FINE-STRUCTURAL ALTERATIONS IN CELL PARTICLES DURING CHEMICAL CARCINOGENESIS

I. INFLUENCE OF THE FEEDING OF AMINOAZO DYES ON THE SWELLING AND SOLUBILIZATION OF RAT-LIVER MICROSOMES*

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Much research has been done on the relation between the morphology and structure of isolated mitochondria and their enzymic activities. The correlation between swelling and the uncoupling of oxydative phosphorylation in rat liver mitochondria has been known for some time[1,2,3]. It was visualized[4] that a reversible alteration of the mitochondrial structure may be a basis of the regulation of the enzymic pattern. Recent investigations have shown that during experimental liver carcinogenesis, by the feeding of aminoazo dyes, the activities of some mitochondrial enzymes are influenced, possibly through the modification of the structural organization of this cell particle[5]. The existence of such structural modification was demonstrated later by comparing the swelling of mitochondria isolated from the liver of normal and dye-fed rats, and from various strains of hepatomas[6].

Similar ideas were reached by us recently through an entirely different approach, by the physico-chemical consideration of stereochemical factors governing carcinogenesis. These studies[7,8,9] suggested, that the irreversible change in the cell, during chemical carcinogenesis, would start in the spatial arrangement of the molecular network of a key enzyme or enzyme system. Since the high RNA content and the rapid rate of amino acid incorporation[10,11] suggest that the microsomes are the main centers of protein synthesis in the cell[12], our attention was focused on these particles.

In the present study swelling has been used to detect structural alterations in the microsomes of the liver, during chemical carcinogenesis. It was found as with mitochondria[6], that there is a gradual increase in structural rigidity, until an irreversibly altered stage is reached, leading finally to the emergence of tumors. The effect of variables on the swelling of microsomes has also been studied. A group of soluble proteins, released from the microsomes, was resolved in two fractions by starch electrophoresis.

* Supported in part by grants from the National Cancer Institute, U. S. Public Health Service, the American Cancer Society, and the Damon Runyon Fund.

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MATERIALS AND METHODS

Care of animals

Male albino rats (Holtzman Rat Co., Madison, Wis.) with an initial weight of 150–200 g were fed ad libitum a semi-synthetic diet, essentially[13] (for medium tumor incidence), but containing 18% casein and 1 g/kg of choline chloride. 0.06% of the hepatic carcinogen 3'-methyl-*p*-dimethylaminoazobenzene, or of the relatively non-carcinogenic 2-methyl-*p*-dimethylaminoazobenzene were incorporated in the diet. The animals used for the control experiments were fed at least 1 week the same semi-synthetic diet (basal diet) containing no dye. For the study of the influence of the diet, 2 rats were fed the basal diet for 2 weeks, and for the same period 2 rats were fed Purina Laboratory Chow. The rats were sacrificed by decapitation, the livers perfused through the superior vena cava *in situ*, with ice-cold isotonic sodium chloride, excised and kept in ice until homogenization. The firm white hepatomas, which were used, have been carefully dissected to eliminate the adhering liver tissue and some necrotic material. These operations were carried out at approx. 4°C.

Preparation of the microsomal fraction

The livers and the tumors were homogenized (40% w/v homogenate) in an all-Lucite Elvehjem-Potter homogenizer, in 0.25 *M* ice-cold sucrose, at approx. 4°C. This first sucrose solution contained also 0.001 *M* ethylenediamine tetraacetate (EDT). Nuclei, mitochondria, and unbroken cells were removed in an International refrigerated centrifuge, at 4°C. The microsomal fraction, used in these experiments, was isolated from the supernatant fluid by centrifugation at 105,000 *g* for 50 min in a Spinco L ultracentrifuge, at the same temperature. The resulting pellet was washed once by resuspension and homogenization in 0.25 *M* sucrose containing no EDT, and recentrifuged in the same conditions. The final pellet was resuspended in 0.25 *M* sucrose so, that 1 ml contained the microsomes from 1 g of fresh tissue. All the operations were carried out at approx. 4°C. This standard stock suspension, kept in ice, was used in less than 20 min after it had been prepared, except when "aged" microsomes were studied.

Swelling test

The standard swelling test was patterned after the method which TAPLEY[14] applied to the mitochondria. The basic test system consisted of 4.7 ml of 0.02*M* barbital buffer at pH = 8.1, containing 0.055 *M*/l KCl, and 0.3 ml of the microsomal stock suspension. The same buffer was used for the study of the pH dependence, by adjusting the pH from 6.8 to 11.0.

The percentage of swelling was measured by following the decrease of the optical density at 515 mμ in a Lumetron photometer and relating the decrement to the zero-time reading. This was found to be more convenient in the earlier phases of this study[15], than to record the swelling in units of optical density, since the relative positions of the curves are not affected by the variations of the zero-time reading. This first reading was always done within 15 seconds after the stock suspension was added to the buffer.

