The Effect of a Single Intraperitoneal Dose of the Hepatocarcinogen 4-Dimethylaminoazobenzene on the Rough-Surfaced Endoplasmic Reticulum of the Liver of the Rat

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1. Changes in the structure and function of the rough-surfaced endoplasmic reticulum of the rat liver as deduced by electron microscopy, polysome analysis and the amino acid-incorporating activity of microsome fractions have been followed at various time-intervals after a single intraperitoneal dose of the hepatocarcinogen 4-dimethylaminoazobenzene. 2. The earliest effect observed was detachment of polysomes and disorganization and vesiculation of the cisternae of the rough-surfaced endoplasmic reticulum. This occurred after 6 hr. 3. Subsequent to this was a phase of polysome disaggregation accompanied by impaired amino acid-incorporating activity by microsomes together with a much enhanced stimulating effect of polyuridylic acid on amino acid uptake. This reached a maximum at 24 hr. 4. By 40 hr. polysomes had re-formed and the amino acid-incorporating activity, together with the polyuridylic acid effect, were similar to those in controls. 5. It was not until 112 hr. that something like the normal structure of the rough-surfaced endoplasmic reticulum was re-established. 6. It is not yet possible to relate these changes specifically with the process of azo-dye carcinogenesis.

One of the earliest effects on the rat liver of a number of hepatotoxins and carcinogens is to produce alterations in the rough-surfaced endoplasmic reticulum of the parenchymal cells (Porter & Bruni, 1959; Emmelot & Benedetti, 1961; Steiner, Phillips & Miyai, 1964; Lafontaine & Allard, 1964; Baglio & Farber, 1965). For example, a single dose of dimethylnitrosamine causes a breakdown of the characteristic lamellar organization of this reticulum and a marked increase in the number of free ribosomes (Emmelot & Benedetti, 1961). Similar effects are caused by feeding the carcinogenic azo-dye 3'-Me-DAB* (Porter & Bruni, 1959) or its non-carcinogenic isomer 2-Me-DAB (Lafontaine & Allard, 1964).

Alterations in the function of the rough-surfaced endoplasmic reticulum, as judged by the amino acid-incorporating activity of microsome fractions, are also caused by such substances. Magee (1958) and later Mukherjee, Gustafsson, Afzelius & Arrhenius (1963) have shown that when rats are treated with dimethylnitrosamine the microsome fraction of the liver has a diminished ability to

incorporate labelled amino acids. This effect of dimethylnitrosamine has been found by Mizrahi & Emmelot (1964) to be associated with an increased sensitivity of the microsome fraction to the stimulating effect of the synthetic messenger RNA, polyU, on [¹⁴C]phenylalanine incorporation. These results are in accord with the breakdown of polysomes to free ribosomes (Henshaw, Bozarski & Hiatt, 1963). Hultin & Arrhenius (1965) have shown that the period of depressed amino acid incorporation is followed by a second phase where incorporating activity is enhanced. Such a dual effect also results from treatment with other carcinogenic amines.

The effect of a single intraperitoneal dose of the azo-dye 3'-Me-DAB on amino acid incorporation by microsome fractions has been studied by Hawtrey, Schirren & Dijkstra (1963). They, too, have observed a dual effect, but only when microsomes are incubated with whole cell sap. When the cell sap is replaced by pH 5 enzyme no initial phase of impairment occurs. Polysome fractions behave similarly towards cell sap, and pH 5 enzyme, after 3'-Me-DAB (Hawtrey & Nourse, 1966).

The present study concerns the effect of a single intraperitoneal injection of the azo-dye DAB on the structure of the rough-surfaced endoplasmic reticulum, as judged by polysome analysis and electron

* Abbreviations: DAB, 4-dimethylaminoazobenzene; 3'-Me-DAB, 4-dimethylamino-3'-methylazobenzene; 2-Me-DAB, 4-dimethylamino-2-methylazobenzene; PolyU, polyuridylic acid.

microscopy, and on its function, as judged by the amino acid-incorporating activity of microsome fractions. An initial phase of impaired incorporating ability has been observed, followed by a period of recovery. These effects, obtained with washed microsomes and pH 5 enzyme, appear to be related to changes in the state of the rough-surfaced endoplasmic reticulum.

