Similarities between the Biochemical Actions of Cycasin and Dimethylnitrosamine

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1. Rats were given the hepatotoxin and carcinogen cycasin by stomach tube. In one experiment, rats whose RNA had previously been labelled with [^{14}C]-formate were given the acetate ester of the aglycone form of cycasin, methyl-azoxymethanol, by intraperitoneal injection. 2. Incorporation of ^{14}C from L-[U-^{14}C]leucine into the proteins of some organs was measured in cycasin-treated rats. Cycasin inhibited leucine incorporation into liver proteins but not into kidney, spleen or ileum proteins. This inhibition was not evident until about 5 hr. after cycasin administration, but once established it persisted for the next 20 hr. 3. Methylation of nucleic acids was detected in some organs of rats treated with cycasin or methylazoxymethanol. The purine bases of RNA and DNA were isolated by acid hydrolysis followed by ion-exchange column chromatography. The resulting chromatograms showed an additional purine base that was identified as 7-methylguanine. It was shown that, in animals treated with the toxin, liver RNA was methylated to a greater extent than was either kidney or small-intestine RNA. Also, as a result of cycasin administration, liver DNA guanine was methylated to a greater extent than was RNA guanine. 4. These results are discussed in relation to comparable experiments with dimethylnitrosamine. It is suggested that cycasin and dimethylnitrosamine are metabolized to the same biochemically active compound, perhaps diazomethane, but that various tissues differ in their capacity to metabolize the two carcinogens.

The water-soluble hepatotoxin and carcinogen cycasin, found in the nuts and leaves of the gymnosperm *Cycas circinalis* L. and other cycad plants, has been identified as β-D-glucosyloxy-azoxymethane (Nishida, Kobayashi & Nagahama, 1955; Riggs, 1956), and has been shown to produce both liver and kidney tumours in rats (Laqueur, Mickelsen, Whiting & Kurland, 1963) and liver tumours in guinea pigs (Spatz, 1964; Spatz & Laqueur, 1965). The active component accounting for both the acute toxicity and the carcinogenic activity has been shown to be the aglycone, methyl-azoxymethanol (CH$_3$·NO:N·CH$_2$·OH) (Matsumoto & Strong, 1963; Kobayashi & Matsumoto, 1965; Laqueur, 1965; Laqueur & Matsumoto, 1966). This is believed to be produced from cycasin by the microbial β-glucosidase found in the gastrointestinal tract of the animal (Laqueur, 1964).

The pattern of tumour production by cycasin closely resembles that by dimethylnitrosamine [(CH$_3$)$_2$N·NO], another water-soluble hepatotoxin and carcinogen. When either compound is given for short periods, renal tumours but few if any hepatic tumours result. The reverse is true after prolonged administration of low doses, when tumours are induced in the liver but not in the kidney. Dimethylnitrosamine inhibits the incorporation of labelled amino acids into protein in rat liver *in vivo* and *in vitro*, but not in rat kidney or spleen (Magee, 1958; Hultin, Arrhenius, Löw & Magee, 1960). Magee & Farber (1962) have shown that dimethylnitrosamine also methylates rat liver DNA and RNA at the 7-position of guanine *in vivo*. Because of the similarities in structure and pathological action between methylazoxymethanol and dimethylnitrosamine, it was decided to investigate the action of methylazoxymethanol (acetate ester) on protein synthesis and its ability to methylate nucleic acids in rat liver cells *in vivo*. The results are discussed in the present paper in relation to the suggestion (Miller, 1964) that cycasin and dimethylnitrosamine are metabolized to the same biochemically active product.

MATERIALS AND METHODS

Cycasin. Cycasin was obtained through the courtesy of Dr Marjorie G. Whiting and Dr Gert L. Laqueur of the National Institutes of Health, Bethesda, Md., U.S.A.

Methylazoxymethyl acetate. Methylazoxymethyl acetate

was kindly given by Professor Hiromu Matsumoto of the Department of Agricultural Biochemistry, University of Hawaii, Honolulu, Hawaii, U.S.A.

Radioactive chemicals. Sodium [³H]formate, sodium [¹⁴C]formate and L-[U-¹⁴C]leucine were obtained from The Radiochemical Centre, Amersham, Bucks.

Animals. Albino rats of the Porton strain were maintained on M.R.C. diet no. 41 (Bruce & Parkes, 1956).