Since it is known that the amount of microsomes in the liver varies with dye feeding, we have shown that the swelling curves obtained by using increasing volumes of a same stock suspension vary within the usual experimental errors, when the zero-time optical densities are between 0.120 and 0.500.

All swelling curves were determined in duplicate, with non-pooled stock suspensions from two different rats.

The compounds, the action of which on swelling was studied, were either directly dissolved in a pH = 8.2 buffer, or a concentrated stock suspension was prepared and a small aliquot of it (0.1–0.2 ml) was added to the test system. The pH of the buffers or the stock solutions containing the compounds to be tested were adjusted to 8.2 after dissolution. The stock solutions of the N2H mustard (Merck mustargen) were not adjusted to avoid hydrolysis, but it was observed that the added amounts do not diminish the pH of the test system below 8.0. N2H was the only compound which was added to the test system *after* the stock suspension.

The optical densities were read against a blank of distilled water, except for tannic acid, 2,4-dinitrophenol, cytochrome *c*, and alloxan. Since the optical densities of these solutions change with time at this pH, the blank consisted of the same test system but without added microsomal suspension.

The test system for thyroxine contained only trace amounts of this compound, since it is only scarcely soluble at this pH. A suspension of 4 mg of finely powdered thyroxine in 4.9 ml barbital buffer was shaken on a mechanical shaker for 2 h. The insoluble part was sedimented by centrifugation and the clear supernatant used as test system.

The determination of dye bound in the microsomes

The stock suspensions, in excess after the swelling tests, were precipitated with equal volumes of 20% trichloroacetic acid (TCA). The precipitates were washed once with pH = 4.6 acetate

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buffer (57.4 g NaOAc + 40.2 ml AcOH/l), once with water, twice with 95% ethanol at 70–80° for 10 min, once with ether, and dried. The determination of bound 3'-methyl-*p*-dimethylaminoazobenzene was carried out as already described by MILLER AND MILLER[16,17]. The net optical densities were recalculated to μmoles[17] and plotted against the time of dye-feeding.

Proteins released in pH 8.2 barbital buffer

Gravimetry. The microsomes, from 35 g perfused liver tissue in each case, were isolated in the usual way, but in the absence of EDT. The final pellet was homogenized at approx. 4°C, in 38.5 ml pH 8.2 standard barbital buffer. Three 11-ml aliquots were quickly pipetted in 12-ml Erlenmeyers, stoppered, and incubated for 4 h, with occasional shaking, at approx. 4°C, at room temperature (23–25°C), and at approx. 38°C, respectively. The suspensions were sedimented in the usual conditions, and the supernatants carefully decanted. The sediments were homogenized in a few ml of water and both sediments and supernates precipitated with equal volumes of 20% TCA. These protein samples were washed as already described for the samples used for bound dye determination, but in strictly standardized conditions in centrifuge tubes. The washed samples were thoroughly dried, first in air, then *in vacuo* over $CaCl_2$ and NaOH, overnight. The percentage extraction is given by the weight of the supernatant proteins over the sum of the supernatant and the residual sediment.

For the experiment with the hepatoma only 20.3 g tissue was used and two aliquots were taken, incubated at approx. 4°C, and at room temperature.

Preparation of samples for starch electrophoresis. For the comparative study of the electrophoretic patterns on starch, the microsomes obtained from rats fed the normal and the 3'-methyl-*p*-dimethylaminoazobenzene-containing diet, were used. In each case the microsomes from 19.8 g of perfused liver tissue were extracted in 33–34 ml buffer at approx. 4°C for 4 h. The supernatant fluids were concentrated by dialysis against a 15% solution of polyvinylpyrrolidone in the same barbital pH 8.2 buffer, at approx. 4°C for about 12 h. The sedimented microsomes were precipitated washed, and weighed, as described. By assuming proportionality of the released proteins with the amount of microsomes present, the electrophoretic pattern of the extract of the dye-containing microsomes is corrected on the basis of the relative weights of the sediments.

Solubilization of microsomes by desoxycholic acid

Microsomes of normal and dye-containing liver and of hepatoma were solubilized by a modification of the method of LITTLEFIELD *et al.*[10]. The microsomes were obtained from the livers of rats fed: (a) the usual semi-synthetic diet, (b) the same diet, each of which received by stomach tube 50 mg of 3'-methyl-*p*-dimethylaminoazobenzene in 2 ml corn oil 24 h before sacrifice, and (c) from hepatoma tissue. 12 g were used of each tissue. To the microsomal fraction of each tissue 180 mg desoxycholic acid (DCA) was added in a 5% solution (3.6 ml) and it was homogenized at approx. 4°C. The suspensions were allowed to stand for 3–4 min and then diluted with ice-cold water to bring the final DCA concentration to approx. 0.5%. The suspension was mixed by shaking, and centrifuged at 105,000 *g* for 2 h. All these operations were carried out conveniently in the tubes of the ultracentrifuge.