MATERIALS AND METHODS

Chemicals. Sodium deoxycholate was obtained from E. Merck A.-G., Darmstadt, Germany, the dipotassium salt of ATP and the disodium salt of GTP were from Sigma Chemical Co., St Louis, Mo., U.S.A., pyruvate kinase was from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, and polyU was from Miles Laboratories, Stoke Poges, Slough, Bucks. Solutions of phosphoenolpyruvate were made up before use from the silver-barium salt of phosphoenolpyruvic acid prepared by the method of Clark Kirby (1963).

Animals. Male Wistar-strain rats inbred in this Laboratory and maintained on the Rowett diet 86 containing 20% of protein were used. Rats weighing about 200 g. were selected and given 50 mg. of DAB in 2 ml. of corn oil by intraperitoneal injection. At various time-intervals after injection they were stunned and bled to death. Control animals received 2 ml. of corn oil alone. All animals were kept without food overnight before being killed.

Polysome analysis. Polysome analysis by sucrose-density-gradient centrifugation was performed on the C-ribosome fraction described by Wettstein, Staehelin & Noll (1963) as follows. The livers were perfused with 0.25 M-sucrose in TKM buffer (composed of 50 mM-tris, 25 mM-KCl and 5 mM-MgCl₂) at pH 7.5, and then excised and passed through a tissue press (Climpex Ltd., Mill Hill, London, N.W. 7). Each 4 g. sample of the resulting mince was homogenized in 11 ml. of 0.25 M-sucrose in TKM buffer by using 3 strokes of the pestle of an all-glass Potter-Elvehjem homogenizer rotating at a speed of 1200 rev./min. In general homogenates from the livers of six treated rats and from those of three control rats were respectively pooled for simultaneous C-ribosome analysis and determination of microsomal amino acid uptake.

A post-mitochondrial supernatant fraction was prepared by centrifuging the homogenate at 10000 *g*_{av} for 10 min. in the Spinco model L centrifuge (no. 40 rotor). After Webb, Blobel & Potter (1964), two types of C-ribosome fraction were prepared; for one type the post-mitochondrial supernatant was made 1.25% (w/v) with respect to sodium deoxycholate and for the other it was left untreated. In either case the post-mitochondrial supernatant was sedimented through 4 ml. of 0.5 M-sucrose layered over 3 ml. of 2.0 M-sucrose by centrifugation for 4 hr. at 105000 *g*_{av} in the Spinco model L centrifuge (no. 40 rotor). The resulting pellet was the C-ribosome fraction. An amount of the C-ribosome fraction derived from 0.6 g. of liver suspended in 1 ml. of TKM buffer was sedimented through a 10–40% (w/v) sucrose gradient for 2 hr. at 63000 *g*_{av} in the Spinco model L centrifuge (SW 25 rotor). Fractions (0.5 ml.) were then removed from the bottom of the gradient with a syringe, made up to 3 ml. with water and the extinction of each was determined at 260 m μ . In most experiments C-

ribosomes were prepared both with and without deoxycholate and compared with controls prepared with deoxycholate.

Incorporation of [¹⁴C]phenylalanine into microsomes. Microsomes were prepared from the post-mitochondrial supernatant fraction described in the preceding section by centrifugation for 90 min. at 105000 *g*_{av} in the Spinco model L centrifuge (no. 40 rotor). They were washed by resuspending them in more 0.25 M-sucrose in TKM buffer and centrifugation for 30 min. at 105000 *g*_{av}. The washed pellet was resuspended in a volume of TKM buffer sufficient to give a microsomal protein concentration of 14–20 mg./ml. Protein content was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard.

The pH 5 enzyme was prepared by titrating 10 ml. of the soluble cell supernatant fraction to pH 5.2 with N-acetic acid and collecting the precipitate that formed by centrifugation at 10000 *g*_{av} in the Spinco no. 40 rotor. The sediment was washed by resuspending it in TKM buffer and recentrifugation. It was then dissolved by neutralization with 0.5 ml. of M-tris and made up to volume of 5 ml. with water.

