vate and α-oxoglutarate in the blood and urine but none in glyoxylate. In thiamine-deficient rats, the glyoxylate in blood and urine contained up to 6·9 % of the radioactivity from the injected glycine, and radioactivity was not found in the pyruvate or α-oxoglutarate.

6. The present study suggests that the glyoxylate found in blood and urine of thiamine-deficient rats is derived from the glycine liberated by excessive tissue breakdown.

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Toxic Liver Injury and Carcinogenesis

METHYLATION OF PROTEINS OF RAT-LIVER SLICES BY DIMETHYLNITROSAMINE *IN VITRO*

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Dimethylnitrosamine is a powerful liver poison in several mammalian species (Barnes & Magee, 1954; Jacobsen, Wheelwright, Clem & Shannon, 1955). It is carcinogenic in the rat, inducing tumours of the liver (Magee & Barnes, 1956; Schmähl & Preussmann, 1959) and of the kidney (Magee & Barnes, 1959, 1962; Zak, Holzner &

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Popper, 1960). It has been suggested (Magee, 1956; Stoner & Magee, 1957; Brouwers & Emmelot, 1960; Hultin, Arrhenius, Löw & Magee, 1960) that dimethylnitrosamine must be metabolically activated before it becomes hepatotoxic and that a metabolite or metabolites may be responsible for toxicity and carcinogenicity. The possibility that the active molecule might be diazomethane was suggested by Dr Regina Schoental (personal communication) and by Rose (1958). Support for such an alkylation hypothesis has come from the work of Druckrey and his colleagues. After an earlier report (Schmähl, Preussmann & Hamperl, 1960) that diethylnitrosamine is carcinogenic in rat liver, the same group (Druckrey, Preussmann, Schmähl & Müller, 1961*a*) have reported the results of carcinogenicity tests on a series of nitrosamines. Only those compounds which were expected to yield diazoalkanes proved to be carcinogenic, and those which were unlikely to do so, on chemical grounds, failed to induce tumours. Diazomethane itself has been shown to be carcinogenic in the lung in mice and rats by Schoental (1960), who also showed that nitroso-*N*-methylurethane induces stomach tumours in the rat. This latter result has been recently confirmed by Druckrey *et al.* (1961*b*) in the rat. Brouwers & Emmelot (1960) have demonstrated the formation of formaldehyde from dimethylnitrosamine by a microsomal enzyme system in rat liver and have suggested that this may be the actively carcinogenic molecule. Reasons against this have been advanced by Hultin *et al.* (1960).

In the present paper experiments are described in which rat-liver slices were incubated with [^{14}C]dimethylnitrosamine *in vitro*, and the incorporation of radioactivity into proteins and nucleic acids was studied.

MATERIALS AND METHODS

Chemical reagents

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

Radioactive compounds. [^{14}C]Dimethylnitrosamine was synthesized by Dr D. F. Heath as described by Dutton & Heath (1956). [^{14}C]Paraformaldehyde was supplied by The Radiochemical Centre, Amersham, Bucks.

Special reagents. A mixture containing 1-methyl-, 3-methyl-, and 1,3-dimethyl-histidine was prepared by methylation of histidine by Dr A. R. Mattocks according to Sheehan & Frank (1949) as modified by Tallan, Stein & Moore (1954). Authentic samples of 1-methyl- and 3-methyl-histidine were supplied by the California Corp. for Biochemical Research, Los Angeles 63, California, U.S.A.

Experiments on tissue slices

Albino rats of the Wistar strain were maintained on a diet of vitamin-enriched rat-bread (Lindberg, Älvsjö, Sweden) or on M.R.C. diet 41 B (Bruce & Parkes, 1956), and were killed by decapitation. Rats of either sex, usually weighing about 250 g., were starved overnight before use. Tissue slices were prepared and incubated essentially as described previously (Hultin *et al.* 1960) except that Krebs–Ringer phosphate medium (Umbreit, Burris & Stauffer, 1957) was sometimes used instead of the bicarbonate medium. At the end of the incubation period the vessels were plunged into ice-cold water and the medium was removed from the slices and discarded. The slices were washed twice with ice-cold medium containing a large excess of non-radioactive dimethylnitrosamine and were disintegrated in water, again containing excess of the unlabelled compound, in a homogenizer of the type described by Potter & Elvehjem (1936). An equal volume of 10% (w/v) trichloroacetic acid was then added to the tissue suspension with stirring and the precipitate was centrifuged down. In some experiments the supernatant acid-soluble fraction was retained for isolation of formaldehyde.