Experiments on protein synthesis. Sixty-five male rats each weighing 95–105g. were starved overnight. Five rats served as 'zero-time' controls; they were given 1·67μc of L-[U-¹⁴C]leucine in 0·1ml. of 0·1M-phosphate buffer, pH7·4, by intraperitoneal injection. Then, 30min. later, the rats were stunned and decapitated, and their livers, kidneys, spleens and ilea were removed, frozen in liquid nitrogen and stored at −25°. Thirty rats were given 50mg. of cycasin in 1ml. of water by stomach tube, and 30 control rats were given water only. At 0·5, 2, 4, 5·5, 8 and 24hr. after administration of the toxin, five rats from both the control and the cycasin-treated groups were given 1·67μc of L-[U-¹⁴C]leucine intraperitoneally. Then, 30min. after the injection of the radioactive tracer, the animals were killed and their tissues were removed and frozen. Protein was isolated by a slight modification of the Siekevitz (1952) procedure.

Experiments on methylation of nucleic acids. Two 200g. female rats were given 0·1ml. of sodium [³H]formate in 0·9% NaCl (1000μc/ml.; 4·8mg./ml.) by intraperitoneal injection daily for 6 days. Similarly, two rats received 0·1ml. of sodium [¹⁴C]formate (100μc/ml.; 4·8mg./ml.) daily for 6 days. In this way the tissue RNA became labelled with the radioactive tracer (Craddock & Magee, 1966). After the sixth formate injection the animals were starved overnight. The next morning the ³H-labelled rats were each given 0·25ml. of 0·9% NaCl by intraperitoneal injection and the ¹⁴C-labelled rats were given 10μl. (approx. 12mg.) of methylazoxymethyl acetate in 0·25ml. of 0·9% NaCl. Then, 5hr. later, one ¹⁴C-labelled and one ³H-labelled rat were stunned and decapitated. The livers, kidneys and small intestines were removed, rinsed with cold 0·9% NaCl, blotted, frozen in liquid nitrogen, weighed and stored overnight at −25°. The remaining two rats were treated similarly 10hr. after receiving the methylazoxymethyl acetate or 0·9% NaCl.

The livers from the two rats killed at 5hr. were pooled, and the RNA was isolated and purified according to the method of Kirby (1962). The other organs were treated similarly. In this manner the RNA from the ³H-labelled animals served as an internal control for any artificial change that may have taken place in the RNA isolated from the poisoned animals.

Up to 50mg. of RNA was hydrolysed in N-HCl (1ml./ 10mg. of RNA) at 100° for 1hr. The cooled hydrolysate was transferred to a column (1cm.×10cm.) of Dowex 50 (X12; H⁺ form). The hydrolysate was eluted by an exponential gradient of 1–4N-HCl, and the eluate was collected in 10ml. fractions at a flow rate of 12·5ml./hr. After measurement of its E_{260} value, each fraction was evaporated to dryness and its radioactivity assayed.

An additional rat was given six daily injections of sodium [¹⁴C]formate and killed 24hr. after the last injection. Nuclear RNA, ribosomal RNA and 'soluble' RNA were isolated from the liver by the methods of Hoagland, Stephenson, Scott, Hecht & Zamecnik (1958) and Barondes,

Dingman & Sporn (1962), and their specific radioactivities were determined.

In a second experiment, 12 female rats, each weighing 200g., were given 20mg. of cycasin in 0·5ml. of water orally after overnight starvation. Similarly, 12 rats were given 0·5ml. of water to serve as controls. Then, 10hr. later, the rats were killed and their livers removed, washed in cold 0·9% NaCl and frozen in liquid nitrogen. The livers of the 12 treated rats were pooled, as were the control livers. RNA and DNA were isolated by the method of Kirby (1962), and were hydrolysed and fractionated as described above for RNA, except that a Dowex 50 (X8; H⁺ form) chromatographic column (2cm.×20cm.) was used. The column was eluted with an exponential gradient of 0·25– 3·0N-HCl at a flow rate of 10ml. every 24min.

Identification of 7-methylguanine. Evidence for the identity of 7-methylguanine was obtained by the chromatographic and absorption-spectral methods described by Magee & Farber (1962).

Radioactive assays. Radioactive samples were dissolved in Hyamine hydroxide [p-(di-isobutylcresoxyethoxyethyl)-dimethylbenzylammonium chloride, hydroxide form] and counted in a toluene scintillation mixture (Vaughn, Steinberg & Logan, 1957) with a Packard Tri-Carb scintillation spectrometer. Internal standards of [¹⁴C]toluene and [³H]toluene were used to correct for quenching and to measure counting efficiencies.