The reaction medium for incorporation studies contained 0.4 ml. of microsomal suspension, 0.1 ml. of pH 5 enzyme, 5 μ moles of MgCl₂, 15 μ moles of phosphoenolpyruvate, 50 μ g. of phosphokinase, 2 μ moles of ATP, 0.25 μ mole of GTP and 0.25 μ C of ¹⁴C-labelled phenylalanine. Incorporation was also studied after the addition of 50 μ g. of polyU to the reaction medium. The total volume of the reaction medium was 1 ml. After shaking for 30 min. at 37° the reaction was stopped with 1 ml. of 10% (w/v) trichloroacetic acid.

Microsomal protein was prepared for the determination of its specific radioactivity in the following manner. The trichloroacetic acid precipitate was washed twice with 5% trichloroacetic acid, heated in 5% trichloroacetic acid for 20 min. at 90° and then washed once more with 5% trichloroacetic acid. The residue was then washed with ethanol, ethanol-chloroform-ether (2:2:1, by vol.), acetone and finally ether. Microsomal protein so obtained was dissolved in 1 ml. of formic acid and dried on to aluminium disks for the counting of radioactivity at infinite thinness with an automatic gas-flow-type counter (model C115; Nuclear-Chicago Corp.).

Electron microscopy. A sample of tissue weighing approx. 0.25 g. was removed from the distal end of the median lobe of the livers of both control and treated animals. It was immediately cut into tiny fragments in ice-cold 4% (w/v) glutaraldehyde buffered with 67 mM-cacodylate to pH 7.3 (Sabatini, Bensch & Barrnett, 1963). After fixation at 0° for 4 hr. the tissue was washed overnight by immersion in 67 mM-cacodylate buffer, pH 7.3, containing 0.25 M-sucrose. It was then post-fixed for 1 hr. at 0° in a 1% (w/v) solution of osmium tetroxide in 0.1 M-phosphate buffer, pH 7.2. After dehydration with graded strengths of aqueous ethanol, the tissue fragments were embedded in Epon (Shell Epikote 812) according to the procedure of Luft (1961). Sections showing silver to light-gold interference colours were cut on a Porter-Blum microtome and stained with uranyl acetate and alkaline lead citrate (Reynolds, 1963). They were then examined in a Philips EM 200 electron microscope at an accelerating voltage of 60 kv.

Observations were made in this way on livers from two to six rats in each of the time groups 6, 24, 40 and 112 hr. after

treatment with DAB, and from corn oil-treated controls and normal rats.

RESULTS

C-ribosome analysis. C-ribosomes were analysed at 6, 8, 10, 16, 24, 40 and 112 hr. after dosage of animals with DAB and compared with controls that received corn oil alone. The results are shown in Fig. 1. C-ribosomes obtained after the use of deoxycholate were referred to as 'total' C-ribosomes. They are believed to represent the total of the free and the bound polysomes in the post-mitochondrial

supernatant together with those single ribosomes that penetrate the 2.0M-sucrose barrier during the 4 hr. centrifugation. Those obtained in the absence of deoxycholate are referred to as 'free' C-ribosomes and are believed to comprise free and loosely bound polysomes and, again, those free or loosely bound single ribosomes that cross the 2.0M-sucrose barrier (Webb *et al.* 1964). On analysis by sucrose-density-gradient centrifugation both monomeric and dimeric ribosomes were found.

In control animals monomers and dimers account for only a small proportion of the 'total' C-ribosome

Fig. 1. Effect of a single intraperitoneal injection of DAB on the 'total'- and 'free'-C-ribosome patterns obtained from the livers of rats. (a) Corn oil control (the control C-ribosome patterns did not differ significantly with time after injection with corn oil); (b) 6 hr. after DAB; (c) 8 hr. after DAB; (d) 16 hr. after DAB; (e) 24 hr. after DAB; (f) 40 hr. after DAB; (g) 112 hr. after DAB; 1 and 2 refer to monomer and dimer respectively; —, C-ribosomes prepared with deoxycholate ('total'); ····, C-ribosomes prepared without deoxycholate ('free').

pattern, most of which is due to polysomes. In the 'free'-C-ribosome pattern the yield of polysomes is 50% or less than that obtained in the 'total'-C-ribosome pattern, showing that at least 50% of the polysomes are firmly bound to membrane.

