Studies on Azo-Dye Carcinogenesis in Rat Liver

THE EFFECT OF 4-DIMETHYLAMINO-3'-METHYLAZOBENZENE ON THE INCORPORATION OF [14 C]LEUCINE INTO RAT-LIVER MICROSOMAL PROTEIN

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Studies on the effect of chemical carcinogens on protein synthesis in rat liver have been carried out by various workers. Hultin, Arrhenius, Löw & Magee (1960), using the water-soluble carcinogen dimethylnitrosamine, showed it to cause considerable inhibition of the incorporation of [14 C]leucine in both rat-liver slices and microsomal preparations a few hours after injection. Brouwers & Emmelot (1960), also using dimethylnitrosamine, obtained similar results at 3 hr. after a single injection of the carcinogen; they also showed that the labelling of transfer RNA with [14 C]leucine was the same for preparations from both treated and untreated animals, thus indicating that the inhibitory effect is at the microsomal level.

Arrhenius & Hultin (1961*a*) studied the effect of 2-aminofluorene and related amines on the incorporation of [14 C]valine into proteins of rat-liver slices, and showed it to cause considerable inhibition. In these experiments *in vitro*, a preincubation of the liver slices with the carcinogen was necessary to obtain maximal inhibition, suggesting that 2-aminofluorene is a precursor of the actual inhibitory agent. Inhibition was also observed in cell-free incorporating systems a few hours after treatment *in vivo* with the same carcinogen (Arrhenius & Hultin, 1959).

Work by Arrhenius & Hultin (1961*b*, 1962) has shown, however, that, at 8–48 hr. after the administration of 2-aminofluorene, isolated microsomal preparations showed an enhanced incorporation of labelled amino acid. This stimulation occurs after the early inhibition previously reported.

We have studied the effects of the carcinogen 4-dimethylamino-3'-methylazobenzene on protein synthesis by rat-liver microsomal preparations, and the results of our investigations which differ to some degree from those of the above workers are reported in the present paper. A preliminary report of part of this work has appeared (Hawtre & Schirren, 1962).

MATERIALS AND METHODS

Chemicals and enzymes. ATP (crystalline disodium salt), phosphoenolpyruvate (silver-barium salt), GSH, ribonuclease and pyruvate kinase [suspended in 2.1 M-(NH₄)₂SO₄

solution, pH 6.0] were obtained from C. F. Boehringer und Soehne, Mannheim, Germany. ATP was neutralized to pH 7.6 with 1 N-KOH before use. Phosphoenolpyruvate was prepared immediately before use from the silver-barium salt by treatment with (NH₄)₂SO₄ under acid conditions and finally adjusted to pH 7.6. GTP and deoxyribonuclease were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. GTP was neutralized to pH 7.6 before use with 1 N-KOH. Uniformly labelled L-[14 C]leucine was obtained from The Radiochemical Centre, Amersham, Bucks. Unlabelled L-leucine and other amino acids were of analytical reagent grade and were supplied by the H.M. Chemical Co. Ltd., Santa Monica, Calif., U.S.A. 4-Dimethylamino-3'-methylazobenzene (m.p. 120.4–121.2°) was prepared as described by Miller & Miller (1948). Formic acid (98%) was supplied by E. Merck, Darmstadt, Germany; it was diluted to 80% (v/v) with water before use. Tris was obtained from British Drug Houses Ltd., Poole, Dorset; tris buffers were adjusted to the required pH with 3 N-HCl. Sucrose was of analytical reagent grade obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex.

Preparation of cell particulates and enzyme fractions. Female rats (200–250 g., unless otherwise stated) were injected intraperitoneally with 4-dimethylamino-3'-methylazobenzene (50 mg./250 g. body wt.) dissolved in 2–2.5 ml. of olive oil and were killed by decapitation at various times thereafter. Livers were quickly removed, placed in ice-cold beakers and weighed. They were carefully washed four or five times in medium A [containing (final concentrations): sucrose (0.25 M), MgCl₂ (5 mM), KCl (25 mM) and tris-HCl, pH 7.6 (50 mM)], blotted dry on Whatman no. 1 filter paper and transferred to a small mortar. The tissue was then minced with scissors, pulped to a fine paste and finally transferred to an all-glass Dounce homogenizer (supplied by Blaessig Glass Specialities, Rochester, N.Y., U.S.A.). Homogenization was carried out in 2 vol. of medium A, with 8–12 strokes of the loose plunger (clearance, 0.0015 in.). The homogenate was centrifuged at 1250*g* for 10 min. to remove nuclei and cell debris, and the resulting supernatant fraction centrifuged at 8500*g* for 15 min. to remove mitochondria. Centrifuging of the mitochondrial supernatant was carried out in the Spinco model L preparative centrifuge (no. 40 rotor) for 60 min. at 105 000*g*. The clear supernatant fraction above the microsomal pellet was removed by means of a Pasteur pipette and stored at 0° for preparation of the pH 5 fraction. The microsomal pellet was then rinsed 6–8 times with medium A, and the sides of the centrifuge tube were cleaned and wiped dry with filter paper. The pellets were washed once by resuspension in medium A (in the Dounce homogenizer, with 4–6 strokes

with the loose plunger) followed by centrifuging at 105 000g for 60 min. They were finally suspended in medium A and kept at 0°.

