A Reconsideration of the Isolation in Presence of EDTA and the Swelling-Contraction Characteristics of Rat Heart Sarcosomes. Metabolic Topography of the Sarcosomal Contractile Protein*

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The yield of rat heart sarcosomes, but not of liver mitochondria, is considerably decreased when ethylenediaminetetraacetate is included in the 0.44 M sucrose isolation medium. The sensitivity of sarcosomes to this agent is also shown by the appreciable inhibition of hypotonicity-initiated swelling and by the inhibition of oxygen uptake. Sarcosomes isolated in a 0.44 M sucrose medium containing no ethylenediaminetetraacetate were found to be intact by phase-contrast microscopic standards. The hypotonicity-initiated swelling of heart sarcosomes, unlike that of liver mitochondria, shows a series of minima and maxima when the total concentration of the medium is varied between 0.01 and 2.00 M. The *p*H dependence of swelling of heart sarcosomes in 0.04 M sucrose shows a sharp maximum at *p*H 7.4, which is absent in 0.44 M sucrose. The kinetics of swelling induced by NAD, Ca^{2+}, dodecylsulfate, and desoxycholate follow the kinetics of drug-receptor complex formation, while those induced by thyroxine, pentachlorophenol, and *p*-mercuribenzoate show linear relationships. The swelling induced in heart sarcosomes by various agents is inhibited and reversed by ATP + Mg^{2+} and by reduced nicotinamide-adenine dinucleotide ($NADH_2$). Both types of inhibition and reversal are strongly *p*H dependent, the optimum being between 4.5 and 3.5. The swelling of liver mitochondria is also reversible by $NADH_2$ at this *p*H. There is no reversal of sarcosomal swelling either by $NADH_2$ or by ATP + Mg^{2+} at *p*H 6.8–7.4, which is the optimum range for swelling. It was shown, by measuring the absorption at 340 mμ and by the ability of acid-treated $NADH_2$ to support sarcosomal diaphorase activity, that large proportions of the nucleotide remain unchanged in the *p*H range of contraction even after incubation for 2 hours. Amytal, azide, and 2,4-dinitrophenol do not affect $NADH_2$-induced reversal of sarcosomal swelling, while antimycin A, pentachlorophenol, 2,3-dimercaptopropanol, and *p*-mercuribenzoate enhance this reversal to various degrees. In the *p*H range optimum for contraction, 2,4-dinitrophenol and *p*-mercuribenzoate do not affect ATP-produced reversal of swelling; fluoride is inhibitory at high levels. The results suggest that the contractile protein of the sarcosomal membrane represents a direct shunt between the particulate ATP pool and the respiratory chain, and furthermore is associated with an intermediate of the coupling sequence.

Studies on the swelling and contraction of mitochondria have been carried out to a great extent with particles isolated from rat liver (Lehninger, 1962). The relative scarcity of information on mitochondria from other tissues may be due in part to the difficulties encountered in isolating the particles from physically more firm tissues. A procedure widely used for the isolation of heart sarcosomes involves the liberation of the particles by manual grinding with acid-washed sand in the presence of EDTA[1] (Cleland and Slater, 1953a,b). Although the first studies in which swelling was measured photometrically were carried out on heart sarcosomes (Cleland, 1952), few reports have appeared subsequently on the swelling and contraction of sarcosomes. Important information on the nature of sarcosomal swelling (Tapley and Cooper, 1956; Cooper and Tapley, 1957) showed that heart sarcosomes, as compared to liver mitochondria, are very resistant to swelling induced by different agents. Paralleling this is the fact that heart sarcosomes, unlike liver mitochondria, can withstand to a great extent the damaging action of prolonged exposure to hypotonic media, since exposure is accompanied by only small changes of enzyme activity (e.g., Slater and Cleland, 1953; Tyler, 1954). Swollen rat heart sarcosomes are contracted by ATP and the contraction is not influenced by a dietary magnesium deficiency, although this condition consistently produced a marked decrease in the P:O ratio but only occasionally increases particulate swelling (Nakamura *et al.*, 1961).

This paper reports a comparative study, with heart sarcosomes and liver mitochondria, on the effect of EDTA on the isolation yield, hypotonicity-initiated swelling, and oxygen uptake, and on the effect on swelling of increasing osmolarities. The *p*H dependence of swelling and the kinetics of the swelling response, using various inducers, were studied with sarcosomes. The reversal of swelling of sarcosomes, the *p*H requirement, and the effect of inhibitors on this phenomenon have been investigated with ATP and $NADH_2$. The latter is a new agent that has now been found to produce contraction of sarcosomes and mitochondria. The findings point to a *p*H requirement for the contraction of sarcosomes by both agents. The relationship of the sarcosomal contractile protein to the electron transport chain and the phosphorylating coupling sequence has been discussed.

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[1] Abbreviations used in this work: EDTA, ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; NAD (also known as DPN), nicotinamide-adenine dinucleotide; $NADH_2$ (also known as DPNH), reduced nicotinamide-adenine dinucleotide; DNP, 2,4-dinitrophenol. *p*-Mercuribenzoate designates the compound previously termed *p*-chloromercuribenzoate or *p*-hydroxymercuribenzoate.

Experimental Procedures

Isolation of Sarcosomes and Mitochondria.—Male Sprague-Dawley rats (Holtzman Co., Madison, Wis.) weighing 300–370 g and maintained on Purina Laboratory Chow were used. The rats were killed by decapitation and the hearts (or livers) were rapidly removed and weighed. By using coarse-surfaced all glass homogenizers[2] for heart tissue and homogenizers with Teflon pestle for liver tissue, 10% homogenates were prepared (at 1–3°) in 0.44 M sucrose containing no EDTA, except when the effect of EDTA on sarcosomal and mitochondrial yield and swelling was studied. After gross breaking up of the tissues, homogenization was completed with 13 passes for heart and 11 passes for liver. The isolation of the liver mitochondria was carried out as previously described (Arcos *et al.*, 1960). For heart sarcosomes the homogenate was first centrifuged at 600 *g* for 12 minutes, and then the nuclei and cell debris sediment was extracted twice by rehomogenization in sucrose and recentrifugation. The three supernatants were pooled and centrifuged for 10 minutes at 13,000 *g* (at 1–3°). The resulting pellet was washed once, with 0.44 M sucrose, by resuspension and homogenization; this sucrose contained no EDTA even when the sarcosomes or mitochondria were isolated in the presence of EDTA. The presence of a "fluffy layer" was not apparent in these preparations. For the swelling and contraction studies the final pellets were resuspended in 0.44 M sucrose so that 1 ml contained the heart sarcosomes (or liver mitochondria) from 1 g fresh tissue. For the oxygen uptake studies the final pellets were resuspended in 2.5 ml of 0.25 M sucrose/g of fresh tissue. The "stock suspensions," kept in ice, were always used within 10 minutes after preparation.

Swelling Tests.—Sarcosomal and mitochondrial volume changes were studied at 520 mμ (at 23–25°) in a Coleman spectrophotometer using 7-ml-capacity matched plane-parallel cells, following an adaptation of the procedure of Tapley (1956). The standard test system used here consisted of 1 ml 0.04 M sucrose solution buffered with 0.03 M Tris, initially adjusted to *p*H 7.4. Hypotonic conditions were used because the swelling of sarcosomes, which are resistant to various types of swelling-inducer agents in more concentrated media, occurs "spontaneously" under these circumstances and is enhanced further by specific swelling-inducer agents to different degrees. For the studies of the effect on swelling of various total molar concentrations or of different *p*H values, the two parameters were changed accordingly. In all swelling assays the initial absorbancy was adjusted closely to 0.300, the volumes of "stock suspension" used were recorded, and the swelling was followed at intervals up to 40 minutes. The first readings were always taken within 10 seconds after the addition of the "stock suspension." When the effect of various agents on swelling was studied, these agents were either directly dissolved in the test system or added to it as 0.04-ml aliquots of neutral concentrated solutions *prior* to the addition of the "stock suspension." In the sole instance of NAD (Sigma, St. Louis, Mo.; 98% purity), the nucleotide, in solid form, was added to the medium after addition of the sarcosomes because of the very rapid swelling produced. Absorbancy changes were plotted either directly or as final per cent decrease (at 40 minutes) relative to the zero-time value. For the kinetic studies net initial rates of absorbancy decrease (%/min) based on the 10-minute absorbancy values were plotted. These net rates were obtained by subtracting the rate of hypotonicity-initiated swelling from the rates with the various swelling-inducer agents.

Contraction Experiments.—Immediately after the 5-minute or the 40-minute absorbancy reading, sarcosomal contraction was induced by adding a 0.02-ml aliquot of an ATP-$MgCl_2$ solution mixture of such concentration as to bring the levels of these compounds in the test systems to 5×10^{-3} or 1×10^{-2} M, and to 3×10^{-3} M, respectively. These solution mixtures were prepared as follows. Disodium ATP was first dissolved either in a *p*H 7.4, 0.04 M sucrose–0.03 M Tris solution (which resulted in a final *p*H of 3.5–4.5 in the test system), or in 1.5 M Tris adjusted initially to *p*H 8 (which gave a final *p*H of 6.8–7.4 in the test system). To these ATP solutions was then added $MgCl_2$ as an aqueous stock solution. The mixtures of ATP-$MgCl_2$ were always freshly prepared immediately before addition to the test systems, and maintained ice cold. When bovine serum albumin was used (only in studies at neutrality), it was combined with the ATP-$MgCl_2$ mixture as an aqueous stock solution to give a final level of 2 mg per test system. When used, ADP was at the concentration of 1×10^{-2} M. Inhibition of sarcosomal swelling by ATP was studied by adding the ATP-$MgCl_2$ mixtures to the test systems immediately following the sarcosomes (Fig. 7), or together with the inducer agent (Fig. 9).

