Fine Structural Alterations in Cell Particles During Chemical Carcinogenesis

II. Further Evidence for Their Involvement in the Mechanism of Carcinogenesis. The Swelling of Rat Liver Mitochondria During Feeding of Amino Azo Dyes*

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ABSTRACT

Swelling under carefully controlled conditions has been used to study alterations in the structure of rat liver mitochondria as a result of feeding azo dyes. The changes of the swelling properties of the mitochondria during feeding of the hepatocarcinogenic 3′-methyl-4-dimethylaminoazobenzene are essentially comparable to those observed previously with the microsomes, under the same dietary conditions. These alterations in mitochondrial swelling are not related to changes in the amount of these cell particulates per unit weight of tissue, during feeding of this azo dye. As with the microsomes, feeding of the isomeric but relatively noncarcinogenic 2-methyl-4-dimethylaminoazobenzene does not affect swelling. The structural differences between liver and hepatoma mitochondria show up not only in the rate and extent of swelling but also in the form of the curves of pH dependence.

The influence of ketones and sulfhydryl compounds on the swelling of normal liver mitochondria were studied, with particular emphasis to the role of sulfhydryl groups in membrane permeability.

The sudden steep rise in the tumor incidence in groups of rats fed 3′-methyl-4-dimethylaminoazobenzene for increasing intervals of time occurs at about 4 weeks. This time correlates with the point of the minimum swelling of microsomes and mitochondria isolated from the livers of rats fed this same dye. Thus, a correlation is established between the alterations of the swelling properties of these particulates and the carcinogenic process.

In previous studies (1) swelling was used to detect alterations in the fine structure of rat liver microsomes during feeding of 3′-methyl-4-dimethylaminoazobenzene. It was observed that the decrease of the swelling ability of the microsomes, which can be detected even during the 1st week of feeding of 3′-methyl-4-dimethylaminoazobenzene, reaches a minimum at *4 weeks*. If the feeding of the dye is continued, the swelling ability gradually returns to a nearly normal level at about 20 weeks. The swelling properties of microsomes from hepatomas which are induced by 3′-methyl-4-dimethylaminoazobenzene, resemble those from the liver of rats after the dye has been fed for *4 weeks*. These findings suggested that after 4 weeks a critical period is reached in which a few surviving cells undergo certain irreversible structural alterations and become tumor cells. In contrast to these findings with the carcinogen, no appreciable change in microsomal swelling could be observed when animals were fed the noncarcinogenic isomer, 2-methyl-4-dimethylaminoazobenzene.

It was felt that a correlation between the changes observed in microsomal swelling and the actual appearance of gross tumors would provide an indication of whether these phenomena are

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involved in the carcinogenic process. For this reason the effect on the tumor incidence of feeding 3′-methyl-4-dimethylaminoazobenzene for various intervals has been studied. The evidence presented now supports the hypothesis that feeding this dye beyond the critical 4 weeks period does, in fact, cause the animals to reach a "point of no return," since the curve of tumor incidence as a function of the time of dye-feeding is not a straight line but shows a point of inflexion followed by a sudden steep rise at about 4 weeks.

A second phase of the present investigation was concerned with the problem of whether during chemical carcinogenesis primary cellular alterations that can be detected by the study of swelling are restricted to the endoplasmic reticulum, as was felt previously (1) and suggested later by the observations of Porter and Bruni (2), or whether the alterations occur in other cell particulates as well. Actually, it has been found that the swelling curves of liver mitochondria show essentially the same alterations at 4 weeks during feeding 3′-methyl-4-dimethylaminoazobenzene, as do the microsomes. As with these latter particulates, feeding of the non-carcinogenic 2-methyl-4-dimethylaminoazobenzene did not influence the swelling ability of the mitochondria either. The results imply that the endoplasmic reticulum is not an exclusive first target in the carcinogenic process, and are in agreement with previous reports (3, 4) that there are changes in the swelling properties of mitochondria during carcinogenesis.

In the third part of the present investigations the effect of variables and of various compounds of biological interest on the swelling of mitochondria was studied, with emphasis on the role of sulfhydryl groups.

Materials and Methods

Care and Feeding of Animals:

Sprague-Dawley male rats (Holtzman Rat Co., Madison, Wisconsin) with an initial weight of 180 to 230 gm., housed two in a cage, were fed *ad libitum* a semi-synthetic diet (5) (diet No. 2), called hereafter basal diet, identical to that previously used (1). The hepatic carcinogen 3′-methyl-4-dimethylaminoazobenzene, or the relatively non-carcinogenic 2-methyl-4-dimethylaminoazobenzene were incorporated in this diet to the extent of 0.06 per cent when the effects of these compounds were studied.

For the tumor incidence experiments, 9 groups of 16 to 17 rats per group were fed basal diet for 1 week, after which they were fed the diet containing 3′-methyl-4-dimethylaminoazobenzene for periods varying from 1 to 12 weeks. Feeding of the basal diet was resumed after the respective periods of feeding the diet containing the dye. All animals were sacrificed a total of 7 months after initiation of the experiment. The macroscopically visible nodules on the livers were fixed in Bouin's fixative and examined histopathologically.[1]

The percentage of tumor incidence in each group was calculated as the ratio of the number of tumor-bearing rats to the sum of all survivors plus those animals sacrificed earlier because of rapid tumor development. The rats having histopathologically benign hepatomas, 4 in the 5 weeks group, 3 in the 6 weeks group, and 2 in the 8 weeks group, have been included in these calculations as tumor bearers.

