Effect of Feeding Amino Azo Dyes on Mitochondrial Swelling and Contraction. Kinetic Evidence for Deletion of Membrane Regulatory Sites

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SUMMARY

In previous investigations on the Ca++, Hg++, and hypotonically-induced swelling of rat liver mitochondria while feeding 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB), a sharp minimum has been observed at 4 weeks; this 4-week minimum corresponds to the onset of irreversibility of tumor induction under the conditions of administration. The present work establishes that large minima at 4 weeks also occur when glutathione, phlorizin, phosphate, or arsenate are used, which provides support that the occurrence of the minimum at 4 weeks is not related to the nature of the swelling-inducer agent. Also at 4 weeks, impressively large minima are seen in the extent of ATP-produced absorbancy rise in 0.30 M sucrose at pH 4.0, following swelling at pH 7.4 prior to ATP addition. ATP is known to bind optimally to the mitochondrial "structural protein" at pH 4.0. On the other hand, in 0.125 M KCl (pH 7.2) test medium, the 4-week minimum of ATP-produced "contraction" is small or absent. In the latter system the extent of ATP-produced "contraction" gradually decreases beginning at 6-7 weeks and approaches zero in 3'-Me-DAB hepatoma mitochondria.

In kinetic studies the sensitivity of the mitochondrial swelling response has now been defined as the ratio of the maximum swelling velocity to the inducer concentration bringing about half-maximum velocity. This ratio gradually decreases during feeding of 3'-Me-DAB and reaches low levels or zero with mitochondria from 3'-Me-DAB hepatoma (irrespective of the nature of the swelling inducer), indicating the deletion of swelling-inducer receptor sites in the mitochondria during 3'-Me-DAB carcinogenesis. Administration of the comparatively inactive 2-methyl-4-dimethylaminoazobenzene for a 4-week period brings about ratio increases or decreases of various magnitudes depending upon the inducer used.

The level of 0.6 M KCl-extractable mitochondrial protein (hitherto regarded as the contractile entity of the membrane) was unaffected by administration of 3'-Me-DAB or 2-methyl-4-dimethylaminoazobenzene for 10 weeks. A fourfold increase was found in 3'-Me-DAB hepatoma mitochondria, which are comparatively nonresponsive in all swelling and contraction assay systems.

INTRODUCTION

Several investigations have demonstrated the drastic decrease in the ability of liver mitochondria to swell as a result of feeding the hepatic carcinogens DAB or o-aminoazotoluene for continuous periods of time (14, 16, 17). Strong impairment of the swelling ability has also been reported with mitochondria from azo dye-induced hepatomas or from transplanted solid tumors (16, 17, 34, 35). In studies on the entire time-course of subcellular alterations during 3'-Me-DAB carcinogenesis, Arcos and coworkers found a large decrease in swelling at 4 weeks with both microsomes (1) and mitochondrion (4); microsomes and mitochondria from 3'-Me-DAB induced hepatomas show low values of swelling, comparable to those of the liver-cell particles at the 4-week minimum. Inactive azo compounds do not bring about the typical swelling changes seen with 3'-Me-DAB (3, 4). The existence of the 4-week minimum with 3'-Me-DAB was confirmed by Yamamoto et al. (53). A tumor-incidence study established that the 4-week minimum correlates with the onset of irreversibility in 3'-Me-DAB carcinogenesis (4). It has been established (2, 5, 19) that certain types of mitochondrial swelling follow the kinetics of drug-receptor complex formation. A preliminary study (19) has shown that the feeding of 3'-Me-DAB for a single 4-week period produces a displacement of the Line

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of rat liver mitochondria. The influence of 3′-Me-DAB administration on the ATP-induced “contraction” of mitochondria was explored. The sensitivity of the mitochondrial swelling response was defined as a combination of kinetic parameters, and the variation of this response was followed during the time-course of administering 3′-Me-DAB and 2-Me-DAB. The level of the 0.6 M KCl-extractable mitochondrial protein was determined during the time-course of administration of the two dyes.