pH 5 fraction. This was prepared from the clear microsomal supernatant by adjustment of the solution to pH 5.2 with 1N-acetic acid, followed by centrifuging of the suspension at 1900g for 15 min. The white pellet of pH 5 amino acid-activating enzymes was rinsed with ice-cold water and then washed once by resuspension in ice-water. It was finally suspended in medium A and kept at 0°.

pH 5 supernatant fraction. This was obtained after removal of the pH 5 precipitate. It was adjusted to pH 7.6 immediately by the dropwise addition of 3N-KOH, and kept at 0°.

System for the incorporation of [¹⁴C]leucine into microsomal and supernatant proteins. The reaction medium used for incorporation studies contained: tris-HCl buffer, pH 7.6 (final concn. 35 mM); KCl (final concn. 18 mM); MgCl₂ (final concn. 9 mM); sucrose (final concn. 0.2M); ATP (potassium salt), pH 7.6 (1.5 μmoles); GTP (potassium salt), pH 7.6 (0.33 μmole); phosphoenolpyruvate (potassium salt) (9.0 μmoles); phosphoenolpyruvate kinase (40 μg); L-[¹⁴C]leucine (0.6 or 1.0 μC). The final reaction volume was 2.0 ml. Incubations were carried out in test tubes or Erlenmeyer flasks under aerobic conditions with shaking at 37° for various times. For studies of total incorporation, the reaction was terminated by the addition of 0.2 ml. of a saturated solution of [¹⁴C]leucine and 2.0 ml. of 10% (w/v) trichloroacetic acid. In experiments where incorporation into both microsomes and supernatant fractions was studied, the following method was used. The reaction mixture (2.0 ml.) was quickly added to 8.0 ml. of ice-cold 0.25M-sucrose in a plastic centrifuge tube containing 0.2 ml. of a saturated solution of [¹⁴C]leucine. The contents were mixed and then centrifuged at 105 000g for 60 min. The resulting supernatant was carefully decanted, and to it was added 1.5 ml. of 30% (w/v) trichloroacetic acid. The microsomal pellet was rinsed ten times with cold water, then resuspended in 2.0 ml. of 0.25M-sucrose and precipitated by the addition of 2.0 ml. of 10% (w/v) trichloroacetic acid. All precipitated nucleoproteins were kept overnight in the refrigerator before being treated further.

Washing and counting of proteins. Washing of the ¹⁴C-labelled proteins was carried out essentially according to the method of Mans & Novelli (1961). The washed labelled proteins were dissolved in 80% (v/v) formic acid, plated on planchets of 5.0 cm. diameter and dried under an infrared lamp. Counting was carried out with a gas proportional counter with appropriate corrections for thickness and background absorption.

Protein content of microsomes and different enzyme preparations. This was determined according to the biuret method of Cleland & Slater (1953), with a bovine albumin standard.

Ribonucleic acid. RNA was determined by the method of Hoagland, Stephenson, Scott, Hecht & Zamecnik (1958).

Determination of azo dye bound to protein of different cellular fractions. The microsomal, pH 5-enzyme and pH 5-supernatant fractions were prepared as described above. Protein plus nucleic acid of the various fractions was precipitated by the addition of trichloroacetic acid to give a final concentration of 5% (w/v). The nucleoprotein precipitates were washed once with 10% (w/v) trichloroacetic

acid and twice with 96% (v/v) ethanol at room temperature, and the protein-bound dye was determined by the method of Dijkstra & Joubert (1961).

RESULTS

Protein synthesis at different times after the administration of 4-dimethylamino-3'-methylazobenzene

The incorporation of [¹⁴C]leucine into microsomal protein from washed-microsomes-pH 5-enzyme and mitochondrial-supernatant systems was examined at various times after a single intraperitoneal injection of 4-dimethylamino-3'-methylazobenzene (Fig. 1). The time-intervals chosen were based on the analysis of protein-bound 4-dimethylamino-3'-methylazobenzene in the microsome, pH 5-enzyme and pH 5-supernatant fractions (Table 1), which showed the same time-course of binding to proteins of the whole liver as reported by Dijkstra & Louw (1962), where maximum binding was found at about 40 hr. after dosing.

