Effect of Dimethylnitrosamine on Enzyme Induction in Rat Liver

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1. The effects of various doses of dimethylnitrosamine on the hydrocortisone induction of tryptophan pyrrolase were studied. A single LD₅₀ dose of dimethylnitrosamine inhibits the synthesis of the enzyme if given within the first 5 hr. of the induction. A quarter of this dose also inhibits the synthesis of the enzyme, but, in addition, retards the rate of decay of the enzyme.
2. A single small dose of dimethylnitrosamine significantly inhibits the hydrocortisone induction of tryptophan pyrrolase for at least 14 days without causing widespread damage to liver substructure.
3. The inhibition by dimethylnitrosamine of induced tryptophan pyrrolase synthesis is probably independent of any action of the toxin on the synthesis of cofactors necessary for full expression of the enzyme's activity. Dimethylnitrosamine appears to act directly on the synthesis of the enzyme protein.
4. The synthesis of that form of the enzyme which is most sensitive to hydrocortisone is apparently also most susceptible to the action of dimethylnitrosamine.
5. It is suggested that the inhibition of protein synthesis by dimethylnitrosamine may be a result of methylation of messenger RNA, which then is unable to code effectively for amino acid polymerization.

Dimethylnitrosamine, a chemical carcinogen, causes acute liver injury and inhibits hepatic protein synthesis shortly after administration (Magee, 1958). It has been suggested that the biological action of dimethylnitrosamine requires metabolic conversion into a toxic intermediate (see Magee & Barnes, 1967), and there is evidence that nucleic acids and proteins become methylated in the liver and some other organs of rats treated with dimethylnitrosamine (Magee & Farber, 1962; Magee & Hultin, 1962; Craddock & Magee, 1963). Villa-Treviño (1967) has shown that inhibition of hepatic protein synthesis by dimethylnitrosamine *in vivo* is accompanied by breakdown of the liver ribosomal aggregates and has suggested that methylation of messenger RNA may occur and be related to the inhibition of protein synthesis.

Garren, Howell, Tomkins & Crocco (1964) have studied the effects of various inhibitors on the induction of tryptophan pyrrolase in the livers of rats treated with hydrocortisone. These authors reported marked differences in the inhibition of the synthesis of the enzyme by actinomycin D, thought to be an inhibitor of the transcription phase of protein synthesis, and of puromycin, thought to be an inhibitor of translation. By carefully choosing the time of administration of actinomycin D after

hydrocortisone, an apparent stimulation in enzyme synthesis resulted, whereas puromycin was capable only of inhibiting this synthesis.

Cycasin, another hepatotoxin and carcinogen, which is thought to be metabolized to the same biochemically active intermediate as dimethylnitrosamine, has been found to inhibit the induction of hepatic tryptophan pyrrolase in the rat (R. C. Shank, unpublished work). Because of the similarities between cycasin and dimethylnitrosamine (Miller, 1964; Shank & Magee, 1967), it was decided to study further the mechanism of action by which alkylating agents, especially dimethylnitrosamine, inhibit protein synthesis. The present paper reports the studies on the mechanism of action by which dimethylnitrosamine inhibits induced protein synthesis, by using the experimental model of Garren *et al.* (1964).

MATERIALS AND METHODS

Dimethylnitrosamine. Dimethylnitrosamine, supplied by British Drug Houses Ltd. (Poole, Dorset), was redistilled before use. It was determined by the polarographic method of Heath & Jarvis (1955) and administered in 0.9% NaCl by intraperitoneal injection.

Hydrocortisone. Hydrocortisone sodium succinate (Efcortelan) was obtained from Glaxo Laboratories (Greenford, Middx.) and was administered by intraperitoneal injection after solution in 0.9% NaCl.

Other chemicals. L-Tryptophan, kynurenine, haematin

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and haemoglobin (containing approx. 85% of methaemoglobin) were supplied by Sigma Chemical Co. (St Louis, Mo., U.S.A.).

