Reaction of the Carcinogen Dimethylnitrosamine with Proteins and with Thiol Compounds in the Intact Animal

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1. [^{14}C]Dimethylnitrosamine was administered to rats, and the formation of free and protein-bound *S*-methylcysteine was investigated. 2. Very little *S*-methylcysteine was found in liver acid-soluble fraction, in liver protein or in urine. 3. The methylhistidines formed in liver protein were found to have largely disappeared by 6 days after injection of dimethylnitrosamine. 4. The significance of these results in the carcinogenic action of dimethylnitrosamine is discussed.

There has been much speculation concerning the involvement of tissue thiol groups in the mechanism of action of chemical carcinogens. On the evidence that carcinogenic hydrocarbons can react with thiol compounds, including cysteine, to form substitution products, it was first suggested by Wood & Fieser (1940) that these carcinogens may exert their biological effects by reacting with protein thiol groups, and by causing the opening of disulphide links in proteins. Since then, there have been several demonstrations of the reactions of carcinogens, anti-carcinogens and mutagens with naturally occurring thiol compounds. Such reactions have been shown to occur with certain carcinogenic alkylating agents, including β -propiolactone (Dickens & Jones, 1961) and *N*-methyl-*N*-nitrosourea (Schoental & Rive, 1963). The experiments were carried out under physiological conditions, with neutral aqueous solutions at physiological temperatures, but were nevertheless performed *in vitro*.

There are groups in addition to the thiol which are susceptible to alkylation; these include carboxyl, imidazole and ring nitrogen atoms of purines. The additional groups must compete with each other for the alkylating agent (Ogston, 1948) in proportion to their reactivities, the fractions of the particular group which is in the reactive form at physiological pH, and to their concentrations. The bond-forming ability towards a saturated carbon atom decreases in the order $\text{S} > \text{N} > \text{O}$ (Ross, 1962, p. 19). However, though about 99% of the carboxyl and imidazole groups in protein are in the reactive nucleophilic form at pH 7.5, only approximately 1.00–0.05% of the thiol groups are in the reactive form at this pH (Ross, 1962, p. 34). In the intact animal, the location of the reactive group in the cell is important, especially when the alkylating agent is

formed *in vivo* by metabolism of a precursor. There is evidence that dimethylnitrosamine is metabolized by an oxidative *N*-demethylase, located in microsomal membranes, with the production of formaldehyde and an unstable alkylating intermediate (Magee & Vandekar, 1958; Brouwers & Emmelot, 1960). In this case, groups in the vicinity of the microsomes are more likely to become alkylated, and the effect of location in the cell may overshadow the effect of the different reactivities of the susceptible groups. The more unstable the alkylating intermediate, the more likely it is to react with water molecules before it has penetrated far from its site of origin, and the more significance has the location of the various reacting groups.

It therefore seemed highly desirable to study the possibility of reaction of alkylating agents with thiol compounds in the intact animal. Although such reactions have been studied in detail *in vitro*, especially the reaction of mustard gas with proteins (e.g. Banks, Boursnell, Francis, Hopwood & Wormall, 1946), there is less evidence that these reactions do occur *in vivo*. Most experiments on the intact animal have been limited to studying the binding of these agents with proteins, without determining which of the reactive groups in the protein molecule were involved. Experiments on the reactions of compounds containing thiol groups with ethyl methanesulphonate and other alkylating agents in the intact animal have been carried out by Roberts & Warwick (1957, 1958, 1959, 1963*a,b*).

Administration of dimethylnitrosamine to rats leads to the development of tumours in the liver and kidney. Incubation of the compound with liver slices brings about methylation of histidine residues in liver protein (Magee & Hultin, 1962), and administration to rats causes methylation of

guanine moieties in liver and kidney nucleic acids (Magee & Farber, 1962; Craddock & Magee, 1963), and there is evidence for the formation of other metabolites (Heath & Dutton, 1958). An attempt is being made in this Laboratory to determine which are the relevant reactions in carcinogenesis, by comparing the reactions undergone *in vivo* by the carcinogenic alkylating agent, dimethylnitrosamine, with those of alkylating agents, such as methyl iodide and dimethyl sulphate, which have not been shown to be carcinogenic.