Fig. 1. Effect of a single intraperitoneal injection of 4-dimethylamino-3'-methylazobenzene on the incorporation of [¹⁴C]leucine *in vitro* at different times by: ●, washed microsomes plus pH 5 enzyme; ○, mitochondrial supernatants; ■, washed microsomes. The composition of the reaction medium was as described for Fig. 3. Reactions were run for 30 min. at 37° with constant shaking. Female rats, weight 100–115 g., were used.

Fig. 1 shows that 4-dimethylamino-3'-methylazobenzene causes a stimulation of the incorporation of [14 C]leucine into microsomal protein from the washed-microsomes-pH 5-enzyme system. The greatest enhancement of stimulation occurred at 40 hr. after the administration of the dye when the microsomes had a specific activity of 4000 counts/min./mg. This represents an approximately four-

Table 1. Concentrations of covalently-bound dye in the microsomes, pH 5 enzyme and pH 5 supernatant at various times after a single injection of 4-dimethylamino-3'-methylazobenzene

Proteins containing covalently-bound dye were processed as described in the Materials and Methods section. All animals received a single intraperitoneal injection of the carcinogen dissolved in 2-2.5 ml. of olive oil (50 mg./250 g. body wt.), and were killed at various times thereafter. Results are expressed as $\log(I_0/I)$ at 520 $m\mu$, e.g. extinction at 520 $m\mu$ of dye/50 mg. of material, dissolved in a mixture of 2.0 ml. of ethanol and 2.5 ml. of 7N-HCl.

Time after injection (hr.) ...	Protein-bound dye [$\log I_0/I$ at 520 $m\mu$ /50 mg. of material]			
	4	17	42	132
Liver fraction				
Microsomes	0.045	0.127	0.211	0.148
pH 5 enzyme	0.023	0.114	0.141	0.055
pH 5 supernatant	0.060	0.227	0.343	0.206

Fig. 2. Time-course of incorporation of [14 C]leucine into microsomal and soluble protein from normal and azo-dye-treated rats (weight 200-220 g.). 4-Dimethylamino-3'-methylazobenzene (50 mg./250 g. body wt.) was administered by intraperitoneal injection 40 hr. previously. (a) $\circ$, 'Azo'-microsomes plus 'azo'-pH 5 enzyme; $\bullet$, normal microsomes plus normal pH 5 enzyme; $\square$, soluble 'azo'-protein; $\blacktriangle$, soluble normal protein. (b) $\bullet$, Normal microsome plus 'azo'-pH 5 enzyme; ∇ , 'azo'-microsomes plus normal pH 5 enzyme; $\blacksquare$, 'azo'-microsomal supernatant protein; $\triangle$, normal microsomal supernatant protein.

fold increase in specific activity over the normal microsomes. In the absence of added pH 5 enzyme the incorporating activity of washed microsomes remained fairly constant (Fig. 1) at different times after the administration of the dye. The time at which the microsomes-pH 5-enzyme system shows its greatest activity is the same as that at which maximal binding of 4-dimethylamino-3'-methylazobenzene to the various cell fractions occurs.

Examination of mitochondrial supernatants (that fraction containing microsomes, pH 5 enzyme and other soluble enzymes) at various times after an injection of 4-dimethylamino-3'-methylazobenzene, on the other hand, gave results different from those obtained with the washed-microsomes-pH 5-enzyme system (Fig. 1). As early as 3-4 hr. after an injection the specific activity of microsomes isolated from mitochondrial supernatant incubations was 600 counts/min./mg. compared with 1100 counts/min./mg. for those of the control. After 4 hr. there was a gradual return to normal, with a slight increase in specific activity at 40 hr. These results suggested that there is an inhibitor of the incorporation of [14 C]leucine present in the soluble fraction of homogenates from livers of rats previously injected with 4-dimethylamino-3'-methylazobenzene.

Studies on rats injected with 4-dimethylamino-3'-methylazobenzene 40 hr. previously

The incorporation of [14 C]leucine by washed-microsomes-pH 5-enzyme systems from rats injected with 4-dimethylamino-3'-methylazobenzene 40 hr. previously was studied in greater detail to try and discover the factors responsible for the stimulation. The time-course of incorporation of [14 C]leucine into microsomal and soluble proteins of the washed-microsomes-pH 5-enzyme systems from normal and azo-dye-treated rats is shown in Fig. 2 (a). In the first 10 min. of reaction the system containing microsomes and pH 5 enzyme from azo-dye-treated rats (referred to below as the 'azo'-microsomes-'azo'-pH 5-enzyme system) incorporates at a rate which is approximately twice that found with the normal system. Thereafter the incorporation rates of both systems decline and at 20 min. are practically zero. Soluble labelled proteins are released at similar rates in both systems. Fig. 2 (b) shows the rates of incorporation of the crossed-over systems containing 'azo'-microsomes plus normal pH 5 enzyme and normal microsomes plus 'azo'-pH 5 enzyme, which are similar in the first 10 min. of reaction, and are greater than that of the normal-microsomes-normal-pH 5-enzyme system but less than that of the 'azo'-microsomes-'azo'-pH 5-enzyme system.