For the swelling experiments the control animals for the azo dye or tumor studies were fed basal diet for at least 2 weeks. The rats fed azo dyes were maintained previously on basal diet for 1 week. The rats used to study the effect of various compounds on mitochondrial swelling, and the corresponding control animals, were fed Purina laboratory chow.

Preparation of the Mitochondrial Fractions:

The rats were sacrificed by decapitation, the livers were perfused through the superior vena cava *in situ* with ice cold isotonic sodium chloride, and were excised. The firm white hepatomas which were used, were carefully dissected to eliminate the adhering liver tissue or any necrotic material. When liver tissues were used after prolonged feeding, all macroscopically visible nodules were eliminated before homogenization. All operations were carried out in the cold room.

The liver or tumor tissues were minced and then homogenized (20 per cent *w./v.* homogenate) in the cold in ice cold 0.44 M sucrose in a Potter-Elvehjem glass homogenizer using a teflon pestle. This sucrose solution also contained 0.001 M ethylenediaminetetraacetate (EDTA). Nuclei and cell debris were sedimented at 700 *g*, for 10 minutes, in the cold. The mitochondrial fraction, used in these experiments, was isolated from the supernatant fluid by centrifugation for 10 minutes at 13,000 *g* (2–3 °C.), according to Siekevitz and Watson (6). The resulting pellet was washed twice by resuspension and homogenization. EDTA was present in the 0.44 M sucrose used in the first washing but not in the sucrose used in the second washing. "Fluffy layer," if present, was removed. The final pellet was resuspended in 0.44 M sucrose so that 1 ml. contained the mitochondria from 1 gram of fresh tissue. This standard stock suspension, kept in ice, was always used within 10 minutes after preparation.

To study the effect of the feeding of 3′-methyl-4-

[1] The authors wish to thank Dr. Victor M. Arean and Dr. Joseph Simon for the histopathological examinations.

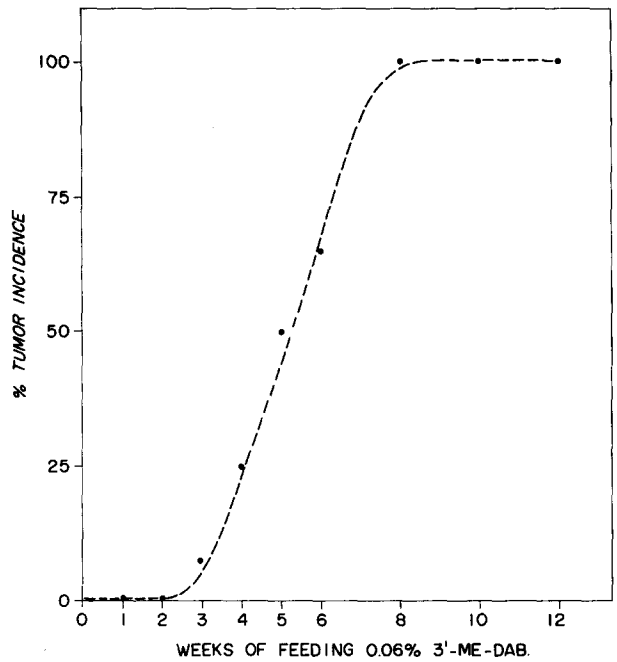

FIG. 1. Hepatic tumor incidence in 9 groups of rats 16 animals each (except 17 for 6 weeks), as a function of the time of feeding a semi-synthetic diet (1, 5) containing 0.06 per cent of 3′-methyl-4-dimethylaminoazobenzene. In all cases the tumor incidence was determined after 7 months.

dimethylaminoazobenzene and of the 2-methyl isomer on the amount of mitochondria present in the tissue, 10 gram samples of liver were used. The final mitochondrial suspensions were precipitated with trichloroacetic acid, washed as already described (1), dried, and weighed. These determinations were carried out in triplicate.

Swelling Tests:

(*a*) *By the Optical Density Method.*—Mitochondrial volume changes were followed at room temperature (~25 °C.) by the optical density method introduced by Cleland (7), as modified by Tapley (8). The basic test system consisted of 4.7 ml. 0.3 M sucrose containing 0.02 M trishydroxy-methyl-amino-methane (Tris) buffer, 0.2 ml. of a stock solution containing thyroxine, calcium chloride, or mercurcic chloride and that volume (0.05 to 0.2 ml.) of mitochondrial standard stock suspension which gave an initial optical density of 0.300. Since the percentage change in optical density during swelling of a given mitochondrial preparation depends to a certain extent on the initial optical density, all studies were made with test solutions with an initial optical density between 0.290 and 0.310. Furthermore, by conducting the assay in this manner, adjustment was automatically made for changes in the concentration of liver mitochondria during feeding of azo dyes. The final concentrations of thyroxine, calcium chloride, and mercuric chloride in the photometer cells were 1×10^{-5} M/l, 5×10^{-3} M/l, and 1×10^{-5} M/l, respectively. Many experiments were also conducted with 0.17 M sucrose which contained the same amount of Tris buffer but was used without added thyroxine, calcium chloride, or mercuric chloride. Here also 5 ml. was the final volume of the test system. The pH of the buffered 0.30 M and of the 0.17 M sucrose solutions was 7.4, except when the pH dependence of mitochondrial swelling was studied. The mitochondrial stock suspension was always added last to the test system. The decrease of optical density was followed at 520 mμ in rectangular absorption cells. The first reading was taken within 10 seconds after addition, and subsequent readings at 5 or 10 minute intervals for 40 minutes. The decrements of optical density were recalculated in "percentage swelling" as previously described (1). All swelling curves were determined in duplicate or triplicate, using mitochondrial fractions of individual rats. The data presented under "Results" in Figs. 2 *a* and *b*, 3 *a*, *b*, and *c*, and in Table I are based on the final state (per cent swelling) at 40 minutes. Since the comparison of the individual swelling curves (showing the rate of swelling) does not give, in this case, different or additional information, the above presentation has been used for the sake of clarity.