For reversal or inhibition of swelling by $NADH_2$, the nucleotide was added to the test systems containing the sarcosomes to obtain levels of 8×10^{-3} or 1.5×10^{-2} M. In specified experiments aliquots of $NADH_2$ solutions, adjusted with HCl to *p*H 4.0 and incubated for 30 minutes, were added to the test systems. Finally, the *p*H of the test systems was adjusted to 3.5–4.5 by adding small aliquots of 1 N HCl after the addition of $NADH_2$.

For studying the effect of inhibitors on the contraction induced by ATP-$MgCl_2$, or by $NADH_2$, the inhibitors were added as small aliquots of neutral, concentrated stock solutions immediately after the 40-minute absorbancy reading, and the test systems were incubated for 1 minute before addition of the contracting agent (in some experiments with *p*-mercuribenzoate incubation was for 10 minutes). Absorbancy changes during reversal were recorded at 5-minute intervals up to 20 minutes. In occasional experiments frequent readings were made during the first 5 minutes.

Determination of the Stability of $NADH_2$ in Acid Media.—The stability of $NADH_2$ in the acid *p*H range required for contraction was determined in two ways: (a) by following the percentage loss of $NADH_2$ in solutions (5 mg/ml) adjusted to the stated acid *p*H values, after incubation for 2 or 7.5 hours and subsequent 60-fold dilution for the absorbancy readings (at 340 mμ in a Beckman DB spectrophotometer); and (b) by measuring the comparative rates of sarcosomal diaphorase activity supported by the above-mentioned acid-treated $NADH_2$ solutions, following a modification of the procedure of Edelhoch *et al.* (1952) and Mahler *et al.* (1952).

Determination of Oxygen Uptake.—Oxygen uptake was measured in a Warburg respirometer. The final reaction mixture contained 50 μmoles phosphate buffer (*p*H 7.4), 10 μmoles magnesium chloride, 6 μmoles ATP, 60 μmoles sodium fluoride, 0.03 μmole cytochrome *c*, 20 μmoles α-ketoglutarate, EDTA at the levels stated, 2 mg hexokinase (Nutritional Biochemicals Corp., Cleveland, Ohio), 10 mg glucose, heart sarcosomes or liver mitochondria from 200 mg fresh tissue, and 0.25 M sucrose to complete a final volume of 3 ml. Oxygen uptake was recorded at 5-minute intervals up to 20 minutes. The particles obtained by the present

[2] Duall Tissue Grinder K-88545, Kontes Glass Co., Vineland, N.J.

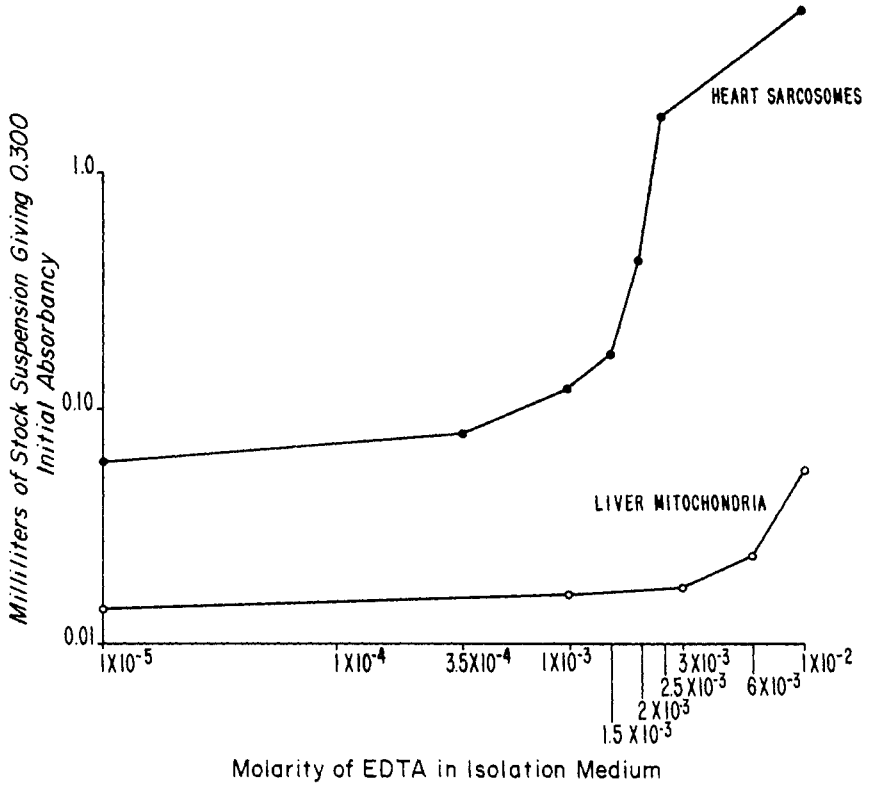

FIG. 1.—Relative yield of mitochondria and sarcosomes from rat liver and heart tissue, respectively, as a function of the molar concentration of EDTA present in the 0.44 M sucrose isolation medium. Relative particle concentration in the "stock suspension" (particles from 1 g of fresh tissue in each ml) was measured by determining the milliliters of this suspension required to obtain 0.300 initial absorbancy when added to 1 ml of a 0.04 M sucrose + 0.03 M Tris (*p*H 7.4) solution. Each point represents the average obtained with five animals.

procedure from 200 mg of fresh tissue correspond to an average of 3.2 mg of dry sarcosomal weight and of 5.8 mg of dry mitochondrial weight as determined by colorimetry of aliquots of the "stock suspensions" following Lowry *et al.* (1951). The protein used for colorimetric standard was a liver mitochondrial fraction precipitated with trichloroacetic acid, washed with water, extracted with acetone, and dried.

RESULTS

Comparative Effect of EDTA on Sarcosomal and Mitochondrial Yields, Hypotonicity-initiated Swelling, and Respiration.—In preliminary experiments for establishing the isolation procedure for heart sarcosomes it was observed that the presence of EDTA in the 0.44 M sucrose isolation medium produces an appreciable decrease of sarcosomal yield. The results of a systematic study of this phenomenon are given in Figure 1. The figure shows that EDTA produces a rapidly increasing "trapping" of the sarcosomes in the nuclear sediment when the EDTA concentration in the isolation medium is beyond 3.5×10^{-4} M. The presence of ten times this level of EDTA is required, however, before a noticeable decrease of mitochondrial yield is observed with liver homogenates. For this reason the use of EDTA in the sarcosomal isolation medium was abandoned.

Furthermore, heart sarcosomes isolated in the presence of EDTA (1×10^{-3} M in the isolation medium) were found to have a significantly decreased ability ($p < 0.001$) to swell when the rate and extent of hypotonicity-initiated swelling of these particles was compared to that of the same particles isolated in the absence of EDTA. Similar observations were made with liver mitochondria, which confirmed the findings of others that EDTA is an inhibitor of swelling of liver mitochondria (reviewed in Lehninger, 1962). This substantiates the interpretation that the increasing volumes of sarcosomal stock suspension required to attain a given absorbancy, as indicated in Figure 1, represent a true decrease of sarcosomal yield and not an increasingly swollen state of the sarcosomes present. Since EDTA inhibits the swelling of sarcosomes and mitochondria, the only possible effect of the presence of this agent in the isolation medium would be to increase the initial absorbancy of sarcosomal and mitochondrial suspensions.

The effect of EDTA on the respiration of sarcosomes and of mitochondria is shown in Figure 2. Figure 2a shows the increasing inhibition of sarcosomal oxygen uptake by EDTA at levels from 1×10^{-6} to 5×10^{-5} M using α-ketoglutarate as substrate. In contrast, the respiratory rate of liver mitochondria (Fig. 2b) was considerably enhanced by 1×10^{-6} M EDTA and not inhibited by 5×10^{-6} M; only at higher concentrations did inhibition become manifest.

Swelling Characteristics of Rat Heart Sarcosomes.—Figure 3a shows a unique and statistically significant swelling maximum at 0.18 M for liver mitochondria, while the heart sarcosomes (Fig. 3b) present a series of maxima and minima between 0.01 and 2.00 M when the final extent of absorbancy decrease (in per cent) is plotted against the total molar concentration of the test system. The probabilities for true differences between these maxima and minima are unequivocal ($p < 0.05$) between 0.23 and 0.63 M and between 0.63 and 2.00 M. In three other instances (between 0.01 and 0.02 M, between 0.02 and 0.07 M, and between 0.13 and 0.23 M) the p values are higher ($0.10 < p < 0.20$)

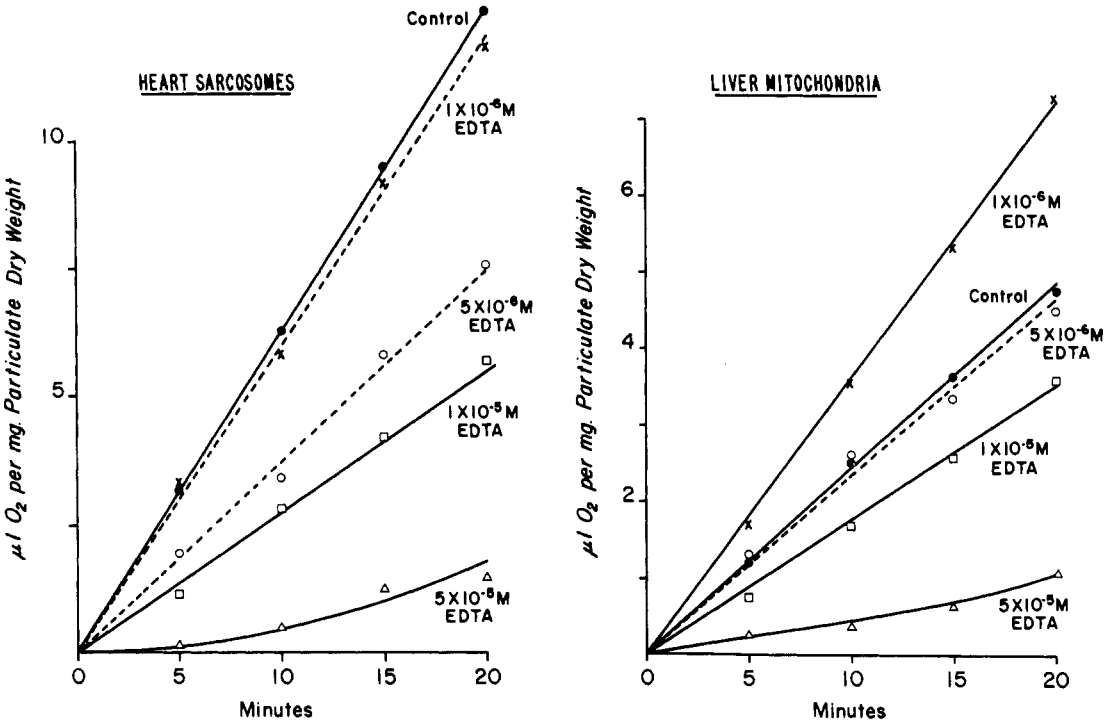

FIG. 2.—Comparative effect of EDTA on the oxygen uptake of heart sarcosomes (a, left) and liver mitochondria (b, right) of the rat. Cell particles were isolated in 0.44 M sucrose without EDTA. The reaction mixture contained the usual cofactors, particles from 200 mg fresh tissue, and α-ketoglutarate as substrate in a 0.25 M sucrose medium at pH 7.4. Each point represents the average obtained with three animals.

but not exclusive of the probability of true difference.