Isolation of [^{14}C]*formaldehyde and periodic acid oxidation of serine*

Isolation of [^{14}C]formaldehyde from the acid-soluble fraction was carried out according to Mitoma & Greenberg (1952). Formaldehyde (4 mg.) was added to 25 ml. of the acid-soluble fraction, made 25% (w/v) with respect to trichloroacetic acid. This mixture was distilled almost to dryness *in vacuo*, the distillate being trapped in 12 ml. of an aqueous solution containing 0·4% of dimedone (5,5-dimethylcyclohexane-1,3-dione) buffered at pH 7·4 (MacFadyen, 1945). The formaldehyde–dimedone compound, methylenebis-(5,5-dimethylcyclohexane-1,3-dione), was purified by repeated solution in alkali containing excess of dimethylnitrosamine and reprecipitation with acid, dried and assayed for radioactivity. Distillation of added carrier formaldehyde was repeated three times to remove residual formaldehyde, and a fourth time with collection of the distillate and preparation of the dimedone derivative to confirm that removal was complete. Serine (20 mg.) was then added to the residue in the distillation flask and periodic acid oxidation carried out as in the procedure of Mitoma & Greenberg (1952) but with the method of Boyd & Logan (1942). Distillation and collection of the formaldehyde–dimedone compound was followed by assay of its radioactivity, which was assumed to be derived from the β-carbon atom of serine.

Isolation of proteins and nucleic acids

Proteins were isolated essentially according to Schneider (1945). After centrifuging, the acid-soluble supernatant was discarded and the precipitate was washed three times with 5% (w/v) trichloroacetic acid on the centrifuge, followed by ethanol, ethanol–ether (3:1, v/v), twice with ether, and then dried. The dry powder was then suspended in 5 vol. of 10% (w/v) NaCl, and heated at 100° for 1 hr. to extract the nucleic acids (Davidson & Smellie, 1952). This procedure was repeated twice by using 2·5 vol. of 10% NaCl and heating for 0·5 hr., and the three extracts were pooled and set aside for precipitation and purification of the mixed sodium nucleates. The protein residue was then extracted twice with 5% (w/v) trichloroacetic acid at 90° for 20 min. to remove any remaining nucleic acids (Schneider, 1945), washed with ethanol and ether, and dried. This material was regarded as the mixed total protein of the

tissue. The nucleic acids were precipitated from the 10% NaCl extract by the addition of 2 vol. of ethanol, and were purified according to Davidson & Smellie (1952).

Hydrolysis and ion-exchange chromatography of proteins

Proteins were hydrolysed with 6 N-HCl either in a sealed tube at 110° for 16 hr. or by refluxing for 20 hr. The neutral and basic amino acids were separated by ion-exchange chromatography on a column (1 cm. × 150 cm.) of Dowex 50 resin (H^+ form) essentially according to Hirs, Moore & Stein (1954) after preliminary removal of the acidic amino acids on a column of Amberlite IR-4B resin. The effluent from the columns was collected by a mechanical fraction collector. The basic amino acids were separated on a column (0·9 cm. × 50 cm.) of Dowex 50 (Na^+ form), eluting with buffers of increasing ionic strength according to Tallan *et al.* (1954) and Moore & Stein (1954). The presence of amino acids in the effluent fractions was detected by spotting on strips of Whatman no. 1 paper followed by development of colour with ninhydrin. Amino acids were determined quantitatively according to Yemm & Cocking (1955). Buffer salts were removed from fractions by the method of Drèze, Moore & Bigwood (1954), and the salt-free acid solutions of the amino acids were evaporated to dryness at room temperature by exposing the contents of the tubes to a stream of compressed air in a fume cupboard. The residual material in each tube was dissolved in about 2 ml. of water and transferred to plastic (polythene) planchets on which it was evaporated to dryness for assay of radioactivity.