Isotopically labelled compounds. [^{14}C]Dimethylnitrosamine (specific radioactivity 4.8 mc/m-mole) was prepared by Dr D. F. Heath by the method of Dutton & Heath (1956). L-[^{14}C]Leucine (specific radioactivity 8.5 mc/m-mole) was obtained from The Radiochemical Centre (Amersham, Bucks.).

Animals. Wistar-derived rats (150–200 g.) of the Porton strain were either bred in the laboratory or were obtained from Scientific Products (Canterbury, Kent). The laboratory-bred animals were derived from a specific-pathogen-free (S.P.F.) colony.

Adrenalectomized male rats (150–160 g. body wt.) were purchased from Carworth Animal Breeding Laboratories (New York, N.Y., U.S.A.) and from Scientific Products (Canterbury, Kent). All rats were maintained on M.R.C. diet 41B (Bruce & Parkes, 1956) and were kept without food overnight before administration of hydrocortisone or dimethylnitrosamine or both. Animals studied more than 24 hr. after drug treatment were again given food *ad libitum*. Adrenalectomized rats were given 1% NaCl in place of drinking water. All animals were killed by a blow on the head and bled from the neck vessels. The livers were rapidly removed, rinsed in ice-cold 0.9% NaCl, blotted and weighed. They were either used fresh or were frozen in solid CO_2 , after being wrapped in aluminium foil, and kept at -25° for up to 5 days before use as described in the text.

Assay of tryptophan pyrrolase. Tryptophan pyrrolase was generally determined on whole-liver homogenates by the method of Knox & Auerbach (1955). In some experiments, as indicated in the text, the enzyme was assayed by three additional methods: on cell sap and on cell sap plus haematin by the methods of Feigelson & Greengard (1961) and on preincubated cell sap by the method of Knox, Piras & Tokuyama (1966).

Amino acid incorporation into proteins. Rats were injected intraperitoneally with 0.2 ml. of a solution containing 1.67 μC of L-[^{14}C]leucine in 0.9% NaCl 30 min. before they were killed. Liver proteins were prepared by a slight modification of the conventional method of Siekevitz (1952) and assayed for radioactivity.

Metabolism of dimethylnitrosamine in vivo. Rats were injected intraperitoneally with 0.2 ml. of a solution containing 1.125 mg. (2.2 μC) of [^{14}C]dimethylnitrosamine (7.5 mg./kg. body wt.) and were placed in metabolism cages. Expired CO_2 was trapped in 2N-NaOH continuously for 6 hr. Duplicate samples of the alkali solution were taken at regular intervals, and BaCO_3 was precipitated after the addition of carrier Na_2CO_3 as described by Heath (1962) and assayed for radioactivity.

Metabolism of dimethylnitrosamine in vitro. Approx. 200 mg. of liver slices, weighing 80–100 mg. each, were incubated in Krebs–Ringer phosphate buffer (Umbreit, Burris, & Stauffer, 1964) containing 35 μg . (0.015 μC) of [^{14}C]dimethylnitrosamine in a conventional Warburg apparatus for 4 hr., CO_2 was trapped in alkali in the centre wells of the flasks and BaCO_3 was precipitated as described above before assay of radioactivity.

Radioactivity assays. A Packard Tri-Carb model 3000 liquid-scintillation spectrometer was used with [^{14}C]toluene internal standards to correct for quenching. Protein samples (5–10 mg.) were dissolved in Hyamine

Fig. 1. Effect of dimethylnitrosamine on hydrocortisone induction of tryptophan pyrrolase. A 5 mg. dose of hydrocortisone (as the sodium succinate) was injected intraperitoneally into male adrenalectomized rats at zero time, and at zero time or 2, 3, 4, 6 or 8 hr. later 30 mg. of dimethylnitrosamine/kg. body wt. was administered intraperitoneally to some of the hormone-treated rats. Control hydrocortisone-treated rats were killed 2, 4, 6, 8 or 12 hr. after hormone administration (●). Dimethylnitrosamine-treated rats were killed 2 or 4 hr. after receiving the toxin (○). Each point represents the mean tryptophan pyrrolase activity (expressed as μmoles of kynurenine formed/g. of liver/hr. at 37°) from individual duplicate assays on five rats measured by the assay method of Knox & Auerbach (1955).