Figure 4 shows the pH dependence of swelling of sarcosomes at two different sucrose molarities, both in the presence of 0.03 M Tris. The figure shows a quite sharp statistically significant optimum at pH 7.4 in the 0.04 M sucrose medium. There is gradual dissolution of the particles at increasing pH values beyond pH 10.4. In the 0.44 M sucrose medium the optimum at pH 7.4 is absent, since the small apparent differences between the swelling values at the different pH values are not statistically significant.

The appreciably greater resistance of heart sarcosomes, as compared to liver mitochondria, to most inducers of swelling (Tapley and Cooper, 1956; Cooper and Tapley, 1957) was confirmed. The kinetics of swelling produced by NAD, Ca^{2+}, dodecylsulfate, and desoxycholate (Fig. 5a,b,c,d, respectively) were found to obey the equation describing the formation of drug-receptor complexes (Ariens *et al.*, 1956, 1957) and which shows a formal analogy with the Michaelis-Menten equation (Ariëns *et al.*, 1956). In contrast, the swelling produced by thyroxine, p-mercuribenzoate, and pentachlorophenol shows a linear relationship between the concentration of the swelling-inducer agent and the initial swelling rate (Fig. 6a). A linear relationship was also found with digitonin which is known to solubilize mitochondrial particles. Figure 6b shows that with digitonin the linearity of increase of the rate is maintained between 1×10^{-5} and 1×10^{-3} M, probably corresponding to increasing dissolution of the sarcosomes with the increasing concentration of the agent. Between 1×10^{-3} and 4×10^{-3} M the initial rate suddenly becomes stationary because of dissolution of the total particle population. That digitonin "titrates" the sarcosomes is also shown by the fact that, at any of the concentrations studied, after the initial very rapid decrease of absorbancy has occurred there is little or no further change and the absorbancy is stationary between 10 and 40 minutes.

Other inducers produced statistically significant sarcosomal swelling (per cent decrease of absorbancy above the control, at 40 minutes) at the following minimum single concentrations: 4% with phlorizin at 1×10^{-3} M ($0.05 < p < 0.10$); 12% with inorganic phosphate at 1×10^{-3} M ($p < 0.001$); 5% with arsenate at 5×10^{-3} M ($0.02 < p < 0.05$). The sarcosomes were completely resistant to mercuric chloride at 1×10^{-5} M (compare Cooper and Tapley, 1957); at this concentration this agent induces a 65% decrease in the absorbancy of liver mitochondria (Arcos *et al.*, 1960). The isomer α-NAD was found to be slightly less active than β-NAD to induce sarcosomal swelling when both were assayed at the single concentration of 1×10^{-2} M.

Reversal of Swelling by ATP and the Acid pH Requirement.—The requirements for inhibition and reversal of swelling of rat heart sarcosomes by ATP are represented in Figures 7, 8, and 9. In these experiments swelling was initiated by the hypotonicity of a 0.04 M sucrose + 0.03 M Tris solution (Fig. 7), or induced by 5×10^{-5} M thyroxine (Fig. 8), or by 1.5×10^{-2} M NAD (Fig. 9). Contraction of swollen sarcosomes and inhibition of swelling were produced by ATP at 5×10^{-3} M or at 1×10^{-2} M, plus $MgCl_2$ at 3×10^{-3} M.[3] Both contraction of swollen sarcosomes (Fig. 8) and inhibition of

[3] That NAD is a potent agent to induce swelling in mitochondrial particles (Corwin and Lipsett, 1959; Arcos *et al.*, 1960; Gosch *et al.*, 1962; and the present report) and that this swelling may be readily reversed or inhibited by ATP suggest that the phenomenon recently observed by Lehninger and Gregg (1963) with digitonin fragments and termed "reverse acceptor control" may be the reflection of the maintenance of coupling to an appreciable degree despite the presence of high levels of NAD, because ATP synthesis proceeds in the presence of ADP and inorganic phosphate. Hence ADP and inorganic phosphate (both required for "reverse acceptor control" in digitonin preparations) produce inhibition of respiration relative to the respiratory rate of more uncoupled control preparations (containing NAD but no ADP or inorganic phosphate).

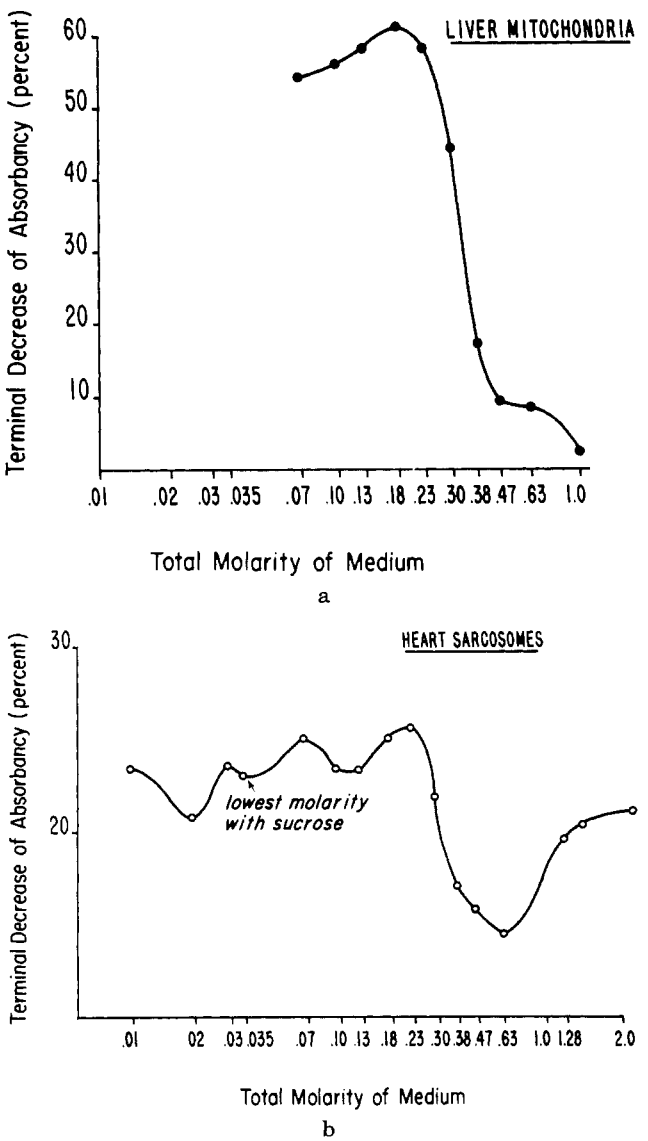

FIG. 3.—The swelling of liver mitochondria and heart sarcosomes of the rat as a function of the total molar concentration of the test system, at *p*H 7.4. Mitochondria (a, upper) and sarcosomes (b, lower) were isolated in 0.44 M sucrose without EDTA. At and above 0.035 M of total molar concentration, the test systems consisted of 0.03 M Tris plus the appropriate amounts of sucrose corresponding to the respective differences; below 0.035 M the test systems contained Tris alone. The absorbancy readings were not consistent below 0.07 M for the mitochondria and 0.01 M for the sarcosomes; there was a tendency for aggregation resulting in small or no change of absorbancy at 40 minutes (relative to the zero time value). Each point represents the mean value obtained with five rats, and corresponds to the terminal decrease of absorbancy (in per cent at 40 minutes). The probabilities for true differences between the minima and maxima obtained with heart sarcosomes are: between 0.01 and 0.02 M, $0.10 < p < 0.20$; between 0.03 and 0.035 M, $p > 0.80$; between 0.02 and 0.07 M, $0.10 < p < 0.20$; between 0.07 and 0.13 M, $p > 0.30$; between 0.13 and 0.23 M, $0.10 < p < 0.20$; between 0.23 and 0.63 M, $p < 0.001$; between 0.63 and 2.00 M, $0.02 < p < 0.05$. For the true difference between 0.07 and 0.18 M, with the liver mitochondria $0.02 < p < 0.05$.

swelling by adding the two agents at the onset of the assay (Fig. 7) were more pronounced with the higher level of ATP. Reversal of swelling by ATP + Mg^{2+}, essentially identical to that with thyroxine-swollen sarcosomes (Fig. 8), was observed when swelling was induced by 5×10^{-4} M $CaCl_2$, 3×10^{-2} M inorganic phosphate, 7×10^{-3} M arsenate, 1×10^{-3} M phlorizin, or 2×10^{-5} M *p*-mercuribenzoate. The reversal is specific to the triphosphate of adenosine, since at 1×10^{-2} M ADP (together with $MgCl_2$) was found to produce only slight reversal of NAD-induced sarcosomal swelling (Fig. 9), which may be attributed to the dismutation of ADP to ATP by adenylate kinase (cf. Lehninger, 1959a).