As part of this investigation, the possibility of methylation of thiol compounds was studied. [^{14}C]Dimethylnitrosamine was administered to rats, which were later killed and preparations of various tissues were made. Carrier *S*-methylcysteine was added to the extracts, and the carrier was re-isolated and assayed for radioactivity. Formaldehyde is known to be formed from dimethylnitrosamine, and this metabolite presumably enters the C_1 pool of the cell, and leads to the radioactive labelling of normal cell components. As a control experiment therefore [^{14}C]formate was administered to rats and the tissues were studied as after treatment with dimethylnitrosamine. A preliminary report of this work has been published (Craddock, 1964).

A possibly helpful factor in assessing the significance of alkylation of different cell components in carcinogenesis is the persistence of the alkylated products in the cell. It was of interest to know whether the abnormal proteins formed by methylation were not metabolized and remained in the cell, or whether they were rapidly broken down and the products excreted. For practical reasons, it was easier to study the persistence of methylhistidines in protein, rather than of *S*-methylcysteine. Experiments were therefore carried out in which rats were killed at varying time-intervals after administration of dimethylnitrosamine, and the amount of methylhistidines in the liver proteins was determined.

EXPERIMENTAL

Materials. Dimethylnitrosamine, supplied by British Drug Houses Ltd., was redistilled before use. [^{14}C]Dimethylnitrosamine was prepared by Dr D. F. Heath, by the method of Dutton & Heath (1956). *S*-Methylglutathione was obtained from L. Light and Co. Ltd. *S*-Methylcysteine sulphoxide was a gift from Dr R. Schoental.

Animals. Wistar albino rats of the Porton strain were maintained on M.R.C. diet 41B (Bruce & Parkes, 1956).

Animal experiments. (1) Experiments on the formation of *S*-methylcysteine after administration of dimethylnitrosamine. Male rats, about 200 g. body wt., were starved overnight. Four animals were given intraperitoneal injections of [^{14}C]dimethylnitrosamine, suitably diluted with carrier, in 0.9% sodium chloride. The dose was 30 mg./kg., 250 μg /kg. body wt. The urine was frozen as collected. After 5 hr. the animals were anaesthetized with

Nembutal, and blood was collected from the aorta. The livers were removed and plunged into liquid nitrogen. The tissue was homogenized in water, an equal volume of 10% trichloroacetic acid added, and the mixture allowed to stand in crushed ice for 30 min. before centrifugation. The supernatant fraction was made 0.1N with respect to hydrochloric acid, and was extracted repeatedly with ether to remove the trichloroacetic acid, then with chloroform to remove the unmetabolized dimethylnitrosamine. After concentration by evaporation, the acid-soluble fraction was made 5N with respect to hydrochloric acid, and heated in a sealed tube at 110° for 16 hr. The hydrolysate was diluted to 1N with respect to hydrochloric acid and carrier *S*-methylcysteine (3 mg.) added before examination by chromatography.

The precipitate from the liver homogenate was washed repeatedly with 5% trichloroacetic acid, and then with ice-cold water, ethanol, chloroform-methanol (2:1, v/v), ethanol-ether (3:1, v/v) and twice with ether. Nucleic acids were removed by repeated extraction with hot 10% sodium chloride, and the protein precipitate was washed with hot and then cold 5% trichloroacetic acid, and dried with ethanol and ether. Protein samples were hydrolysed in 5N-hydrochloric acid at 110° for 16 hr., adjusted to 1N with respect to hydrochloric acid, and carrier *S*-methylcysteine (3 mg.) was added before chromatographic examination.

The urine was hydrolysed with an equal volume of conc. hydrochloric acid at 110° for 16 hr., and the acid then removed by repeated evaporation in a rotary evaporator. Carrier *S*-methylcysteine (3 mg.) and proline (3 mg.) were added, and the preparation was examined by column chromatography.