Hydrolysis and ion-exchange chromatography of nucleic acids

In a preliminary experiment, the whole material extracted by 5% trichloroacetic acid at 90° (Schneider, 1945) was shaken repeatedly with ether until the aqueous phase was barely acid (pH about 5). Hydrochloric acid was then added to a final concentration of 1 N followed by heating at 100° for 1 hr., thus liberating purines but leaving pyrimidine nucleotides intact (Vischer & Chargaff, 1948). This hydrolysate was applied to a column of Dowex 50 (H^+ form) and eluted with increasing concentrations of hydrochloric acid. The extinction at 260 mμ of each fraction was measured and several fractions were combined, evaporated to dryness in a rotary evaporator at 50° and finally taken to dryness on glass planchets for assay of radioactivity. In later experiments the isolated mixed nucleic acids were hydrolysed with alkali by the modification of the method of Schmidt & Thannhauser (1945) due to Davidson & Smellie (1952). The RNA nucleotides, after precipitation of DNA and potassium perchlorate, were separated by chromatography on a column (1·5 cm. × 21 cm.) of Dowex 1 anion-exchange resin (formate form) with gradient elution from 1 to 4 N-formic acid, according to Brumm, Potter & Siekevitz (1956) as modified by Gilbert & Yemm (1958). After the extinction at 260 mμ had been recorded, the fractions were evaporated to dryness individually, as described above, and transferred to polythene planchets for radioactive assay.

Paper chromatography

Amino acids were identified by descending chromatography on Whatman no. 1 paper using phenol saturated with water as solvent. The position of amino acid spots was revealed by spraying with, or dipping in, 0·2% ninhydrin in acetone (Toennies & Kolt, 1951). Special colour reactions used were the Pauly reaction for histidine, as applied to paper chromatograms by Sanger & Tuppy (1951), and the Sakaguchi reaction for arginine according to Jepson & Smith (1953). Products of hydrolysis of RNA were separated by the propan-2-ol–ammonia solvent system of Markham & Smith (1952) on Whatman 3MM paper. The position of ultraviolet-absorbing material on the paper was detected according to Markham & Smith (1949). Aliphatic amines were separated by the method of Schwyzer (1952).

Radioactivity measurements and spectrophotometry

The radioactivity of solid materials was assayed on aluminium planchets (area 1 cm.^{2}) and corrected, when necessary, to 'infinite thickness' with an empirical correction curve. Liquids were assayed as 'infinitely thin' samples after evaporation on to glass or plastic planchets as described above. Measurements of radioactivity were made with end-window counters and automatic sample changers (Tracerlab, or Phillips). Extinction measurements were made with Beckman or Unicam SP. 500 instruments.

RESULTS

Metabolic formation of formaldehyde from dimethylnitrosamine

Incubation of [^{14}C]dimethylnitrosamine with liver slices was shown to give [^{14}C]formaldehyde by the procedure of Mitoma & Greenberg (1952). Carrier formaldehyde added to the acid-soluble fraction of liver slices incubated with [^{14}C]dimethylnitrosamine was isolated as the formaldehyde–dimedone compound and found to be radioactive (422 counts/min.). After removal of almost all the residual formaldehyde, as shown by the very low radioactivity (9 counts/min.) of the dimedone compound obtained with the formaldehyde from the fourth redistillation (see Methods section), carrier serine was added, followed by periodic acid oxidation to convert the β-carbon atom into formaldehyde (Nicolet & Shinn, 1939). This was then isolated as before, and found to be radioactive (51 counts/min.), indicating the incorporation of carbon from [^{14}C]dimethylnitrosamine into serine. The metabolic formation of formaldehyde from dimethylnitrosamine by the liver (Brouwers & Emmelot, 1960) is therefore confirmed, and the presence of radioactivity in the β-carbon atom of serine indicates that it has entered the C_1 metabolic pool.

Incorporation of radioactivity from [^{14}C]dimethylnitrosamine into proteins of liver slices

Rat-liver slices were incubated aerobically and anaerobically with [^{14}C]dimethylnitrosamine and kidney slices were incubated anaerobically. After incubation for 3 hr., proteins were isolated from the

slices and assayed for radioactivity. The specific radioactivities were: liver protein (aerobic incubation), 39 counts/min.; liver protein (anaerobic incubation), 2 counts/min.; kidney protein (aerobic incubation), 4 counts/min. These results suggested that some form of incorporation or binding of dimethylnitrosamine or a metabolite to the liver protein had occurred, that this process required oxygen, and that there was a smaller, but probably significant, incorporation into kidney protein.

With [^{14}C]dimethylnitrosamine of higher specific radioactivity, the time course of incorporation of ^{14}C into protein of rat-liver slices was determined. The rate of incorporation was found to be linear for times of incubation up to 3 hr. Proteins isolated at zero time had no detectable radioactivity, indicating that the washing procedure was adequate to remove unchanged [^{14}C]dimethylnitrosamine. In this experiment the specific radioactivity of the residues before extraction of the total nucleic acids with hot 5% trichloroacetic acid was found to be considerably higher than that of the final nucleic acid-free protein powders. This suggested that incorporation had also occurred into the nucleic acids of the liver slices.