RESULTS

Experiments on protein synthesis. In a preliminary experiment that was carried out by one of the authors (R.C.S.) in collaboration with Dr Maria Spatz and Dr Gert L. Laqueur (National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md., U.S.A.), it was found that cycasin inhibited the incorporation of L-[U-¹⁴C]leucine into rat liver protein. That experiment was extended to the one reported here, the results of which are summarized in Table 1. Inhibition of the incorporation of leucine into liver protein was not observed until approx. 5hr. after the administration of cycasin. Once established, however, this inhibition persisted for the next 20hr. The inhibition is probably specific to the liver, as, in the same study, cycasin had no significant effects on leucine incorporation into protein in kidney, spleen or ileum.

Experiments on methylation of RNA and DNA. Because no radioactively labelled methylazoxymethyl acetate or cycasin was available, and because of the difficulty of detecting minute quantities of alkylated bases in RNA hydrolysates by conventional spectrophotometric means, rats were used with all their liver RNA bases prelabelled. In this way any methylated base, which would be eluted from an ion-exchange column in a different chromatographic fraction from the non-alkylated bases, could be detected in small amounts by its radioactivity.

The various species of liver RNA, labelled with

Table 1. *Inhibition of incorporation of* L-[U-14C]*leucine* in vivo *into the tissue protein of rats given cycasin orally*

The analytical method is described in the text. Values are the means $\pm$ S.D. of five experiments.

Time after cycasin administration (hr.)	Specific radioactivity of protein (disintegrations/min.)							
	Liver		Kidney		Spleen		Ileum	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
0	642 ± 176	642 ± 176	284 ± 48	284 ± 48	413 ± 23	413 ± 23	732 ± 200	732 ± 200
0·5	677 ± 177	517 ± 19	311 ± 34	278 ± 61	371 ± 41	367 ± 70	815 ± 144	881 ± 132
2	586 ± 193	507 ± 163	255 ± 23	254 ± 23	383 ± 86	328 ± 67	700 ± 254	793 ± 171
4	567 ± 63	527 ± 84	331 ± 93	255 ± 44	366 ± 42	338 ± 18	752 ± 125	768 ± 101
5·5	587 ± 94	229 ± 37	243 ± 71	215 ± 29	329 ± 79	407 ± 69	771 ± 194	670 ± 98
8	551 ± 80	160 ± 45	251 ± 24	209 ± 27	321 ± 34	376 ± 99	734 ± 275	762 ± 299
24	567 ± 145	341 ± 140	250 ± 30	246 ± 22	288 ± 110	367 ± 65	683 ± 143	661 ± 175

14C as described in the Materials and Methods section, had approximately equal specific radioactivities (nuclear RNA, 743 disintegrations/min./mg.; ribosomal RNA, 901 disintegrations/min./mg.; 'soluble' RNA, 1076 disintegrations/min./mg.). As shown in Fig. 1, all the bases were labelled with either 14C or 3H. However, there was an additional 14C-labelled peak (peak X), not accompanied by a comparable 3H-labelled (control) peak; at this point in the chromatogram there was only a slight inflexion in the extinction curve. In earlier experiments it was found that 1-methylguanine and 7-methylguanine are eluted from the area of the chromatogram between guanine and adenine. The 14C-labelled peak probably represents 7-methylguanine from the RNA of the methylazoxymethyl acetate-treated rats, since this methylated base is known to be eluted in the central region of the chromatogram between guanine and adenine.

Similarly, ion-exchange chromatograms of hydrolysed RNA from the kidneys and the small intestines of poisoned rats demonstrated trace amounts of suspected 7-methylguanine, and their respective controls did not. These results applied equally to the groups of rats killed 5hr. and 10hr. after receiving cycasin.