Fig. 3 shows the effect of the concentration of pH 5 amino acid-activating enzyme on the in-

corporation of [14 C]leucine into protein of washed microsomal preparations. The 'azo'-microsomal incorporating system was saturated at a concentration of 2.25 mg. of 'azo'-pH 5 enzyme/2 ml. Almost identical results were observed for the normal-microsomes-normal-pH 5-enzyme system. At these saturating values of the concentrations of pH 5 enzyme the specific activity of 'azo'-microsomes was 740 counts/min./mg., whereas that of the normal microsomes was 420 counts/min./mg.

Fig. 3. Effect of the concentration of pH 5 amino acid-activating enzyme on the incorporation of [14 C]leucine into microsomal protein. Each reaction tube contained the following in a final volume of 2.0 ml.: tris-HCl buffer, pH 7.6 (final concn. 35 mM); KCl (final concn. 18 mM); $MgCl_2$ (final concn. 9 mM); sucrose (final concn. 0.2 M); ATP (potassium salt), pH 7.6 (1.5 μ moles); GTP (potassium salt), pH 7.6 (0.33 μ mole); phosphoenolpyruvate (potassium salt) (9 μ moles); pyruvate kinase (40 μ g.); L-[14 C]-leucine (0.66 μ C); normal microsomes or 'azo'-microsomes (2.0 mg. of protein). 'Azo'-microsomes and 'azo'-pH 5 enzyme were obtained from rats injected with 4-dimethylamino-3'-methylazobenzene 40 hr. previously. Reactions were run for 30 min. at 37°. ●, 'Azo'-microsomes plus 'azo'-pH 5 enzyme; ○, normal microsomes plus normal pH 5 enzyme; ■, normal microsomes plus 'azo'-pH 5 enzyme; ◐, 'azo'-microsomes plus normal pH 5 enzyme.

Normal microsomes were saturated with 'azo'-pH 5 enzyme at a concentration of 2.25 mg. of the latter/2 ml. and had a specific activity of 470 counts/min./mg. This is similar to the systems mentioned above. On the other hand, 'azo'-microsomes were saturated with 0.8 mg. of normal-pH 5-enzyme protein/2 ml., and had a specific activity of 350 counts/min./mg. In all the incorporating systems studied, increase of the concentration of pH 5 enzyme above the saturating value had a slight inhibitory effect on incorporation.

A study was made of the effect of increasing amounts of microsomal protein in a system containing excess of pH 5 enzyme on the total incorporation of [14 C]leucine and on the specific activity of the microsomes. The results for the normal and 'azo' systems are shown in Fig. 4, and those for the crossed-over systems in Fig. 5. In all cases the total incorporation increased linearly with the amount of microsomal protein added, as

Fig. 4. Incorporation of [14 C]leucine with a fixed quantity of pH 5 enzyme and increasing amounts of microsomal protein. The reaction mixture was as described in Fig. 3. Rats were injected 40 hr. previously with 4-dimethylamino-3'-methylazobenzene. Reactions were run for 30 min. at 37°. ●, Total incorporation by 'azo'-microsomes; ◐, total incorporation by normal microsomes; ○, specific activity of 'azo'-microsomes; ◐, specific activity of normal microsomes.

long as the pH 5 enzyme was in excess; the specific activity of the microsomes remained constant.

A study of the cofactors needed for the maximal incorporation of [14 C]leucine, by the system consisting of washed microsomes and pH 5 enzyme from rats pretreated with 4-dimethylamino-3'-methylazobenzene (Table 2), showed that both an ATP-generating system and GTP were essential. These requirements are similar to those previously found for normal systems.

Effect of starvation on protein synthesis. As controls in the above experiments we used rats fed *ad lib.* until they were killed. Since it was observed that animals injected with the dye consumed less food than did the controls, it was thought desirable to investigate the effect of reduced food intake on protein synthesis.