(2) Experiments with [^{14}C]formate. Four female rats, starved overnight, were given intraperitoneal injections of [^{14}C]formate, 510 μg /kg., 100 μg /kg. body wt. After 2 hr. the animals were anaesthetized, bled, killed and the acid-soluble fractions of liver and liver protein were prepared as in the experiment on the formation of *S*-methylcysteine after injection of [^{14}C]dimethylnitrosamine.

(3) Experiments on the persistence of radioactivity in protein after administration of [^{14}C]dimethylnitrosamine. Groups of female rats were given intraperitoneal injections of [^{14}C]dimethylnitrosamine, 30 mg./kg., 10 μg /kg. body wt., and were anaesthetized at the time-intervals stated. Blood was collected from the aorta, and the liver, kidney, spleen and pancreas were plunged into liquid nitrogen. Dry samples of the tissue proteins and plasma proteins were prepared as described above, and were assayed for radioactivity.

To study the persistence of methylhistidines in liver protein after administration of dimethylnitrosamine, five female rats, starved overnight, were given intraperitoneal injections of [^{14}C]dimethylnitrosamine, 30 mg./kg., 125 μg /kg. body wt. One rat was killed at each of the time-intervals stated, and a sample of dry liver protein prepared as described above, and examined for methylhistidine.

Stability of *S*-methylcysteine and of *S*-methylglutathione under the conditions of the isolation procedures. Samples of the reference compounds were subjected to treatment with ether and chloroform, and to acid hydrolysis, as described above under the preparation of the acid-soluble fraction of liver and of liver protein, and the products were examined by paper chromatography.

Column chromatography of S-methylcysteine. Preliminary experiments were carried out to investigate the behaviour of *S*-methylcysteine on column chromatography. The reference amino acid was run alone and with a protein hydrolysate on columns (100 cm. $\times$ 0.9 cm.) of Dowex 50 (X4) with 1*N*-hydrochloric acid (Hirs, Moore & Stein, 1954), with 0.2 *M*-ammonium formate buffer, pH 3.4 (Hirs, Moore & Stein, 1952), and with 0.2 *M*-sodium citrate buffer, pH 3.4 (Moore & Stein, 1951). The locations of the amino acids in the effluent were studied by paper chromatography. The percentage recovery of *S*-methylcysteine was determined by column chromatography of standard solutions, followed by quantitative ninhydrin determinations (Cocking & Yemm, 1954) on fractions of the effluent.

Column chromatography of methylhistidines. 1-Methylhistidine (3 mg.) and 3-methylhistidine (3 mg.) were run on columns (50 cm. $\times$ 0.9 cm.) of Dowex 50 (X4) in sodium phosphate buffer, pH 6.6, according to the method of Tallan, Stein & Moore (1954).

Examination of tissue preparations for S-methylcysteine. The hydrolysates of liver acid-soluble fractions, liver protein and urine, from rats treated with [^{14}C]dimethylnitrosamine and [^{14}C]formate, prepared as described above, were examined first by chromatography on Dowex 50 columns, with 1*N*-hydrochloric acid, followed by 4 *N*-hydrochloric acid. Carrier *S*-methylcysteine (3 mg.) was added in each case. Each fraction of the effluent was examined by paper chromatography, and was then concentrated by evaporation in a stream of air, transferred to plastic planchets, dried and assayed for radioactivity.

Pooled fractions were re-run on columns of Dowex 50 with sodium citrate buffer or ammonium formate buffer, as described above. After examination by paper chromatography, the fractions were desalted on columns of Dowex 2, according to the method of Drèze, Moore & Bigwood (1954), before being concentrated, transferred to plastic planchets and assayed for radioactivity.

Examination of tissue preparations for methylhistidines. Samples of liver protein (50 mg.), prepared as described above, were hydrolysed in 5 *N*-hydrochloric acid at 110° for 16 hr. After removal of the hydrochloric acid by repeated evaporation in a rotary evaporator, carrier 1-methylhistidine (3 mg.) and 3-methylhistidine (3 mg.) were added, and the preparations run on Dowex 50 columns,

phosphate buffer being used, as described above. The amino acids in the effluent were located with the ninhydrin reagent, and samples of each effluent were taken for assay of radioactivity in a liquid-scintillation system.