Several rats were then treated with the toxin to isolate sufficient nucleic acid to detect the suspected 7-methylguanine spectrophotometrically in the acid hydrolysates of the RNA and DNA. Since only a very limited amount of the aglycone acetate ester derivative of the carcinogen was available, the glycoside, cycasin, was used. The hydrolysate of the liver RNA from these rats gave a very definite extinction peak (max. $E_{260}^{1\,\text{cm.}}$ 0·300) at the same chromatographic position as the earlier unknown 14C-labelled peak (Fig. 2). The hydrolysate from the RNA of the same number of control rat livers yielded no obvious peak in this area between guanine and adenine. In both groups,

control and cycasin-treated, the fractions of the eluted hydrolysate between guanine and adenine (between arrows, Fig. 2) were pooled (the control group separately from the treated) and evaporated to dryness in a rotary vacuum evaporator. The residue was redissolved in N-hydrochloric acid and refractionated on a small Dowex 50 column, as described above. In the RNA hydrolysate from the cycasin-treated animals a very prominent peak was obtained at the position previously established for 7-methylguanine. In the hydrolysate from control animals a smaller peak also occurred at the 7-methylguanine position (Fig. 3). The fractions contributing to these peaks from both the control and the treated samples were again evaporated to dryness, dissolved in small volumes of water and applied to Whatman no. 1 chromatographic paper. Authentic 7-methylguanine (10 μg.) was spotted on to the same paper. The chromatogram was developed overnight in the propan-2-ol–ammonia solvent system of Markham & Smith (1952). The unknown substance from the cycasin-treated liver RNA hydrolysate separated into one major spot and two trace spots, seen under u.v. light. The major spot had R_F 0·38; one trace spot remained at the origin, the other ran close to the solvent front. The unknown sample from the control RNA hydrolysate separated into one weak spot and two trace spots. The weak spot also had R_F 0·38. The sample of authentic 7-methylguanine ran as a single spot with R_F 0·38.

The spots with R_F 0·38 were eluted from the paper chromatogram with 3ml. of 0·1N-hydrochloric acid. The u.v. spectrum for each sample was determined at pH 1·2 and again at pH 11·0, the pH being adjusted with N-sodium hydroxide. The spectra of the authentic and the suspected 7-methylguanine samples were identical, as shown in Fig. 4. The chromatographic and absorption-spectra data are regarded as sufficient evidence to
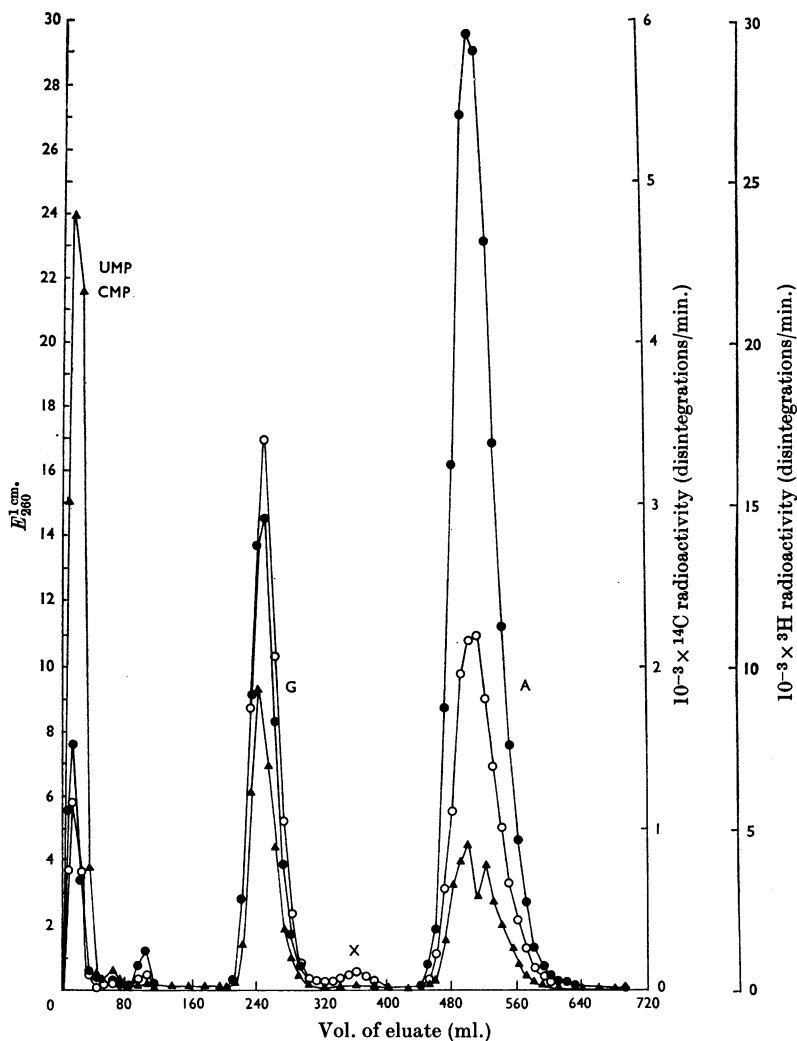
Fig. 1. Ion-exchange chromatography of an acid hydrolysate of RNA (47 mg.) from the pooled livers of rats treated with [^{14}C]formate or [^{3}H]formate daily for 6 days, then 50 mg. of methylazoxymethyl acetate/kg. body wt., 10 hr. before decapitation. The hydrolysate was run on a Dowex 50 (X12; H$^+$ form) column (1 cm. × 10 cm.), with gradient elution with 1–4 N-HCl. ▲, $E_{260}^{1\,cm.}$; ○, ^{14}C radioactivity (disintegrations/min.) (from treated animals); ●, ^{3}H radioactivity (disintegrations/min.) (from control animals). G, Guanine; A, adenine; X, additional peak (see the text).