The solubilized microsomal proteins were decanted and the solutions were concentrated by dialysis at approx. 4°C against a 15% solution of polyvinylpyrrolidone in a barbital–desoxycholic buffer, for about 12 h. This buffer is composed of 0.02 mole barbituric acid, 0.013 mole desoxycholic acid, and 0.042 mole potassium chloride per liter, and adjusted to pH 8.2. The 5% desoxycholic acid solution is prepared by dissolving 0.66 g of glycyl-glycine and 1.25 g DCA in 23 ml water. The solution is adjusted to pH 8.2 and filled up to 25 ml.

Zone electrophoresis on starch of soluble microsomal proteins

The electrophoresis apparatus used for these studies was built in the laboratory from sheets of Lucite[18]. The horizontal starch holder used in these experiments contains six canals holding the starch slurry. These canals are rectangular in cross section and have a size of 1 × 2 × 31 cm. A thick filter paper, held against each end of the starch block by rubber bands, is dipped into the buffer chamber of the double electrode-buffer vessels. These vessels contain two chambers, a smaller electrode chamber and a large buffer chamber. The platinum-sheet electrodes dip into 1.5 *M* phosphate buffer at pH 7 in the electrode chambers and the current is carried to the buffer chambers by means of agar bridges. These bridges are U-tubes filled with a solution prepared by adding an equal volume of 2% melted agar to 1.5 *M* phosphate buffer at pH 7.0. The whole apparatus: the starch holder, the electrode-buffer vessels, and the electrodes are encased in a refrigerated Lucite-case, where the temperature can be maintained between 0° and 4°C.

The starch slurry was prepared by washing starch with water and then with the particular buffer used, until no change in the pH of the filtrate was observed. The holder is filled with a slight excess of the slurry, the excess of buffer removed by filter paper, and the surface of the block leveled with a spatula. The blocks are covered with Parafilm to avoid excessive drying out, and the

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holder is placed in the apparatus so that the filter paper at each end of it is dipped into the respective buffer chamber. The cover of the refrigerated case is closed and the block allowed to equilibrate for at least 4 h.

A 1 cm-wide segment is then exposed by cutting the Parafilm at the 16th cm from the negative pole, and the starch slurry removed from this portion. A slurry, prepared from the protein solution to be studied and starch, which has the approximate consistency of the block, is then placed in the trench. Since such a trench can take up a slurry prepared from about 0.6 to 0.8 ml solution, the time of dialysis has to be regulated consequently. The segment was covered with Parafilm, the case closed, and after equilibrating 10 min more, the current was turned on.

The conditions of the electrophoresis were: 225 V and 11–13 mA per 1 × 2 × 31 cm block, for 12 h.

At the end of the run the block was cut into 1 cm pieces, which were dropped into 2 ml ice cold 0.013 *M* KCl, containing 0.002 *M* phosphate buffer, at pH 8.2. The negative end was always the tube No. 1. The starch pieces were broken up by stirring once, mechanically, with a glass rod. The suspensions were centrifuged in the cold.

0.1 to 0.3 ml from each supernatant fluid were analyzed by the method of LOWRY *et al.*[19]. The optical densities at 660 mμ are plotted against the length in cm of the starch block. The rest of the supernatants are dried down in small beakers. The residues are dissolved each in 2 ml 88% formic acid, the solutions clarified by careful centrifugation, protecting the cuvettes in stoppered plastic tubes. Because of the presence of a liver pigment, absorbing mostly at 400 mμ but also somewhat at 525 mμ, the dye absorption at this last wavelength is corrected. The absorption due to the liver pigment at 525 mμ in the dye-bound protein sample is calculated by the proportionality rule, from the optical densities of both the normal and the dye-bound samples at 400 and 525 mμ. The net absorption due to the dye is obtained by subtracting the pigment absorption from the reading at 525 mμ.

RESULTS

Swelling during the feeding of aminoazo dyes

Table I and its graphical representation (Fig. 1a and 1b) show that the swelling ability of the microsomes decreases successively with the feeding of 3'-methyl-*p*-dimethylaminoazobenzene, and attains a minimum level at about 4 weeks. The data in Table I are mean values of two experiments.

TABLE I

SWELLING OF LIVER MICROSOMES OF RATS FED 3'-METHYL-*p*-DIMETHYLAMINOAZOBENZENE

The rats were fed a semi-synthetic diet containing 0.06% of dye. The swelling of the particles was followed with a Lumetron photometer at 515 mμ. The percentage swelling was calculated with the formula: $\frac{d_o - d_i}{d_o} \times 100$, where d_o and d_i are the optical densities of the suspension, at zero-time and after a given period, respectively. 0.3 ml microsomal stock suspension was used in a test system consisting of 4.7 ml of a pH 8.1 0.02 *M* barbital buffer, containing also 0.055 *M*/l KCl.

