The Effect of 4-Dimethylamino-3'-methylazobenzene on ^{14}C -Labelled Amino Acid Incorporation by Rat-Liver Polysome Preparations

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1. The incorporation of ^{14}C -labelled amino acids into polysomal protein was studied in a system consisting of polysomes and pH 5 enzyme obtained 4 and 40 hr. after a single intraperitoneal injection of 4-dimethylamino-3'-methylazobenzene. Labelling of the polysome fraction of preparations of both the 4 hr.-treated and 40 hr.-treated rats was considerably higher than in the normal control. 2. In further experiments on protein synthesis by polysomes from azo-dye-treated rats, the effect of replacing pH 5 enzyme with cell sap was studied. Incorporation of ^{14}C -labelled amino acids into polysomal protein was similar to that of the control. 3. Aggregate size of polysomes obtained from rats injected previously with 4-dimethylamino-3'-methylazobenzene was studied by sucrose-gradient centrifugation. Polysomes prepared at 4 hr. after azo-dye administration contained a considerable amount of large aggregates (approx. 700s), whereas at 40 hr. after administration of the azo-dye the amount of large aggregates was less than in the control. 4. Determination of the ultraviolet spectra of polysome preparations from both normal and azo-dye-treated rats revealed no difference between the preparations. On the other hand, the ultraviolet spectra of cell-sap fractions from the different preparations showed that there is a definite shift in the absorption maximum from 272 m μ (normal) to 267 m μ , 40 hr. after treatment, with an intermediate value of 270 m μ for the preparation from 4 hr.-treated rats. The absorption minimum changes from 250 m μ (normal) to 245 m μ for the preparation from 40 hr.-treated rats.

In previous reports on the effect of 4-dimethylamino-3'-methylazobenzene on [^{14}C]leucine incorporation into protein by a system consisting of washed microsomes and pH 5 enzyme, it was shown that incorporation was stimulated at 40 hr. after administration of the azo-dye (Hawtre & Schirren, 1962a; Hawtre & Schirren & Dijkstra, 1963). These findings have been confirmed independently by Daudel, Jacquier & Touplain (1965) with the carcinogenic azo-dyes 4-dimethylamino-3'-methylazobenzene and 4-dimethylaminoazobenzene. Of further interest was their observation that the non-carcinogenic azo-dye *p*-aminoazobenzene was without effect on protein synthesis.

In the experiments *in vitro* (Hawtre & Schirren, 1963), stimulation of [^{14}C]leucine incorporation in the treated system was dependent on both microsomes and pH 5 enzyme. When pH 5 enzyme was replaced by cell sap (supernatant remaining after removal of mitochondria), however, very little stimulation of incorporation was observed at 40 hr. after dye administration, whereas at 4 hr. after administration incorporation was inhibited by approx. 50%.

In this connexion, a diffusible inhibitor of [^{14}C]leucine incorporation was found in supernatant fractions from both normal and treated rats after removal of pH 5 enzyme.

Through the studies of Warner, Knopf & Rich (1963), Goodman & Rich (1963), Wettstein, Staehelin & Noll (1963), Noll, Staehelin & Wettstein (1963) and others, it is now established that protein synthesis is carried out by clusters or aggregates of ribosomes known as polysomes. In the rat-liver cell, polysomes are attached to lipoprotein membranes of the endoplasmic reticulum. This material, which is not homogeneous in that it contains a number of different elements, is obtained in the fraction sedimented at high speed (105 000g) by differential centrifugation and is usually referred to as the microsomal fraction. It was considered of importance therefore to carry out experiments similar to those described by Hawtre & Schirren (1963), in which, however, polysomes replaced the microsomal fraction. The results obtained would enable us to establish whether the previously observed effect of the azo-dye on microsomal protein syn-

thesis is located in the polysomes (ribosomes) or lipoprotein membrane of the microsome fraction.

In this paper we present results which appear to indicate that 4-dimethylamino-3'-methylazobenzene exerts some of its effects at the polysome level.