establish that the compound appearing between guanine and adenine in the hydrolysate of liver RNA from cycasin-treated rats is indeed 7-methyl-guanine. After correction had been made for the differences in the amounts of RNA hydrolysate initially chromatographed, approx. 0·1% of the total RNA guanine from the poisoned rats and 0·06% of the total RNA guanine from the control rats was methylated in the 7-position. It is not surprising to find 7-methylguanine in control RNA,

as this compound has been shown to be a normal constituent of human urine (Weissman, Bromberg & Gutman, 1957) and in pig and rat liver (Dunn, 1963; Villa-Trevino & Magee, 1966).

In the same experiment, liver DNA was isolated from both the control and the treated rats. Acid hydrolysis of the DNA from the treated rats was followed by ion-exchange chromatography on a Dowex 50 (X8) column (2 cm. × 20 cm.). The fractions eluted between guanine and adenine were
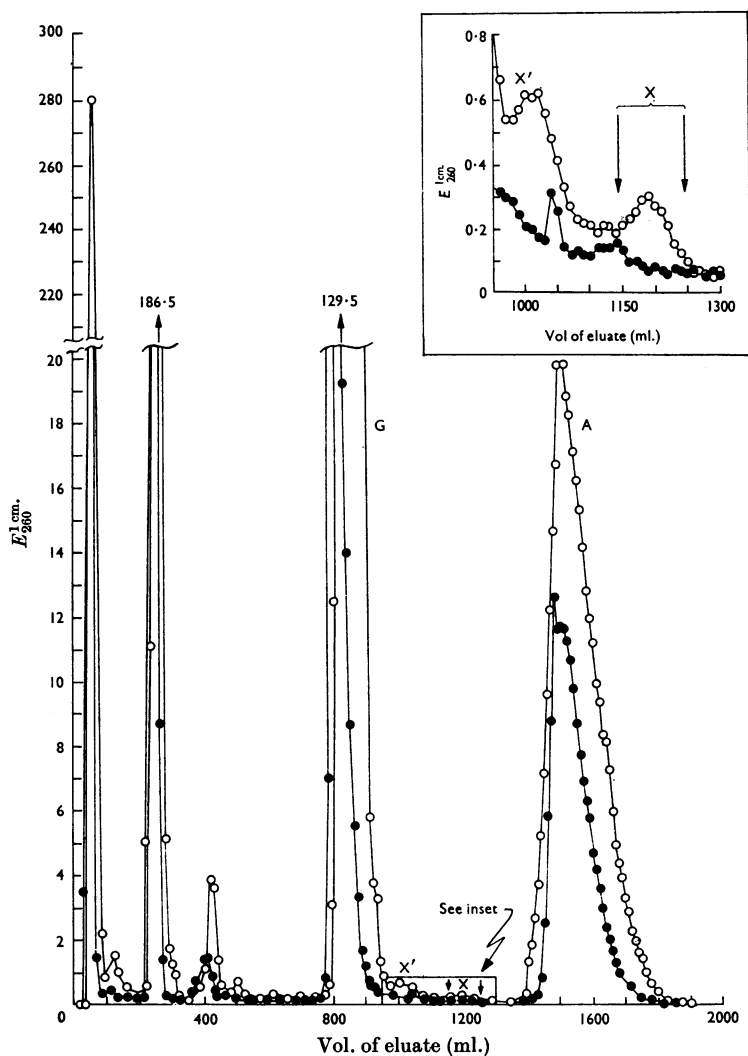
Fig. 2. Ion-exchange chromatography of liver RNA (242 mg.) from 12 control rats and RNA (547 mg.) from 12 rats given 100 mg. of cycasin/kg. body wt. by stomach tube and killed 10 hr. later. The hydrolysates were run on a Dowex 50 (X8; H⁺ form) column (2 cm. × 20 cm.), with gradient elution with 0·25–3·0 N-HCl. ●, $E_{260}^{1\,cm.}$ of control RNA hydrolysate fractions; ○, $E_{260}^{1\,cm.}$ of cycasin-treated rat-liver RNA hydrolysate fractions. G, Guanine; A, adenine; X and X', additional peaks (see the text). The inset is a magnification of a portion of the complete chromatogram. The fractions between the arrows were pooled (see the text).