MATERIALS AND METHODS

Care and Feeding of Animals. Sprague-Dawley male rats (Holtzman Co., Madison, Wis.) weighing 180–230 gm at the beginning of the experiments and housed two in a cage were used. Control animals were fed, for 8 to 10 days before sacrifice, a semisynthetic diet previously used (1, 4). Experiments received, for varying lengths of time, 3′-Me-DAB or 2-Me-DAB incorporated into the same diet at the level of 0.06 percent. Food and water were ad libitum.

Preparation of Mitochondrial Fractions. The rats were killed by decapitation. When liver tissues were used after prolonged feeding of 3′-Me-DAB, all macroscopically visible nodules were eliminated before homogenization. The firm white 3′-Me-DAB-induced hepatomas which were used were rapidly dissected to eliminate the adhering liver tissue and any necrotic material. During dissection, pieces of tumor tissue for homogenization were collected in ice-cold sucrose medium. In the experiments with liver mitochondria, the tissues of single animals were used; in each experiment with tumor mitochondria, tumor tissues originating from 2 to 4 animals were pooled. All operations were carried out in the cold.

For the sake of comparability with earlier studies, the isolation of liver and tumor mitochondria in 0.44 M sucrose in the presence of EDTA exactly followed the procedure previously described (4).

Extent of Swelling and Contraction. Mitochondrial volume changes were studied at 520 mμ (23–25°C) in a Coleman spectrophotometer using 7-mI capacity matched plane-parallel cells. The terminal extent of mitochondrial swelling induced by various agents was measured, as previously described (4), by following the decrease of absorbancy up to 40 min in a 0.30 M sucrose medium buffered with 0.02 M Tris (pH 7.4), using standard amounts of mitochondria.

In the studies on ATP-induced reversal of swelling, mitochondrial “contraction” was induced after the swelling had preceded for 40 min, at which time absorbancy had become practically stationary. In these studies the swelling prior to “contraction” was induced by the agents (and at the concentrations) indicated in Charts 2a–f, in 0.30 M sucrose or 0.125 M KCl, both buffered with 0.02 M Tris (pH 7.4); the volume of these systems was 5.0 ml during the swelling phase of the assay. Immediately after the 40-min absorbancy reading, “contraction” was induced by adding to each test system a 0.10 ml aliquot of an ATP + MgCl₂ solution mixture of such concentrations as to bring the levels of these compounds in the test systems to 5 X 10⁻³ M and 3 X 10⁻³ M respectively; the ATP + MgCl₂ solution that was added to the KCl systems also contained 2 mg bovine serum albumin per aliquot. The pH of this ATP + MgCl₂ + bovine serum albumin solution was adjusted to pH 7.2 when added to the KCl test systems. The pH of the ATP + MgCl₂ solution that was added to the sucrose systems was not adjusted so as to result in a final pH of 3.9–4.3 in the test systems. “Contraction” of mitochondria by ATP is inhibited by sucrose at neutrality (27). Low pH conditions similar to the above were used previously to study the “contraction” of heart sarcomeres in sucrose-containing media (2), the “contraction” of liver mitochondria following triamcinolone-induced swelling (18), and the swelling of rat liver mitochondria in various conditions of osmolarity, composition of the medium, and pH (12).

During “contraction,” increase of absorbancy was followed at 5-min intervals for up to 20 min; the absorbancy values were corrected for the dilution involved in adding the aliquot of ATP + cofactor(s) solution. “Contraction” was expressed as percent of the change of absorbancy between 40 and 60 min relative to the change of absorbancy between 0 and 40 min. The experiments on the effect of 3′-Me-DAB feeding on terminal extent of swelling and on reversal of swelling (Charts 1 and 2) were carried out in quintuplicate.