MATERIALS AND METHODS

Chemicals and enzymes. Both the source and the details of the use of the following substances were as described by Hawtrey *et al.* (1963): ATP (disodium salt), GSH, ribonuclease, GTP (disodium salt), 4-dimethylamino-3'-methylazobenzene (m.p. 120.4–121.2°), formic acid (98%), tris and sucrose. Phosphoenolpyruvate (tricyclohexylammonium salt) and pyruvate kinase were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Phosphoenolpyruvate was prepared immediately before use from the tricyclohexylammonium salt by dissolving in water without neutralization (pH 7.0). [We studied the effect of using the tricyclohexylammonium salt of phosphoenolpyruvate as compared with the potassium salt (obtained from the barium-silver salt) on protein synthesis, and found that it is inhibitory to a certain extent. However, no difference in inhibition was observed between incorporating systems from normal and azo-dye-treated rats.] The ^{14}C -labelled amino acids were used in the form of a yeast hydrolysate (850 $\mu\text{C}/\text{mg.}$) obtained from Schwarz Bio-Research Inc., Orangeburg, N.Y., U.S.A. It was dissolved in water, neutralized to pH 7.6, and stored at -15° . Sodium deoxycholate was supplied by General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A. Bovine albumin (crystalline) and yeast RNA (sodium salt) were obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

Preparation of cell particles and enzyme fractions. Female rats (100–120 g.) were injected intraperitoneally with 4-dimethylamino-3'-methylazobenzene (50 mg./250 g. body wt.) dissolved in 2.0 ml. of olive oil and killed at 4 and 40 hr. thereafter. Animals killed 40 hr. later were deprived of food 24 hr. before death. This starvation period was also applied to the normal control animals, but the rats injected 4 hr. before death were starved for 20 hr. before injection.

Polysomes. Rat-liver homogenates were prepared in medium A [containing (final concentrations): sucrose (0.25 M), MgCl_2 (5 mM), KCl (25 mM) and tris-HCl buffer, pH 7.6 (50 mM)], as described by Hawtrey *et al.* (1963). Nuclei and cell debris were removed by centrifuging at 1400 g for 10 min. (all g values are given for the bottom of the centrifuge tube), and the resulting supernatant was centrifuged at 14370 g in the Spinco model L centrifuge (no. 30 rotor) for 20 min. to remove mitochondria. The supernatant after sedimenting mitochondria was used for the preparation of polysomes, which was carried out essentially by the method of Wettstein *et al.* (1963). In applying this method to centrifuge tubes of the no. 30 rotor, the following procedure was adopted: Supernatant after sedimenting mitochondria (13 ml.) was mixed with 1.5 ml. of 13% (w/v) sodium deoxycholate (freshly prepared in water), and allowed to stand in ice for 5–7 min. before carefully layering on to a preformed double layer consisting of 9 ml. of 2 M-sucrose (bottom) and 12 ml. of 0.5 M-sucrose (upper). Both layers contained tris-HCl buffer, pH 7.6 (50 mM), KCl (25 mM) and MgCl_2 (5 mM). Centrifugation was carried out at 99000 g (no. 30 rotor) for 240 min. After

centrifugation, the solution above the polysomes was carefully removed by means of a large Pasteur pipette, and the sides of the tube were carefully wiped with filter paper. The pellet of polysomes was resuspended in 1.0 ml. of medium A by means of a glass rod with gentle stirring, and the resulting suspension allowed to stand at 0° for 1 hr. with occasional stirring. It was then centrifuged at 2000 rev./min. (1400 g) for 10 min. to remove aggregated and denatured material. The final clear suspension of polysomes was kept at 0° .

Cell sap. This was prepared from a portion of the mitochondrial supernatant by centrifuging at 105000 g for 60 min.

pH 5 fraction. This was prepared from a sample of the cell sap as described by Hawtrey *et al.* (1963), except that the final suspension of washed pH 5 enzymes in medium A was centrifuged at 20000 g for 10 min. (no. 40 rotor) to remove denatured material.

Preparation of sucrose gradients. Linear sucrose gradients (15–30%, w/w) were prepared in no. 25 rotor tubes by using a modified mixing apparatus of the type described by Britten & Roberts (1960). Sucrose-gradient centrifugation was carried out in the no. SW 25.1 swinging-bucket rotor at 25000 rev./min. (90000 g) for the times given in the text. All gradients were run at a 20°F temperature setting.