Minutes	0	10	20	30	45	60	120	180
Weeks of dye feeding				% *Swelling*				
0	0	8.3	13.0	16.4	21.9	24.8	35.0	39.4
1	0	11.3	15.9	20.0	23.3	25.4	31.9	33.7
2	0	10.2	14.5	16.9	19.2	20.9	26.6	28.3
3	0	7.8	10.5	12.6	15.7	17.8	22.9	26.7
4	0	9.2	11.3	14.7	15.4	16.2	17.2	18.1
6	0	9.8	14.9	18.2	22.0	24.9	29.3	30.5
8	0	8.9	12.2	14.3	20.5	22.1	26.3	29.0
20	0	7.6	12.8	15.1	20.2	22.4	31.9	37.5
24	0	11.0	19.0	22.1	27.0	29.3	34.4	37.4
Tumor	0	6.4	10.3	11.4	13.2	14.9	16.3	18.0

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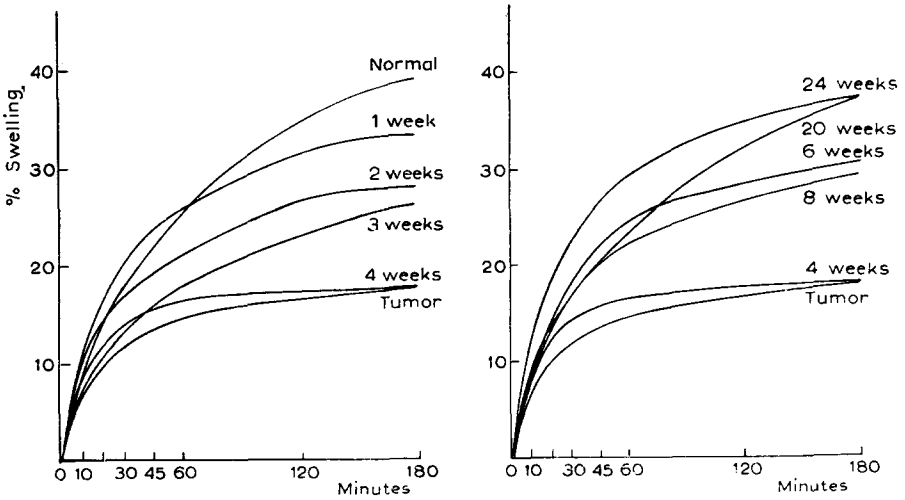

Fig. 1a and 1b. Graphical presentation of Table I by omitting the determining points of the curves for the sake of clarity.

However, after about 4 weeks apparently important changes take place in the microsomal structure, causing a strong reversal of this decrease. Although the feeding of the dye was continued, the swelling ability of the liver microsomes increased again and finally reached the normal level at a stage where the tumors become macroscopically visible. On the other hand the swelling of the microsomes isolated from the emerging hepatoma was very low, comparable to that of the liver microsomes after 4 weeks of dye feeding. "Aging" of the standard stock suspension for 1 ½ h before use did not influence noticeably the swelling curve.

The feeding at the same level, of the relatively non-carcinogenic 2-methyl-*p*-dimethylaminoazobenzene, did not seem to influence swelling to a large extent, even after 4 weeks, as shown in Table II.

TABLE II

SWELLING OF LIVER MICROSOMES OF RATS FED 2-METHYL-*p*-DIMETHYLAMINOAZOBENZENE

Same experimental conditions as in Table I

Minutes		0	10	20	30	45	60	120	180
Weeks of dye feeding					% *Swelling*				
2		0	8.5	12.7	16.1	21.6	29.6	37.9	37.5
		0	6.3	9.7	14.3	16.0	18.6	26.4	30.6
4		0	5.5	12.8	18.3	23.2	25.4	31.7	34.7
		0	4.9	9.0	11.8	14.7	17.6	25.1	33.0

The pH dependence of the swelling

The structural differences between liver microsomes and hepatoma microsomes show up not only in the extent of swelling but also in its pH dependence.

Thus Fig. 2 shows, for the liver microsomes, an optimum at pH 8.0–8.2, with a

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steady decrease toward lower and higher pH values. The curve obtained with hepatoma microsomes has only an inflexion point at this pH, then it rises toward pH 11.0.

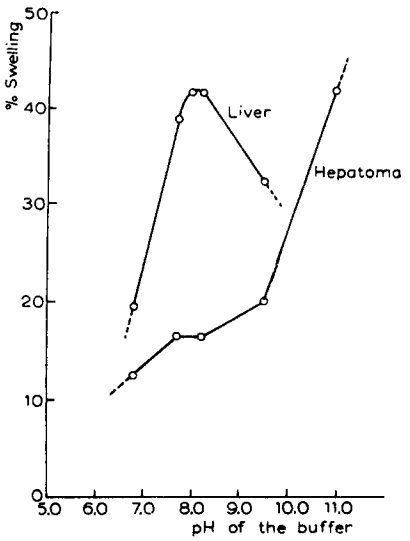

Fig. 2. Dependence on pH of the microsomal swelling. Same experimental conditions as in Table I, but buffers adjusted with KOH to various pHs. Percentage swelling after 3 h incubation was plotted against the pH.