The nature of the binding of the radioactivity to the protein was then studied by dissolving samples of the protein in formic acid and in alkali, adding a large excess of non-radioactive dimethylnitrosamine to the solutions, and reprecipitation followed by determination of radioactivity. Protein with initial specific activity of 72 counts/min. gave 71 counts/min. after precipitation from solution in formic acid, indicating that the radioactivity was not due to unchanged [^{14}C]dimethylnitrosamine and probably not to other small molecular metabolites adsorbed on to the protein precipitate. Solution in alkali, on the other hand, caused reduction in the specific radioactivity of the protein to 19 counts/min. The conditions of hydrolysis were adequate to hydrolyse any methyl esters of carboxylic groups of the protein which might have been formed.

Since formaldehyde is produced by the metabolic breakdown of dimethylnitrosamine, the observed radioactivity in proteins and other cell constituents could be explained by incorporation via normal metabolic pathways. This possibility was explored by acid hydrolysis of the radioactive protein and subsequent ion-exchange chromatography, according to Hirs *et al.* (1954). The chromatographic profile is shown in Fig. 1. There are three main peaks of radioactivity. The first, in the region of serine, and the second smaller peak, in the region of methionine, could probably be explained by normal incorporation via [^{14}C]formaldehyde. The large peak of radioactivity in the region of the basic amino acids lysine and histidine could not be explained in this way. For example, rat-liver slices were incubated with [^{14}C]formaldehyde with subsequent isolation, hydrolysis and chromatography of the proteins. Peaks of radioactivity were again observed in the regions of serine and of methionine but there was no radioactivity in relation to the basic amino acids. The possibility that some unchanged dimethylnitrosamine remained occluded in the protein was further tested by the addition of a small amount of [^{14}C]dimethylnitrosamine to non-radioactive normal rat-liver protein, followed by hydrolysis and chromatography in the usual way. Almost all the radioactivity appeared in the region between methionine and leucine and there was none with the basic amino acids. This radioactive material was found to be volatile in alkali and to migrate with carrier dimethylamine on paper chromatography by the method of Schwyzer (1952). Since dimethylnitrosamine is converted into dimethylamine by concentrated hydrochloric acid (Hickinbottom, 1948), it seems probable that the radioactive material under examination was in fact dimethylamine.

Methylation of histidine residues of wheat proteins by methyl bromide was demonstrated by Bridges (1955), and Alexander & Cousens (1958) showed that the imidazole group of histidine was rather readily, and to some extent preferentially, alkylated in native proteins by several biologically active alkylating agents. A preliminary experiment suggested that a similar reaction might occur after metabolism of [^{14}C]dimethylnitros-

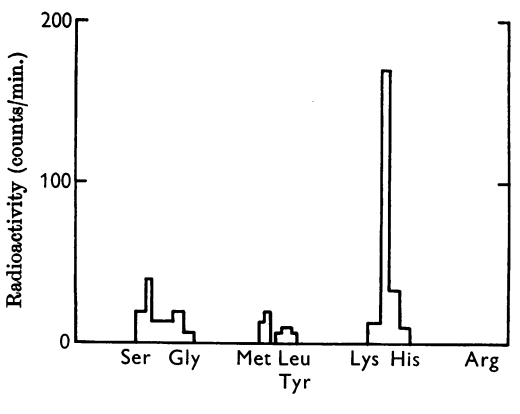

Fig. 1. Ion-exchange chromatography of an acid hydrolysate of proteins (293 mg.) prepared from rat-liver slices incubated *in vitro* with [^{14}C]dimethylnitrosamine. Dowex 50 (H^+ form) column (1 cm. × 150 cm.) essentially according to Hirs, Moore & Stein (1954). Elution sequence, first 1000 ml. with N-HCl, followed by 4N-HCl. Position of amino acids shown by conventional abbreviations, radioactivity as histogram. Acidic amino acids were removed initially on a column (1 cm. × 30 cm.) of Amberlite IR-4B resin.