then applied to a Dowex 50 (X12) column (1 cm. × 10 cm.), which yielded a small extinction peak in the exact position of 7-methylguanine. This substance was shown to be 7-methylguanine as described above. Approx. 0·24% of the total DNA guanine was methylated in the 7-position. No 7-methylguanine could be detected in the DNA isolated from control rats.

In Fig. 2, there is another extinction peak in addition to the 7-methylguanine peak between guanine and adenine (peak X'). It has previously been established (Weissman et al. 1957) that N^2-methylguanine and 1-methylguanine may be extracted in this area of the chromatogram. It is apparent from the chromatograms that cycasin treatment almost doubles the amount of this unknown substance in liver RNA. The compound has not been identified; however, because of its
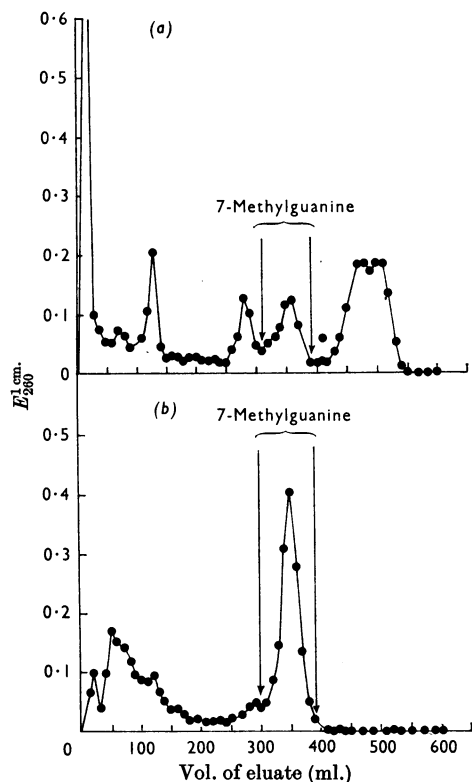
Fig. 3. Ion-exchange chromatography of the combined fractions (between the arrows in Fig. 2) obtained from the liver RNA hydrolysate from (a) control and (b) cycasin-treated rats. Column conditions were the same as in Fig. 1.

Fig. 4. Ultraviolet spectra of authentic 7-methylguanine at pH 1·2 and 11·0 (———) and suspected 7-methylguanine at pH 1·2 (●) and pH 11·0 (○). The spectra of authentic 7-methylguanine are represented as observed. The spectra of the unknown material are superimposed by multiplication of the extinction values of the unknown by the ratio of the extinctions of the authentic to the unknown. This adjustment corrects only for the difference in concentration between the two samples.

chromatographic position, it is suggested that it is N^2-methylguanine, or 1-methylguanine, or a mixture of the two.

DISCUSSION

Inhibition of protein synthesis. It has been shown (Magee, 1958; Hultin *et al.* 1960) that dimethylnitrosamine, a substance structurally similar to the aglycone of cycasin, inhibits the incorporation of amino acids into liver protein *in vivo* and in rat liver slices and cell-free preparations. This effect of a necrotizing dose of dimethylnitrosamine was not demonstrable until 2–3 hr. after its administration, and was not detectable at all in kidney or spleen.