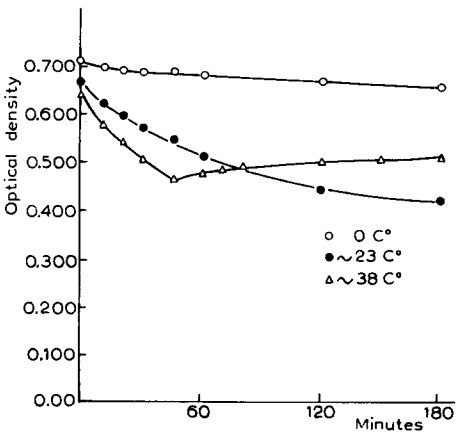

Fig. 3. Change in swelling with the temperature of incubation. The swelling was followed in the usual pH 8.1 barbital buffer, and recorded in optical density. The samples were maintained, between readings, at 0°C, room temperature and 38°C, respectively.

Influence of the diet, the temperature and X-rays on swelling

Since the change in swelling might have been attributed to the depletion of some essential nutrients in the liver due to the feeding on the semi-synthetic diet, the dependence of swelling on the type of diet was briefly studied. Freshly received rats were put on semi-synthetic diet or on Purina Laboratory Chow, for two weeks. The swelling of the microsomes was found somewhat higher (42.0 %) when the animals were fed the Laboratory Chow, against 37.2 % with the semi-synthetic diet.

Fig. 3 illustrates the influence of the temperature on swelling. The microsomes swell to a much higher extent at room temperature, than at 0° C (samples kept in melting ice). At 38°C however, after a rapid swelling until 45 min, the optical density starts to increase again. It will be shown in a later heading (*The release of soluble microsomal proteins*) that this increase is not due to the particles themselves but to the aggregation of some released proteins, at this temperature.

X-ray irradiation of a standard stock suspension, made up in isotonic sodium chloride, seems not to influence measurably the extent of swelling. The conditions of the irradiation were: 48 R/min; 175 KV; filters: ½ mm Al + 1 mm Cu. On the other hand the microsomes showed a slight tendency to initial aggregation (increase of the zero-time optical density) when the total dose was 800 R, an average lethal dose to rats. Not so, when irradiated with 400 R.

Influence of various compounds on swelling

The action of various inorganic cations and organic compounds on microsomal swelling

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was explored. Each set of swelling curves, determined in presence of the compounds to be studied and obtained with the same standard stock suspension, was accompanied each time by a normal control curve. The Inhibition Indexes: test swelling over control swelling, were determined after 3-h incubation. Control level: 1.00. 100% inhibition: 0.00.

TABLE III

ACTION OF VARIOUS COMPOUNDS ON THE SWELLING OF LIVER MICROSOMES

The action of various inorganic and organic compounds on the swelling of liver microsomes, in the usual pH 8.1 barbital buffer. The "Inhibition Index" was obtained by relating the percentage of swelling in presence of the compound, to the swelling of the control, at 3 h. Control corresponds to 1.00, 100% inhibition to 0.00.

Inorganic cations		
Compound	*Concentrations*	*Inhibition index*
$CaCl_2$	$1 \cdot 10^{-3} M$	0.69
$CaCl_2$	$1 \cdot 10^{-2} M$	0.00
$MgCl_2$	$1 \cdot 10^{-3} M$	0.78
$MnSO_4$	$1 \cdot 10^{-3} M$	0.40
$CuCl_2$	$1 \cdot 10^{-3} M$	0.20
$CoCl_2$	$1 \cdot 10^{-3} M$	aggregation
$HgCl_2$	$1 \cdot 10^{-6} M$	1.02
$HgCl_2$	$1 \cdot 10^{-4} M$	0.00
KCN	$1 \cdot 10^{-5} M$	0.96
KCN	$1 \cdot 10^{-3} M$	0.98
Organic compounds		
Compound	*Concentrations*	*Inhibition index*
EDT	$1 \cdot 10^{-3} M$	0.46
Tannic acid	$2 \cdot 10^{-4} M$	0.24
Alloxan	$5 \cdot 10^{-3} M$	0.85
Alloxan	$1 \cdot 10^{-2} M$	0.63
Indoleacetic acid	$1 \cdot 10^{-3} M$	0.96
Iodoacetic acid	$2 \cdot 10^{-2} M$	0.44
Histamine	$1 \cdot 10^{-3} M$	0.94
DNP	$1 \cdot 10^{-3} M$	0.99
N2H mustard	$1 \cdot 10^{-5} M$	1.03
N2H mustard	$1 \cdot 10^{-4} M$	0.83
N2H mustard	$1 \cdot 10^{-3} M$	0.70
α-α-Dipyridyl	$1 \cdot 10^{-3} M$	0.99
Cytochrome C	$3 \cdot 10^{-5} M$	1.00
Colchicine	$5 \cdot 10^{-4} M$	0.83
Thyroxine	$\ll 1 \cdot 10^{-3} M$	0.75
Glutathione	$5 \cdot 10^{-3} M$	1.08
Heparine	200 mg/l	1.04
Protamine sulfate	600 mg/l	0.79
Polyvinylpyrrolidone	600 mg/l	1.00