System for incorporation of ^{14}C -labelled amino acids into polysomes. The reaction medium used for incorporation studies contained: tris-HCl buffer, pH 7.6 (final concn. 50 mM); KCl (final concn. 25 mM); MgCl_2 (final concn. 8.5 mM); sucrose (final concn. 0.25 M); ATP (potassium salt), pH 7.6 (1.25 μmoles); GTP (potassium salt), pH 7.6 (0.5 μmole); phosphoenolpyruvate (tricyclohexylammonium salt) (10 μmoles); phosphoenolpyruvate kinase (60 $\mu\text{g.}$); ^{14}C -labelled yeast hydrolysate of specific activity 850 $\mu\text{C}/\text{mg.}$ (1.4 μC); polysomes (0.8–2.0 mg. of RNA); cell sap (4–6 mg. of protein) or pH 5 fraction (2.5–5.0 mg. of protein). The final reaction volume was 1.3 ml. Incubations were carried out in test tubes at 37° for various times. In agreement with Sargent & Campbell (1965), the tricyclohexylammonium salt of phosphoenolpyruvate partly inhibited protein synthesis, but the use of this salt in no way invalidated the present results (see above). In experiments where the reaction mixture was to be analysed by sucrose-gradient centrifugation, it was first cooled in ice for 15 min., then carefully layered on to a precooled sucrose gradient. Centrifugation was then carried out as described above. Tubes were punctured with a no. 21 syringe needle and 13-drop fractions collected (approx. 1 ml.). Each fraction was then diluted by the addition of 1 ml. of water and its extinction at 260 μ measured. The same fraction was later analysed for the incorporation of ^{14}C -labelled amino acids into protein as described below.

Isolation of ^{14}C -labelled proteins. To each fraction (2 ml.) from a sucrose gradient was added 0.1 ml. of a solution containing 0.25 mg. of yeast RNA and 0.5 mg. of albumin (carrier), followed by 0.2 ml. of 60% (w/v) trichloroacetic acid. After mixing, the tubes were allowed to stand for 24–36 hr. at 4° . This procedure allows hydrolysis of the transfer RNA with 90% release of labile counts (^{14}C -labelled amino acids attached to transfer RNA). The transfer RNA from both normal and azo-dye-treated rats was broken down by identical amounts. The resulting precipitates were washed successively three times with 5% (w/v) trichloroacetic acid (5 ml./tube). During the first

wash, 0.1 ml. of a complete unlabelled amino acid mixture was added to each tube. After washing, the precipitates were dissolved in 0.5 ml. of 2.5% (w/v) NH_3 and plated. The planchets were dried under infrared lamps. Samples were counted with a Philips electronic counter (tube PW41491) and appropriate corrections for thickness and background made.

Protein and RNA. These were determined as described by Hawtrej *et al.* (1963).

RESULTS

Incorporation of ^{14}C -labelled amino acids into polysomes at 40 hr. after the administration of 4-dimethylamino-3'-methylazobenzene. In the system

Fig. 1. Sucrose-gradient centrifugation of polysomes labelled with ^{14}C -labelled amino acids in the presence of cell sap. Polysomes were prepared from livers of both normal and azo-dye-treated rats. 4-Dimethylamino-3'-methylazobenzene (50 mg./250 g. body wt.) was administered by intraperitoneal injection 40 hr. previously. Labelling of the polysomes was carried out in the following reaction medium: tris-HCl buffer, pH 7.6 (final concn. 50 mM); KCl (final concn. 25 mM); MgCl_2 (final concn. 8.5 mM); sucrose (final concn. 0.25 M); ATP (1.25 μmoles); GTP (0.5 μmole); phosphoenolpyruvate (tricyclohexylammonium salt) (10 μmoles); phosphoenolpyruvate kinase (60 $\mu\text{g.}$); ^{14}C -labelled yeast hydrolysate (1.4 μC); polysomes (1.2 mg. of RNA) from normal or treated rats; cell sap (4.9 mg. of protein) from normal rats or cell sap (5.0 mg. of protein) from treated rats. The final reaction volume was 1.3 ml. Reactions were run at 37° for 10 min. Centrifugation was carried out at 25000 rev./min. (no. 25.1 rotor) for 150 min. [gradients were 15–30% (w/w) sucrose in the medium A buffer system]. (a) Polysomes from normal rats plus cell sap from normal rats; (b) polysomes from treated rats plus cell sap from treated rats. —, E_{260} ; ●, incorporation (stable counts).