Paper chromatography. Fractions from the chromatography columns were examined by paper chromatography in butan-1-ol-acetic acid-water (12:3:5, by vol.). Fractions containing the *S*-methylcysteine carrier were further examined by paper chromatography in the solvent systems shown in Table 1. Two-dimensional chromatograms were run first in the butan-1-ol-acetic acid-water system, then in the phenol-water system.

To convert *S*-methylcysteine into *S*-methylcysteine sulphone, samples were treated with hydrogen peroxide according to the method described by Smith (1960, p. 101), before application to the paper chromatogram.

Amino acids were located by means of the ninhydrin reagent (Smith, 1960, p. 95).

Radioactivity on paper chromatograms was located by the following methods. (1) Radioautography, with Ilford X-ray film, with exposure times of up to 4 weeks. (2) After treatment with ninhydrin the chromatograms were cut into narrow strips, which were eluted with 60% ethanol, and the eluates with washings transferred to plastic planchets, dried and counted. (3) Chromatograms were scanned for radioactivity by use of an automatic two-dimensional radiochromatogram scanner (Gauging Systems Ltd., Baldock, Herts.).

Determination of radioactivity. Material from column chromatography, after being desalted where necessary, was counted on plastic planchets (area 4 cm.²) at 'infinite thinness'. Dry solid protein preparations were counted at 'infinite thickness' on aluminium planchets (area 1 cm.²). Measurements were made on an end-window counter with an automatic sample changer (Nuclear-Chicago Corp.), to an accuracy of at least 3%.

Samples from the phosphate buffer columns were assayed in a liquid-scintillation system, using a modification of the method of Bruno & Christian (1961): 1.6 ml. samples were mixed with 9.0 ml. of ethylCellosolve, and 9.0 ml. of scintillator solution [2,5-diphenyloxazole (5 g./l.) and 1,4-bis-(2-phenyloxazol-5-yl)benzene (0.3 g./l.) in toluene], and counted in a Packard Tri-Carb liquid-scintillation spectrometer model 314EX-2.

Table 1. *Solvent systems used in paper chromatography*

Direction of flow was ascending, except for solvents 1 and 10. Time allowed for development was 16 hr., except for solvent 2, where it was 3 days.

Solvent no.	Composition of solvents (by vol.)	R_F	
		<i>S</i> -Methylcysteine	<i>S</i> -Methylcysteine sulphone
1	Butan-1-ol-acetic acid-water (12:3:5)	0.56	0.33
2	Butan-1-ol-water-ammonia (sp.gr. 0.88) (10:10:1)	0.20	
3	Butan-1-ol-pyridine-3 <i>N</i> -ammonia (4:3:3)	0.52	0.42
4	Propan-1-ol-ammonia (sp.gr. 0.88)-water (6:3:1)	0.86	0.64
5	Butan-1-ol-formic acid-water (7:2:1)	0.49	0.16
6	Propan-1-ol-water (7:3)	0.72	0.32
7	<i>tert.</i> -Butanol-formic acid-water (14:3:3)	0.45	
8	Butan-1-ol-ethanol-water (2:2:1)	0.63	0.17
9	Butan-1-ol-pyridine-water (1:1:1)	0.55	0.49
10	Phenol-water (500 g. in 125 ml. of water)	0.66	

RESULTS

Experiments on the stability of S-methylcysteine and S-methylglutathione. After treatment with ether and chloroform, after acid-hydrolysis treatment, and after evaporation to dryness in a stream of air, S-methylcysteine was found to give only one spot, with R_f identical with that of the untreated compound, on chromatography in different solvents. Comparison with reference S-methylcysteine sulphoxide showed that very little oxidation took place during isolation.

S-Methylglutathione, when subjected to the procedures used in the isolation of S-methylcysteine, was found to be completely hydrolysed to S-methylcysteine, glycine and glutamic acid, with very small amounts of other products.