amine, with the formation of radioactive methylated histidines. Radioactive protein from slices incubated with [^{14}C]dimethylnitrosamine was hydrolysed and subjected to ion-exchange chromatography of the basic amino acids according to Tallan *et al.* (1954). Three peaks of radioactivity emerged with the basic amino acids, one merging into histidine, one after lysine and well separated from it, and one emerging later just after ammonia. It seemed likely that the first two peaks were 3[^{14}C]-methyl- and 1[^{14}C]-methyl-histidine respectively, but there was no reaction with ninhydrin, probably because the amounts present were too small. When a small amount of a synthetic mixture of methylated histidines was added to the protein hydrolysate to act as carriers, the chromatographic profile shown in Fig. 2 was obtained. In this experiment the 3-methylhistidine merged into the histidine, but the 1-methylhistidine was well separated. The first radioactive peak appeared to correspond to the 3-methylhistidine and was not associated with histidine itself. Lysine was free from radioactivity and the small peak of 1-methylhistidine corresponded closely with the second radioactive peak. The third radioactive peak emerging after ammonia was not identified and the fourth very small peak coincided with a small ninhydrin-positive one which was probably 1,3-dimethylhistidine, since this is its expected position (R. Bridges, personal communication). The final peak of radioactivity coincided with arginine. The radioactive material apparently coinciding with 3-methylhistidine and overlapping histidine was rechromatographed under different conditions according to Moore & Stein (1954) to obtain separation of the two amino acids. This was achieved, as shown in Fig. 3, where all the radioactivity was found to be associated with 3-methylhistidine, the profiles of amino nitrogen concentration and radioactivity coinciding well apart from one point. No radioactivity was detected in the histidine. Similar rechromatography of the 1-methylhistidine showed very good coincidence between the profiles. Rechromatography of the arginine on Dowex 50 (H^+ form), with hydrochloric acid as eluent, also showed close coincidence of the profiles of concentration and radioactivity. It was concluded that a large part of the radioactive material appearing in the region of the basic amino acids was made up of 1-methyl- and 3-methyl-histidine.

Incorporation of radioactivity from [^{14}C]*dimethylnitrosamine into nucleic acids of liver slices*

As mentioned above, incorporation of radioactivity from [^{14}C]dimethylnitrosamine into the nucleic acids as well as into the proteins of the slices appeared likely. The total nucleic acids extracted from the slices were chromatographed, after

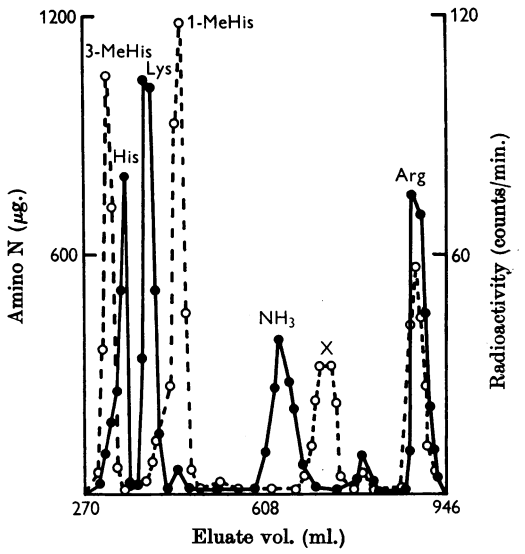

Fig. 2. Ion-exchange chromatography of an acid hydrolysate of proteins (250 mg.) prepared from rat-liver slices incubated *in vitro* with [^{14}C]dimethylnitrosamine. Dowex 50 (Na^+ form) column (1 cm. × 50 cm.) essentially according to Tallan, Stein & Moore (1954). ●, Amino N (μg.); ○, radioactivity (counts/min.). The radioactive material, X, was not identified.

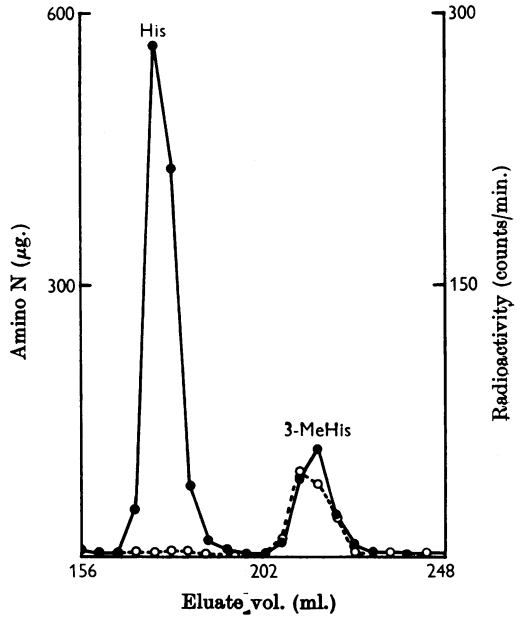

Fig. 3. Rechromatography of the 3-methylhistidine and overlapping histidine obtained previously (Fig. 2). Ion-exchange chromatography on a Dowex 50 (Na^+ form) column essentially according to Moore & Stein (1954). Symbols as in Fig. 2.