consisting of polysomes and cell sap from rats injected with azo-dye 40 hr. previously, polysomes are labelled after a 10 min. incubation to an extent similar to that of the normal control (Fig. 1). In this and all subsequent experiments labelling of the polysomes is reported as total counts and as such these are not specific activities. In experiments with incubation for 20 min. (Fig. 2), the treated and normal polysomes are again labelled to similar extents.

Further experimental work was carried out with a system consisting of polysomes but with pH 5 enzyme instead of cell sap. Results of experiments at 40 hr. after azo-dye administration are shown in Fig. 3. Incorporation of ^{14}C -labelled amino acids into both polysomes and supernatant protein occurs to a greater extent in the system with fractions from treated rats than in the system with fractions from normal rats: total counts of polysomes from treated rats, 1886 counts/min.; supernatant protein from treated rats, 2730 counts/min.; polysomes from normal rats, 1208 counts/min.; supernatant protein from normal rats, 1945 counts/min. Equal amounts of both polysomes and cell sap or pH 5

Fig. 2. Sucrose-gradient centrifugation of polysomes labelled with ^{14}C -labelled amino acids in the presence of cell sap for 20 min. at 37° . Incubation conditions were as described for Fig. 1. Centrifugation was carried out for 150 min. at 25000 rev./min. (a) Polysomes from normal rats plus cell sap from normal rats; (b) polysomes from treated rats plus cell sap from treated rats. —, E_{260} ; ●, incorporation (stable counts). The azo-dye was administered 40 hr. previously as described for Fig. 1.

Fig. 3. Analysis of polysomes (prelabelled in the presence of pH5 enzyme) by sucrose-gradient centrifugation. The reaction medium and conditions of labelling and centrifuging were as described in Fig. 1. Azo-dye was administered to rats 40 hr. previously. Polysomes (1.2 mg. of RNA) from normal or treated rats were incubated with pH5 enzyme (2.7 mg. of protein) from normal or treated rats for 10 min. at 37°. Centrifugation was at 25000 rev./min. for 150 min. (a) Polysomes from normal rats plus pH5 enzyme from normal rats; (b) polysomes from treated rats plus pH5 enzyme from treated rats. —, E_{260} ; ●, incorporation (stable counts).

Fig. 4. Sucrose-gradient centrifugation of polysomes prelabelled either in the presence of cell sap of pH5 enzyme. 4-Dimethylamino-3'-methylazobenzene was administered by intraperitoneal injection 4 hr. previously. Conditions for the experiment were as described in Fig. 1. Polysomes (1.4 mg. of RNA) from treated rats were incubated with cell sap (5.2 mg. of protein) from treated rats or pH5 enzyme (2.8 mg. of protein) from treated rats for 10 min. at 37°. (a) Polysomes from treated rats plus cell sap from treated rats; (b) polysomes from treated rats plus pH5 enzyme from treated rats. —, E_{260} ; ●, incorporation (stable counts).

enzyme were used whenever a comparison was made between systems of fractions from normal and treated rats.

Incorporation of ^{14}C -labelled amino acids into polysomes at 4 hr. after the administration of 4-dimethylamino-3'-methylazobenzene. Results presented in Fig. 4 show the incorporation of ^{14}C -labelled acids into polysomes and supernatant protein in systems consisting of polysomes from treated rats plus cell sap from treated rats (Fig. 4a), and polysomes from treated rats plus pH5 enzyme from treated rats (Fig. 4b) after 10 min. of incubation at 37°. The labelling of polysomes and supernatant protein of the system containing cell sap as a source of activating and transfer enzymes is low when compared with the system in which pH5 enzyme replaces cell sap. Total counts for different polysome and supernatant proteins are as follows: in Fig. 4(a), polysomes, 799 counts/min.; supernatant protein, 365 counts/min.; in Fig. 4(b), polysomes, 2044 counts/min.; supernatant protein, 1652 counts/min.