hydroxide [*p*-(di-isobutylcresoxyethoxyethyl)dimethylbenzylammonium chloride, hydroxide form] and counted in a toluene scintillation mixture (Vaughn, Steinberg & Logan, 1957). The $\text{Ba}_2^{14}\text{CO}_3$ was assayed in suspension in a liquid-scintillation solution containing a thixotropic gel (Cab-O-Sil; Packard Instrument Co., La Grange, Ill., U.S.A.).

RESULTS

The effect of dimethylnitrosamine (30 mg./kg. body wt.) on the induction of tryptophan pyrrolase by hydrocortisone in adrenalectomized rats is shown Fig. 1. Dimethylnitrosamine given within the first 4 hr. after hydrocortisone inhibited the induction of tryptophan pyrrolase. When given 6 hr. or later after hydrocortisone, when synthesis of the enzyme was finished, dimethylnitrosamine had no effect on the induction curve. When dimethylnitrosamine and the hormone were given at the same time, the effect of the carcinogen was not apparent until after 2 hr. However, this effect of dimethylnitrosamine was detected within 2 hr. after its administration when the toxin was given 2–4 hr. after the hydrocortisone.

The dose of 30 mg./kg. body wt. is near to the median lethal level and causes severe necrosis of the liver. Therefore experiments were carried out to determine whether the inhibition of induction of hepatic tryptophan pyrrolase by hydrocortisone could be detected with lower doses of dimethylnitrosamine.

Fig. 2. Dose-response curve for the inhibition by dimethylnitrosamine of tryptophan pyrrolase induction. Rats were given 1, 5, 7.5, 10 or 30 mg. of dimethylnitrosamine/kg. body wt. immediately after receiving 5 mg. of hydrocortisone by intraperitoneal injection. Five rats were given hydrocortisone only. The rats were killed 4 hr. after hydrocortisone administration and tryptophan pyrrolase activities on whole-liver homogenates were measured. The percentage inhibition of induction was plotted against dose of dimethylnitrosamine on log-probability paper by the method of Litchfield & Wilcoxon (1949).

Fifteen male rats were given hydrocortisone (5mg.) and dimethylnitrosamine at the same time by separate intraperitoneal injections. The dose range of dimethylnitrosamine was 0–30 mg./kg. body wt. and the control animals received saline only. The degree of inhibition of enzyme induction with different doses of dimethylnitrosamine was determined and, when plotted on log-probability paper by the procedure of Litchfield & Wilcoxon (1949), gave an estimate of the ED_{50} , i.e. the dose of dimethylnitrosamine that is 50% effective in inhibiting the induction of tryptophan pyrrolase (Fig. 2). The ED_{50} with 95% confidence limits was 9.6 (4.6–20.2) mg. of dimethylnitrosamine/kg. body wt.

The livers of rats killed at increasing intervals after a dose of 7.5 mg. of dimethylnitrosamine/kg. body wt. were examined histologically. Within the first 6 hr. after treatment there was some loss of cytoplasmic basophilia in the cells of the centrilobular areas, but no necrosis. At 24 hr. the centrilobular areas were congested and the parenchymal cells had indistinct outlines with eosinophilic cytoplasm. In some animals there was haemorrhagic centrilobular necrosis at 48 hr., but the lesions were less severe than those seen after a dose of 30 mg. of dimethylnitrosamine/kg. body wt.

The effect of 7.5 mg. of dimethylnitrosamine/kg.