acid hydrolysis, on a cation-exchange column, and the major part of the radioactivity appeared in the effluent fraction between guanine and adenine, though there was no detectable ultraviolet-absorbing material corresponding to this fraction. In view of subsequent work (Magee & Farber, 1962), it is probable that this radioactive material was 7-methylguanine, present in amounts inadequate for spectroscopic detection. In later experiments the mixed nucleic acids were hydrolysed with alkali (Schmidt & Thannhauser, 1945), and the resulting RNA nucleotides were chromatographed by anion-exchange chromatography. A large peak of radioactivity appeared immediately before the guanylic isomers, merging into them to a greater or lesser extent in different experiments (Fig. 4). There was also a smaller peak of radioactivity corresponding exactly to that of the two isomers of adenylic acid. The radioactive material in the region of guanylic acid could be well separated from it by paper chromatography with the propan-2-ol–ammonia solvent system. It is probable that this material was a pyrimidine derivative arising by ring opening induced by alkaline hydrolysis of RNA methylated on the 7-position of some guanine moieties (Lawley & Wallick, 1957). On cation-exchange chromatography of the same alkaline hydrolysate of RNA, after further hydrolysis with N-hydrochloric acid to liberate the purine bases, the radioactive material appeared close to and shortly after the pyrimidine nucleotides. In no instance was enough material present to obtain ultraviolet-absorption spectra.

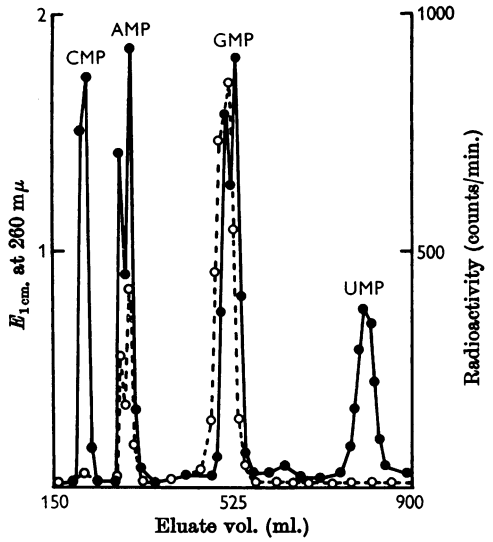

Fig. 4. Ion-exchange chromatography of an alkaline hydrolysate of RNA prepared from rat-liver slices incubated *in vitro* with [^{14}C]dimethylnitrosamine. Dowex 1 (formate form) column (1·5 cm. × 21 cm.) with gradient elution from 1 to 4N-formic acid. ●, $E_{1\,cm.}$ at 260 mμ; ○, radioactivity (counts/min.).

DISCUSSION

The metabolic formation of formaldehyde from dimethylnitrosamine and its subsequent entry into the C_1 metabolic pool must be followed by labelling of all the cell constituents on the normal metabolic pathways of this compound. An attempt must therefore be made to distinguish between isotopic incorporation by this process and the formation of abnormal labelled tissue constituents which could not be derived from formaldehyde, since only the latter are likely to be related to the pathological action of dimethylnitrosamine.

The linear rate of incorporation of radioactivity from [^{14}C]dimethylnitrosamine into liver slices and the requirement for oxygen suggest that this process is enzymic. This probably involves the system present in the microsome + cell sap fraction of the liver which catalyses the decomposition of dimethylnitrosamine (Magee & Vandekar, 1958) and which has been recently shown to be a microsomal *N*-demethylase by Brouwers & Emmelot (1960). The absence of detectable radioactivity at zero incubation time in this experiment confirms that unchanged dimethylnitrosamine is very efficiently removed from the protein preparations by the washing procedures used, as would be expected from its solubility properties. It is possible, of course, that [^{14}C]dimethylnitrosamine might become more firmly adsorbed to the protein on incubation with the slices, but this is made unlikely by the failure to remove radioactivity by solution and reprecipitation of the protein in formic acid containing excess of non-radioactive carrier. This procedure has been shown by Gurnani, Kumta & Sahasrabudhe (1955) to make proteins more readily hydrolysed by acid, probably because the coiled form of the molecule is transformed into the extended form. Persistence of unchanged [^{14}C]-dimethylnitrosamine in any significant quantity is also ruled out by the absence of a radioactive peak in the chromatographic profile of the labelled protein (Fig. 1) in the expected region, i.e. that of dimethylamine. Solution in alkali, however, did remove a large amount of radioactivity from the protein. This method is widely used to remove [^{14}C]amino acids not bound by peptide bonds from protein preparations (Siekevitz, 1952), and the loss of radioactivity observed suggests that the alkali treatment splits off labelled groups chemically combined with the protein. This would be quite consistent with hydrolysis of methyl esters formed by methylation of protein carboxyl groups during incubation of the slices with dimethylnitrosamine.