The incorporation of the polysomes from rats treated 4 hr. previously supplemented with cell sap is similar to that found with the polysomes from

40 hr.-treated and normal rats, the results of which are shown in Fig. 1. There was high labelling of polysomes in the system consisting of polysomes and pH5 enzyme (Fig. 4b), which is similar to that of the system of fractions from 40 hr.-treated rats, but greater than that of the system of fractions from normal rats (Fig. 3). With regard to the polysome-pH5 enzyme system, with both fractions from 4 hr.-treated rats (Fig. 4b), breakdown of polysomes is not extensive, yet the labelling of both the polysomes and supernatant protein is high. On the other hand, there appears to be a more extensive breakdown of polysomes in the system where pH5 enzyme is replaced by cell sap (Fig. 4a), as shown by the height of the 73s monomer peak, and labelling is relatively small.

Size and stability of polysomes obtained from rats injected 4 and 40 hr. previously with 4-dimethylamino-3'-methylazobenzene. In view of the results obtained above on the stimulation of incorporation of ^{14}C -labelled amino acids into polysomal and supernatant protein of the systems of fractions from azo-dye-treated rats, it was considered important to study the size and stability of the different polysome preparations.

Fig. 5. Extinction profiles of polysome preparations after sucrose-gradient centrifugation at 25000 rev./min. (no. 25.1 rotor) for 105 min. ●, Polysomes from normal rats; ○, polysomes from 40hr.-treated rats.

Results in Fig. 5 show the extinction profiles of polysome preparations after sucrose-gradient centrifugation at 25000 rev./min. for 105 min. The polysome preparation from 40hr.-treated rats has a peak to the right of the normal polysome peak, suggesting that it has a higher proportion of smaller aggregates than the normal.

To obtain a clearer picture of the polysome distribution in the different preparations, centrifugation was carried out for the shorter time of 50 min. (Fig. 6). The polysomes obtained at 4hr. after azo-dye treatment contain a higher percentage of large aggregates (approx. 700s) than the preparations from either the normal or 40hr.-treated rats.

Stability of the polysome preparations from normal and treated rats was studied by allowing the polysomes (in medium A) to age for 1 week at 0°. Thereafter, they were examined as to their aggregate size by sucrose-gradient centrifugation. The polysomes from 4hr.-treated rats were broken down to smaller aggregates to a greater extent than the preparation made 40hr. after azo-dye administration. Results intermediate between those obtained with the preparations from treated rats were observed with the polysomes from normal rats.

Ultraviolet spectra of enzyme and polysome fractions. During the present work, ultraviolet measurements were always carried out on both the enzymes and different polysome preparations. It was noticed that the E_{260}/E_{280} ratio varied between different samples of cell sap taken at different times

Fig. 6. Sucrose-gradient centrifugation of different polysome preparations. Centrifugation was carried out at 25000 rev./min. for 50 min. ○, Polysomes from 4hr.-treated rats; ●, polysomes from normal rats; ○, polysomes from 40hr.-treated rats. Sucrose gradients were 15–30% (w/w) in the buffer system of medium A.

after administration of the azo-dye. Hence complete ultraviolet spectra of the different cell-sap fractions were determined (Fig. 7). The results show clearly a progressive shift of the absorption maximum from 272m μ (normal) through 270m μ (4hr.-treated) to 267m μ (40hr.-treated). There is also a simultaneous shift in the absorption minimum (Fig. 7).

Determination of the ultraviolet spectra of the polysome preparations from normal and azo-dye treated rats, on the other hand, revealed little difference between their spectral curves (Fig. 8).