Kinetics of Swelling. These studies were all carried out in the Tris-buffered 0.125 M KCl medium (pH 7.4) with the exception of the determinations with thyroxin, which were performed in the Tris-buffered 0.30 M sucrose (pH 7.4), because reproducibility with this inducer is very poor in the KCl medium (5). In these studies absorbancy was recorded at 1-min intervals for up to 10 min. The greatest absorbancy change occurring in any 1-min interval was taken as the “initial

![Chart 1. Swelling of rat liver mitochondria in 0.30 M sucrose containing 0.02 M tris-(hydroxymethyl)-aminomethane (pH 7.4) as a function of the time of feeding 0.06 percent 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) in a semisynthetic diet. Hepatomas were induced with 3′-Me-DAB. The agents used to induce swelling and their concentrations are given in the chart. Mitochondrial volume changes were measured by determining the percent decrease of absorbancy (at 520 mμ; 23–25°C) at 40 min. Each point plotted is the mean value of 5 determinations. The probabilities for the true differences between the 0- and 4-week values, and between the 4-week and 7-week values are 0.01 > P > 0.001 or P < 0.001.](attachment:chart1.png)
Charts 2a–f. ATP-induced "contraction" of swollen rat liver mitochondria in two different media as a function of the time of feeding 0.06 percent 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) in a semisynthetic diet. In the first phase of this assay, swelling was induced by the agents and at the concentrations indicated in the charts, in 0.125 M KCl and in 0.30 M sucrose both buffered with 0.02 M tris-(hydroxymethyl)-aminomethane (pH 7.4), and measured by following the decrease of absorbancy at 520 mÅ (23–25°C). After swelling had proceeded for 40 min, mitochondrial "contraction" was induced by adding a small aliquot of ATP + Mg++ so as to establish respective levels of 5 x 10^-3 M and 3 x 10^-3 M in the assay systems; the ATP + Mg++ solution added to the KCl systems also contained bovine serum albumin at the level of 2 mg per aliquot. The ATP + Mg++ + bovine serum albumin solution added to the KCl systems was adjusted to pH 7.2. On the other hand, the pH of the ATP + Mg++ solution added to the sucrose systems was not adjusted to neutrality (so as to result in a final pH of 3.9–4.3 in the test systems). Increase of absorbancy during reversal was followed up to 20 min and expressed as percent change relative to the absorbancy decrease which occurred during the swelling phase of the assay. In the sucrose-phosphate and sucrose-arsenate systems at 4 weeks and in the KCl-digitonin system with hepatoma mitochondria, the addition of ATP + cofactor(s) resulted in further swelling rather than "contraction"; this is expressed here as "negative contraction." All points on these charts represent the mean values of 4 to 5 determinations each. The sharp decreases, at 4 weeks, in the sucrose system are statistically significant. With thyroxin, 4-chloromercuribenzoate, phosphate, arsenate, and phlorizin, the probability for true differences between 0 and 4 weeks and between 4 and 5 weeks is 0.05 > P > 0.02 or better, except between 0 and 4 weeks for phosphate and arsenate for which 0.10 > P > 0.05. With digitonin, the differences between 0 and 4 weeks and between 0 and 7 weeks P < 0.001. The increase at 2 weeks, relative to 0 week, gave P = 0.05 for 4-chloromercuribenzoate and 0.10 > P > 0.05 for phlorizin; with the other inducers the probabilities were 0.20 > P > 0.10. In the KCl systems and with all inducers the probability for the true difference between control and hepatoma mitochondria is P < 0.001.
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rate" for the particular inducer concentration (cf. 5); however, the lag period, if any, was generally short and overwhelm-
ingly the highest rates were observed in the 2nd or 3rd min. Sufficient amount of mitochondrial suspension was prepared in each experiment so that the rates, for the whole range of inducer concentrations for any given kinetic curve, were ob-
tained with the mitochondria from the same homogenate. The ranges of concentrations required for establishing the kinetic curves, and the number [in brackets] of determinations, within these ranges, used for exploring the concentration-dependence of the rate, were as follows: phlorizin 5 X 10^-4 to 2 X 10^-2 M [12]; hydrocortisone 8 X 10^-4 to 1.2 X 10^-2 M [11]; phosphate 2 X 10^-3 to 6 X 10^-2 M [11]; PCMB 1 X 10^-5 to 1 X 10^-3 M [12]; Ca^++ 5 X 10^-6 to 1 X 10^-3 M [13]; deoxycholate 5 X 10^-6 to 1 X 10^-3 M [11]; thyrroxin 5 X 10^-6 to 1 X 10^-4 M [11]; pentachlorophenol 1 X 10^-5 to 2 X 10^-3 M [11]; Hg^++ 1 X 10^-6 to 1 X 10^-4 M [13]. (With phlorizin and phosphate, higher concentrations are required for swelling in the KC1 medium than in the sucrose medium; compare the respective ranges to Chart 1). The rates were plotted against the concentrations of the respective inducer, individually for each mitochondrial preparation, for the evaluation of the maximum rate attainable and of the inducer concentration bringing about the half-maximum velocity. The sensitivity of the mitochondrial swelling response was calculated as the ratio of the maximum swelling velocity and of the half-maximum inducer concentration. The kinetic results represent the mean values of 4 to 6 experiments.