It seems reasonable to conclude that the peak of radioactivity in the region of lysine and histidine (Fig. 1) is largely due to the presence of 1-methyl- and 3-methyl-histidine. Insufficient material was present to obtain a positive reaction with ninhydrin, but the close agreement between the chromatographic profiles of the added carrier compounds and the radioactivity (Figs. 2 and 3), including the small peak in the expected region for 1,3-dimethylhistidine, are all consistent with this. Also, since good chemical evidence for the presence of methylated nucleic acids is presented in the next paper (Magee & Farber, 1962), the intracellular formation of a methylating agent is strongly implied. The possibility that free histidine is methylated before incorporation into protein is unlikely. Although 1-methyl- and 3-methyl-L-histidine are excreted in the urine of man and other animals (Searle & Westall, 1951; Tallan *et al.* 1954) and 1-methylhistidine is known to occur in muscle as the dipeptide anserine (Behrens & du Vigneaud, 1939), methylhistidines have not been reported as constituents of protein (Greenstein & Winitz, 1961). Harms & Winnick (1954) treated rats with DL-1-methyl[1-^{14}C]histidine and found no significant labelling of tissue proteins, and Cowgill & Freeburg (1957) administered 1[^{14}C]-methyl-, 3[^{14}C]-methyl- and 1,3[$^{14}C_2$]-dimethyl-L-histidine to rats and found no detectable incorporation of these compounds into anserine or into other peptides or proteins of liver and other tissues. It seems highly probably, therefore, that the imidazole groups of some of the histidine residues in the protein molecules are methylated when rat-liver slices are incubated with dimethylnitrosamine. This, together with the probable methylation of the protein carboxyl groups, provides direct evidence in support of the methylation hypothesis of the mechanism of action of dimethylnitrosamine. The absence of methylated histidines from the proteins isolated from rat-liver slices incubated with [^{14}C]formaldehyde makes it unlikely that the observed methylation is mediated via this compound, although its metabolic formation from dimethylnitrosamine has been confirmed. Among the many reactions of formaldehyde with proteins *in vitro* (French & Edsall, 1945; Fraenkel-Conrat, 1951) methylation of histidine residues has not been reported.

The biological significance of the methylation of proteins in the liver is difficult to assess. Binding of carcinogens or their metabolites to tissue proteins has been investigated and discussed very extensively since the original observation by Miller & Miller (1947) of the combination of azo dyes with rat-liver proteins. Numerous correlations between protein binding and carcinogenesis have been established (Miller & Miller, 1953) and the enzyme deletion hypothesis, recently restated by Potter (1958), was formulated on the basis of combination between the carcinogen and cellular proteins as the initial reaction in chemical carcinogenesis. A similar protein–carcinogen interaction was postulated by Green (1954) in his immunological theory of cancer. Difficulties in acceptance of a primary protein change in carcinogenesis have been discussed by Anderson & Law (1960) and Magee (1962).

Since the cellular alteration in cancer must presumably be heritable, it seems more likely that the initial action of the carcinogen is on cellular nucleic acids, which are known to be capable of transmitting hereditary information, rather than on protein, which is not known to transmit such information. The possible relevance of methylation of nucleic acids to carcinogenesis by dimethylnitrosamine will be discussed in the following paper (Magee & Farber, 1962).