At 6hr. after the administration of DAB the 'total'-C-ribosome pattern is similar to the control pattern, but the yield of polysomes in the 'free'-C-ribosome pattern is somewhat greater than in controls. Between 8 and 10hr., amounts of monomer and dimer exceed the control amounts. At 16hr. the peak due to monomers has increased in magnitude at the expense of that part of the pattern due to polysomes. This change progresses to a maximum at 24hr. when the monomer-dimer peak is predominant and the quantity of polysomes in the 'total'-C-ribosome pattern has fallen to 35% of that found in controls. Among the polysomes remaining, smaller polysomes preponderate.

The 'total'-C-ribosome pattern after 40hr. has a considerably decreased monomer-dimer peak, polysomes have increased in quantity, and heavy polysomes once again preponderate. By comparison with the 'total'-C-ribosome pattern the yield of polysomes in the 'free'-C-ribosome pattern is now 80%.

After 112hr. there is very little monomer and dimer and the difference between the 'total'- and 'free'-C-ribosome patterns suggests that membrane-bound polysomes have begun to increase in number again.

Incorporation of [¹⁴C]phenylalanine into micro-

somes. Incorporation of [¹⁴C]phenylalanine into microsomes in the presence and absence of polyU has been studied at 8, 16, 24, 40 and 112hr. after the administration of DAB. C-ribosome patterns were also obtained from the same post-mitochondrial supernatants (see Table 1). At 8hr., when polysome breakdown first becomes evident, amino acid-incorporating activity in the absence of polyU has fallen to 76% of that of controls and the enhancement of incorporating activity obtained in the presence of polyU is twice that found in controls. At 24hr., when polysome disaggregation is maximal, incorporating activity in the absence of polyU is only 46% that of controls whereas in the presence of polyU the enhancement of incorporation is six times that found in the controls.

At 40hr. a recovery in incorporating activity in the absence of polyU has taken place. Incorporation at this stage is a little higher than that of controls, but not markedly so. The response to polyU is unexpected since it is very little different from that of controls despite the presence of monomer and dimer in C-ribosome patterns in amounts comparable with those found in patterns from rats 8hr. after DAB, when a definite effect of polyU is obtained.

At 112hr. the results are similar to controls.

In general, pH 5 enzyme from DAB-treated rats was incubated with microsomes from DAB-treated rats and pH 5 enzyme from control rats with control microsomes. However, incorporation was not significantly different when control microsomes

Table 1. *Incorporation of [¹⁴C]phenylalanine by microsomes from livers of rats at various time-intervals after a single intraperitoneal injection of 4-dimethylaminoazobenzene compared with corn oil-treated controls*

Experimental details are given in the text. Each result is the mean obtained from two experiments. The microsomes from DAB-treated rats are from the pooled homogenates of the livers of six rats and those from corn oil-treated control rats from pooled liver homogenates from three rats.

Time after injection (hr.)	PolyU	Control		DAB-treated		Incorporation ratio: DAB-treated (- polyU) control (- polyU)
		Incorporation (counts/min. mg. of protein)	Incorporation ratio: + polyU - polyU	Incorporation (counts/min./mg. of protein)	Incorporation ratio: + polyU - polyU	
8	-	559	2.6	442	4.9	0.76
	+	1442		2150		
16	-	446	2.5	321	6.4	0.72
	+	1102		2063		
24	-	373	2.3	172	14.7	0.46
	+	1923		2526		
40*	-	436	4.4	536	4.9	1.2
	+	1923		2621		
112	-	438	2.9	494	2.9	1.0
	+	1264		1432		

* At the 40hr. stage the sample of polyU was different from that used in the other experiments.

were incubated with pH5 enzyme from DAB-treated rats and vice versa.

Electron microscopy. (a) Control animals. The fine structure of liver parenchymal cells from animals receiving corn oil alone (Plate 1A) was indistinguishable from that of untreated animals. In both cases overnight starvation caused some depletion of glycogen deposits and some formation of intracellular lipid droplets in centrilobular areas. The rough-surfaced endoplasmic reticulum formed characteristic stacks of flattened cisternae studded with ribosomes. Free polysomes and perhaps some free ribosomes were present in the intercisternal spaces, and what smooth membranes were present were mainly those of the Golgi region or those associated with areas containing glycogen.

(b) Treated animals. Deviations from normal cytology were observed soon after the administration of DAB. Most cell organelles undergo some alteration in form or distribution, but those involving the rough-surfaced endoplasmic reticulum and its derivatives are particularly relevant in the present context.