The *p*H ranges required for the swelling and for the contraction of sarcosomes are different. The optimum *p*H for the hypotonicity-initiated swelling is 7.4 (Fig. 4). The *p*H required for optimum contraction of the sarcosomes is between 3.5 and 4.5. This is the *final* *p*H of the swelling test systems after disodium ATP was added and in which contraction actually occurred. When ATP + Mg^{2+} neutralized with Tris (see Contraction Experiments, under Experimental Procedure) was added to swelling test systems, no reversal resulted with or without bovine serum albumin (e.g., Figs. 7 and 8). The final *p*H of these test systems was 6.8–7.4. However, the addition of sufficient hydrochloric acid to bring the *p*H of these ATP-containing (no bovine serum albumin) test systems to 3.5–5 resulted in rapid contraction. The use of bovine serum albumin was abandoned, because this adjuvant is precipitated by ATP + Mg^{2+} in the *p*H range required for contraction of sarcosomes. There was a rapid contraction of sarcosomes by ATP + Mg^{2+}, in any of the swelling-inducer systems, when the final *p*H of the complete assay system was between 3.5 and 5. Although a systematic study to determine the exact positions of the *p*H optima for the reversals has not been carried out, they appear to lie between 3.5 and 4.5; the contractive response to a given concentration of ATP is fairly constant in this zone.

The reversals observed are not due to the low *p*H itself. Addition of hydrochloric acid in absence of ATP produces, depending on the type of swelling, only very small increase in absorbancy (Fig. 7), no change (Fig. 9), or considerable further decrease of absorbancy (Fig. 8). Nor is the inhibition of swelling by ATP (Figs. 7 and 9) caused by the low *p*H for the following reasons: (a) hypotonic swelling, even at *p*H 4, is as much as 10% (Fig. 4), while in presence of ATP and at the same *p*H the absorbancy of the test systems is always higher than the initial 0.300 (Figs. 7 and 9); (b) the ATP-produced increase in absorbancy of the test systems above the zero-time value (Figs. 7 and 9) is consistent with the contraction of nonswollen sarcosomes to very small, dense granules by 1×10^{-2} M ATP (*vide infra* Phase-Contrast Microscopic Examination of Sarcosomal . . .).

Reversal of Swelling by $NADH_2$ and the Acid pH Requirement.—Figures 7, 8, and 10 give the requirements for reversal of swelling by $NADH_2$, which has now been found to be a new agent for producing the contraction of sarcosomes. These requirements appear to be very similar to those of ATP-induced reversal. In the neutral *p*H range the hypotonic swelling of sarcosomes is not modified by the presence of 8×10^{-3} M $NADH_2$ (Fig. 10). In the optimum low *p*H range, however, $NADH_2$ is a potent agent to inhibit (Fig. 10) or reverse (Figs. 7 and 8) sarcosomal swelling. The importance of *p*H is dramatically illustrated by the experiments of $NADH_2$ reversal of thyroxine-induced swelling as given in Figure 8. Swelling induced by Ca^{2+}, inorganic phosphate, arsenate, phlorizin, and *p*-mercuribenzoate (all at the concentrations given under Reversal of Swelling by ATP . . .) was also reversed by $NADH_2$. While in the experiments in Figures 7 through 10 sarcosomal contraction is represented as being completed only at about 5 minutes after additions, other

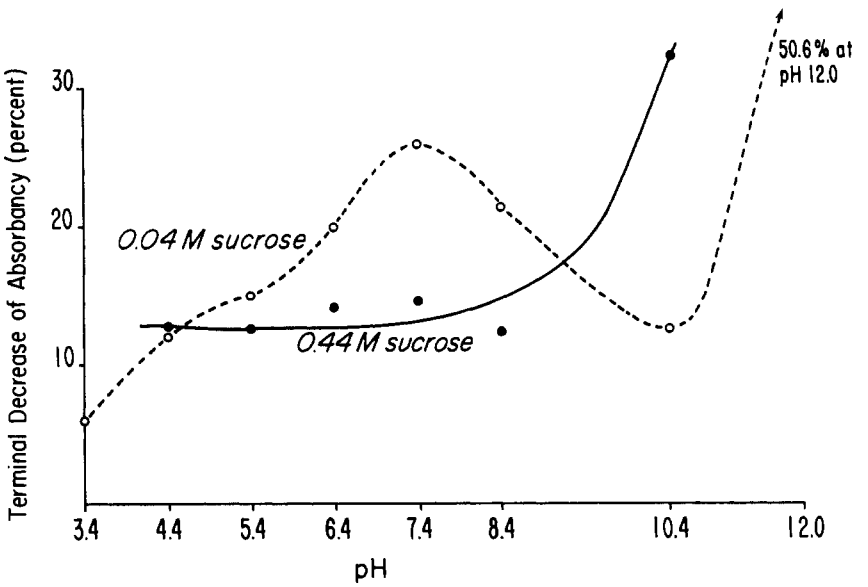

FIG. 4.—The *p*H dependence of swelling of rat heart sarcosomes in 0.04 and 0.44 M sucrose solutions both in presence of 0.03 M Tris. Each point is the mean value obtained with five rats, and represents the terminal decrease of absorbancy (in per cent at 40 minutes). The *p*H values on the figure represent final values of the complete assay mixtures, measured at the termination of the runs. The probabilities for true differences between the values at *p*H 7.4 and extreme *p*H values are: in 0.04 M sucrose for *p*H 4.4, $p < 0.001$, and for *p*H 10.4, $p < 0.001$; in 0.44 M sucrose for *p*H 4.4, $p > 0.30$, and for *p*H 8.4, $p > 0.30$.

TABLE I

EXTENT OF LOSS OF $NADH_2$ AS A FUNCTION OF THE *p*H AND THE ABILITY OF ACID-TREATED PREPARATIONS TO SUPPORT DIAPHORASE ACTIVITY[a]

*p*H of Incubation	Per Cent Loss of $NADH_2$	Initial Rate of Diaphorase Activity
7.4	0	0.400
4.9	10.0	0.377
4.0	22.8	0.420
3.5	61.2	0.380
2.5	95.5	0.030
1.0	92.5	0.030

[a] For the *p*H 7.4 stock solution, $NADH_2$ was dissolved immediately before use in water adjusted with a few drops of dilute Tris buffer. All other $NADH_2$ stock solutions were adjusted to the *p*H values indicated with HCl at room temperature and then incubated in ice for 2 hours before the determinations were carried out. For the absorbancy measurements at 340 mμ, 0.05-ml aliquots of these stock solutions (all 5 mg/ml) were added to 3 ml water. The absorbancy of the *p*H 7.4 solution was 0.625. The per cent loss at the low *p*H values was calculated from the decrements of absorbancy. The diaphorase system contained 0.1 ml (500 μg) of the foregoing $NADH_2$ stock solutions, 0.1 μmole (0.03 ml) of 2,6-dichlorophenolindophenol, heart sarcosomes from 25 mg fresh tissue (0.03 ml), 0.4 ml 0.2 M *p*H 7.4 Tris buffer, and 2.5 ml water (for the *p*H 1.0 determination, 0.8 ml Tris and 2.1 ml water). Absorbancy was measured at 600 mμ and the rates were calculated from the 30-second values as change of absorbancy per minute. The *p*H of the complete diaphorase systems measured at the termination of the determinations was always between 7.1 and 7.4. Spectrophotometric $NADH_2$ determinations were carried out in duplicate and diaphorase activity measurements in triplicate.

results (not shown in the figures) indicate that actually with both ATP and $NADH_2$ reversal of swelling is nearly complete in about 1 minute. Preliminary experiments indicate that the swelling of liver mitochondria is also reversible by $NADH_2$.

Stability of $NADH_2$ in the pH Zone of Contraction.—The contraction of the sarcosomes by $NADH_2$ in the acid *p*H zone is caused by the nucleotide itself and not by its products of acidity-induced transformation. Table I shows the effect of incubation at acid *p*H on the loss of $NADH_2$ (as measured by the absorption at 340 mμ) and on the ability of $NADH_2$ to support sarcosomal diaphorase activity. The table indicates that a substantial amount of $NADH_2$ remains unchanged after incubation for 2 hours at *p*H values as low as 3.5. Practically total loss of the nucleotide results when the incubation is at or below *p*H 2.5. That the 340-mμ-absorbing material is, in fact, unchanged $NADH_2$ is shown by the observation that these acid-treated preparations can support a high rate of sarcosomal diaphorase activity. Since the level of $NADH_2$ is not limiting down to *p*H 3.5, the rate of diaphorase activity is constant down to this value. Then at almost total loss of $NADH_2$ at *p*H 2.5 the rate suddenly decreases to close to zero. Other results not tabulated here show that even after incubation of $NADH_2$ for 7.5 hours, 44% remains unchanged at *p*H 4.0 and 24% at *p*H 3.5. This is consistent with a high rate of diaphorase activity supported by these solutions.

Paralleling these findings, $NADH_2$ stock solutions adjusted to *p*H 4.0 and incubated for 30 minutes at 23–25° were found to be as active to contract swollen sarcosomes as fresh neutral $NADH_2$ solutions followed by hydrochloric acid.

The $NADH_2$-induced contraction does not appear to be due to the formation of the "inhibitor substance" described by Fawcett *et al.* (1961) since reduced nucleotide samples of various origins and used freshly, or frozen and thawed repeatedly, gave comparable results.