Table 3 shows that the specific activity of microsomes from uninjected rats starved for 40 hr. was 424 counts/min./mg. compared with 400 counts/

min./mg. for microsomes from uninjected animals fed *ad lib.* Animals injected with olive oil or 4-dimethylamino-3'-methylazobenzene in olive oil, and fed *ad lib.* for 40 hr., gave microsomal specific activities of 372 and 710 counts/min./mg. re-

Table 2. Cofactor requirements for incorporation of [14 C]leucine by a microsomal system from rats injected with 4-dimethylamino-3'-methylazobenzene 40 hr. previously

The reaction medium for incorporation was as described in the Materials and Methods section. Each tube contained also 0.6 μ C of [14 C]leucine, 3.13 mg. of microsomal protein and 1.90 mg. of pH 5-enzyme protein. Reactions were run for 30 min. at 37°. Female rats were injected with 4-dimethylamino-3'-methylazobenzene (50 mg./250 g. body wt.) 40 hr. before being killed.

Omissions from complete system	Total incorporation (counts/min.)
None	1630
ATP	53
GTP	214
Phosphoenolpyruvate	218
Phosphoenolpyruvate and pyruvate kinase	128
Pyruvate kinase	442
Microsomal particles	35
pH 5-enzyme fraction	177

Fig. 5. Incorporation of [14 C]leucine with a fixed quantity of pH 5 enzyme and increasing amounts of microsomal protein. The experimental conditions were as described for Fig. 3. Reactions were run for 30 min. at 37°. Rats were injected 40 hr. previously with 4-dimethylamino-3'-methylazobenzene. ■, Total incorporation by normal microsomes supplemented with 'azo'-pH 5 enzyme; ○, total incorporation by 'azo'-microsomes with normal pH 5 enzyme; ●, specific activity of 'normal' microsomes; □, specific activity of 'azo'-microsomes.

Table 3. Effect of nutritional states on the incorporation of [14 C]leucine by rat-liver microsomal systems

Female rats weighing 150–175 g. were used. All treatments of the animals were commenced 40 hr. before the experiment. The standard reaction medium was as described for Fig. 3. Each tube contained 0.6 μ C of [14 C]leucine. Reactions were run for 30 min. at 37°.

Pretreatment	System	Incorporation (counts/min./mg. of microsomal protein)
Intraperitoneal injection of 4-dimethylamino-3'-methylazobenzene (50 mg./250 g. body wt.) in 2.5 ml. of olive oil; food and water given <i>ad lib.</i>	Microsomes (2.78 mg. of protein) + pH 5 enzyme (1.51 mg. of protein)	710
Intraperitoneal injection of 2.5 ml. of olive oil; food and water given <i>ad lib.</i>	Microsomes (2.60 mg. of protein) + pH 5 enzyme (1.53 mg. of protein)	372
Starved, but free access to water	Microsomes (2.28 mg. of protein) + pH 5 enzyme (1.28 mg. of protein)	424
No pretreatment; food and water given <i>ad lib.</i>	Microsomes (2.20 mg. of protein) + pH 5 enzyme (1.33 mg. of protein)	400

spectively. These results show that the stimulation observed with the system from dye-treated rats is not the result of injection with olive oil or reduction of food intake.

Effect of pH 5 supernatant on protein synthesis

Fig. 1 shows that microsomes isolated after incubation of an 'azo'-mitochondrial supernatant have a lower specific activity than those of a washed microsomal preparation incubated with pH 5 enzyme. This finding suggested that there is an inhibitor of the incorporation of [14 C]leucine in the soluble cell sap. Evidence of this was obtained in titration experiments with neutralized pH 5 supernatant. Results (Fig. 6) showed that the incorporation of [14 C]leucine by a washed-'azo'-microsomes-pH 5-enzyme system was progressively inhibited by increasing amount of 'azo'-pH 5 supernatant (24 mg. of supernatant protein/2 ml. produced 70 % inhibition). The addition of normal pH 5

supernatant to the 'azo'-microsomal incorporating system also brought about inhibition, but to a smaller extent (24 mg. of supernatant protein/2 ml. produced 40 % inhibition).

Normal pH 5 supernatant and 'azo'-pH 5 supernatant also inhibited the incorporation of [14 C]leucine into protein of a normal microsomal system, but to a much smaller extent than that found for the 'azo'-microsomes (Fig. 6).

To characterize further the substances responsible for the inhibitory activity of the pH 5 supernatant, material obtained from rats injected with dye 40 hr. previously was neutralized to pH 7.6 and fractionated with ammonium sulphate into fraction A (precipitated at 40 % saturation), fraction B (precipitated between 40 % and 60 % saturation) and fraction C (soluble at 60 % saturation). The effect of these different ammonium sulphate fractions, after they had been dialysed, on the incorporation of [14 C]leucine into protein of a washed-microsomes-pH 5-enzyme system is shown in Table 4.