The unused portions of the mitochondrial stock suspension of the rats fed 3′-methyl-4-dimethylaminoazobenzene were precipitated with trichloroacetic acid,

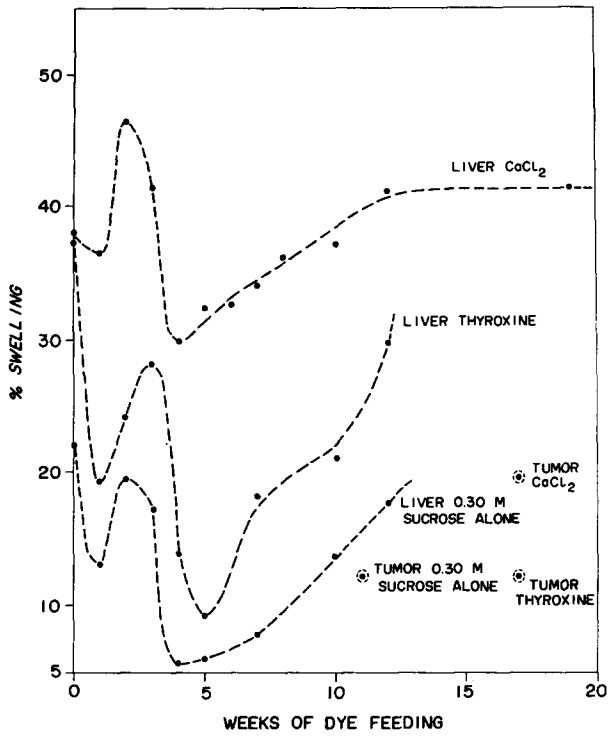

FIG. 2 *a*

FIGS. 2 *a* and *b*. Swelling of rat liver mitochondria in 0.30 M and 0.17 M sucrose containing Tris buffer (pH 7.4) as a function of the time of feeding 0.06 per cent 3′-methyl-4-dimethylaminoazobenzene. The agents used to induce swelling, calcium chloride, thyroxine, and mercuric chloride, were always employed in 0.30 M sucrose, and their respective concentrations were 5×10^{-3} M, 1×10^{-5} M and 1×10^{-5} M. The 0.17 M sucrose was used without added inducer of swelling. Each point of these curves corresponds to the swelling at 40 minutes. Lead nitrate at 1×10^{-5} M was found to produce a 90 per cent inhibition of the swelling of normal rat liver mitochondria, while cupric nitrate gives only a very slight inhibition of swelling even at 1×10^{-2} M.

washed as described (1), and resuspended in 3 per cent trichloroacetic acid for direct observation of the bound dye.

The compounds tested for possible action on swelling were either directly dissolved in the pH 7.4 sucrose buffer solution or added to the test system as small aliquots (0.1 to 0.2 ml.) of a concentrated stock solution adjusted to pH 7.4. The N2H mustard (Merck mustargen) solution was not neutralized to avoid hydrolysis and was the only compound which was added to the test system *after* the mitochondrial stock suspension.

The optical densities were always read against a blank of distilled water, except for tannic acid, bilirubin, and biliverdine, in which the blank consisted of the same complete test system but without added mitochondrial stock suspension.

(*b*) *By Gravimetry.*—The change in the swelling of mitochondria during feeding of 0.06 per cent 3′-methyl-4-dimethylaminoazobenzene was followed also by determination of the percentage dry weight of mitochondrial pellets. The method used was essentially that of Price, Fonnesu, and Davies (9).

The final mitochondrial pellets obtained from 10 gram tissue samples (in tared centrifuge tubes) were resuspended in the same tubes in 0.17 M Tris buffered pH 7.4 sucrose solution, and incubated for 20 minutes at room temperature. The swollen mitochondria were sedimented as usual and the weight of the wet pellets determined after the outer and inner walls of the tubes had been carefully wiped with "celluwipe." The tubes containing the pellets were then dried overnight at 90 °C., placed in desiccator and weighed after cooling. These experiments were carried out in duplicate.