Fig. 3. Effect of dimethylnitrosamine on tryptophan pyrrolase induction. A 5 mg. dose of hydrocortisone was injected intraperitoneally into male adrenalectomized rats at zero time, and at zero time or 2, 4, 5, 6 or 8 hr. later 7.5 mg. of dimethylnitrosamine/kg. body wt. was administered intraperitoneally to some of the hormone-treated rats. Control hydrocortisone-treated rats were killed 2, 4, 5, 6 or 8 or 12 hr. after hormone administration (●). Dimethylnitrosamine-treated rats were killed 2 or 4 hr. after receiving the toxin (○). Each point represents the mean tryptophan pyrrolase activity (expressed as μ moles of kynurenine formed/g. of liver/hr. at 37°) from individual duplicate assays on five rats measured by the whole-homogenate method of Knox & Auerbach (1955).

body wt. on hepatic tryptophan pyrrolase induction of hydrocortisone was studied in more detail in adrenalectomized rats. Male rats treated with hydrocortisone only were killed at intervals after injection to establish the control enzyme-induction curve and other animals received dimethylnitrosamine at various times after injection of the hormone as shown in Fig. 3. The pattern of inhibition of enzyme induction by the lower dose of dimethylnitrosamine (7.5 mg./kg. body wt.) was similar to that with the higher (30 mg./kg. body wt.) when the toxin was given at 0 and 2 hr. after the hormone, but it was smaller in degree. At the later times, however, the effect of the lower dose was different, especially in the 6–8 hr. period, when the usual falling-off in the enzyme activity was considerably less. The inhibitory effect of dimethylnitrosamine on tryptophan pyrrolase induction was confirmed by using the three other methods for assay of tryptophan pyrrolase described in the Materials and Methods section (Table 1).

Since the nitroso carcinogens can induce cancer in some organs after only one dose (see Magee & Barnes, 1967), the duration of the inhibition of tryptophan pyrrolase induction after treatment with dimethylnitrosamine was studied. Rats received 7.5 mg. of dimethylnitrosamine/kg. body wt. and the response of tryptophan pyrrolase to hydrocortisone induction was measured at intervals up to 14 days after the carcinogen had been given. As shown in Fig. 4 inhibition of tryptophan

Table 1. *Inhibition by dimethylnitrosamine of induced tryptophan pyrrolase synthesis measured by using four methods of assay of the enzyme*

All rats received 5 mg. of hydrocortisone; treated animals in addition received 7.5 mg. of dimethylnitrosamine/kg. body wt. at the same time as the hormone. All rats were killed 5 hr. after hormone administration. Tryptophan pyrrolase activities were measured on fresh liver by four methods: with whole homogenate (Knox & Auerbach, 1955), with cell sap and with cell sap plus haematin (Feigelson & Greengard, 1961), and with preincubated cell sap (Knox *et al.* 1966). Activities are given as means $\pm$ S.D. for five rats.

Group	Method of enzyme assay ...	Tryptophan pyrrolase activity (μ moles of kynurenine formed/g. of liver/hr. at 37°)			
		Whole homogenate	Cell sap	Cell sap + haematin	Preincubated cell sap
Control		15.1 $\pm$ 0.4	6.9 $\pm$ 0.2	13.6 $\pm$ 2.1	42.8 $\pm$ 5.6
Treated		10.6 $\pm$ 1.3	4.3 $\pm$ 0.1	9.3 $\pm$ 1.1	21.4 $\pm$ 4.7
Inhibition of induction (%)		29.8	37.7	31.6	50.0

Fig. 4. Duration of the inhibition by dimethylnitrosamine of tryptophan pyrrolase induction. Forty male rats were given a single intraperitoneal injection of dimethylnitrosamine (7.5 mg./kg. body wt.). At 12 hr. and 1, 2, 3, 4, 5, 9 and 14 days later these rats were compared with controls for response to hydrocortisone administration as measured by the increase in tryptophan pyrrolase activity. The percentage inhibition of induction was calculated from the average activities from five control and five treated rats for each point on the curve. Tryptophan pyrrolase activity was assayed by the whole-homogenate method of Knox & Auerbach (1955). Experimental details are given in the text.

pyrrolase induction was still present 14 days after the single dose of dimethylnitrosamine. The livers at the 14-day interval appeared histologically normal.