All three fractions stimulated the incorporation of [14 C]leucine into microsomal protein in a system consisting of 'azo'-microsomes saturated with 'azo'-pH 5 enzyme. Further, the three fractions could all replace the pH 5 enzyme, but were much less active than it on a protein basis. Inhibition of incorporation was never encountered with the dialysed ammonium sulphate fractions of the pH 5 supernatant. 'Azo'-pH 5 supernatant dialysed for 4 hr. against medium A inhibited the incorporation of [14 C]leucine less than did undialysed material (Table 4).

Table 5 shows the results of the effects of GSH, ribonuclease and chloramphenicol on the incorporation of [14 C]leucine by 'azo'-microsomes and the ammonium sulphate fractions of the pH 5 supernatant. GSH inhibited the incorporation of [14 C]leucine by these systems much less than in the system containing microsomes and pH 5 enzyme (Table 4). Ribonuclease inhibited incorporation with all the fractions used. Chloramphenicol caused some inhibition of incorporation with fraction A.

The ultraviolet-absorption spectra of the pH 5 enzyme and of the different ammonium sulphate fractions are shown in Fig. 7. Fractions A and B have typical protein spectra, whereas fraction C has a nucleoprotein spectrum. Fraction C contains considerable quantities of RNA extractable with phenol.

DISCUSSION

The present work on the effect of a single large dose of the carcinogen 4-dimethylamino-3'-methylazobenzene on protein synthesis by rat-liver microsomal systems has produced evidence that the

Fig. 6. Effect of pH 5 supernatant on the incorporation of [14 C]leucine by rat-liver microsomal preparations. The experimental conditions were as described for Fig. 3. Reactions were run for 30 min. at 37°. Rats were injected 40 hr. previously with 4-dimethylamino-3'-methylazobenzene. ●, 'Azo'-microsomes plus 'azo'-pH 5 supernatant; ○, 'azo'-microsomes plus normal pH 5 supernatant; □, normal microsomes plus normal pH 5 supernatant; ■, normal microsomes plus 'azo'-pH 5 supernatant.

Table 4. *Effect of different supernatant fractions on the incorporation of [¹⁴C]leucine into microsomal protein*

Each reaction tube contained the following in a final volume of 2.5 ml.: tris-HCl buffer, pH 7.6 (final concn. 30 mM); KCl (final concn. 20 mM); MgCl₂ (final concn. 9 mM); sucrose (final concn. 0.2 M); ATP (potassium salt), pH 7.6 (1.5 μ moles); GTP (potassium salt), pH 7.6 (0.33 μ mole); phosphoenolpyruvate (potassium salt) (9 μ moles); pyruvate kinase (40 μ g.); L-[¹⁴C]leucine (0.6 μ C); microsomes (2.20 mg. of protein); pH 5 enzyme (1.35 mg. of protein) (these last two fractions were from rats injected 40 hr. previously with 4-dimethylamino-3'-methylazobenzene). Reactions were run for 30 min. at 37°. The fractions of the pH 5 supernatant that were precipitated at 40% saturation with (NH₄)₂SO₄ (fraction A) and at between 40% and 60% saturation with (NH₄)₂SO₄ (fraction B) were dissolved in medium A and dialysed against the same for 16 hr., with four changes of dialysing medium. The pH 5 supernatant fraction soluble at 60% saturation with (NH₄)₂SO₄ (fraction C) was dialysed against 50 mM-tris-HCl buffer, pH 7.6, with eight to ten changes of buffer, for 18–20 hr. The (NH₄)₂SO₄ fractions were prepared from the pH 5 supernatant of a homogenate obtained from rats injected with 4-dimethylamino-3'-methylazobenzene 40 hr. previously.

Expt. no.	System	Total microsomal incorporation (counts/min.)
1 A	Microsomes + pH 5 enzyme	1270
	Microsomes + pH 5 enzyme + (NH ₄) ₂ SO ₄ fraction A (0.73 mg. of protein)	1424
	Microsomes + pH 5 enzyme + (NH ₄) ₂ SO ₄ fraction A (7.32 mg. of protein)	1736
	Microsomes + pH 5 enzyme + (NH ₄) ₂ SO ₄ fraction A (10.98 mg. of protein)	1496
	Microsomes + pH 5 enzyme + (NH ₄) ₂ SO ₄ fraction B (10.10 mg. of protein)	1579
	Microsomes + (NH ₄) ₂ SO ₄ fraction A (3.66 mg. of protein)	451
	Microsomes + (NH ₄) ₂ SO ₄ fraction B (5.05 mg. of protein)	660
	(NH ₄) ₂ SO ₄ fraction A (3.66 mg. of protein)	51
1 B	(NH ₄) ₂ SO ₄ fraction B (13.20 mg. of protein)	40
	Microsomes + pH 5 enzyme	1497
	Microsomes + pH 5 enzyme + (NH ₄) ₂ SO ₄ fraction C (1.03 mg. of protein)	2094
	Microsomes + pH 5 enzyme + (NH ₄) ₂ SO ₄ fraction C (1.03 mg. of protein) + GSH (20 μ moles)	464
	Microsomes + pH 5 enzyme + GSH (20 μ moles)	63
1 C	Microsomes + (NH ₄) ₂ SO ₄ fraction C (1.03 mg. of protein)	550
	Microsomes + pH 5 enzyme	1480
	Microsomes + pH 5 enzyme + pH 5 supernatant (20 mg. of protein)	540
	Microsomes + pH 5 enzyme + pH 5 supernatant (dialysed)* (20 mg. of protein)	832