Effect of Inhibitors on Reversal of Swelling by ATP and $NADH_2$.—Figure 9 shows that the ATP reversal of NAD-induced sarcosomal swelling is inhibited only slightly by 2×10^{-3} M sodium azide. Pronounced inhibition is produced, however, with very high levels (2×10^{-2} M and 5×10^{-2} M) of sodium fluoride (Fig. 9). At the concentrations of 1×10^{-4} M and 2×10^{-4} M, DNP did not inhibit the ATP + Mg^{2+} induced contraction of sarcosomes in which swelling was initiated by hypotonicity or by NAD. Neither did *p*-mercuri-

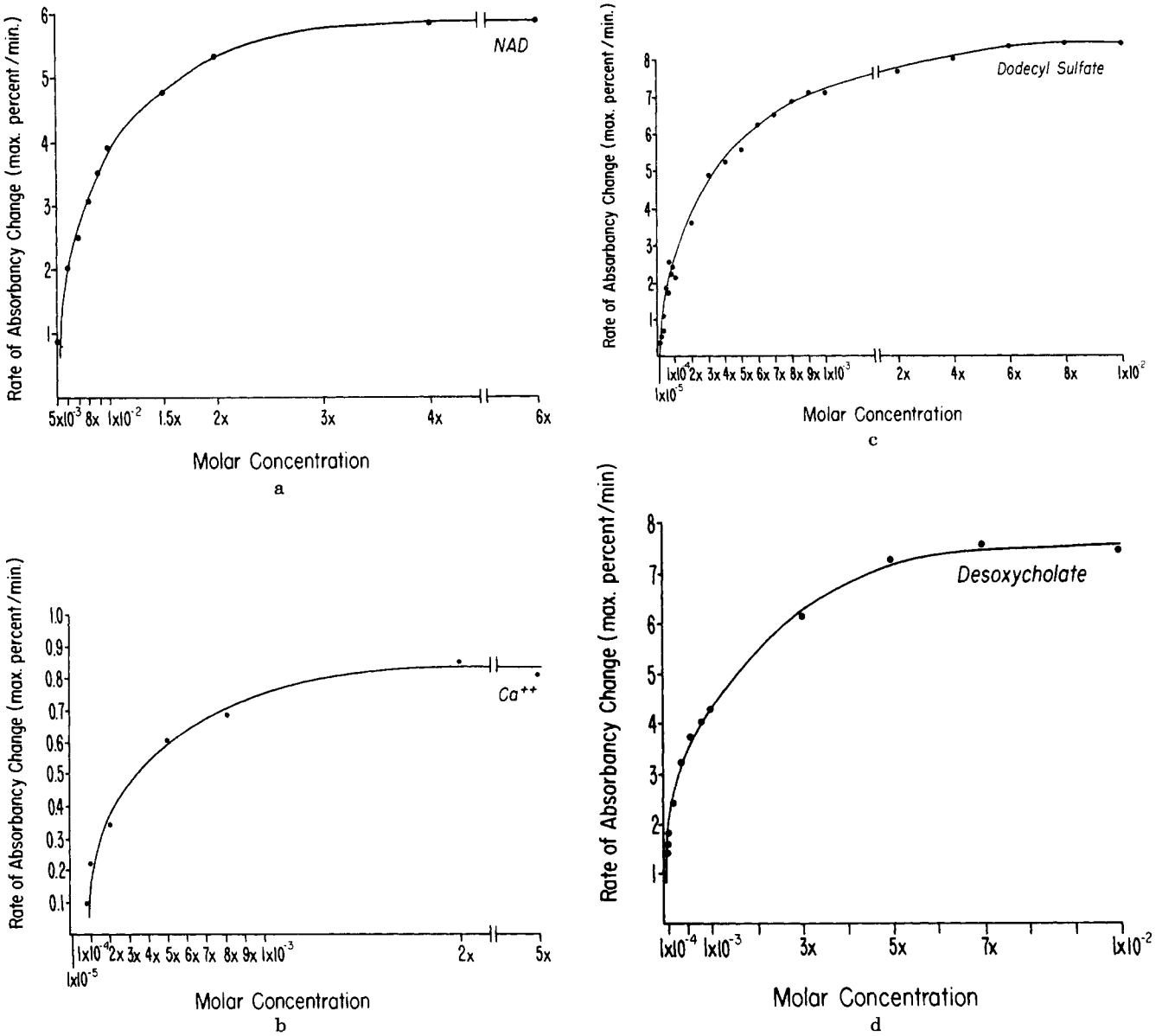

FIG. 5.—Kinetics of swelling of rat heart sarcosomes induced by NAD, Ca^{2+}, dodecylsulfate, and desoxycholate. (From the top: a, NAD; b, Ca^{2+}; c, dodecylsulfate; d, desoxycholate.) The test medium was 0.04 M sucrose buffered with 0.03 M Tris at *p*H 7.4. Each point represents the mean value obtained with ten rats, and corresponds to the initial rate of absorbancy decrease (max per cent/minute).

benzoate, a potent inhibitor of the ADP-ATP exchange enzyme and of the P_i-ATP exchange (Lehninger *et al.*, 1958), inhibit these reversals when present at the concentrations of 1×10^{-4} or 2×10^{-4} M provided that the *p*H was carefully adjusted to 4.0 Contraction was not inhibited at this *p*H by *p*-mercuribenzoate whether the ATP + Mg^{2+} solution (ATP at 5×10^{-3} M) was added 1 minute after *p*-mercuribenzoate, or following 10 minutes' incubation with the inhibitor.

Like ATP-induced contraction, the $NADH_2$-induced contraction of sarcosomes, in which swelling was initiated by hypotonicity or by NAD, is insensitive to various inhibitors. Contraction by $NADH_2$ appears rather to be enhanced by pentachlorophenol, 2,3-dimercaptopropanol, *p*-mercuribenzoate, and antimycin A to different degrees (Fig. 10). Control experiments have shown that without $NADH_2$ these inhibitors do not produce contraction when tested at the same concentrations. It is of importance for later discussion that DNP at 1×10^{-4} and 2×10^{-4} M does not inhibit the reversal by $NADH_2$ of NAD-induced swelling (Fig. 10) and of hypotonicity-initiated swelling. The action of *p*-mercuribenzoate and DNP in these experiments was not different when contraction was induced after swelling for only 5 minutes.

Phase-Contrast Microscopic Examination of Sarcosomal Kinetics and of Contraction by ATP and $NADH_2$.—Examination by phase-contrast microscopy (970×) under green-blue light indicated that the sarcosomes obtained in 0.44 M sucrose without EDTA are small spheres when observed in occasional resting stages; this is essentially in agreement with the observation of Cleland and Slater (1953b). However, the sarcosomes in the present preparations, which appeared to be devoid of cell debris, had a highly dynamic and mobile appearance, as they could be observed moving about in the field while undergoing very rapid reversible shrinking-swelling motions and elongations.[4] The

[4] The integrity of the sarcosomes has also been ascertained by the determination of the Q_{O_2} values, and the P:O and respiratory control ratios. The reaction mixture used to determine the first two parameters was identical to the one used in the experiments with EDTA (see Determination of Oxygen Uptake), except that only 40 μmoles of sodium

addition of a minute drop of a 1×10^{-2} M ATP solution, with or without 3×10^{-3} M $MgCl_2$, to the preparation on the slide caused immediate cessation of all sarcosomal movement and shrinkage to very small and dense granules. An identical effect was observed with a drop of 1.5×10^{-2} M $NADH_2$ followed by hydrochloric acid to bring the *p*H to about 4.0. The effect of the two nucleotides may not be ascribed to the lowering of the *p*H, since addition of acid alone, even in excess (a drop of 0.2 N HCl), causes swelling and the mobility of the sarcosomes is maintained to some extent.

Discussion

Effect of EDTA on Sarcosomes and Mitochondria.—The present results suggest that the chelation of Ca^{2+} may be only one facet in the maintenance of sarcosomal integrity by EDTA when isolating in 0.25 M sucrose. Intraparticulate cross-linking of the sarcosomal membrane, by multipoint attachment of EDTA to basic groups or to groups capable of hydrogen bonding, may be a concurrent mechanism. This may be evidenced by the here observed decreased swelling ability of sarcosomes which are isolated in the presence of EDTA. In agreement with this is the tight binding of [^{14}C]-EDTA to mitochondrial particles (cited by Lehninger, 1962). Consistent with multipoint attachment is also the decreasing relative sarcosomal yield with increasing EDTA concentration, which may be accounted for by interparticulate cross-linking in the homogenate causing the formation of aggregates sedimented with the nuclear fraction.

The considerable inhibition of the respiration of heart sarcosomes by EDTA does not appear to be in agreement with the results of Slater (1957), the latter indicating strong enhancement of sarcosomal respiration by EDTA. We are at loss to explain this discrepancy but it may be pointed out that: (a) The sarcosomes used here were obtained in 0.44 M sucrose which must be considered closer to isotonicity with sarcosomal and mitochondrial particles than 0.25 M sucrose, since there is a much lower level of "spontaneous" swelling in the former solution. (b) The mechanical homogenization employed in the present experiments probably gives preparations of more uniform quality than the procedure of liberating sarcosomes by manual grinding with acid-washed sand (Cleland and Slater, 1953a,b). Essentially identical mechanical homogenizing technique in 0.44 M sucrose yields liver mitochondria of excellent morphology (Siekevitz and Watson, 1956), although liver mitochondria are less resistant to mechanical stress than are sarcosomes. (c) The physical and kinetic integrity of the sarcosomes used here has been ascertained by microscopic observation; they also gave usual Q_{O_2} values with different substrates, P:O ratios close to the theoretical ones, and high respiratory control levels.[4]

Swelling of Sarcosomes.—The appearance of maxima and minima in the extent of sarcosomal swelling when the total molarity of the medium is varied (Fig. 3b)

fluoride was present here and substrates other than α-ketoglutarate have also been used (all at the level of 20 μmoles). The following values (averages of three experiments) were obtained: with pyruvate (and 2 μmoles of fumarate as "sparker") Q_{O_2} = 86.0, P:O = 2.80; with α-ketoglutarate Q_{O_2} = 63.5, P:O = 2.81; with succinate Q_{O_2} = 71.1, P:O = 1.73; with malate Q_{O_2} = 49.8, P:O = 2.81. The respiratory control ratios were determined using the reaction mixture of Holton *et al.* (1957), with ADP as acceptor, and substrates at the level of 20 μmoles. The following values (averages of four experiments) were obtained: 2.65 with glutamate, 1.78 with pyruvate (and 2 μmoles of fumarate), 1.40 with α-ketoglutarate.