Laqueur (1964, 1965) has shown that cycasin must be hydrolysed to the aglycone before it shows its acute toxic and carcinogenic actions, and that some ingested cycasin can be excreted in the urine and faeces unchanged. The delayed inhibition of the incorporation of leucine into liver protein by
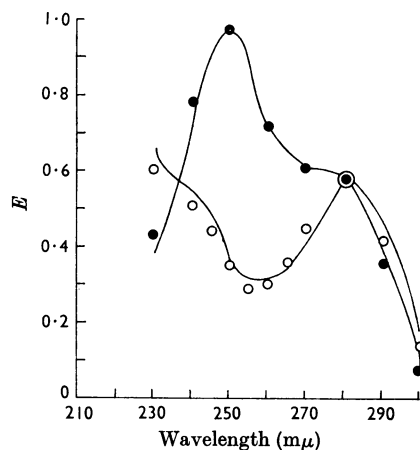
cycasin is not unexpected, since some time would be necessary for the intestinal flora to hydrolyse the glycoside.

Methylation of RNA and DNA. It is probable that an alkylating intermediate is formed from dimethylnitrosamine *in vivo* (Magee & Farber, 1962). Matsumoto & Higa (1966) have shown that methylazoxymethyl acetate alkylates DNA and RNA at the 7-position of guanine *in vitro* at pH 7·4.

In the present experiments, doses of methylazoxymethyl acetate and cycasin were chosen to be equimolar to the doses of dimethylnitrosamine used by Magee & Farber (1962), namely 0·4 m-mole of toxin/kg. body wt. The amount of 7-methylguanine resulting from a single administration of dimethylnitrosamine is about 1% of the guanine in liver RNA. In control liver RNA about 0·06% of the total guanine was recovered as 7-methylguanine, but no 7-methylguanine could be detected in control DNA. It is estimated that approx. 0·1% of the liver RNA guanine and 0·24% of the liver DNA guanine is recovered as 7-methylguanine from rats treated with a single dose of cycasin. The possibility that the increased proportion of 7-methylguanine in the RNA of the cycasin-treated animals might have resulted from a change in the relative proportion of 'soluble' RNA to total RNA cannot be excluded. In the double-label experiment with the aglycone derived from cycasin, approx. 2% of the total ^{14}C-labelled guanine in

liver RNA was methylated in the 7-position. This value is high compared with the results in the cycasin experiment. The lower level of methylation induced by cycasin compared with that by methylazoxymethyl acetate may be, in part, because less of the administered dose reached the target organ, owing to absorption of the unhydrolysed glycoside from the intestinal tract and its excretion in faeces and urine (Laqueur, 1964, 1965; Kobayashi & Matsumoto, 1965). The possibility that the higher level of methylation induced by methylazoxymethyl acetate is a result of a specific methylation of a species of RNA that had been labelled to a greater extent by [^{14}C]formate is unlikely because the nuclear RNA, ribosomal RNA and 'soluble' RNA of these rats had approximately equal specific radioactivities.

One difference between dimethylnitrosamine and cycasin is that dimethylnitrosamine does not induce tumours in the intestine whereas cycasin does. No significant amount of methylation of intestinal DNA and RNA was detected after the administration of dimethylnitrosamine, whereas a very small but detectable amount of 7-methylguanine was found in intestinal RNA after the administration of methylazoxymethyl acetate. In recent experiments (P. N. Magee, unpublished work), no metabolism of dimethylnitrosamine by rat small intestine *in vitro* could be detected. It is known that cycasin is hydrolysed to the aglycone by the intestinal flora and that this aglycone is unstable (Kobayashi & Matsumoto, 1965). Also, the tumours caused by cycasin in rat intestine occur in the large intestine, the site where most of the hydrolysis of cycasin occurs. These observations might suggest that the production of intestinal tumours by cycasin is dependent on the metabolism of the glycoside in the lumen of the gut.

It has been proposed (Brouwers & Emmelot, 1960) that dimethylnitrosamine is demethylated by liver microsomes to form formaldehyde and monomethylnitrosamine. Monomethylnitrosamine is unstable at body temperature and is thought to decompose to yield a methylating agent, possibly diazomethane. Diazomethane is known to be a potent alkylating intermediate, and it induces tumours (Schoental & Magee, 1962). Miller (1964) has suggested a similar metabolic pathway for cycasin leading to the formation of diazomethane. This involves hydrolysis of cycasin by a β-glucosidase in the intestinal flora to form the unstable aglycone, methylazoxymethanol. This decomposes, losing formaldehyde, to produce $CH_3 \cdot N(O){:}NH$, which could then lose water to form diazomethane.

The data presented here on inhibition of protein synthesis and methylation of nucleic acids support the proposal for a common intermediate in the metabolism of dimethylnitrosamine and cycasin by the liver.

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