RESULTS

Tumor Incidence as a Function of the Time of Feeding 3′-Methyl-4-Dimethylaminoazobenzene:

Fig. 1 shows that a sudden steep rise in the hepatic tumor incidence in groups of rats fed under our experimental conditions 3′-methyl-4-

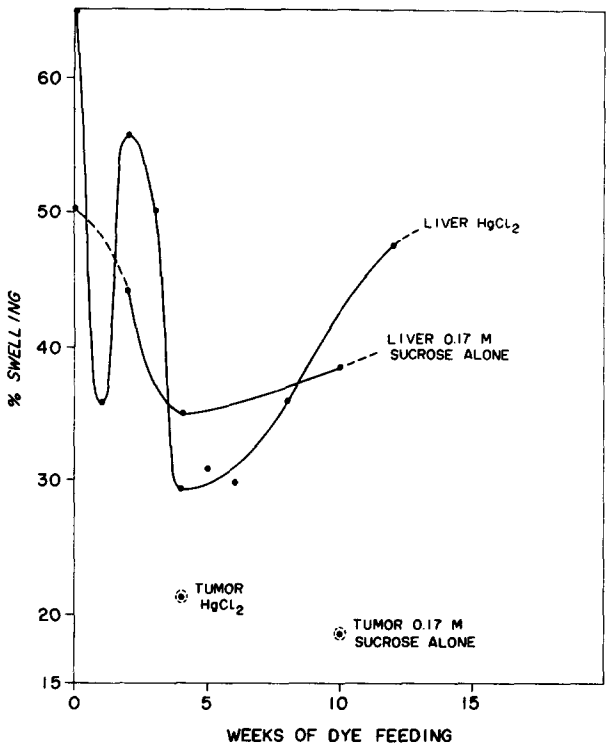

FIG. 2 *b*

dimethylaminoazobenzene for increasing time intervals, occurs at about 4 weeks. The ratios of the number of tumor-bearing rats over the effective totals have been given for most groups in a preliminary report (10). The curve is linear between 4 and 7 weeks and a plateau is reached at about 8 weeks. The curve shows that if the feeding of the dye is continued beyond 4 weeks, the animals rapidly reach a point of no return. The critical time coincides with the time of minimal microsomal and mitochondrial swelling.

The non-linearity of the curve near the origin is not surprising since several authors have already shown (11–13), that in order to induce liver tumors in rats with various agents, the carcinogen must be administered for a certain critical period, after which tumors may appear without further treatment. This period may vary with the experimental conditions and the nature of the carcinogen. It has been shown for one particular case that the end of this critical period coincides with minimum cellular levels of protein, pentosenucleic acid, and riboflavin (14), and with a sudden rapid cell proliferation (13).

The sudden sharp rise of the tumor incidence shown in Fig. 1 suggests that once beyond the critical period, certain irreversible fine-structural alterations may become rapidly established in some liver cells by further feeding of the carcinogen. This aspect of the malignant transformation recalls the breakdown in the cell of the steric conformation of highly organized macromolecular regions, which is akin to a first order phase-transition of oriented polymer molecules (15, 16) in the sense described in detail by Flory (15). This phenomenon can be compared to the partial breakdown of simple crystal lattices; *e.g.*, during thawing or irradiation. Following statistical thermodynamics the entropy of a system is proportional to the number of possible conformations of its constituting units. Thus, the loss of preferential structural orientations, that is, from ordered toward random alterations of highly organized macromolecular regions, corresponds to the increase of the entropy of the cell considered as a closed system. Similar conclusions were reached earlier by Ambrose (17) on the basis of the comparison of histological patterns, and by Rondoni (reviewed in reference 18) who, on correlating various experimental data, observed similarities between the process of carcinogenesis and protein denaturation.

Mitochondrial Swelling during Feeding of Aminoazo Dyes:

Figs. 2 *a* and *b* show the changes in the swelling ability of rat liver mitochondria (determined by the optical density method), as a function of the time of feeding 3′-methyl-4-dimethylaminoazobenzene. These figures complete the data given in a preliminary report (10). As with the microsomes (1), all of these curves show a minimum level at 4 weeks (except for thyroxine at 5 weeks) followed by a phase of apparent recovery. The swelling of mitochondria from hepatoma is generally comparable to or lower than the swelling of these same particulates from liver at the minimum point of the curve, in the same experimental conditions. Thus, the mitochondria from hepatoma seem to be very resistant to swelling (*cf.* 3, 4).

The change in the swelling ability of the mitochondria as a result of feeding this azo dye was similar when determined by the percentage dry weight of the mitochondrial pellet. The swelling after 20 minutes in 0.17 M sucrose containing Tris buffer (pH 7.4) was measured by this method on mitochondria isolated from the livers of normal rats, from rats fed 0.06 per cent 3′-methyl-4-dimethylaminoazobenzene for 2, 4, 6, 8 and 10 weeks, and from hepatoma. As with the optical density method, there was a minimum in the swelling at 4 weeks, which was then followed by a recovery phase. Very little swelling was observed with the mitochondria from hepatoma.

As with the microsomes (1), feeding at the same level of the relatively non-carcinogenic 2-methyl-4-dimethylaminoazobenzene did not seem to influence noticeably mitochondrial swelling even after 6 weeks, as shown in Table I.

Thus, mitochondrial swelling shows essentially the same alterations during azo-dye carcinogenesis as does microsomal swelling.