Column chromatography of S-methylcysteine. S-Methylcysteine was heated in a sealed tube under the conditions used in the isolation procedure, and was then run on a column of Dowex 50 (X4) with 1N-hydrochloric acid. The amino acid emerged as a sharp peak, quantitative ninhydrin determinations showing a recovery of 69%.

On chromatography with 1N-hydrochloric acid in the presence of a protein hydrolysate, S-methylcysteine overlapped with the end of the valine and the beginning of the proline peaks. With sodium citrate or ammonium formate buffers, the S-methylcysteine was well separated from valine, but overlapped with proline.

Column chromatography of methylhistidines. Under the conditions described above, 3-methylhistidine

was well separated from other amino acids of a protein hydrolysate. 1-Methylhistidine travelled with other basic amino acids.

Formation of S-methylcysteine in the acid-soluble fraction of liver. The results of the initial chromatographic investigation of the acid-soluble fraction of liver of rats given [^{14}C]dimethylnitrosamine is shown in Fig. 1. The first two peaks of radioactivity, A and B, correspond to the locations of naturally occurring amino acids which could have become labelled by ^{14}C from the C_1 pool. These include serine, alanine, glutamic acid and aspartic acid. Paper chromatography of these effluents in different solvents gave evidence that these naturally occurring amino acids accounted for some, though not necessarily all, of the radioactivity. The effluents of peak C, containing the S-methylcysteine, were pooled. A sample was taken, the hydrochloric acid removed, carrier S-methylcysteine (3 mg.), proline (3 mg.) and valine (3 mg.) were added, and the preparation was run on a Dowex 50 (X4) column with 0.2 M-sodium citrate buffer, pH 3.4, as described. The results are shown in Fig. 2. Paper chromatography gave evidence that the radioactivity in peak D was due to methionine sulphoxide, formed during the isolation procedures from methionine which had become labelled by metabolism of the [^{14}C]formaldehyde formed from the [^{14}C]dimethylnitrosamine. Paper chromatography of peak E, with different solvents, showed that all the radioactivity accompanied the S-methylcysteine carrier, and no radioactivity was present in the proline. Peak F did not correspond exactly to the position of valine.

Another sample from peak C, Fig. 1, was treated as above, and was re-run on a column of Dowex 50

Fig. 1. Chromatography on Dowex 50 (X4) in 1N-hydrochloric acid, of an acid hydrolysate of the acid-soluble fraction of rat liver, after administration of [^{14}C]dimethylnitrosamine (30 mg./kg., 250 μC /kg. body wt.) Details are given in the text. Locations of relevant amino acids: V, valine; P, proline; M, S-methylcysteine.

Fig. 2. Rechromatography of a sample of peak C, Fig. 1, after addition of further carrier S-methylcysteine, valine and proline, on a Dowex 50 (X4) column, with sodium citrate buffer, pH 3.4, 0.2 M. Locations of amino acids: P, proline; V, valine; M, S-methylcysteine.

(X4) with 0.2 M-ammonium formate buffer, pH 3.4. The results are shown in Fig. 3. Further experiments with paper chromatography gave evidence that the radioactivity in peak *G* was due to *S*-methylcysteine sulfoxide, while all the radioactivity in peak *H* accompanied the *S*-methylcysteine carrier in each solvent system used.

Portions from peak *C*, Fig. 1, were run on paper chromatograms with the solvent systems listed in Table 1. In each case a peak of radioactivity accompanied the *S*-methylcysteine carrier. Valine and proline were found not to be radioactive, but other labelled compounds were present in the preparation. Samples from the same peak were treated with hydrogen peroxide before application to the paper chromatogram. Preliminary experiments with reference compounds showed that *S*-methylcysteine was oxidized to the corresponding sulphone under the conditions used. In chromatograms run in each of the solvent systems listed, radioactivity disappeared from the position on the paper previously occupied by *S*-methylcysteine, and accompanied the *S*-methylcysteine sulphone that was formed.