It is interesting, while mitochondrial swelling is increased by Ca^{++} and inhibited by EDT, which chelates Ca^{++}, both these compounds inhibit microsomal swelling. Iodoacetic acid, alloxan, nitrogen mustard, all of which react with sulfhydryl groups, are inhibitors, although relatively high concentrations are required. Since it is known that tannic acid exerts its specific action on proteins by cross-linking, mainly by the

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establishment of hydrogen bonds, it is not surprising that tannic acid profoundly affects swelling. The action of colchicine can be interpreted in a similar way.

The bound dye level in the microsomes

The determination of 3'-methyl-*p*-dimethylaminoazobenzene bound by covalent linkages in the microsomes, has shown a maximum level between about 2 and 3 weeks. This distribution is in good correspondence with the findings of MILLER AND MILLER[17] on the binding of azo dyes in whole liver homogenates.

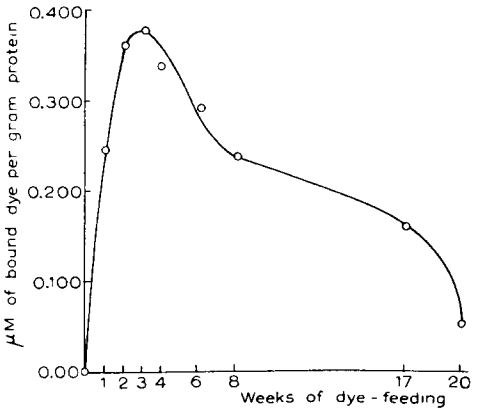

Fig. 4. Levels of bound dye in the microsomes, plotted against the time of dye feeding. The nonextractible bound dye was measured by colorimetry in TCA precipitated microsomes, after alkaline hydrolysis, as described by MILLER AND MILLER[16,17]. The levels of bound dye are expressed in μmoles.

It would appear from Fig. 4 that the time corresponding to the maximum level of dye does not coincide with the time of minimum swelling ability (4–6 weeks). At about 20 weeks, the microsomes contain practically no bound dye.

The release of soluble microsomal proteins

The swelling of liver microsomes is paralleled by the release in the buffer of a group of soluble proteins non-sedimentable at 105,000 *g*. However, the microsomes isolated from hepatoma do not follow this pattern, since their low swelling is accompanied by a relatively high protein release. Table IV shows the percentage of released soluble proteins as a function of the time of dye feeding, at three different temperatures.

TABLE IV

SOLUBLE PROTEINS RELEASED FROM THE LIVER MICROSOMES OF RATS FED 3'-METHYL-*p*-DIMETHYLAMINOAZOBENZENE

Microsomes were incubated in the usual pH 8.2 barbital buffer for 4 h and then centrifuged. The proteins in the supernatant fluid and the sedimented microsomes were precipitated with TCA, washed with acetate buffer, ethanol, ether. Then dried and weighed.

Weeks of dye feeding	*% Release*		
	4°C	*23°C*	*38°C*
0	13.2	15.9	14.0
1	10.1	12.4	9.8
2	9.1	11.8	9.5
4	9.4	10.4	8.4
8	11.5	13.6	11.0
17	16.4	17.8	13.9
Tumor	12.0	14.2	—

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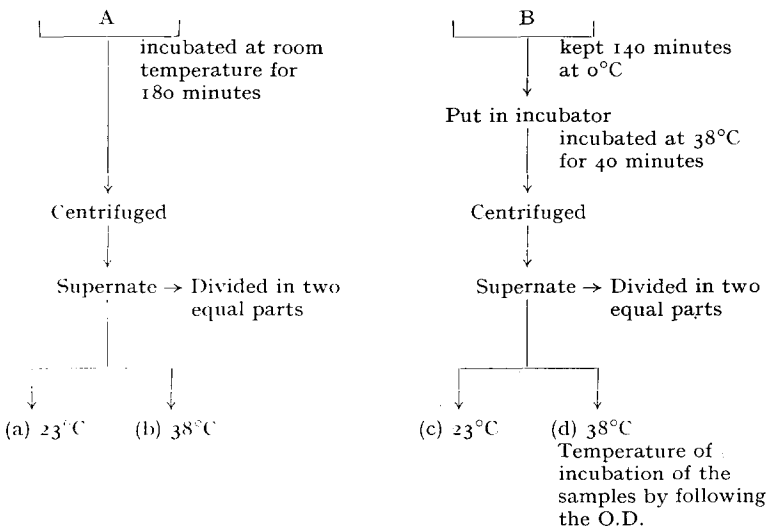
It will be noted that at 38°C the release was always lower than at 23°C (room temperature). This is not due actually to a lower release at 38°C, but to the denaturation and aggregation of a part of the released proteins during incubation, sedimented together with the microsomes. It is this same slow aggregation which causes the apparent reversal in the swelling curve of normal microsomes, taken at 38°C, as we have seen previously in Fig. 3.