The only noticeable feature of the mitochondrial swelling curves, which could not be observed with the microsomes (1), is that there is an early recovery phase of the swelling with a maximum at 2 weeks. It may not be excluded that there may be a relation between this early recovery phase of swelling and the time of maximum dye-binding at about 2 weeks in these particles. That the protein-bound azo dyes first observed by Miller and Miller (19) are distributed all over the cell (20–22), is now well established. Quantitative studies have shown already that the maximum binding of 3′-methyl-4-dimethylaminoazobenzene by microsomes occurs between 2 and 3 weeks (1), as with the total liver (19). Although these quantitative studies (1) could not be extended yet to the mitochondria, observation of the trichloroacetic precipitates of the mitochondrial fractions in 3 per cent trichloroacetic acid (see Swelling Tests under Materials and Methods) clearly shows that as with microsomes the maximum level of dye binding to mitochondria occurs between 2 and 3 weeks. This early recovery phase of the swelling at 2 weeks suggests a response of the mitochondrial fine-structure, compensating for the loss at 1 week of the initial swelling ability of these particulates. In contrast, the *late* recovery of the swelling that takes place beyond the critical 4 weeks is likely to be of a different nature. In fact, this *late* recovery of the swelling coincides in time with the large and rapid decrease of the bound 3′-methyl-4-dimethylaminoazobenzene, in spite of the continuous feeding of this carcinogen. In this connection, the existence in the early phase of azo dye feeding of other compensatory responses in the soluble cytoplasmic proteins may be suggested by the work of Sorof *et al.* (23).

TABLE I

Swelling of Liver Mitochondria of Rats Fed 0.06 Per Cent 2-Methyl-4-Dimethylaminoazobenzene

Same experimental conditions as in legend of Figs. 2 *a* and *b*.

	Normal	2 weeks	4 weeks	6 weeks
5×10^{-3} M $CaCl_2$	40.8	36.7	42.8	36.3
1×10^{-5} M $HgCl_2$	65.1	65.5	62.4	63.8

The pH Dependence of Mitochondrial Swelling:

The important differences between the macromolecular organization of liver mitochondria and of hepatoma mitochondria are indicated not only by changes in the extent of swelling, but also by drastic alteration of the pH dependence curves when passing from the liver to the hepatoma (Figs. 3 *a*, *b*, and *c*). Significant differences were also found previously with the microsomes (1).

All curves obtained with the liver mitochondria have relatively sharp maxima at pH = 7.5–8.0 (*cf.* 24, 25), while the mitochondria from hepatoma showed always much less swelling and gave rather flat pH dependence curves with no clear cut maxima. The pH dependence curve with calcium chloride of liver mitochondria shows a sharp minimum at pH = 9.0, which could not be seen in any other case. A difference can be observed

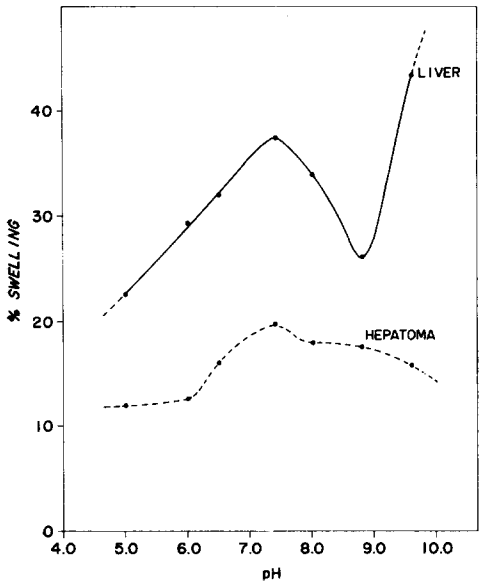

FIG. 3 *a*

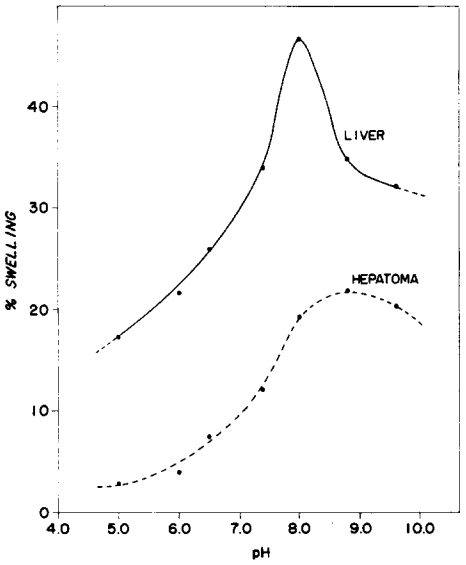

FIG. 3 *b*

between the positions of the maxima of the liver (pH = 8.0) and of the hepatoma (pH = 8.8) when thyroxine is used as inducer.