* Dialysed against medium A in the cold for 4 hr.

carcinogen affects protein synthesis in a number of ways.

The incorporation of [¹⁴C]leucine into protein of a washed-microsomes pH 5-enzyme system studied at different times after a single intraperitoneal injection of 4-dimethylamino-3'-methylazobenzene showed that in the first 17 hr. the incorporation rate and specific activity of the microsomes remained unchanged. However, after this time incorporation was greatly stimulated (Fig. 1), reaching a maximum at about 40 hr., and thereafter returning to normal. The maximum binding of 4-dimethylamino-3'-methylazobenzene to protein of the microsomes, pH 5 enzyme and pH 5 supernatant occurs also at about 40 hr. after injection (Table 1). This suggests a definite relationship between dye-binding and maximum stimulation of protein synthesis. Exactly what the detailed significance of this finding is, however, remains to be determined.

Titration experiments with microsomes and pH 5 enzyme (Fig. 3) have shown that the stimu-

lation of the incorporation of [¹⁴C]leucine by the system from dye-treated rats at lower concentrations of pH 5 enzyme is due to increased activities of both the microsomal and pH 5-enzyme fractions. Mans & Novelli (1961) have assumed that each ribosomal particle of their maize-seedling incorporating system possesses a fixed and limited number of sites available for the incorporation of leucine which are rapidly filled in the presence of excess of pH 5 enzyme. According to this, the addition of more particles to a system in the presence of an excess of pH 5 enzyme should give particles of equal specific activity independent of particle concentration. They obtained results that appeared to support this assumption. We carried out similar experiments (Fig. 4) and obtained results not unlike those of Mans & Novelli (1961). However, in the crossed-over systems, results at variance with their hypothesis were obtained. 'Azo'-microsomes saturated with normal pH 5 enzyme had a specific activity of 400 counts/min./mg. (Fig. 5) compared with 630 counts/min./mg.

Table 5. *Effect of glutathione, ribonuclease and chloramphenicol on the incorporation of [14 C]leucine into microsomal protein*

The reaction medium was as described in Table 4. Each tube contained 1.80 mg. of microsomal protein, and the following fractions as stated: $(\text{NH}_4)_2\text{SO}_4$ fraction A (8.1 mg. of protein); $(\text{NH}_4)_2\text{SO}_4$ fraction B (10.1 mg. of protein). Microsomes and the various fractions were from livers of rats treated with 4-dimethylamino-3'-methylazobenzene 40 hr. previously. Other additions (where stated) were: GSH (20 μ moles); ribonuclease (200 μ g.); chloramphenicol (100 μ g.). Reactions were run for 30 min. at 37°.

System	Total microsomal incorporation (counts/min.)
Microsomes + $(\text{NH}_4)_2\text{SO}_4$ fraction A	469
Microsomes + $(\text{NH}_4)_2\text{SO}_4$ fraction A + GSH	260
Microsomes + $(\text{NH}_4)_2\text{SO}_4$ fraction A + ribonuclease	83
Microsomes + $(\text{NH}_4)_2\text{SO}_4$ fraction A + chloramphenicol	326
Microsomes + $(\text{NH}_4)_2\text{SO}_4$ fraction B	964
Microsomes + $(\text{NH}_4)_2\text{SO}_4$ fraction B + GSH	460
Microsomes + $(\text{NH}_4)_2\text{SO}_4$ fraction B + ribonuclease	136
Microsomes + $(\text{NH}_4)_2\text{SO}_4$ fraction B + chloramphenicol	1006

when saturated with 'azo'-pH 5 enzyme (Fig. 4). As the microsomes were saturated with respect to either pH 5 enzyme, their specific activities should theoretically be equal. Clearly this is not the case, indicating that the problem is not merely a question of available sites. Results of the titration experiments with pH 5 enzyme (Fig. 3) are also at variance with the concept of Mans & Novelli (1961).

Time-incorporation experiments showed that the 'azo' system incorporated [14 C]leucine at a more rapid rate than did the normal system. This suggests a possible alteration in the state of equilibrium of one of the reactions connected with protein synthesis.