The long duration of inhibition of enzyme induction (Fig. 4) was shown by using the enzyme assay method of Knox & Auerbach (1955) on whole homogenate of liver. Essentially the same results were obtained when the same method was repeated with fresh unfrozen livers and with the three other methods for holoenzymes, apoenzymes and 'latent' enzymes, as described in the Materials and Methods section. Rats killed at 7 and 14 days after treatment with dimethylnitrosamine had enzyme activities 83–84%, 95–97%, 83–87% and 80–97% of the control values when determined by the whole-homogenate method, the cell-sap method, the cell-

Table 2. *Effect of hydrocortisone on the inhibition by dimethylnitrosamine of L-[¹⁴C]leucine incorporation into hepatic protein*

At zero time rats received by intraperitoneal injection either 1 ml. of saline, or 5 mg. of hydrocortisone, or hormone followed by 7.5 mg. of dimethylnitrosamine/kg. body wt.; each rat received 1.67 μ C of L-[¹⁴C]leucine in 0.2 ml. of saline 3.5 hr. later. All rats were killed 30 min. after receiving the intraperitoneal injection of leucine. The specific radioactivities are given as means $\pm$ S.D. for five rats. The *P* value was determined by Student's *t* test and is a measure of the significance between the mean specific activities of protein from dimethylnitrosamine-treated and dimethylnitrosamine-plus-hydrocortisone-treated animals.

Treatment	Sp. radioactivity of hepatic proteins	
	(disintegrations/min./mg.)	(% of control)
None (control)	738 $\pm$ 38	100
Hydrocortisone (5mg./kg. body wt.)	800 $\pm$ 34	108.4
Dimethylnitrosamine (7.5 mg./kg. body wt.)	624 $\pm$ 61 (<i>P</i> = 0.01)	84.6
Hydrocortisone and dimethylnitrosamine	504 $\pm$ 20	63.0

sap-plus-haematin method and the preincubated-cell-sap method respectively.

The above experiments clearly show that dimethylnitrosamine could inhibit hepatic protein synthesis profoundly, as measured by enzyme induction, at a dose of 7.5 mg./kg. body wt. Previous work had reported inhibition at 30 mg./kg. body wt. as measured by incorporation of labelled amino acids into liver proteins *in vivo* (Magee, 1958; Villa-Treviño, 1967). The effect of the lower dose of dimethylnitrosamine on amino acid incorporation was therefore tested with and without simultaneous administration of hydrocortisone. As shown in

Time after dimethylnitrosamine administration (hr.)

Fig. 5. Effect of hydrocortisone on the metabolism of dimethylnitrosamine *in vivo*. A female rat was given 7.5 mg. (2-2 μ C) of [14 C]dimethylnitrosamine/kg. body wt. intraperitoneally (●). A second rat was treated similarly except that 5 hr. before being given dimethylnitrosamine it received 5 mg. of hydrocortisone intraperitoneally (○). Expired $^{14}\text{CO}_2$ was trapped for 5 hr. and was assayed for radioactivity. Experimental details are given in the text.

Fig. 6. Effect of hydrocortisone on the hepatic metabolism of dimethylnitrosamine *in vitro*. Three male rats were killed 2, 4 or 6 hr. after receiving 5 mg. of hydrocortisone. Approx. 200 mg. of slices prepared from the livers of these rats were incubated in Krebs-Ringer phosphate buffer containing 35 μ g. (0-15 μ C) of [14 C]dimethylnitrosamine. The production of $^{14}\text{CO}_2$ (●) and removal of dimethylnitrosamine (○) were measured; details are given in the text.

Table 2, hydrocortisone alone had a slightly stimulatory, if any, effect, and dimethylnitrosamine was slightly inhibitory. The hormone and carcinogen combined caused a more definite inhibition.