Yield of 0.6 M KC1-extractable Mitochondrial Protein. For these determinations the mitochondria were isolated from 15 percent liver homogenates made up with 0.25 M sucrose con-
taining 0.002 M EDTA (pH 7.4). The mitochondria were washed, once in the same sucrose + EDTA solution and a second time in 0.25 M sucrose alone. The mitochondria were then submitted to osmotic rupture by suspension in 0.01 M Tris (pH 7.2). A small aliquot of this suspension was taken for the determination of the total protein content of the mito-
ochondria by Folin colorimetry following Lowry et al. (31). The osmotically ruptured mitochondria were sedimented at 15,000 X g for 12 min and washed once more in the 0.01 M Tris buffer. The particulate sediment was suspended in 0.1 M KC1 and the suspension, packed in ice, was sonicated for 1.5 min (Raytheon sonic oscillator, 200 watts). The KC1 concen-
tration of the suspension was raised to 0.6 M and the prepara-
tion was incubated at 1—3°C for 20 hr. Insoluble material was sedimented at 30,000 X g for 30 min and the extracted protein recovered from the supernatant fluid by tenfold dilution. The precipitated protein was redissolved in 0.6 M KC1 for the determination of the amount of extracted protein, following Lowry et al. (31). The percent yield of this 0.6 M KC1-extract-
able protein was calculated relative to the total mitochondrial protein. These experiments were carried out in quadruplicate.

RESULTS

Terminal Extent of Swelling and “Contraction” of Mitochon-
dria during Feeding of 3'-Me-DAB. In previous studies (4) the 4-week decrease of the terminal extent of mitochondrial swelling (percentage 40-min absorbancy decrease) during feeding 0.06% 3'-Me-DAB in a semisynthetic diet has been ob-
erved with thyroxin, Ca^{++}, Hg^{++} (in 0.30 M sucrose), and hypotonicity (0.17 M sucrose alone) as inducers of swelling. Chart 1 shows that under identical dietary conditions, using the same 0.30 M sucrose test system, the 4-week minimum is also seen with glutathione 5 X 10^-3 M, phlorizin, or arsenate both at 5 X 10^-4 M, and phosphate 4 X 10^-4 M. With these inducers the swelling of 3'-Me-DAB-induced hepatoma mitochon-
dria was very low in comparison with normal liver mitochon-
dria, which was also noted with thyroxin, Ca^{++}, Hg^{++}, and hypotonicity (4). The previously observed (4) pronounced swelling maximum at 2 weeks (which coincides with the time of maximum binding of 3'-Me-DAB in the mitochondria), preced-
ing the 4-week minimum, was not observed with the present inducers, except for the small peak at 2 weeks with glutathione. The 4-week minimum is statistically significant with all four inducers with respect to both the control and the 7-week populations (0.01 > P > 0.001 or P < 0.001). Thus, the occurrence of the 4-week decrease of the terminal extent of mitochondrial swelling during feeding 3'-Me-DAB under the standard dietary conditions appears to be independent from the nature of the inducer.