Changes in the endoplasmic reticulum appear to be degenerative during the first 24 hr. after the administration of a single dose of DAB and resemble those described by Porter & Bruni (1959), who fed rats with 3'-Me-DAB for periods up to 15 days. By 40 hr. after administration of the single controlled dose by the intraperitoneal route used here, distinct signs of a return to normal can be detected, a process that is well advanced 112 hr. after the animals received the azo-dye.

There are marked differences in response to DAB of parenchymal cells in different regions of the liver lobules. Those in the centrilobular areas are affected more rapidly and more extensively than those in the portal areas. Changes in the portal areas are recorded here for preference since the changes in the centrilobular areas are frequently complicated by extensive cell death and phagocytosis of debris by proliferating cells of the reticulo-endothelial system.

At 6 hr. after the administration of the DAB (Plate 1B) regular stacks of endoplasmic reticulum are rarely encountered, although many individual cisternae with attached ribosomes are present, generally interspersed with mitochondria, and sometimes closely associated with them. Many free polysomes are present and already the rough-surfaced endoplasmic reticulum shows regions that are free from ribosomes and others that appear to be undergoing a process of vesiculation. Very little glycogen remains.

After 24 hr. (Plate 2A) the cytoplasm now contains a variety of membranous structures, most of which present smooth profiles. Some of these appear to resemble rough-surfaced reticulum which

has become denuded of ribosomes, and others are present as vesicles that seem to be forming from the endoplasmic reticulum by a process of budding. Interspersed in the intercisternal matrix and between vesicles are numbers of free ribosomes; characteristic polysomes were rarely encountered. Abnormally shaped mitochondria are frequently present at this stage, and glycogen is virtually absent from most of the cells.

A trend back to normal cytology is apparent at 40 hr. (Plate 2B). There is a marked change in the character of the ribosomal particles, most of which now form typical polysomal clusters. Strands of double membranes are still interspersed with mitochondria and they once again have groups of attached ribosomes, often separated by short lengths of smooth membrane. Small deposits of glycogen are now present in most of the cells.

By 112 hr. recovery is well advanced (Plates 3A and 3B). New stacks of rough-surfaced cisternae are forming and many polysomes are present. The terminal portions of the rough-surfaced cisternae are often distended and accompanied by small smooth vesicles, particularly in the neighbourhood of the Golgi region of the cells. Deposits of glycogen are increasing in number.

DISCUSSION

The combined results from electron microscopy and C-ribosome analysis, which both throw light on the structure of the rough-surfaced endoplasmic reticulum, and the amino acid-incorporation studies, which throw light on its function, establish that the following sequence of events occurs after a single dose of DAB.

The earliest effect that has been observed in this study is a detachment of polysomes and a derangement in the normal structure of the rough-surfaced endoplasmic reticulum. This is strikingly apparent in electron micrographs obtained from livers 6 hr. after DAB and is supported by C-ribosome analyses performed at the same time-interval, when it is found that the 'free'-C-ribosome patterns contain more polysomes than the same patterns from controls.

Next comes a phase of polysome breakdown. This first becomes apparent in C-ribosome analyses at 8 hr. after DAB and progresses to a maximum at 24 hr. when only 35% of the 'total'-C-ribosome pattern is due to polysomes. Parallel with this polysome disaggregation is an impairment in amino acid incorporation by microsomes. This is to be expected since polysomes are believed to be the site of protein synthesis (Wettstein *et al.* 1963). At the same time there is a marked increase in the stimulating effect of polyU on [¹⁴C]phenylalanine incorporation, presumably due to large amounts of

EXPLANATION OF PLATE I

(A) Part of the cytoplasm of a parenchymal cell from a control rat receiving corn oil alone. The appearance is essentially normal, except that there is some loss of glycogen from the cell. Remaining deposits of glycogen (g) are associated with smooth membranes (sm). Free polysomes can be seen between the packed cisternae of the rough-surfaced endoplasmic reticulum (rer). Magnification $\times 26\,800$. (B) At 6 hr. after a single dose of DAB the rough-surfaced endoplasmic reticulum is present as isolated strands of double membranes, some tracts of which (sm) are denuded of ribosomes. Smooth-surfaced vesicles appear to be forming from intermediate (sv) and terminal regions (tsv) of the membranous system. The intercisternal matrix contains many scattered groups of free polysomes. Part of a microbody (mb) and a lysosome (l) are present. Very little glycogen remains. Magnification $\times 23\,600$.