Since the biological action of dimethylnitrosamine may be dependent on its metabolic conversion into an active metabolite (see Magee & Barnes, 1967),

the effect of hydrocortisone treatment on this process was studied. A comparison of the rate of oxidative demethylation of dimethylnitrosamine in hydrocortisone-treated and control rats showed that the corticosteroid slightly accelerated the initial rate of metabolism of dimethylnitrosamine, but the effect was not marked (Fig. 5). The rate of metabolism of dimethylnitrosamine by rat liver slices *in vitro* was also slightly greater after pretreatment of the rats with hydrocortisone, but again the increase was not large (Fig. 6).

DISCUSSION

The work of Garren *et al.* (1964) on the hydrocortisone induction of tryptophan pyrrolase illustrated how this induction system could be used to study the mechanisms of action of inhibitors, and perhaps indicate whether an inhibitor was effective at the levels of transcription or translation in protein synthesis. These authors suggested that the translation of the messenger RNA associated with tryptophan pyrrolase synthesis could be inhibited through a cytoplasmic repressing mechanism that itself is dependent on continued RNA and protein synthesis (Garren *et al.* 1964; Tomkins, Garren, Howell & Peterkofsky, 1965). Kenney & Albritton (1965) have reported evidence to suggest that this repressing mechanism is under hormonal (somatotrophin) control. Basically, the suggested mechanism states that, when the rate of enzyme synthesis is maximal, the liver cell is stimulated to produce and transcribe a 'repressor' messenger RNA that acts not at the operator site but rather at some site outside the nucleus (perhaps the endoplasmic reticulum). Either this new messenger can act directly on the tryptophan pyrrolase messenger or it can be translated into a protein that acts on the enzyme messenger. In either case the result would be that further translation of the tryptophan pyrrolase messenger RNA is inhibited and synthesis of the enzyme is arrested.

Large doses of dimethylnitrosamine seem to elicit a response similar to that of puromycin (a translation inhibitor) according to the work of Garren *et al.* (1964). However, when a smaller dose of dimethylnitrosamine is given, the puromycin-like response is not seen. Instead, the toxin appears to retard the rate of decay of tryptophan pyrrolase when given 6-8 hr. after hormone administration. If given earlier, dimethylnitrosamine inhibits the synthesis of the enzyme but to a smaller extent than the higher dose. Fig. 7 illustrates a semi-logarithmic plot of data given in Fig. 3. In this way the rate of decrease of tryptophan pyrrolase in hydrocortisone-stimulated rats is compared with that of hydrocortisone-plus-dimethylnitrosamine-treated rats. It can be seen that the enzyme activity

Fig. 7. Effect of dimethylnitrosamine on the rate of decay of tryptophan pyrrolase in hydrocortisone-treated rats. Data were taken from Fig. 3; values are for enzyme activities at 6, 8 and 12 hr. after hydrocortisone for control rats (●) and at 8 and 10 hr. after hydrocortisone for rats given dimethylnitrosamine 6 hr. after the hormone (○). The data were plotted on semi-logarithmic paper to determine the half-lives of the enzyme in control ($t_{1/2}$ 2.6 hr.) and treated ($t_{1/2}$ 6.1 hr.) animals.

decays at a rate 2.4-fold lower in toxin-treated rats. There are several possible explanations for this delay in the decrease of enzyme activity, but, in the light of the suggestion that dimethylnitrosamine methylates messenger RNA (Villa-Treviño, 1967) and that the suggested cytoplasmic 'repressor' involved in tryptophan pyrrolase synthesis may depend on continued RNA and protein synthesis, it seems plausible to suggest that methylation of this 'repressor' messenger RNA might occur in dimethylnitrosamine-treated rats and result in de-repression of the tryptophan pyrrolase synthesis. Full de-repression, however, would not be expected because some of the pre-existing tryptophan pyrrolase messenger RNA would also be methylated and therefore would be inactive in translation. The result would be a partially hindered repression of the enzyme synthesis superimposed on a partially active synthesis of new enzyme, rather than a completely inactive synthesis of the enzyme as seen in the hydrocortisone-stimulated control animals. Very important to this argument is the work of Wilhelm & Ludlum (1966). These authors prepared a synthetic template for polypeptide synthesis containing 7-methylguanine and obtained evidence to suggest that 7-methylguanine renders coding triplets inactive.