To confirm that the radioactivity accompanying the *S*-methylcysteine was not due to contamination by a normal metabolite, or its breakdown product, which had become labelled from the C_1 pool, a control experiment was carried out in which [^{14}C]formate was administered to rats. The acid-soluble fraction of liver was examined in the same way as after treatment with dimethylnitrosamine. On chromatography on Dowex 50 with 1N-hydrochloric acid, the first two peaks of radioactivity, *A* and *B*, as in Fig. 1, due to serine, glutamic acid and other metabolites of formate, were obtained

as expected. However, there was no large peak of radioactivity, *C*, in the position of the *S*-methylcysteine carrier. When samples from the effluents containing carrier *S*-methylcysteine were run on paper chromatograms, there was no radioactivity in the position of *S*-methylcysteine. A small amount of radioactive compound, travelling more slowly than *S*-methylcysteine in the butanol-acetic acid solvent, was present. This therefore confirmed that the radioactivity accompanying the *S*-methylcysteine after administration of [^{14}C]dimethylnitrosamine was an abnormal metabolite, and was not derived from metabolism of the [^{14}C]formaldehyde formed.

From these results an approximate calculation can be made of the extent of methylation of thiol compounds in the acid-soluble fraction of liver. It can be assumed that the specific radioactivity of the methyl group of *S*-methylcysteine is equal to that of the methyl group of the administered dimethylnitrosamine. Therefore, from the counts/min. in the carrier *S*-methylcysteine, a calculation can be made of the amount of labelled *S*-methylcysteine. This value was found to be 81.2 μ g. of *S*-methylcysteine in 100 g. wet wt. of liver. Assuming that all the *S*-methylcysteine derived from the methylation of glutathione, this corresponds to a methylation of approximately 0.08% of the glutathione of rat liver. It has been shown (W. N. Aldridge, unpublished work) that there is no significant change in the glutathione concentration of rat liver, as measured by the method of Leaf & Neuberger (1947), at 3 hr. or 24 hr. after oral administration of dimethylnitrosamine (50 mg./kg. body wt.).

Formation of S-methylcysteine in liver protein. The result of chromatography of a protein hydrolysate from rats treated with [^{14}C]dimethylnitrosamine is shown in Fig. 4. Peaks *A* and *B* correspond

Fig. 3. Rechromatography of a sample of peak *C*, Fig. 1, after addition of further carrier *S*-methylcysteine, valine and proline, on a Dowex 50 (X4) column, with ammonium formate buffer, pH 3.4, 0.2 M. Locations of amino acids: P, proline; V, valine; M, *S*-methylcysteine.

Fig. 4. Chromatography on Dowex 50 (X4) of a liver protein hydrolysate (50 mg.) from rats treated with [^{14}C]dimethylnitrosamine (30 mg./kg., 250 μ C/kg. body wt.). 1N-Hydrochloric acid was used for the first 624 ml., followed by 4N-hydrochloric acid.

Fig. 5. Radioactivities of protein preparations from organs of rats injected with $[^{14}\text{C}]$ dimethylnitrosamine (30 mg./kg., 10 $\mu\text{C}/\text{kg. body wt.}$) intraperitoneally and killed after increasing time-intervals. $\blacktriangle$, Liver; $\blacksquare$, pancreas; $\bullet$, plasma proteins; $\circ$, spleen.

to the naturally occurring amino acids, serine, alanine, glutamic acid and aspartic acid, which would be expected to become labelled by metabolism of the $[^{14}\text{C}]$ formaldehyde formed. Paper chromatography confirmed that some, though not necessarily all, of the radioactivity was in these amino acids. Corresponding peaks were found in a protein hydrolysate from rats given $[^{14}\text{C}]$ formate. Peak *C* corresponded to the position of the *S*-methylcysteine carrier. On paper chromatography, all the radioactivity was found in the *S*-methylcysteine spot. No radioactivity was found in this position in the protein hydrolysate of $[^{14}\text{C}]$ formate-treated animals. Peak *D* was not found in the protein hydrolysate from the $[^{14}\text{C}]$ formate-treated animals, and may be due to another abnormal amino acid, formed by methylation, and stable to acid hydrolysis. Peak *E* is probably due chiefly to 1- and 3-methylhistidine (Magee & Hultin, 1962).