Influence of the Feeding of Azo Dyes on the Amount of Mitochondria in the Liver:

Although Price, Miller, and Miller (20) have shown that there is a significant decrease in the mass of mitochondria which can be isolated from the livers of rats fed various carcinogenic azo dyes

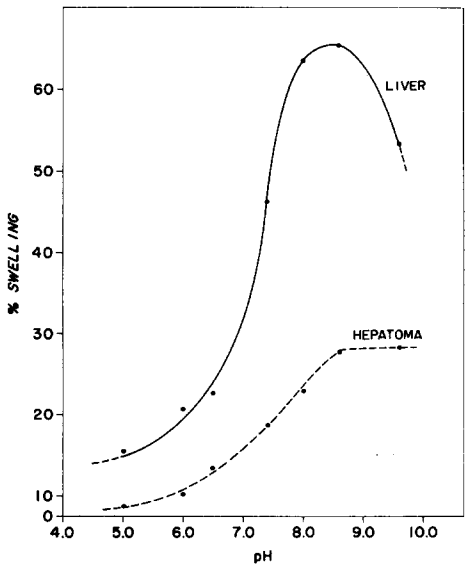

FIG. 3 *c*

FIGS. 3 *a*, *b*, and *c*. Effect of pH on the swelling of mitochondria from liver and hepatoma of the rat. Fig. 3 *a* in 0.30 M sucrose containing Tris buffer and 5×10^{-3} M $CaCl_2$; Fig. 3 *b*, the same sucrose, but with 1×10^{-5} M thyroxine; Fig. 3 *c* in 0.17 M sucrose containing Tris buffer, without added inducer. All points on these curves correspond to the swelling at 40 minutes.

for 4 to 5 weeks, a study of the time course of this reduction has not been made. Moreover Schneider (26) and Schneider and Hogeboom (27) found that the mass of mitochondrial material which can be isolated from a hepatoma is much less than that isolated from the normal liver. In contrast to these findings, feeding of the relatively non-carcinogenic 2-methyl-4-dimethylaminoazobenzene causes a large increase in the amount of mitochondria in the liver (25). These variations probably correspond to changes in the number of mitochondria per cell (28).

The data presented in Fig. 4 constitute further confirmation of the previous observations (20, 28) and indicate that the diminution of the amount of mitochondria reaches a plateau after about 4 to 5 weeks. The results suggest that the recovery of the swelling of liver mitochondria after 4 weeks does not depend upon a return to normal of the *amount* of material in this fraction. On the other hand, feeding the same level of 2-methyl-4-dimethylaminoazobenzene, produced a 67 per cent increase in the dry trichloroacetic precipitate after 2 weeks and 86 per cent after 6 weeks, in agreement with previous results (20).

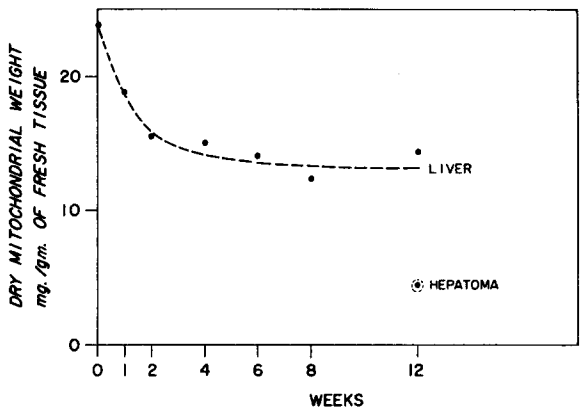

FIG. 4. Variation of the dry weight of the trichloroacetic acid precipitated mitochondrial fraction as a function of the time of feeding 0.06 per cent 3′-methyl-4-dimethylaminoazobenzene.

Influence of Various Compounds on Mitochondrial Swelling:

Keto Compounds.—Carbonyl groups of various organic compounds are known to interact with proteins. For example, the diabetogenic action of alloxan (29) and glyoxal (30) may well result from the irreversible blocking of critical sulfhydryl groups of the beta cells (31).

Consequently, we felt that the study of the effect of carbonyl compounds on mitochondrial swelling may shed some light on the steric correlation or the relative distance of the sulfhydryl groups which seem to play an important role in the permeability of the mitochondrial membrane (8).

Fig. 5 shows that at least two adjacent carbonyl groups are required for the inhibition of mitochondrial swelling, since at the same molar concentration only alloxan and diacetyl are active, while acetone, chloroacetone, acetylacetone, and acetonylacetone are inactive.

Tapley reported (8) that another sulfhydryl reagent, iodoacetamide, at 1×10^{-2} M is as potent an enhancer of swelling as mercuric chloride at 1×10^{-5} M. However, in our experiments, fluoroacetamide or sodium fluoride at various concentrations from 1×10^{-5} M to 1×10^{-2} M had no effect on mitochondrial swelling.

The effect of some of these compounds on the swelling of the mitochondria of livers from rats fed 3′-methyl-4-dimethylaminoazobenzene was also studied. It was found that the inhibition of swelling caused by diacetyl is abolished when 0.06 per cent 3′-methyl-4-dimethylaminoazobenzene is fed for 10 weeks, but not when it is fed for only 2 or 4 weeks. On the other hand, the absence of inhibition by acetylacetone and acetonylacetone is not affected by feeding this dye for the same period of time.

Sulfhydryl Compounds.—Fig. 6 shows the effect of three sulfhydryl compounds on mitochondrial swelling. The observation that reduced glutathione at 1×10^{-2} M causes considerable enhancement of swelling confirms the recent data of Lehninger and Schneider (32). Freshly prepared sodium thiosulfate or sodium hydrosulfite, however, did not affect swelling at 1×10^{-2} M or 1×10^{-3} M. Another sulfhydryl agent, the 2, 3-dimercaptopropanol (BAL) caused nearly total inhibition at 1×10^{-2} M.