EXPLANATION OF PLATE 2

(A) At 24 hr. after treatment with DAB. Loss of ribosomes and vesiculation of endoplasmic reticulum is very extensive. The ground substance of the cell contains many individual ribosomes, but only an occasional polysome (p). No glycogen is present. Magnification $\times 24\,000$. (B) At 40 hr. after the administration of DAB. Numerous polysomes are present in the cellular matrix and there appears to be a beginning of reconstitution of endoplasmic reticulum with attached ribosomes (rer). The cytoplasm presents an appearance far from normal, and in some respects resembles that seen 6 hr. after DAB treatment (Plate 1B). Magnification $\times 24\,800$.

EXPLANATION OF PLATE 3

(A) and (B) are both from liver 112hr. after DAB. Both fields show extensive reconstitution of the rough-surfaced endoplasmic reticulum with the formation of new stacks of cisternae, particularly marked in (B). Numerous vesicles (sv) fill the space between distended ends of the newly forming rough-surfaced cisternae and the Golgi regions (G) seen in the upper right of both micrographs. Newly formed glycogen (g) can be seen in the form of both monomers and polymers, in some cases associated with regions containing smooth-surfaced membranes (sm). Magnification (A) $\times 27\,600$, (B) $\times 23\,600$.

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free ribosomes that are now present. It has been shown that polyU will combine with free ribosomes and provide a template for phenylalanine incorporation (Henshaw *et al.* 1963).

Thus it is firmly established that polysome disaggregation has occurred in homogenates obtained from livers of rats 8–24 hr. after a single intraperitoneal dose of DAB. However, it is important to be sure that this is an effect *in vivo*, rather than the result of the cell fractionation procedure as is the case shortly after a large dose of actinomycin D (Revel, Hiatt & Revel, 1964). Electron micrographs of the liver at the 24 hr. stage show that most of the ribosomes are free in the intercisternal spaces with a random distribution and little apparent polysome structure. Thus the C-ribosome analyses and the incorporation studies reflect a situation *in vivo* and not a fractionation artifact.

At 40 hr. a return to polysome formation becomes apparent from both electron micrographs and C-ribosome analysis. This is accompanied by a recovery in amino acid incorporation to levels that are a little higher than those found in controls together with a loss of the enhanced polyU effect.

It is not until 112 hr. after DAB that something approaching the normal structure of the rough-surfaced endoplasmic reticulum is seen in electron micrographs. In C-ribosome analyses, monomer and dimer have now fallen to control levels.

Thus two changes in this membrane system take place. First, its derangement and the detachment of polysomes, and secondly, the disaggregation of polysomes. During recovery, this sequence appears to be reversed. During the phase of polysome disaggregation, amino acid incorporation by microsome fractions is impaired and presumably, in the intact cell, protein synthesis is severely disturbed. On the other hand, from the results at 40 hr., it is clear that amino acid incorporation can proceed well in microsome fractions that have been derived from a grossly deranged rough-surfaced endoplasmic reticulum containing very few bound ribosomes, provided that polysomes are present. It is not necessary for the polysomes to be membrane-bound. This is not to say, however, that the protein synthesis that occurs *in vivo* at this stage follows the normal pattern.

The effects that we have reported to occur after the administration of DAB differ in two ways from those described by Hawtrey *et al.* (1963) after administration of 3'-Me-DAB. First, we find a clear-cut impairment of the amino acid-incorporating activity of microsomal fractions in the presence of pH 5 enzyme. This is associated with an increased polyU effect and is related to the polysome disaggregation apparent in C-ribosome patterns and from electron microscopy. Hawtrey *et al.* (1963), on

the other hand, find an initial phase of impairment only when microsomes are incubated in the presence of whole cell sap and not in the presence of pH 5 enzyme. Thus the impairment observed by these authors after 3'-Me-DAB is due to a factor in the cell sap and not the result of a change in the microsome fraction. Secondly, whereas they have reported a marked enhancement of the amino acid-incorporating activity of microsome fractions 40 hr. after the administration of 3'-Me-DAB, we find that 40 hr. after DAB the incorporating activity of microsomes has returned to only a little above normal levels.