This was demonstrated as follows. Microsomes from 11.7 g perfused normal liver tissue were rapidly homogenized in 22 ml barbital buffer in the cold, and the suspension was divided in two equal parts: A and B. Both A and B were then incubated for 3 h to obtain soluble proteins. A was incubated at room temperature for the entire period. B was first maintained at 0°C for 140 min, and then incubated at 38°C for 40 min. So the total incubation periods of A and B were equal. It has to be recalled that the apparent reversal of the swelling at this temperature starts after about 40 min of incubation. Both suspensions were then centrifugated and the supernatant fluids divided in two equal parts. One of each (a) and (c) were incubated at room temperature (23°C) and the other two fractions (b) and (d) at 38°C. The optical densities, during incubation, of the four samples of supernatant fluids were followed at 515 mμ. Even after 150 minutes (a) and (c) remained constant, while the initial optical densities of (b) and (d) increased respectively by 200 and 150%.

TABLE V

DEMONSTRATION OF THE MECHANISM OF THE APPARENT REVERSAL OF MICROSOMAL SWELLING AT 38°C



Therefore, the apparent reversal of the swelling, and the lower protein release at 38°C, can not be attributed to the adsorption by the microsomes of a part of these proteins, after a certain extent of swelling. Neither is it influenced by the temperature of incubation at which the soluble proteins were obtained, since the optical densities of (a) and (c) remained constant, while those of (b) and (d) increased in the same order

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of magnitude. The only remaining possibility is, what was actually demonstrated, the heat denaturation and aggregation of a fraction of the released proteins at 38°C.

The electrophoretic patterns of soluble microsomal proteins

The electrophoretic patterns of the proteins released in barbital buffer or obtained by drastic solubilization with desoxycholic acid, were determined (Fig. 5 and Fig. 6).

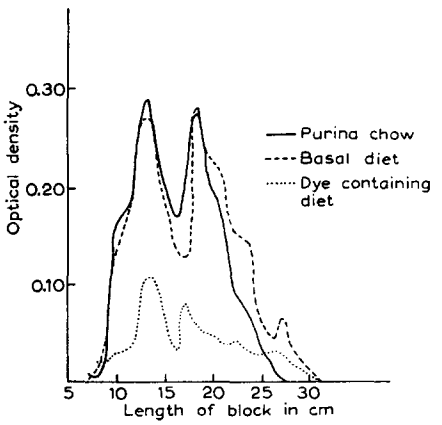

Fig. 5. Electrophoretic patterns of soluble microsomal proteins released in barbital buffer. The solutions of soluble proteins, obtained in barbital buffer from the microsomes from 19.8 g liver tissue, were concentrated to 0.6–0.8 ml by dialysis against a 15% solution of polyvinylpyrrolidone in the same buffer. These concentrated solutions were electrophorized on starch block. No. 1 is the negative end. Origin was at the 16th cm.

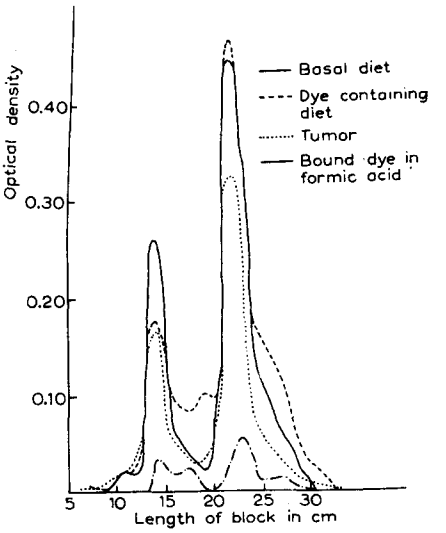

Fig. 6. Electrophoretic patterns of soluble microsomal proteins obtained by desoxycholic acid. The proteins obtained from microsomes by solubilization with 0.5% desoxycholic acid were electrophorized on starch. In each case, 12 g tissue was used. Otherwise the procedure was similar to that given in Fig. 5.

Unfortunately this work had to be terminated before the electrophoresis of proteins, released in barbital buffer from tumor microsomes, could be carried out. The resolution of the proteins, either obtained by release in buffer or by solubilization with desoxycholic acid, gave always two main groups. Although the method is too crude to obtain further separation, the low electroosmotic flow on starch, when compared to other supporting media, makes the interpretation of the pattern possible. Since No. 1 is the negative end of the block and the origin is at the 16th cm, it may be suggested from the relative positions of the two protein groups, that the peak to the left from the origin is made up of basic proteins.