Methylation of proteins may be related to the acute hepatic injury induced by necrogenic doses of dimethylnitrosamine (Barnes & Magee, 1954). Although the extent of methylation of the total mixed proteins is very small indeed it must be remembered that certain specific protein molecules might be more extensively methylated. The imidazole groups of histidine residues in some enzyme proteins are known to be essential for activity (Barnard & Stein, 1958; Koshland, 1960). It is also established that minute quantities of an inhibitor combined irreversibly at the active centre can abolish enzymic activity, as in the phosphorylation of the serine hydroxyl group in esterases by organophosphorus compounds (Aldridge, 1956) and in the alkylation of protein sulphydryl groups by iodoacetate (Dixon, 1948). Such inactivation of an essential enzyme might play a part in the injury and subsequent death of liver parenchymal cells caused by dimethylnitrosamine. The possible role of reaction with sulphydryl groups in carcinogenesis by dimethylnitrosamine has been suggested by Emmelot & Benedetti (1961). Another possibility arises from the capacity of diazomethane to induce hypersensitive states (Fairhall, 1957), presumably following reaction with cellular proteins. Reiss & Tayeau (1959) have shown that methylation *in vitro* by diazomethane produces immunochemical changes in albumin, and similar changes have been shown to follow alkylation of proteins by sulphur mustard (Watkins & Wormall, 1952). In poisoning by dimethylnitrosamine, with local metabolic production of a methylating agent within the parenchymal cell, sufficient abnormal protein might accumulate to induce some form of immune reaction. The methylating agent would probably also methylate other cell constituents as well as protein and nucleic acid (Magee & Farber, 1962) and the cumulative effects of such reactions might well play a part in the cellular injury.

SUMMARY

1. The hepatotoxin and carcinogen dimethylnitrosamine, labelled with radioactive carbon, was incubated with rat-liver slices *in vitro*, and the fate of the label in some components of the acid-soluble protein and nucleic acid fractions of the tissue was studied.

2. Added carrier formaldehyde, isolated from the acid-soluble fraction as the dimedone derivative, was found to be radioactive; carrier serine, after periodate oxidation, also yielded a radioactive formaldehyde derivative.

3. Radioactivity was incorporated into total protein of liver slices and, probably, also into kidney protein. The incorporation process appeared to be enzymic with a requirement for oxygen. The radioactivity could not be removed from the protein by solution in formic acid but solution in dilute alkali led to considerable loss of radioactivity.

4. The radioactive liver protein was hydrolysed to free amino acids by hydrochloric acid, followed by ion-exchange column chromatography of the hydrolysate. Radioactivity appeared in the effluent in association with serine and methionine, and there was a peak of activity associated with lysine and histidine. An hydrolysate of liver-slice protein labelled by incubation with radioactive formaldehyde showed peaks of radioactivity associated with serine and methionine but none with the basic amino acids. An hydrolysate of non-radioactive protein with added radioactive dimethylnitrosamine showed virtually a single peak of radioactivity appearing between methionine and leucine which was probably dimethylamine.

5. Ion-exchange chromatography with better resolution of the basic amino acids showed that histidine and lysine were not labelled. Much of the radioactivity associated with them was resolved into two peaks which corresponded closely with carrier 1-methyl- and 3-methyl-histidine added to the protein hydrolysate before chromatography. Rechromatography of the 1-methyl- and 3-methylhistidine peaks gave very close agreement between the profiles of radioactivity and concentration of amino nitrogen.

6. Radioactivity was incorporated into the mixed nucleic acids of the liver slices. Ion-exchange chromatography of acid and alkaline hydrolysates of the labelled nucleic acids revealed most of the radioactivity in a single peak which did not correspond to any of the major nucleic acid components and was not identified. Most of the remaining radioactivity appeared to be incorporated into adenine.

7. These results are considered to be consistent with the hypothesis that dimethylnitrosamine, after enzymic oxidative *N*-demethylation, releases a methylating agent which methylates the imidazole group of some histidine residues and possibly also some free carboxyl groups of proteins. The carbon of the methyl group removed by oxidative demethylation appears to enter the general metabolic pool. The probable chemical alteration of tissue proteins by dimethylnitrosamine is discussed in relation to some current theories of tissue injury and carcinogenesis.

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Toxic Liver Injury and Carcinogenesis

METHYLATION OF RAT-LIVER NUCLEIC ACIDS BY DIMETHYLNITROSAMINE *IN VIVO*

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In the preceding paper (Magee & Hultin, 1962) it was shown that methylation of proteins of rat-liver slices occurred during incubation of the slices with the hepatotoxin and carcinogen dimethylnitrosamine. This finding supports the hypothesis that dimethylnitrosamine undergoes enzymic oxidative *N*-demethylation in the liver with subsequent intracellular formation of a methylating agent, possibly diazomethane. Evidence for reaction with nucleic acids was also obtained. The reactions of nucleic acids and their components with alkylating agents have been studied very extensively *in vitro*

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