The amount of *S*-methylcysteine present in the rat liver protein was calculated from the results of two chromatographic experiments similar to that illustrated in Fig. 4. Assuming that mixed rat liver protein contains 0.58% of cystine (Schweigert, Guthneck, Price, Miller & Miller, 1949), the result in each case was approximately 0.02 mg. of *S*-methylcysteine formed from each 100 mg. of cystine.

Experiments on urine collected after administration of dimethylnitrosamine. Column chromatography of the urine hydrolysate on Dowex 50 with 1*N*-hydrochloric acid showed that a very small proportion of the total radioactivity present travelled with the *S*-methylcysteine carrier. Paper chromatography of the fractions containing *S*-methylcysteine gave evidence that some of this radioactivity was in the methylated amino acid. The amount of *S*-

Table 2. Radioactivity remaining in amino acid fractions isolated from a hydrolysate of liver protein from rats given $[^{14}\text{C}]$ dimethylnitrosamine (30 mg./kg., 125 $\mu\text{C}/\text{kg. body wt.}$)

Results are expressed as radioactivity of the particular amino acid fraction remaining at the time stated, as percentages of that present at 6½ hr. after injection.

Days after injection	Mixed amino acids	3-Methylhistidine	Normal basic amino acids and 1-methylhistidine
1	43	75	107
2	41	87	96
6	16	13	5

methylcysteine present in the urine collected over the 5 hr. period was calculated to correspond to 1.1 $\mu\text{g.}$ per rat.

Persistence in vivo of labelled protein. The radioactivity of protein isolated from various organs at increasing time-intervals after administration of $[^{14}\text{C}]$ dimethylnitrosamine is shown in Fig. 5. The results obtained with kidney protein followed very closely the curve for liver protein. The radioactivity of liver protein was found to be at a maximum at about 6½ hr. after injection of $[^{14}\text{C}]$ dimethylnitrosamine at a dose of 30 mg./kg. body wt. When a higher dose (90 mg./kg. body wt.) was administered, the peak of radioactivity occurred later, at approximately 1 day after injection, owing presumably to the more prolonged metabolism of dimethylnitrosamine.

To study the persistence *in vivo* of abnormal methylated protein it was not sufficient to measure only the total radioactivity, but it was necessary to determine the extent of methylation of the amino acids. A comparison was made of the rate of disappearance of radioactivity from the mixed neutral and acidic amino acids, which emerged first from the chromatography column, with the rate of disappearance of radioactivity from the 3-methylhistidine peak, and with the rate of loss from the peak of basic amino acids containing the 1-methylhistidine. The results are shown in Table 2. It is seen that there is a difference in the rate of loss of methylated histidines from that of the mixed neutral and acidic amino acids. However, at 6 days, only approx. 13% remains of the 3-methylhistidine present at 6½ hr. after injection of dimethylnitrosamine.

DISCUSSION

The concentration of thiol compounds in liver has been shown to remain relatively unchanged after administration of dimethylnitrosamine (W. N. Aldridge, unpublished work), so that it seemed improbable that extensive methylation of thiol

Table 3. Amount of *S*-methylcysteine found in acid-soluble fraction of liver, in liver protein and in urine from rats given [^{14}C]dimethylnitrosamine (30 mg./kg., 250 μC /kg. body wt.)

	Percentage of total available thiol groups methylated	Percentage of dose of dimethylnitrosamine accounted for
Acid-soluble fraction	0.08	0.008
Protein	0.02	0.001
Urine		0.002