Charts 2a—f show the extent of ATP-produced reversal of mitochondrial swelling in 0.125 M KC1 and 0.30 M sucrose test systems. In these assays, before the addition of ATP + Mg^{++} at the levels specified, swelling of the mitochondria was induced at pH 7.4 with thyroxin, PCMB, phosphate, arsenate, phlori-
zin, and digitonin, and allowed to proceed for 40 min. During the “contraction” phase of the assay (20 min), the pH was 7.2 in the KC1 system; the pH was 3.9—4.3 in the sucrose system. The absorbancy increase following addition of ATP was expressed as the percent reversal, due to ATP + Mg^{++}, of the absorbancy decrease which occurred during swelling. Control experiments were carried out which: (a) confirmed the absence of “contraction” in the sucrose medium with ATP + Mg^{++} at neutrality (cf. 27) and (b) demonstrated that without ATP + Mg^{++} no absorbancy increase, but rather further decrease, occurs when adjusting the pH of the test system to 3.9—4.3 with HCl, after swelling proceeded for 40 min (cf. 2, 18).

Chart 2 shows that in the sucrose systems there is a large, sometimes dramatic drop of reversal, sharply at the critical 4 weeks. With all six inducers this minimum is statistically significant with respect to both the controls and the 5-week (7-week for digitonin) populations. The probability for true differences is 0.05 > P > 0.02 or better, except between 0 and 4 weeks for phosphate and arsenate for which 0.10 > P > 0.05. The “con-
traction” maxima in the sucrose systems at 2 weeks coincide with the time of maximum level of bound 3'-Me-DAB in the microsomes and mitochondria (1, 4). The probabilities for the true difference at 2 weeks, relative to the control, are P ≈ 0.05 for PCMB and 0.10 > P > 0.05 for phlorizin; for thyroxin, phosphate, and arsenate, 0.10 > P > 0.20.

In the KC1 systems the ATP-produced reversal is generally lower than in the sucrose systems. The minimum around 4 weeks is very small or absent; beginning at 6—7 weeks, how-
ever, there is a gradual decrease of “contractility.” “Contractil-
ity” is practically lost in the 3'-Me-DAB hepatoma mitochon-
dria. With all inducers, the probability for the true difference between the control and the hepatoma is P < 0.001.
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Effect of Feeding 3'-Me-DAB and 2-Me-DAB on the Kinetics of Mitochondrial Swelling; the $V_{\text{max}}/C_{1/2\ max}$ Ratio. A different insight into the nature of mitochondrial membrane alterations during 3'-Me-DAB carcinogenesis is gained by measuring the initial swelling velocities at increasing inducer concentrations, rather than the terminal extent of swelling. In a preliminary account on kinetic studies with rat liver mitochondria, 3'-Me-DAB was administered and the changes at only the 4-week interval were analyzed by means of the Lineweaver-Burk plot (19). It became evident, however, in extending the assays to a larger number of inducers and to other periods of administration, that this treatment is impracticable because, with certain inducers and normal control mitochondria and/or after feeding 3'-Me-DAB or 2-Me-DAB for different periods of time, the swelling velocity plots do not follow the kinetic equation. Chart 3 (left) illustrates the different types of swelling velocity plots obtained. Therefore, these data may not be analyzed by means of the Lineweaver-Burk treatment and the half-maximum velocity concentrations ($C_{1/2\ max}$) which may be obtained from the direct plots are not meaningful as $K_m$ values in terms of the kinetic theory. Moreover, it was observed that during the time-course of azo dye feeding not only $C_{1/2\ max}$ but also the maximum swelling velocity ($V_{\text{max}}$) varies with the length of administration. Hence, in order to compare the “sensitivity of the mitochondrial swelling response” in these different conditions, this parameter was defined as the ratio $V_{\text{max}}/C_{1/2\ max}$. This definition combines the notions that the higher the maximum velocity brought about by a given swelling inducer agent and the lower the concentration necessary to bring about the half-maximum velocity, the greater is the sensitivity of the mitochondrial swelling response to that inducer. The $V_{\text{max}}/C_{1/2\ max}$ ratios for different inducers in normal liver mitochondria are given in Chart 3 (right).