The slight enhancement of swelling caused by α-lipoic acid (6,8-dithio-octanoic acid) cannot be attributed to the surface active properties of this compound, since the unsubstituted n-octanoic acid causes total inhibition of swelling at the same concentration. This finding on the effect of α-lipoic acid is consistent with the observation of Biesele (33) that mouse fibroblasts treated with this same agent in tissue culture show somewhat enlarged mitochondria.

The effect on mitochondrial swelling of a large number of compounds of other classes has also been tested, such as: cross-linking and surface active agents, narcotics, antibiotics, substituted anilines and phenols, purine analogs, and derivatives; moreover, diphosphopyridinenucleotide (DPN), histamine, bilirubin, biliverdine, heparin, and alginic acid. These swelling tests have also been carried out in the 0.17 M sucrose assay system. The whole spectrum of the possible effects

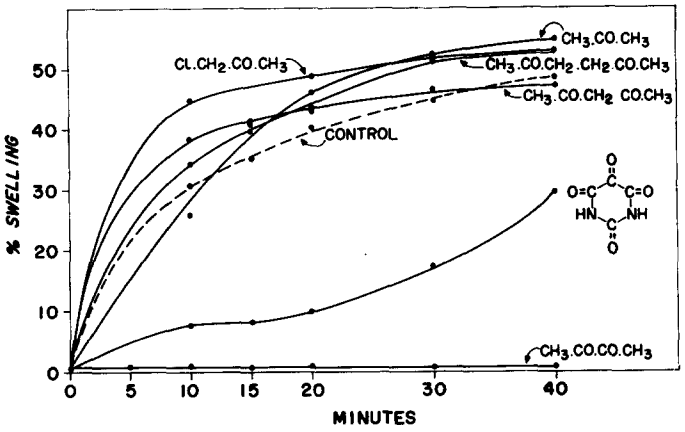

FIG. 5. Effect of keto compounds on the swelling of rat liver mitochondria in 0.17 M sucrose, containing Tris buffer, pH 7.4. All compounds were at 1×10^{-2} M. Diacetyl, acetylacetone, acetonylacetone, and chloroacetone were freshly tri-distilled samples. At 1×10^{-3} M, alloxan caused 45 per cent enhancement of swelling.

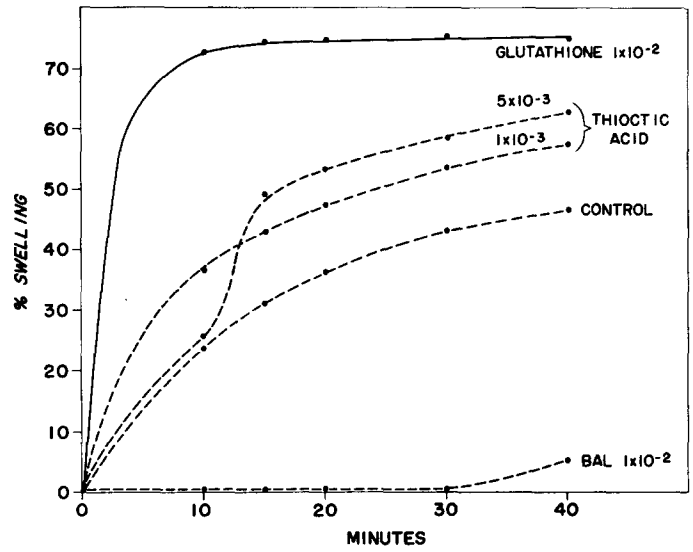

FIG. 6. Effect of sulfhydryl compounds on the swelling of rat liver mitochondra, conditions as in Fig. 5. 2,3-Dimercaptopropanol had no effect on swelling at 1×10^{-3} and 1×10^{-4} M, and glutathione at 1×10^{-3} M.

on mitochondrial swelling has been observed in these tests. The only consistent pattern of the mechanism of action that emerges from these tests is that the cross-linking agents, the surface active agents at lower concentrations, and the substituted anilines having an electronegative group in para position inhibit mitochondrial swelling, possibly through two- or multipoint attachment such as in the model proposed for *p*-aminobenzoic acid (34). It is also noteworthy that under our experimental conditions 5×10^{-3} M DPN caused considerable enhancement of swelling and biliverdine gave strong inhibition at 5×10^{-4} M, while the presence of the same concentration of bilirubin resulted in a marked aggregation of the mitochondria.

The fact that various compounds can affect the swelling so differently suggests that it is not the mere presence of specific functional groups that are the determining factors, but rather the general structural pattern of the whole molecule. This, in turn, may indicate that the various structurally

unrelated chemical agents that affect mitochondrial swelling, act in the membrane structure possibly on different macromolecular sites (*cf.* reference 24).

DISCUSSION

Evidence has been available to the effect that there are changes of the macromolecular organization of rat liver mitochondria during chemical carcinogenesis. Clerici and Cudkovicz (3) have observed by means of electron microscopy an increase of rigidity as a consequence of feeding 4-dimethylaminoazobenzene. Emmelot and Bos reported that the swelling of rat liver mitochondria, induced by thyroxine, decreases after this same dye has been fed for 5 months (4), or by incubating normal mitochondria *in vitro* with the carcinogens 4-monomethylaminoazobenzene or *o*-aminoazotoluene (35). However, no stepwise time study for the successive changes has been reported, such as described for the microsomes (1).