Different results from the above were obtained when we studied the incorporation of [14 C]leucine into microsomal protein of mitochondrial supernatants from livers of rats previously treated with 4-dimethylamino-3'-methylazobenzene. As early as 4 hr. after the administration of the dye, considerable inhibition of incorporation occurred. After this time, however, inhibition was relieved, and at 40 hr. a slight stimulation of incorporation occurred. The specific activity of the microsomes in these experiments was considerably less than that of the washed microsomes from the same homogenate (Fig. 1). Further experiments showed that there is a soluble inhibitor of the incorporation of [14 C]leucine in the pH 5-supernatant fraction,

Fig. 7. Ultraviolet-absorption spectra of different 'azo' supernatant fractions in 50 mM-tris-HCl buffer, pH 7.6. (a) pH 5 enzyme; (b) $(\text{NH}_4)_2\text{SO}_4$ fractions A or B of the pH 5 supernatant; (c) $(\text{NH}_4)_2\text{SO}_4$ fraction C of pH 5 supernatant. The fractions were prepared from the pH 5 supernatant of a rat-liver homogenate obtained from animals injected with 4-dimethylamino-3'-methylazobenzene 40 hr. previously.

which appears to be diffusible and of small molecular weight. There is no correlation between the time of greatest inhibition of the mitochondrial supernatant and that of maximum dye-binding.

The above results differ to some degree from those reported by Arrhenius & Hultin (1962) with aminofluorene. These workers showed that microsomal preparations from rats previously treated *in vivo* with 2-aminofluorene possessed an enhanced rate of protein synthesis 8–48 hr. after administra-

tion of the carcinogen. Their incorporating system, however, differed from ours in that they used microsomes plus cell sap, whereas we used microsomes plus pH 5 enzyme. In our work with mitochondrial supernatants (microsomes plus cell sap) we obtained early inhibition followed by a return to normal, with a slight stimulation in incorporating activity at 40 hr. after administration of the dye.

A stimulation of protein synthesis *in vitro* by rat-liver microsomal systems after the administration of methylcholanthrene *in vivo* has been observed (Decken & Hultin, 1960; Gelboin & Sokoloff, 1961). This has been considered to be associated with the increase in activities of certain microsomal enzymes after the administration of methylcholanthrene (Conney, Miller & Miller, 1956, 1957; Decken & Hultin, 1960). Whether a similar series of events is happening after the administration of 4-dimethylamino-3'-methylazobenzene is as yet unknown.

The presence of a soluble inhibitor of protein synthesis which affects the 'azo' incorporating system to a considerable extent has been demonstrated. Inhibitory activity is lost to some extent on dialysis, and fractionation of the pH 5 supernatant with ammonium sulphate gave fractions that, after dialysis, stimulated the incorporation of [^{14}C]leucine. Further detailed studies are necessary before a proper interpretation of the above results can be advanced.

The present work on the effect of a single dose of 4-dimethylamino-3'-methylazobenzene on protein synthesis in rat liver has produced results that demonstrate that the carcinogen affects a multiplicity of factors concerned in, and controlling, protein synthesis.

SUMMARY

1. The incorporation of [^{14}C]leucine into protein by a system consisting of washed microsomes plus pH 5 enzyme was studied at various times after a single intraperitoneal injection of 4-dimethylamino-3'-methylazobenzene. Both the rate of incorporation and the specific activity of the microsomes showed an increase after 20 hr., reaching a maximum at 40 hr., and thereafter returned to normal.

2. The binding of 4-dimethylamino-3'-methylazobenzene to microsomes, pH 5 enzyme and pH 5 supernatant was studied at different times after a single injection of the carcinogen. Maximum binding occurred at 40 hr. after the administration of the dye.

3. The incorporation of [^{14}C]leucine into the microsomal protein of mitochondrial supernatants was also studied after the administration of 4-

dimethylamino-3'-methylazobenzene. Considerable inhibition of incorporation was found at 4 hr. after the administration of the dye, with a return to normal after this time. The specific activities of microsomes isolated from mitochondrial supernatants were always considerably lower than those of washed preparations.

4. An inhibitor of the incorporation of [^{14}C]leucine was found in both the normal pH 5-supernatant and 'azo'-pH 5-supernatant fractions. The 'azo'-microsomal incorporating system was affected by both supernatant fractions to a much greater extent than was the normal microsomal incorporating system. The inhibitor was diffusible.

5. Fractionation of the pH 5 supernatant of homogenates from azo-dye-treated rats with ammonium sulphate gave three fractions that all stimulated the incorporation of [^{14}C]leucine. These fractions were also able to replace the pH 5-enzyme fraction to a certain extent.

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