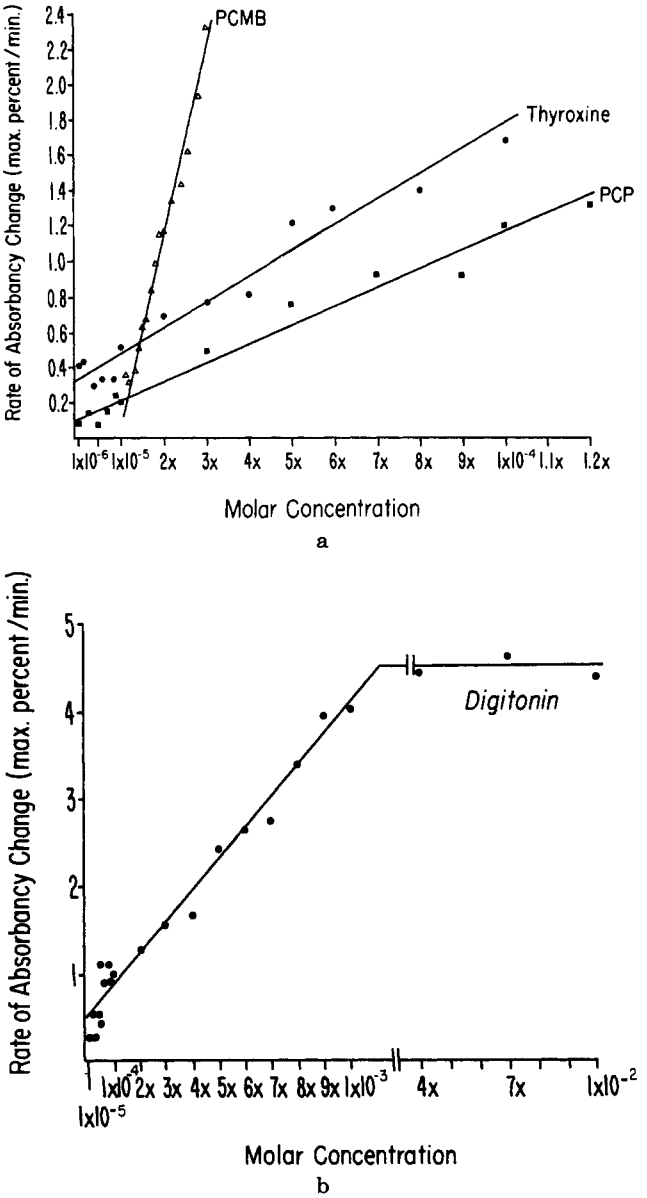

FIG. 6.—Kinetics of swelling induced by thyroxine, PCP, and PCMB (a, upper), and of solubilization by digitonin (b, lower), of rat heart sarcosomes. The test medium was 0.04 M sucrose buffered with 0.03 M Tris at *p*H 7.4. Each point represents the mean value obtained with ten rats and corresponds to the initial rate of absorbancy decrease (max per cent/minute). Abbreviations used: PCMB, *p*-mercuribenzoate; PCP, pentachlorophenol.

suggests that the state of the sarcosomal membrane (cf. Lehninger, 1962) and particulate morphology (cf. Harman and Feigelson, 1952) are subject to intracellular regulation by means of small over-all changes or gradients of osmolarity. The substrate-selective inhibition of mitochondrial oxidations by enhanced tonicity (Johnson and Lardy, 1958) is of interest in this connection. The fact that the sharp *p*H-dependent swelling maximum (at *p*H 7.4) in 0.04 M sucrose is abolished when the concentration is raised to 0.44 M (Fig. 4) may be construed as further suggestive evidence for regulation of the membrane by osmolarity and *p*H gradient changes, and the interrelationship of these regulatory modalities (cf. Lehninger, 1962).

In view of the interesting fact that NAD induces sarcosomal swelling it may be recalled that this nucleotide is also a potent agent to induce the swelling of rat liver mitochondria (Corwin and Lipsett, 1959; Arcos

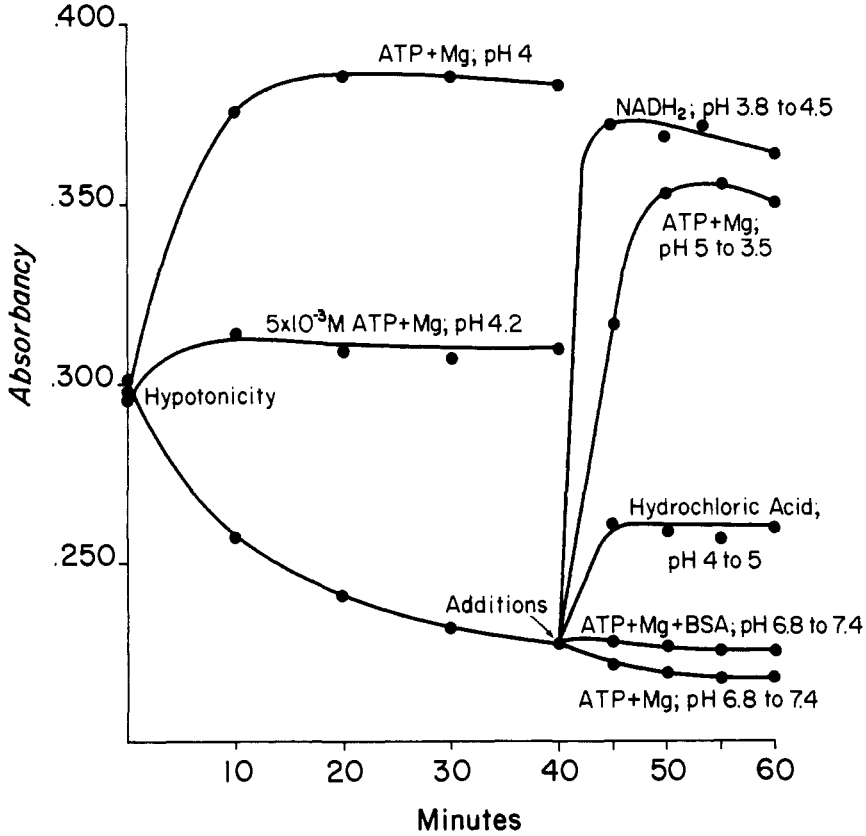

FIG. 7.—Inhibition by ATP and reversal by ATP and $NADH_2$ of hypotonicity-initiated swelling of rat heart sarcosomes in 0.04 M sucrose + 0.03 M Tris solution, adjusted initially to *p*H 7.4. Unless otherwise specified, ATP was at the concentration of 1×10^{-2} M and $NADH_2$ at 1.5×10^{-2} M. Magnesium, added as $MgCl_2$, was at 3×10^{-3} M, and bovine serum albumin (abbreviated as BSA) was at the level of 2 mg/test system. The *p*H values on the figure represent final values of the complete assay mixtures, measured at the termination of the runs. All points are averages obtained with four to five animals.

et al., 1960). That sarcosomal swelling induced by NAD and Ca^{2+} (Fig. 5a,b) follows the kinetics of drug-receptor complex formation is in agreement with analogous observations made with liver mitochondria (Gosch *et al.*, 1962). The specific loci upon which these agents act in the sarcosomes in order to initiate swelling are "sites" in the pharmacological or enzyme-kinetic sense. Since swelling produced by dodecylsulfate and desoxycholate follow similar kinetics (Fig. 5c,d), the action of these agents must involve well-defined sites and is probably more specific than labilization of lipid constituents of the membrane by surface-active action.

The linear relationship found with the uncouplers of oxidative phosphorylation, *p*-mercuribenzoate and pentachlorophenol, possibly reflects the formation of stable combinations with the respective sites. For *p*-mercuribenzoate it is well known that this reagent combines covalently with various —SH-containing proteins, and there is strong indication for the presence of —SH and —S—S— groups in the mitochondrial membrane and for their importance in swelling (Arcos *et al.*, 1960; Neubert and Lehninger, 1962). For pentachlorophenol evidence has been provided that this halophenol is firmly bound by mitochondrial protein (Weinbach, 1963) and by the contractile protein, myosin (Weinbach and Bowen, 1958). The linear relationship found with thyroxine is unexpected in view of the Michaelis-Menten type kinetics of the swelling of liver mitochondria induced by this agent (Gosch *et at.*, 1962). This apparent anomaly of thyroxine-induced swelling of heart sarcosomes is now being further investigated.

The Acid pH Requirement for Reversal and Inhibition of Swelling by ATP.—Previous reports on the ATP-produced contraction of mitochondrial particles (pigeon breast sarcosomes [Chappell and Perry, 1954], rat liver mitochondria [e.g., Lehninger, 1959a,b; Lehninger *et al.*, 1959], and rat heart sarcosomes [Nakamura *et al.*, 1961]) state neither if the ATP added to the swollen particles was neutralized, nor the *final* *p*H of the medium in which the contraction actually occurred. The importance of these experimental details is stressed by the present results which show that to obtain contraction of heart sarcosomes by either ATP or $NADH_2$ it is critical that the *p*H be between 3.5 and 5. The effect is specific for ATP and $NADH_2$ in that addition of hydrochloric acid does not reverse swelling induced under various conditions, with the exception of hypotonicity-initiated swelling in which addition of acid alone produced a small increase of absorbancy. Other workers have indicated some extent of acid-induced reversal of hypotonicity-initiated swelling of *liver* mitochondria (Novelli *et al.*, 1962). While no explanation may be offered for the now observed *p*H requirement for contraction, it likely bears a relation to the proton uptake by mitochondria at *p*H 5 in the absence of active electron transport and the strong stimulation of this uptake by DNP and other uncoupling agents as observed by Mitchell (1961) and confirmed by Greville (1963).

