magee, P. N. (1957). *Biochem. J.* **65**, 31P.
Magee, P. N. & Barnes, J. M. (1956). *Brit. J. Cancer*, **10**, 114.
Magee, P. N. & Vandekar, M. (1958). *Biochem. J.* **70**, 600.
Miller, E. C. & Miller, J. A. (1947). *Cancer Res.* **7**, 468.
Miller, J. A. & Miller, E. C. (1953). *Advanc. Cancer Res.* **1**, 340.
Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
Orr, J. W. & Price, D. E. (1948). *J. Path. Bact.* **60**, 461.
Popják, G. (1950). *Biochem. J.* **46**, 560.
Popper, H., de la Huerga, J. & Koch-Weser, D. (1954). *Ann. N.Y. Acad. Sci.* **57**, 936.
Porter, K. R. (1954). *J. Histochem. Cytochem.* **2**, 346.
Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
Schneider, W. C. (1948). *J. biol. Chem.* **176**, 259.
Simkin, J. L. & Work, T. S. (1957*a*). *Biochem. J.* **65**, 307.
Simkin, J. L. & Work, T. S. (1957*b*). *Nature, Lond.*, **179**, 1214.
Simpson, M. V., Farber, E. & Tarver, H. (1950). *J. biol. Chem.* **182**, 81.
Stoner, H. B. (1956). *Brit. J. exp. Path.* **37**, 176.
Stoner, H. B., Threlfall, C. J. & Green, H. N. (1952). *Brit. J. exp. Path.* **33**, 398.
Van Slyke, D. D., MacFadyen, D. A. & Hamilton, P. (1941). *J. biol. Chem.* **141**, 671.
Zamecnik, P. C. & Keller, E. B. (1954). *J. biol. Chem.* **209**, 337.