Charts 4a—d show the change in the $V_{\text{max}}/C_{1/2\ max}$ ratio during the time-course of feeding 3'-Me-DAB. With all inducers tested, phlorizin, hydrocortisone, phosphate, PCMB, Ca$^{2+}$, deoxycholate, thyroxin, pentachlorophenol, and Hg$^{2+}$, the ratio invariably decreases during administration of the dye and reaches comparatively low levels or zero in 3'-Me-DAB-induced hepatoma. The probability for the true difference between normal and hepatoma mitochondria is $P < 0.001$ in all systems, except with Ca$^{2+}$ and deoxycholate for which $P \approx 0.01$. With the common surface-active agent, dodecyl sulfate, the $V_{\text{max}}/C_{1/2\ max}$ ratio remains constant during the period of administration (not shown in the charts). Since the amount of mitochondria used in all swelling velocity determinations was kept constant, the likely significance of the decrease of the

![Chart 3](chart3.png)

**Chart 3.** On the left: Diversity of types of swelling kinetic curves obtained with rat liver mitochondria using different inducers, and at different stages of feeding 3'-methyl- and 2-methyl-4-dimethylaminoazobenzene; this illustrates the unsuitability of the apparent $K_m$ of swelling as a parameter for comparing the mitochondrial swelling response under varying conditions. $V_{\text{spont}}$ represents the velocity of spontaneous swelling in the KCl or sucrose medium without added inducer agent. On the right: Sensitivity of swelling response of normal rat liver mitochondria to different inducers.

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Charts 4a–d. Decrease of the sensitivity of swelling response of rat liver mitochondria during feeding 0.06 percent 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) in a semisynthetic diet. Hepatomas were induced with 3'-Me-DAB. For the calculation of the sensitivity of swelling response, all determinations of swelling velocities (at 520 μA; 23–25°C) were carried out in 0.125 M KCl, except the determinations with thyroxin which were carried out in 0.30 M sucrose (both test systems were buffered with tris-(hydroxymethyl)-aminomethane, pH 7.4). All results with liver mitochondria are the mean values of 6 experiments each; the results with hepatoma mitochondria are the mean values of 4 experiments each. The probability of the true difference between the control and hepatoma mitochondria is $P < 0.001$ or $P \leq 0.01$. 

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ratios is the gradual deletion of the mitochondrial receptor sites for the inducer compounds during administration of 3′-Me-DAB, the extent and rate of deletion being different for the different types of sites. Identical conclusion was reached on the basis of the Lineweaver-Burk treatment of mitochondrial swelling kinetics following a 4-week period of administration of 3′-Me-DAB and using PCMB, phosphate, and Ca2+ as inducers (19). Unlike 3′-Me-DAB, administration of the comparatively inactive 2-Me-DAB for a 4-week period does not bring about an invariable decrease of the ratio, but the ratio increases or decreases to different degrees depending on the nature of the inducer (Chart 5). It may be inferred that during carcinogenesis the processes of deletion of different control sites of mitochondrial swelling progress at different rates, depending on the structural type and activity of the carcinogenic agent (cf. 3); it is significant in this respect that the highly active 3′-Me-DAB brought about the decrease of the ratio with all inducers tested.