No dye is present in the proteins released in the buffer. Practically all the dye was found in the protein solubilized by desoxycholic acid, since the insoluble part did not give the typical halochromism with TCA. In relation to the findings of PALADE AND SIEKEVITZ[20] on the DCA-solubilization of microsomes, it is tempting to suggest that the dye in the microsomes is bound to the building blocks of the limiting membrane of the vesicles.

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DISCUSSION

The present work has provided evidence for the modification of the macromolecular organization of the microsomes during chemical carcinogenesis. Similar observations on mitochondria have been made recently by CLERICI AND CUDKOWICZ[5] and EMMELOT AND BOS[6,21]. However, since the microsomes are presumably the main centers of protein synthesis in the cell, the writers feel that the structural alterations reported here are more directly involved in the carcinogenic process than changes which may be revealed in other cell particles.

The modifications in the structural organization of cell particles have been followed by the study of swelling. Since the mechanism of the swelling of macromolecular systems is interpreted as the distancing of structural elements by the penetration of solvents or solutions[22,23], changes in the spatial arrangement of these elements will show up in the rate or extent of swelling, provided conditions of temperature, ionic strength and pH are kept constant.

The present results are in agreement with previous studies on the molecular geometry of polycyclic hydrocarbons in relation to chemical carcinogenesis[7,8,9]. The idea was advanced that, through the formation of molecular inclusions, these compounds may bring about or interfere with changes in the spatial conformation of the finestructure of a key enzyme or enzyme system, changes which may be directly related to the functional activity of this system in the cell. However the emphasis lies more on the *changes in the spatial conformation* of the finestructure than on the formation of molecular inclusions, since such changes may be brought about by cross-linking on the external surface of biological macromolecules. Such cross-links may be established, depending on the compound involved, by interactions which may vary from covalent bonds to weak secondary valence forces. Some evidence for the possibility of cross-linking has been presented for the carcinogenic aminostilbenes[24] and azo dyes[25].

It is immaterial, from the standpoint of the *mechanism* of chemical carcinogenesis, whether the changes in the macromolecular pattern of a key enzyme system are brought about by the inclusion of certain small molecules or by external cross-linking. What is important in this respect, is the modified pattern which will determine the altered or new functional activity of the enzyme system, as well as its integration with other systems in the cell metabolism. Once established, the changes in the macromolecular pattern may be transmitted by the duplication mechanisms of the cell. A possible corollary of this concept is, that if during the interaction with carcinogenic molecules some macromolecular systems in the cell undergo stable finestructural changes, then these systems may carry, in somes cases, an "imprint" of the geometry of the molecule (size, shape, and distribution pattern of reactive centers) which brought about these changes. Thus, the very structure of the carcinogenic molecules may help in the designing of specific compounds of chemotherapeutic value. The paradox of the inhibition of tumor growth by carcinogenic compounds[26] may be rationalized in the same outline.

ACKNOWLEDGEMENTS

The authors are greatly indebted to Dr. HAROLD P. RUSCH and Dr. FREDERICK E. SHIDEMAN for support during the course of this investigation. We wish to thank also Dr. ELIZABETH C. MILLER for technical advice during the protein-release experiments,

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Mr. HARRY V. GELBOIN for help in performing the electrophoretic studies, Dr. JOHN W. ANDEREGG for his assistance during the experiments with X-rays, Mrs. YVANNE JONES for carrying out the bound-dye determinations and Dr. CHARLES F. CRAMPTON for helpful criticism and discussion.

SUMMARY

1. Changes have been observed in the structure of rat-liver microsomes as a result of feeding azo dyes. Swelling of the isolated microsomes under carefully controlled conditions has been used to study these changes.

2. When 3'-methyl-4-dimethylaminoazobenzene is fed, there results a progressive decrease in both the rate and the extent of swelling of the isolated microsomes. This decrease reaches a minimum value after 4 weeks of feeding.

Although the feeding is continued, the original susceptibility to swelling gradually reappears. Thus, microsomes from the livers of rats fed with the dye for 20–24 weeks show approximately the same rate and degree of swelling as microsomes from the livers of normal rats.

3. Microsomes from dye-induced hepatoma are as resistant to swelling as are microsomes from the livers of rats fed for 4 weeks.

4. The feeding of the isomeric, but relatively non-carcinogenic, 2-methyl-4-dimethylaminoazobenzene has shown no such action on swelling.

5. In liver microsomes, the swelling is directly proportional with the release of a small group of soluble proteins in the surrounding buffer. However, the hepatoma microsomes do not follow this pattern, and there the low swelling is accompanied by a relatively great release of proteins.

6. The structural difference between liver and hepatoma microsomes shows up not only in the rate and extent of swelling, but also in the dependence on pH.

7. The influence of the diet, of the temperature, of X-rays, and of various inorganic and organic compounds, on the swelling of microsomes was studied.

8. The soluble proteins released from the microsomes in buffer, or obtained by treatment with desoxycholic acid were resolved into two main groups by electrophoresis on starch.

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Received August 21st, 1957