groups took place. The amount of alkylation was in fact found to be very small. As seen from Table 3, whether expressed as a percentage of the available thiol groups alkylated, or as a percentage of the dose of dimethylnitrosamine given, the formation of *S*-methylcysteine is very limited. This does not appear to be due to loss of the methylated amino acid, either *in vitro* during the isolation procedures, or *in vivo*. Control experiments showed that loss of *S*-methylcysteine during isolation is not very extensive. This agrees with previous work (Morris & Thompson, 1956), which showed that *S*-methylcysteine is less easily oxidized than is methionine. Very little *S*-methylcysteine was found to have been excreted in the urine. The other way in which any *S*-methylcysteine formed may have escaped detection would have been by further metabolism *in vivo* as it was formed. There is evidence (Du Vigneaud, Loring & Craft, 1934; Cantoni, 1951) that *S*-methylcysteine does not take part in trans-methylation reactions. It is known to be metabolized in the rat, as injection of *S*-methylcysteine labelled in the methyl group leads to the formation of labelled carbon dioxide, choline and creatine, the last-named compounds presumably being formed by oxidation and reduction, rather than by direct transfer of the intact methyl group (Horner & Kuchinskas, 1959). However, *S*-methylglutathione has been found in the liver after administration of methyl iodide (M. K. Johnson, unpublished work), and conjugates of *S*-methylcysteine have been found in human urine, presumably derived from the amino acid in the diet (Tominaga, Kobayashi, Muta, Takei & Ichinose, 1963), and in rat urine after administration of methyl methanesulphonate (Pillinger, Craig & Jackson, 1964). These experiments indicate that if *S*-methylcysteine is formed *in vivo*, or is administered gradually in the diet, it can be detected in liver and in urine. Extensive loss by further metabolism therefore seems to be unlikely.

The results therefore give evidence that very little alkylation of thiol groups does in fact take place. Alkylation of cysteine in protein, which amounts to about 0.02% of the cystine, is less extensive than

that of histidine, where the corresponding figure is about 0.08%. This would be expected from chemical considerations, and from previous evidence for the alkylation of histidine moieties rather than thiol groups in native proteins by several alkylating agents (Alexander & Cousins, 1958). It is possible that the methylated amino acids found in protein were derived from amino acids which had become methylated in the free state, and were then incorporated into protein during biosynthesis, rather than to methylation of protein-bound amino acids.

The formation of very small amounts of free *S*-methylcysteine seems less likely to account for the carcinogenic activity of dimethylnitrosamine than alkylation of the amino acid incorporated into protein, especially if the abnormal protein so formed persists in the liver. For this reason, the persistence of labelled protein was studied. The total radioactivity is due partly to incorporation of normal amino acids labelled by metabolism of [^{14}C]formaldehyde formed from the administered [^{14}C]dimethylnitrosamine, and partly to the methylation of histidine, cysteine and possibly other amino acids present in the protein. Study of the nucleic acids of different organs showed that methylation occurred in liver and kidney, whereas radioactivity was present only in the normal bases in nucleic acids from pancreas and spleen (Magee & Farber, 1962). It is highly probable therefore that the radioactivity of pancreas and spleen protein is due to the incorporation of normal labelled amino acids during protein biosynthesis, whereas liver and kidney proteins become labelled by this means and also by methylation. In liver, the protein containing methyl-histidines was found to have largely disappeared by 6 days after administration of dimethylnitrosamine. The possibility remains that methylation of protein may be involved in carcinogenesis by causing deletion of a liver protein, or by setting up an autoimmune response while present in the liver.

In comparison with other alkylating agents therefore it appears that methyl iodide, which is not a carcinogen as far as is known, causes methylation of liver glutathione, but not of nucleic acids (V. M. Craddock & M. K. Johnson, unpublished work). Dimethylnitrosamine, on the other hand, causes very much less alkylation of glutathione (0.08% compared with up to 80% methylation by methyl iodide), whereas it does bring about alkylation of RNA and DNA. Most of the alkylated nucleic acids disappear from the liver within a few days (Craddock & Magee, 1963). Disappearance of methylated protein and RNA may be due to catabolism of the whole alkylated molecule, leaving no abnormal residue in the cell. However, loss of the methyl group from DNA may be brought about by splitting out the methylated base, possibly with

consequent scission of the sugar phosphate chain, as has been shown to occur *in vitro* (Lawley, 1957). In this case an abnormal DNA would be left in the cell, and such a situation might explain the carcinogenic and mutagenic action of dimethylnitrosamine.

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