The 0.6 M KCl-extractable mitochondrial protein, which was hitherto regarded as the contractile entity of the membrane responsible for the dynamic morphology of the mitochondria, appears to have no relation with the swelling and “contraction” changes during feeding 3′-Me-DAB or 2-Me-DAB. Chart 6 shows that the level of this protein in the mitochondria remains unchanged during feeding of either of the two dyes for up to 10 weeks. On the other hand, in hepatoma mitochondria, which in all systems showed considerably impaired swelling and “contraction” ability, there was a fourfold increase in the level of this protein. These data are consistent with recent investigations (9) which show that this 0.6 M KCl-extractable mitochondrial protein is not contractile by the same criteria as actomyosin is.

**DISCUSSION**

Significance of the Terminal Extent of Swelling Compared to the \( \frac{V_{\text{max}}}{C_{1/2}^{\text{max}}} \) Ratio. The foregoing results show that the changes in the terminal extent of swelling and the changes in the \( \frac{V_{\text{max}}}{C_{1/2}^{\text{max}}} \) ratio reflect different types of alterations in the mitochondrial membrane during 3′-Me-DAB carcinogenesis. The magnitude of the terminal extent of swelling is a measure of the maximum expansion of the particles under given experimental conditions. At the critical 4 weeks there is only a temporary loss in the magnitude of terminal extent of swelling of liver mitochondria; there is an irreversible loss in 3′-Me-DAB-induced hepatoma mitochondria. On the other hand, the \( \frac{V_{\text{max}}}{C_{1/2}^{\text{max}}} \) ratios decrease *uninterruptedly* throughout and beyond the critical period during the feeding of 3′-Me-DAB and reach the lowest values in tumor mitochondria. These ratios are based on the initial swelling velocities. Thus they are independent from the limit set by the maximum expansion of the mitochondria and measure the compounded result of the affinity of the inducers to the receptor sites and of the rate of the “effector system” (7, 32) of swelling in the membrane.

Although mitochondria from azo dye-induced tumors and from transplanted solid tumors were found resistant to swelling by a number of workers when assayed in the absence of an energy source, Wenner (46) and Wenner et al. (47) observed that, in the presence of ATP, valinomycin produced in Ehrlich ascites tumor mitochondria light scattering changes indicative of swelling. The significance of the latter finding will require further investigation since Hawtrey and Silk (22) and Utsumi et al. (45) found earlier that the “passive” swelling (in the absence of ATP) of Ehrlich ascites tumor mitochondria is very low.

Recent investigations rule out the possibility that the presence of a membrane-localized “contractile protein” or the chemiosmotic hypothesis can account for the swelling-
of such inner membrane-linked functional parameters as the respiratory control index (6) and swelling-“contraction”, until, around the critical 4-week period, deletion of dye-binding segment(s) from the mitochondrialy coded (49—51; reviewed in Ref. 41) “structural protein” and/or the restructuring of the “catalytic protein” surrounding it, would allow the return of membrane mobility toward normal. In occasional cells, however, this process may lead either to the deletion of “structural protein,” or to the coding for “structural protein(s)” which are nonfunctional for conformational change. The mitochondria of these rare cells are the carriers of the impaired swelling and “contraction” pattern seen later with the tumor mitochondria.

**Difference of ATP-produced “Contraction” in Sucrose and in KCl.** Insofar as the absorbancy increase in the KCl medium can be regarded as true contraction (cf. 36), this reversal is assumed to be actuated by ATP hydrolysis (reviewed in Ref. 28) by way of the ATPase located in the headpiece of the repeating units of the mitochondrial inner membrane (cf. 20). However, this will require clarification for the following reasons. Rendi and Ginsburg (40) have shown that urea treatment (which inactivates 70% of the ATPase) does not affect the rate or extent of “contraction,” and at concentrations at which “contraction” is inhibited PCMB affects ATPase activity only to a small extent. Moreover, while Rendi and Ginsburg reported that 2,4-dinitrophenol activates “contraction,” Kopaczyk et al. (25) found that the headpiece-localized ATPase is not inhibited by this uncoupler but is sensitive to PCMB.