This lack of a second phase of clearly enhanced amino acid-incorporating activity may be a dose effect. Daudel, Jaquier & Touplain (1965) have published results that suggest that higher doses of DAB than 3'-Me-DAB are required to give a clear-cut enhancement of amino acid-incorporating activity at 40 hr. after administration of the azo-dye.

The evidence suggests that the impairment of the incorporating activity of the microsome fraction is due to polysome breakdown. DAB could bring this about in one or several of three ways: (a) it could alter the ribosomes so that they are no longer able to combine with messenger RNA; (b) it could react with messenger RNA, causing instability; (c) it could act at the nuclear level by inhibiting synthesis of messenger RNA.

Considering the first possibility, the effect of polyU in enhancing amino acid uptake during the phase of polysome disaggregation shows that many, but not necessarily all, of the released ribosomes are capable of combining with a synthetic messenger RNA. On the other hand, the lack of an enhanced polyU effect at 40 hr., when the C-ribosome pattern shows that significant numbers of free ribosomes are present, could be because these particular ribosomes are damaged and unable to take part in the reformation of polysomes taking place at this stage. Roberts & Warwick (1966) have shown that DAB or metabolites of DAB bind to ribosomal RNA. Thus alteration to the ribosomes may be a contributory factor, but it is not the sole cause of polysome disaggregation.

Secondly, the preponderance of smaller polysomes during the period of maximal polysome breakdown could signify instability in messenger RNA. Such instability in messenger RNA has been postulated to occur after poisoning with dimethylnitrosamine (Mizrahi & Emmelot, 1964).

It is not yet possible to decide whether the third possibility, that DAB affects the synthesis of messenger RNA, is correct or not, since a study of DNA-dependent RNA synthesis during the period of polysome breakdown has yet to be made. However, such an effect has been shown to occur in the rat liver after the administration of actinomycin D (Reich, Franklin, Shatkin & Tatum, 1961) and

aflatoxin B (Clifford & Rees, 1966). In both these cases polysome breakdown is associated with a decrease in the uptake of [^{14}C]orotate into nuclear RNA in the presence of DNA primer. Dimethyl-nitrosamine, on the other hand, has no such effect (Villa-Trevino, 1965).

It is noteworthy that there are striking similarities between the state of the rough-surfaced endoplasmic reticulum at certain stages after a single dose of DAB and the condition of this system in various hepatomas. For example, slow-growing tumours may have rough-surfaced endoplasmic reticulum similar in appearance to that seen in rats both 6 and 40 hr. after a dose of DAB. On the other hand, some fast-growing tumours have a membrane system very similar in appearance to that found 24 hr. after the administration of DAB, but polysomes are more plentiful in these tumour cells (Dalton, 1964; Porter & Bruni, 1964; Ma & Webber, 1966).

The induction of changes in the rough-surfaced endoplasmic reticulum may therefore be an important factor in carcinogenesis. The work of Pitot and his collaborators is relevant in this respect. After a study of the effects of actinomycin D on inducible enzymes in liver and hepatomas, Pitot, Peraino, Pries & Kennan (1965) have drawn the conclusion that a common feature of tumour cells is an alteration in the stability of messenger RNA. Pitot (1966) has concluded that template stability depends among other things on interaction between messenger RNA and the rough-surfaced endoplasmic reticulum. It is clear from the present work that a single dose of DAB has a profound effect on the structure of the rough-surfaced endoplasmic reticulum and also on the interaction between polysomes and membranes. It may also have a direct effect on the stability of messenger RNA, as discussed above. However, it should also be emphasized that apparently similar changes with respect to both structure and function can be brought about by a substance such as carbon tetrachloride, which, although carcinogenic in the mouse, is not a carcinogen in the rat (Smuckler & Benditt, 1965; Mager, Bornstein & Halbreich, 1965; Steiner, *et al.* 1964). Also, 2-Me-DAB, though not a carcinogen, brings about similar but perhaps less extensive changes in the rough-surfaced endoplasmic reticulum (Lafontaine & Allard, 1964).

Thus not all the changes caused by short-term treatment with carcinogens are necessarily related to the process of carcinogenesis. It is hoped that changes specifically related to carcinogenesis may become apparent by extending this study to rats receiving DAB for prolonged periods.

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