The *p*H zone in which the contraction of sarcosomes by ATP occurs indicates that the phenomenon requires very small if any electron transport. While the extent of electron transport in sarcosomes at acid *p*H does not appear to have been studied, it is indicative that at *p*H 4 liver mitochondria oxidize β-hydroxybutyrate

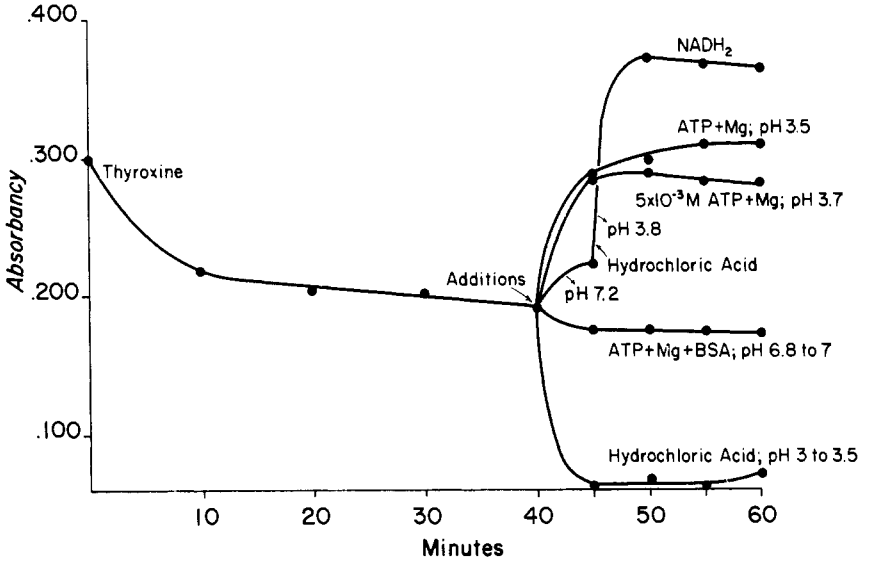

FIG. 8.—Reversal by ATP and $NADH_2$ of thyroxine-induced swelling of rat heart sarcosomes. Experimental conditions were identical to those given in the legend to Fig. 7, except that the test system used here contained, in addition, *l*-thyroxine at the level of 5×10^{-5} M. All points represent averages obtained with three to five rats. Abbreviation: BSA, bovine serum albumin.

only to a very small extent and there is no measurable phosphate uptake (Cooper and Lehninger, 1956). Electron transport is not required for reversal of swelling of liver mitochondria by ATP, since contraction is not inhibited by cyanide (Lehninger, 1959a,b). It must be noted, however, that the cited latter results require confirmation since extensive loss of cyanide may have occurred under the *p*H conditions of the experiment (*vide supra* in this section). Contraction of sarcosomes by ATP does not appear to involve the phosphorylative coupling sequence either, for two reasons: (1) In the optimum *p*H zone for reversal, *p*-mercuribenzoate does not inhibit contraction at concentrations at which it is inhibitory to ADP-ATP or P_i-ATP exchange; (2) DNP-activated ATPase activity, which presumably reflects the reverse action of a segment of the coupling sequence (Hunter, 1956), appears to have multiple *p*H optima which are all higher (Myers and Slater, 1957) than the *p*H zone required for contraction.

Swelling by NAD and Contraction by $NADH_2$.—An attractive explanation for the induction of rapid swelling by NAD and contraction by $NADH_2$ is that the nucleotide brings about the extreme oxidized or reduced state of the respiratory chain without the occurrence of sustained electron transport. The membrane of sarcosomes is permeable to both NAD and $NADH_2$ and the initially high permeability of sarcosomes to the latter is increased further by hypotonic treatment or environment (Boxer and Devlin, 1961; Arcos *et al.*, 1964a). Conditions which increase the accessibility of extrasarcosomal NAD and $NADH_2$ to particle-bound NAD may, however, also increase the accessibility to other members of the respiratory carrier (cf. Green *et al.*, 1954). Thus, segments of the "open" carrier may be oxidized or reduced, without sustained electron transport, by more direct interaction of the exogenous oxidized or reduced nucleotide with individual carrier enzymes, made possible by the increased permeability of the lipoprotein envelope. The kinetics of NAD-induced swelling (Fig. 5a) is actually indicative of direct interaction of the nucleotide with receptor sites in the pharmacological or enzyme-kinetic sense. The involvement of the coupling sequence in $NADH_2$-induced contraction is ruled out by the finding that various uncouplers of phosphorylation do not inhibit

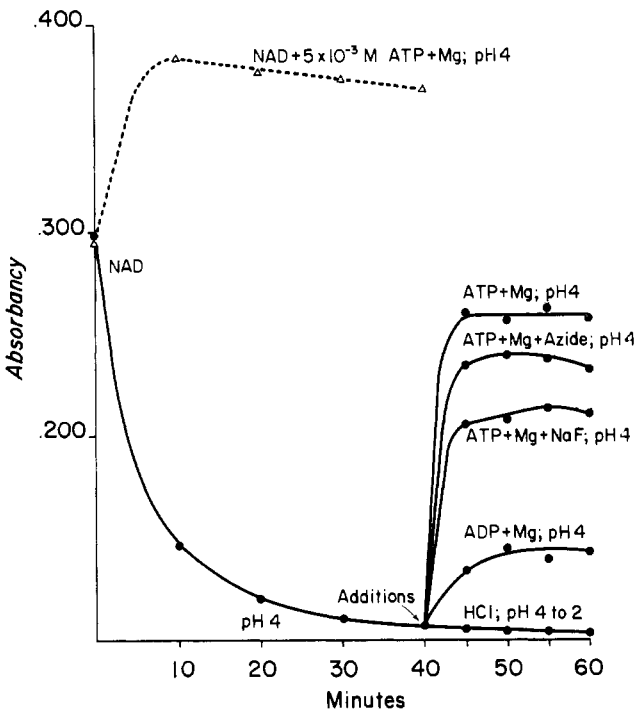

FIG. 9.—Inhibition and reversal by ATP of NAD-induced swelling of rat heart sarcosomes. Experimental conditions were identical to those given in the legend to Fig. 7, except that the test system used here contained, in addition, NAD at the level of 1.5×10^{-2} M. When specified, azide was present at 2×10^{-3} M, NaF at 2×10^{-2} M or at 5×10^{-2} M, and ADP at 1×10^{-2} M. All points represent averages obtained with five animals.

this contraction (Fig. 10). Therefore, since the oxidation of the respiratory chain leads to swelling (e.g., Hunter *et al.*, 1959) and reduction of the chain to the inhibition of swelling or to the contraction of mitochondrial particles (e.g., Lehninger and Ray, 1957; Holton, 1957, 1959; Packer, 1960), the swelling and contracting effect of NAD and $NADH_2$, respectively, is apparently brought about by way of the respiratory chain. This requires structural association between the "contractile protein" in the membrane (Ohnishi and

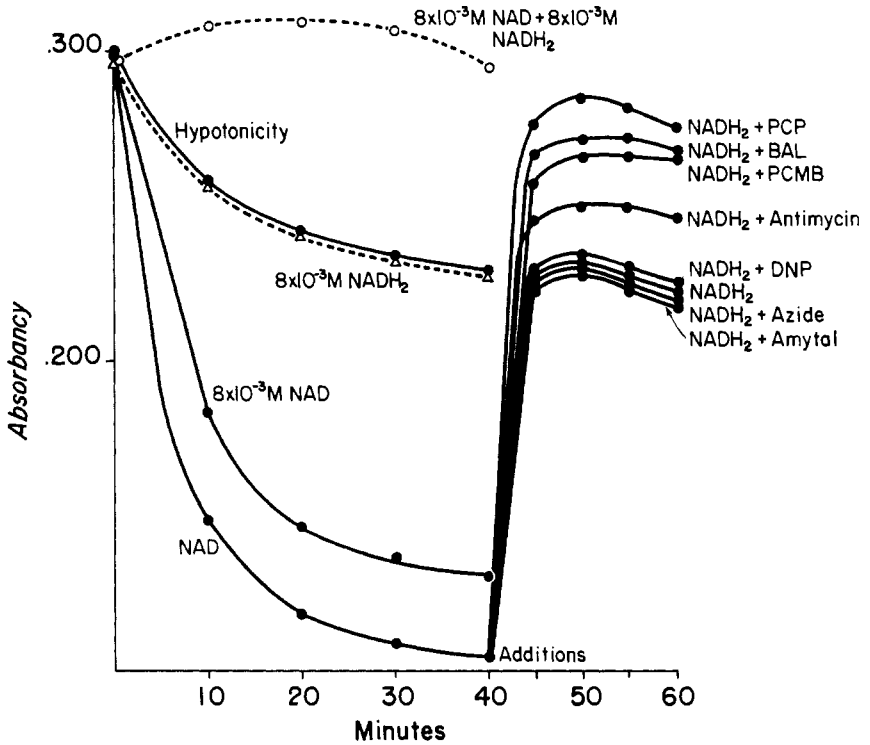

FIG. 10.—Inhibition and reversal by $NADH_2$ of NAD-induced swelling of rat heart sarcosomes. The test system was as in the legend to Fig. 7. Except when otherwise stated both NAD and $NADH_2$ were at 1.5×10^{-2} M. The inhibitors were at the following concentrations: amytal 5×10^{-3} M, sodium azide 5×10^{-3} M, 2,4-dinitrophenol (DNP) 1×10^{-4} M or 2×10^{-4} M, antimycin A 2×10^{-5} M, *p*-mercuribenzoate (PCMB) 1×10^{-4} M, 2,3-dimercaptopropanol (BAL) 1×10^{-2} M, and pentachlorophenol (PCP) 1×10^{-4} M. Except for hypotonicity-initiated swelling in the absence or presence of 8×10^{-3} M $NADH_2$ (both at *p*H 6.8–7.2), all terminal *p*H values were between 3.5 and 4.6. All points represent averages of three to five experiments.