DISCUSSION

Polysomes prepared at 4 and 40hr. after a single intraperitoneal injection of 4-dimethylamino-3'-methylazobenzene exhibited a higher degree of

Fig. 7. Ultraviolet spectra of different cell-sap fractions. All spectra were determined in medium A. Each cuvette contained 1.6mg. of protein. *A*, Cell sap from normal rats; *B*, cell sap from 4hr.-treated rats; *C*, cell sap from 40hr.-treated rats.

labelling in comparison with polysomes from normal rats, when each incorporating system was supplemented with its own pH5 enzyme fraction (Figs. 3 and 4). In particular, polysomes prepared at 4hr. after injection were labelled to the greatest extent. This latter finding is in contrast with previous results (Hawtrey, *et al.* 1963) on the microsomal pH5 enzyme system obtained at 4hr. after azo-dye administration, where incorporation was little different from that observed for the control. The present results, and those obtained previously, suggest therefore that the lipoprotein membrane of the microsome complex may have some controlling influence on protein synthesis. Further, the results appear to indicate that, at 4hr. after administration, 4-dimethylamino-3'-methylazobenzene interferes

Fig. 8. Ultraviolet spectra of different polysome preparations. All spectra were determined in medium A. *A*, Polysomes (0.25mg. of ribonucleoprotein) from normal rats; *B*, polysomes (0.20mg. of ribonucleoprotein) from 40hr.-treated rats; *C*, polysomes (0.19mg. of ribonucleoprotein) from 4hr.-treated rats.

with certain functions in the lipoprotein membrane which are associated with protein synthesis. In this connexion, Hoagland, Scornik & Pfefferkorn (1964) have demonstrated the presence in rat-liver microsomes of a heat-labile inhibitory factor of protein synthesis. It is tempting to suggest that this factor is present in the membrane of the microsome complex. Hawtrey & Schirren (1962*b*) have reported on the inhibition of [^{14}C]leucine incorporation into rat-liver ribosomes (nucleoprotein particles) by a lipoprotein fraction (membrane) obtained from microsomes by iso-octane extraction. Further detailed study on the membrane fraction of rat-liver microsomes is necessary to substantiate these ideas.

Little difference was observed in the labelling patterns of polysome preparations from normal and treated rats when supplemented with cell sap as a source of enzymes (Figs. 1 and 2). In particular, polysomes prepared 4hr. after treatment were labelled to an extent similar to that of the normal control preparation. This result is also in contrast with previous work (Hawtrey *et al.* 1963) where considerable inhibition of incorporation was

Table 1. *Summary of the effects of 4-dimethylamino-3'-methylazobenzene on protein synthesis by rat-liver microsomes and polysomes*

Results on the polysomes are taken from the present work, and those on the microsomes are from a previous investigation (Hawtrey, *et al.* 1963).

System	Effect of 4-dimethylamino-3'-methylazobenzene on protein synthesis	
	Time after azo-dye treatment.....	
	4 hr.	40 hr.
Microsomes plus pH 5 enzyme	Normal	Stimulation
Microsomes plus cell sap	Inhibition	Normal
Polysomes plus pH 5 enzyme	Stimulation	Stimulation
Polysomes plus cell sap	Normal	Normal

observed in a system consisting of microsomes and cell sap (supernatant remaining after removal of mitochondria) obtained at 4 hr. after azo-dye administration. At present, it is difficult to interpret exactly how the azo-dye is affecting protein synthesis, but in terms of the experimental findings of this and a previous paper (Hawtrey *et al.* 1963) we can state that at the early time of 4 hr. after azo-dye administration the rate and amount of protein synthesis is somehow affected by the lipoprotein membrane of the microsomal fraction, and that the effect is influenced by the presence of cell sap or pH 5 enzyme. To describe the effects of the azo-dye on protein synthesis in the various systems studied, we have very briefly summarized the results of the present and previous work (Hawtrey *et al.* 1963) in Table 1. At 40 hr. after azo-dye administration it is clear that stimulation of incorporation into either microsomes or polysomes is brought about through the use of pH 5 enzyme.

The change in the ultraviolet-absorption maximum of the cell sap after 4-dimethylamino-3'-methylazobenzene administration is noteworthy. A progressive shift from 272 m μ (normal) to 267 m μ 40 hr. after treatment was observed (Fig. 7), and is probably related to some change in the RNA/protein ratio of the cell sap or increase in nucleotide material.

The present work shows that 4-dimethylamino-3'-methylazobenzene can exert effects on protein synthesis in rat liver at the level of both the polysome and lipoprotein membrane of the microsome fraction.

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