In the light of the suggestion of Craddock & Magee (1967) that methylated RNA in the liver cell may be stripped of its 7-methylguanine

(24–48 hr. for DNA from dimethylnitrosamine-treated rats; Craddock & Magee, 1965), it would seem that the liver should respond normally to hydrocortisone when the hormone is given to rats several days after toxin administration. This is seen not to be the case in Fig. 6. Even 14 days after a single dose of dimethylnitrosamine, which disturbs very little the architecture of the liver, the liver cell is not able to respond fully to hormone administration as compared with livers from control animals. Strauss & Wahl (1964) have shown that the administration to bacteria of the alkylating agent methyl methanesulphonate results in the production of an altered DNA. The stripping of 7-methylguanine from DNA may also alter the nucleic acid (Lawley & Brookes, 1963); this might suggest an involvement of an altered DNA that has become less effective in serving as a template for the transcription of messenger RNA corresponding to tryptophan pyrrolase synthesis. Should this be the case it would not be difficult to see how a compound like dimethylnitrosamine could alter a living cell in such a way as to endow it (through its DNA) with the property of producing daughter cells that have a diminished capacity to control a specific aspect of protein synthesis. Similar mechanisms could lead to carcinogenesis.

Smuckler, Arrhenius & Hultin (1967) have shown that dimethylnitrosamine decreases the quantity of cytochrome P-450 in liver microsomes. Since tryptophan pyrrolase requires haematin for activation, it might be possible that the reason for the delay in the return of full inducibility of tryptophan pyrrolase in dimethylnitrosamine-treated rats was not a problem in protein synthesis, but rather a lack of a cofactor for the activation of the enzyme protein. Thus it was decided to study more carefully the method in which tryptophan pyrrolase activity was measured. The standard assay method used throughout these experiments was one with whole homogenate of the liver as a source of the enzyme, with no added haematin. Feigelson & Greengard (1961) reported an assay method to distinguish between the apoenzyme (with cell sap as the source of the enzyme) and the holoenzyme (with haematin added to the cell sap). Knox *et al.* (1966) reported a method (with cell sap preincubated with haemoglobin, ascorbic acid and tryptophan) that measures not only the holoenzyme and apoenzyme forms of tryptophan pyrrolase, but also a 'latent form', which appears to be the form that responds greatest to hydrocortisone administration. By using all four methods of assay simultaneously (with whole homogenate, with cell sap, with cell sap plus haematin and with preincubated cell sap) it was found that qualitatively the results were the same, namely that dimethylnitrosamine inhibited the induction of tryptophan pyrrolase by hydro-

cortisone for as long as 14 days. Quantitatively dimethylnitrosamine was more effective against an increase in the 'latent form' of the enzyme, the form most sensitive to hydrocortisone, and therefore may be more effective against the synthesis and translation of the new messenger RNA.

Preliminary studies (R. C. Shank, unpublished work) indicate that when liver homogenates from hydrocortisone-treated rats are incubated for 1 hr. at 37° with concentrations of dimethylnitrosamine as high as 10mM (final concentration in incubation medium) there is no direct effect of the toxin on the activity of tryptophan pyrrolase. Therefore it is unlikely that the inhibition of the induced synthesis of this enzyme seen in the experiments reported here is due to a direct reaction between the inhibitor and the enzyme itself.

It has been established that prolonged starvation of rats results in a decrease in the number of liver cytoplasmic ribosomes and the rate at which the ribosomes are replaced during starvation (Hirsch & Hiatt, 1966; Wilson & Hoagland, 1967). Because the rats in the studies reported in this paper were starved for up to 24 hr., the possibility exists that the ribosomes of these animals may be rendered more susceptible to dimethylnitrosamine by starvation; this aspect has not yet been examined.

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