The present work establishes that these modifications of the mitochondrial swelling occur *simultaneously* with similar alterations undergone by the microsomes. The alterations that can be detected by the study of swelling in the microsomal and mitochondrial fine structure seem to be directly involved in the carcinogenic process since (*a*) these alterations cannot be produced in either particles by feeding a non-carcinogenic azo dye of similar structure[2] and (*b*) there is a close correlation between the minimal feeding period required for the appearance of gross tumors and the time of minimum swelling in these two types of particles.

The present results suggest that the carcinogenic process does not start, as was felt previously (1), within a single type of cellular component but rather occurs at more than one subcellular locus. The result of the structural alterations of the subcellular particles may well be that the rates of flow of nucleotides, various organic cofactors, and inorganic ions among the cell components (36) are modified, thereby introducing disturbances in various feedback mechanisms which may possibly control the rhythm of cell division (37).

One of the simplest explanations of the higher structural rigidity of the tumor mitochondria as compared to that of the liver mitochondria is that the membrane of the former has a more extensively cross-linked lipoprotein structure. Since disulfide bonds are undoubtedly the most common covalent bonds cross-linking the polypeptide chains in proteins (*e.g.* 38), it is reasonable to suppose that one of the structural bases of greater membrane rigidity may be the increase of the cystine content of these organelles during carcinogenesis. *In fact,* it has been reported (39) that mitochondria of the rat hepatoma contain 44 per cent more cystine than the mitochondria of the rat liver.

Although the writers feel that the above explanation constitutes the most likely mechanism of the observed increase in structural rigidity, the possible role of other factors may not be disregarded, such as *e.g.*: (*a*) change in the character of liver cell-population because of extensive bile-duct proliferation in the early periods of feeding (40); (*b*) the possible decrease in the availability of oxidizable substrate necessary for mitochondrial swelling (*e.g.* reference 41); and (*c*) depression of the level of DPN (42) possibly influencing the state of oxygenation of the respiratory carrier. It should be added, however, that the phenomena (*b*) and (*c*) may be, in fact, the consequences of structural alteration of the mitochondrial membrane, respectively through decreasing the steric availability of substrate at critical sites, and by altering the protein carrier to which DPN is bound. Views similar to the latter were expressed earlier by Greenstein in relation to the low B vitamin levels in tumor tissues (43).

In conclusion the following mechanism is tentatively suggested for the action of azo dyes at the level of the mitochondrial membrane:

The azo carcinogens act at this level by inhibiting swelling (*cf.* 3, 4, 35) possibly by cross-linking macromolecular elements that can undergo reversible structural changes and, thus, condition the dynamic behavior of the mitochondrial membrane. That, in the conjugated aromatic carcinogens, the 4 and 4′ (or equivalent) positions are the points of interaction, has been suggested first under the term of "para principle" by Druckrey (44) and later by Buu-Hoi (45), and extensively discussed on the basis of physicochemical considerations by one of us (46). An important evidence that can be cited in favor of this concept is the observation of Cheesman (47) that the elasticity of monomolecular layers of globin (as measured by the pressure-area relationship) is strongly depressed by derivatives of the carcinogenic

[2] Preliminary results of experiments conducted in our Laboratory with several other agents: two carcinogenic and two non-carcinogenic azo dyes, 2-acetylaminofluorene, ethionine and tannic acid, are consistent with the findings described in the present report.

4-dimethylaminostilbene, a close structural analog of the azo dyes. The considerable enhancement of the carcinogenic activity of 4-dimethylaminoazobenzene by introduction in 4′ of certain substituents (such as —F, $—C_2H_5$, or $\equiv N \rightarrow O$ as pyridine *N*-oxide in the ring), which can establish interactions by the various types of secondary valence forces, constitutes another supporting evidence.[3]

The structural rigidity of the mitochondrial membrane, which is maintained in the early periods of feeding by the continuous presence of the cross-linking carcinogens, may become, beyond a critical period of administration, an irreversible characteristic of a few cells. Once established in the form of a new macromolecular pattern, this new property may be transmitted to subsequent generations of cells. Such observed continuity of an acquired extra nuclear property does not presuppose or imply, however, any specific mechanism of transmission of cellular characteristics.

Such drastic alterations in the membrane structure cannot fail to affect the enzymes of the respiratory chain for which it serves as an "embedding core" (48). Thus, by modification of the distribution of the enzymic sites (*cf.* 43) new or alternative metabolic pathways may be established or favored, channeling substrates and electrons in new directions. It is possible that the aerobic glycolysis coexisting with the high rate of respiration in the tumor tissue is the functional expression of these specific structural alterations of the mitochondrial membrane, in conformity with the views voiced years ago by Rondoni (reviewed in reference 18).

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[3] Recent results in our Laboratory have shown that by introducing an ethyl-group in the 4′ position, the inactive azo dyes, 2-methyl-4-dimethylaminoazobenzene and 4-di*ethyl*aminoazobenzene, are transformed to potent hepatic carcinogens (unpublished). Similar observation was made with the latter compound when an $\equiv N \rightarrow O$ group was introduced in 4′ as pyridine *N*-oxide (E. V. Brown, personal communication).

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