The nature of the ATP-produced reversal of absorbancy decrease in 0.30 M sucrose at pH 4.0 has not been explored. It is likely that this phenomenon is in close relation with (cf. 18) the 2,4-dinitrophenol-sensitive binding of ATP by the “structural protein” at pH 4.0 (23, 38). That the sharp 4-week decrease of the ATP-produced reversal occurs only in the sucrose medium is reminiscent of the selective permeability-limiting effects of sucrose (8, 24). During the critical period, accessibility of certain mitochondrial compartments to ATP may be highly limited in sucrose medium.

**Decline of the V_{max}/C_{1/2 max} Ratios.** The continuous and uninterrupted decrease of these ratios during the feeding of 3’-Me-DAB, from the normal to the tumor, shows the gradual deletion of control sites of mitochondrial membrane conformational states. The positions of the receptor sites, which are clearly specific for the induction of swelling by different agents, are not known. However, because of the intimate relationship between mitochondrial size, shape, and particulate energy level, it is of possible importance that several swelling-inducers have been identified as inhibitors of intermediates involved in ATP synthesis (13, 29, 30, 37, 54). The objection may be advanced, of course, that some of the inducers in in vitro assays are used at levels higher than physiologic and/or are nonphysiologic substances. The role of inducers in the in vitro assays is that of probes for detecting the alteration of and measuring the deletion of receptor sites which may respond, in the cell, to specific regulatory substances and/or at substantially lower levels.

The gradual decline of the swelling and “contraction” response during 3’-Me-DAB carcinogenesis, culminating in the loss of these functions in tumor mitochondria, cannot fail to
affect other parameters linked to the dynamic functioning of the membrane. Cereijo-Santalo (11) has given convincing demonstration that mitochondria which can undergo active swelling are potent inhibitors of aerobic glycolysis, whereas mitochondria which are resistant to swelling (originating either from normal or from malignant tissues) are not. This is not inconsistent with the views that in vivo the enzymes involved in glycolysis are linked to the mitochondrial membrane and that the balance between glycolytic and tricarboxylic cycle activities are regulated by the orientation of these enzymes on and the permeability of the mitochondria (reviewed in Ref. 48). It is, thus, not impossible that the high level of aerobic glycolysis of advanced tumors may ultimately be the result of alterations of mitochondrial membrane dynamics.

**ADDENDUM**

Recent results by L. A. Sordahl, Z. R. Bladlock, A. G. Liebelt, G. H. Kraft, and A. Schwartz (Some Ultrastructural and Biochemical Characteristics of Tumor Mitochondria Isolated in Albumin-containing Media. Cancer Res., in press, 1969) show that the reversibility of the electron microscopically observable, respiratory state-dependent "orthodox" vs. "condensed" ultrastructural transition—which is always exhibited by normal liver mitochondria—is absent in mitochondria isolated from a transplantable rat hepatoma and a transplantable mouse mammary adenocarcinoma. This is in good agreement with our findings that tumor mitochondria are resistant to both swelling and contraction as measured by the photometric method. Yet, in their polarographic oxygen monitoring system (using an assay medium high in K+ and devoid of sucrose), Sordahl et al. measured, with mitochondria of their tumor lines, theoretical ADP:O values and respiratory control indexes which were substantial although always lower than those of normal rat liver mitochondria. The totality of these results is consistent with the hypothesis (2) that the mechanism responsible for swelling-contraction and other morphologic changes in mitochondria involves a structural entity different from the electron transport chain and the coupling sequence but linked to them in normal mitochondria. Hence, one locus of mitochondrial alterations in hepatic carcinogenesis may be the site(s) of linkage(s) between the swelling-contraction effector system and the electron transport chain and/or coupling sequence.

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