Ohnishi, 1962; Vignais *et al.*, 1963; Packer, 1963) and the respiratory chain, whereby conformational changes in the contractile macromolecules are brought about by reversible conformational changes in carrier enzymes during oxidation and reduction. There is increasing evidence for conformational changes during various types of enzyme action (reviewed in Okunuki, 1961; Koshland, 1963).

Relation of the Contractile Protein to the Coupling Sequence and the Electron Transport Chain.—The results discussed in the previous two sections suggest that the "contractile protein" constitutes an alternate pathway between the sarcosomal ATP pool and the respiratory chain,[5] "shunting" the coupling sequence. This apparent relationship is shown schematically in Figure 11 and is summarized as follows.

In a mechanistic conception of the respiratory chain the individual carriers are alternately oxidized and reduced during electron transport. Consider a particular instant when Carrier 1 is in the oxidized form. This is the high-energy state of Carrier 1 with respect to phosphorylation, since ADP-ATP exchange proceeds at maximal rate when the respiratory chain is fully oxidized (Lehninger *et al.*, 1958). This means that it is the oxidized form of Carrier 1 which is in equilibrium with ATP. At this same time Carrier 2 is in the reduced form. This is the high-energy state of Carrier 2 with respect to producing sarcosomal and mitochondrial contraction because when the respiratory chain is fully reduced (as in anaerobiosis, in the presence of cyanide or of large excess of $NADH_2$) inhibition or reversal of swelling occurs (Lehninger and Ray, 1957; Holton, 1957, 1959; Hunter *et al.*, 1959; Packer, 1960; and the present results).

It must be recalled that swollen mitochondria are in a low-energy state because swelling is concomitant with the depletion of the intraparticulate ATP pool (Raaflaub, 1953; Brenner-Holzach and Raaflaub, 1954). Contracted mitochondria are in the high-energy state because: (a) contraction also results by the action of ATP, as well as by reduction of the respiratory chain; (b) no ATP-induced contraction is known which does not involve its hydrolysis (Lehninger, 1962; Weinbach *et al.*, 1963); and (c) conditions in which mitochondria and sarcosomes can carry out oxidative phosphorylation also protect against swelling (Harman

[5] Preliminary results of other experiments in this Laboratory indeed provide support for the existence of a pathway, alternative to the coupling sequence, between ATP and the electron transport chain: When glucose-hexokinase is omitted *concurrently with* NaF from the respiratory reaction mixtures of sarcosomes (using 20 μmoles pyruvate plus 2 μmoles fumarate as substrate) there occurs no appreciable lowering of the oxygen uptake, presumably because the high level of Mg-activated ATPase activity of "intact" heart sarcosomes (Azzone *et al.*, 1960) functions as "ATP-trap." Since DNP increases further this ATPase activity (Azzone *et al.*, 1960), the Mg-activated ATPase is apparently distinct from the DNP-activated ATPase. This has been supported recently by the observation that in digitonin fragments of mitochondria, Mg^{2+} greatly stimulates ATPase activity but not respiratory release (Lehninger and Gregg, 1963). In our sarcosomal preparations, where oxygen uptake proceeds freely when both NaF and phosphate acceptor are omitted, addition of high levels of ATP produces inhibition of the respiratory rate (attaining 50–60% inhibition with 200 μmoles) which is expected because of the coupled nature of these sarcosomes. In contrast, in the presence of 1×10^{-4} M DNP which establishes a block in the coupling sequence, addition of 80–120 μmoles ATP produces 25% *enhancement* of the respiratory rate as compared to the control containing DNP alone.

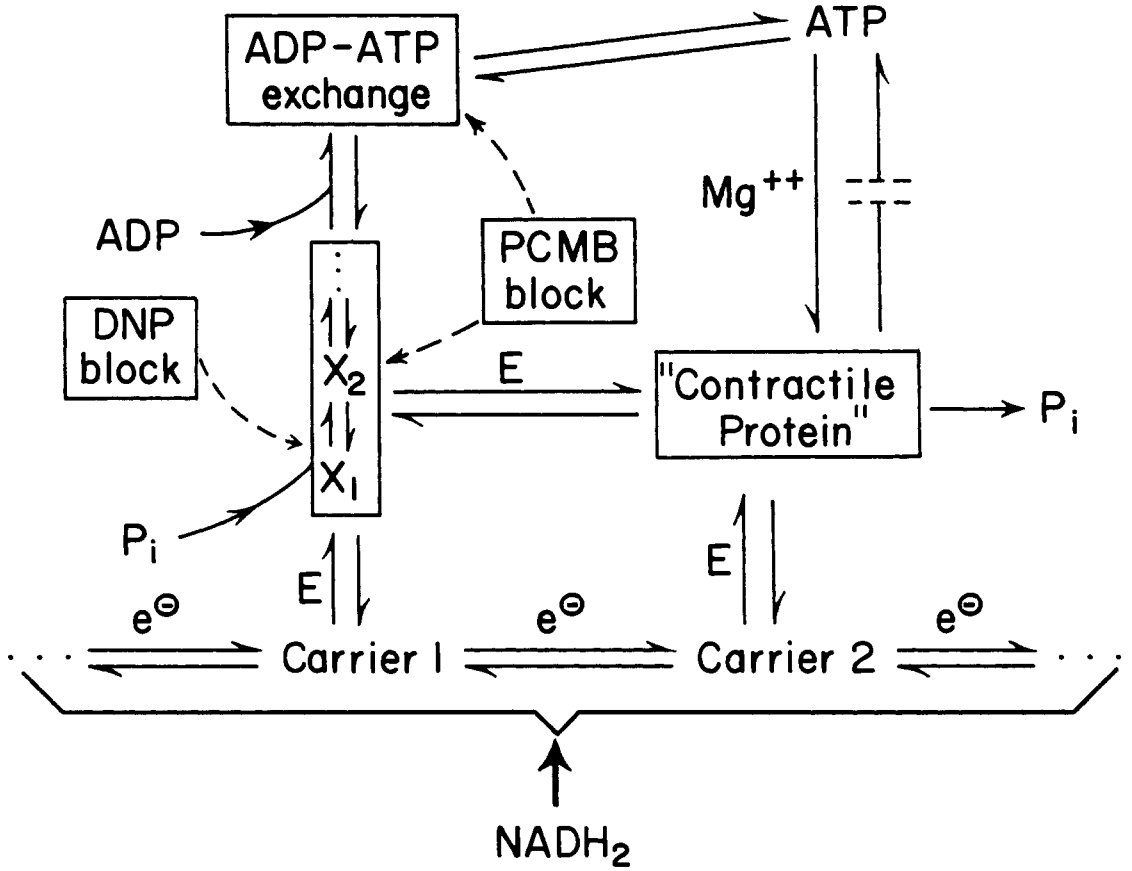

FIG. 11.—Scheme relating the sarcosomal "contractile protein" to the coupling sequence and the respiratory chain. Components "x_1," "x_2," etc., represent members of the coupling sequence, "E" the energy required to drive a reversible change of steric conformation in the structurally connected macromolecule, and "$e^{\ominus}$" the electron flux in the respiratory chain. Carrier 1 and Carrier 2 are subsequent members of the electron transport chain at one or more sites of ATP synthesis. The broken arrow between ATP and the "contractile protein" represents the situation that the forward reaction comes to a stop when the two energy levels are equalized, i.e., in at least one instance ATP hydrolysis ceases when mitochondria reach the fully contracted state (Lehninger, 1962).

and Feigelson, 1952; Price *et al.*, 1956; DiSabato and Fonnesu, 1959). Therefore, with respect to ATP, it is the reduced form which represents the high-energy state of Carrier 2, because both ATP and reduction of the respiratory chain can bring about contraction.

At a second instant during electron transport, Carrier 1 is in the reduced form, which is its low-energy state with respect to phosphorylation because ADP-ATP exchange is minimal when the respiratory chain is fully reduced (Lehninger *et al.*, 1958). At this same time Carrier 2 is in the oxidized form. This is the low-energy state of Carrier 2 with respect to producing sarcosomal and mitochondrial contraction because oxidation of the respiratory chain brings about swelling (e.g., swelling is produced by NAD; or the presence of terminal electron acceptor is required for swelling; see Lehninger and Ray, 1957, and Hunter *et al.*, 1959). In this second instant of electron transport, consequently, no energy is released into either chain-linked pathway. Therefore, in a quantized fashion, it is only every half-cycle (of receiving and giving up electrons) which results in the release of packets of energy-equivalent simultaneously into the coupling sequence and the "contractile protein." During actual electron transport the energy level of the coupling sequence and of the "contractile protein" adjust accordingly to the oxido-reduction steady-state of Carrier 1 and of Carrier 2.

When contraction is brought about by carrier reduction, some "leakage" of the energy content of the "contractile protein" may occur through the energy pathway to the coupling intermediates. Such "leakage" may not occur when contraction is brought about by ATP because forward reactions of the coupling sequence are minimized by excess ATP. This is indicated by the fact that in coupled sarcosomes excess ATP inhibits respiration[5] which in coupled particles is dependent on forward reactions of the coupling sequence. Therefore certain uncouplers of oxidative phosphorylation enhance $NADH_2$-produced contraction (Fig. 10) presumably because the "leak" of energy from the "contractile protein" via the coupling sequence is blocked. The degree of enhancement possibly depends on the activity of the inhibitor and on the position of the block relative to the level of the connection of the "contractile protein" and the coupling sequence. The same conclusion, that structural association exists between intermediate(s) of respiratory chain phosphorylation and macromolecules which determine swelling and contraction, has been reached on different grounds by Packer and Tappel (1960).

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