

On the Mechanism of Oxidative Phosphorylation. IV. Mitochondrial Swelling Caused by Arsenite in Combination with 2,3-Dimercaptopropanol and by Cadmium Ion*

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Previous work has shown either arsenite in combination with 2,3-dimercaptopropanol, or cadmium ion alone, to be a general uncoupler of mitochondrial oxidative phosphorylation. Such uncoupling might result either directly from the action of these reagents on the enzymatic reactions of coupled phosphorylation, or indirectly from their action on the structural integrity of the mitochondria. The swelling activity of these uncoupling agents has been examined and both have been found to cause rapid and extensive decrease in the light-scattering ability of mitochondrial suspensions. Simultaneous measurements of oxygen consumption, oxidation state of pyridine nucleotides, and light scattering have shown that the first events following addition of either arsenite and 2,3-dimercaptopropanol or cadmium to rat liver mitochondria in the presence of β -hydroxybutyrate are the oxidation of endogenous reduced pyridine nucleotides and the loss of respiratory control. Light-scattering changes generally lag behind oxidation of reduced pyridine nucleotide. With mitochondria which have been allowed to swell under the influence of these uncoupling agents, phosphorylative ability can be restored by the addition of excess 2,3-dimercaptopropanol, while swelling is not reversed. The results establish the independence of the uncoupling from the swelling which occurs as the result of the action of arsenite in combination either with 2,3-dimercaptopropanol or with cadmium ion in rat liver mitochondria.

Previous work has demonstrated that cadmium ion alone and arsenite in combination with a dithiol compound will uncouple oxidative phosphorylation in rat liver mitochondria (Jacobs *et al.*, 1956; Fluharty and Sanadi, 1960, 1961). When an excess of 2,3-dimercaptopropanol (BAL) was added to mitochondria which had been exposed previously to the above uncoupling agents, the phosphorylation was recoupled. The uncoupling was attributed to the binding by these reagents of hypothetical dithiol groupings functional in oxidative phosphorylation.

It has been recognized generally that extensive disruption of mitochondrial integrity leads to the uncoupling of phosphorylation from oxidation (Lehninger, 1953-54). Conversely, interference with phosphorylation often, but not always, leads to extensive structural modification (DiSabato and Fonnesu, 1959; Lehninger, 1958). Thus it is difficult to assess whether the primary effect of an added compound is to produce uncoupling or to distort the structure. Both the combined arsenite-2,3-dimercaptopropanol reagent and cadmium ion have been found to produce considerable light-scattering change in mitochondrial suspensions. In these instances it has been possible to show that the structural modification is independent of and follows uncoupling.

EXPERIMENTAL PROCEDURE

The preparation of rat liver mitochondria, the manometric assays for oxidative phosphorylation,

* Paper III: Fletcher and Sanadi (1961).

and the standard experimental conditions for demonstration of uncoupling by cadmium ion or arsenite-2,3-dimercaptopropanol¹ have been described previously (Jacobs *et al.*, 1956; Fluharty and Sanadi, 1961). The trial experiments on mitochondrial swelling were carried out as described by Hunter *et al.* (1959). The absorbancy change at 520 m μ observed in the Beckman DU spectrophotometer at a fixed slit width has been taken as a measure of the swelling, although changes in hydration have not been determined. In order to evaluate swelling under active phosphorylation conditions, parallel measurements of the ratio of phosphate esterification to oxygen consumption (P/O) and light scattering were carried out in independent flasks as described in the legend to Figure 4. The P/O calculations have been made over the entire period of measurement, although the oxidations in the presence of arsenite-2,3-dimercaptopropanol or cadmium ion prior to the addition of the reversing agent are expected to be uncoupled (Jacobs *et al.*, 1956; Fluharty and Sanadi, 1960, 1961).

For the simultaneous measurement of respiration, oxidation of reduced pyridine nucleotides, and light scattering, the methods developed by Chance and co-workers (Chance and Williams, 1955a; Chance, 1959) were used. The vibrating platinum

¹ Arsenite in the presence of 2,3-dimercaptopropanol will be referred to as arsenite-2,3-dimercaptopropanol. The compounds are usually present in nearly equivalent amount. The concentration given will be that of the reagent present in lower concentration, which would approximate the concentration of the cyclic dithioarsenite.

microelectrode was used for the determination of oxygen consumption. Oxidation of reduced pyridine nucleotide was determined fluorometrically with 365 m μ excitation and 450 m μ measurement. The light scattering was measured simultaneously by the absorbancy change at 540 m μ .

RESULTS

A typical experiment on the effect of arsenite-2,3-dimercaptopropanol on respiration, concentration of endogenous DPNH, and light scattering by rat liver mitochondria are shown in Figure 1. Mitochondria diluted in a reaction medium containing phosphate, but no ADP, are initially in state 1 (ADP and substrate limiting) as defined by Chance and Williams (1955b). Under these conditions, the rat liver preparation used in these experiments (Fig. 1) showed a slow decrease of fluorescence due to oxidation of endogenous pyridine nucleotides, *i.e.*, a slow transition from state 1 to 4 (ADP limiting) (Chance and Williams, 1955b). Addition of 4 mM β -hydroxybutyrate caused a downward deflection of the fluorescence trace, indicating a reduction of pyridine nucleotide and a slow decrease of light scattering by the suspension. One minute later 38 μ M 2,3-dimercaptopropanol was added, producing a slight decrease of fluorescence, a cessation of light-scattering change, and a small burst of respiratory activity. The subsequent addition of 55 μ M arsenite produced no immediate alteration in the situation. After 1 minute the fluorescence began to decrease rapidly, but the respiration and light scattering changes

commenced only after an additional delay of 30 to 40 seconds, at which time the fluorescence change was already at its maximum rate and the observable net change in DPNH was nearly half completed. When maximum oxidation of the pyridine nucleotides was achieved, as evidenced by no further fluorescence decrease, the respiration rate declined almost to zero and the light-scattering change continued at a diminished rate. At this point succinate was added, causing an immediate acceleration of respiration to the rapid rate characteristic of an uncoupled system. No change of fluorescence was observed, indicating that the system was sufficiently uncoupled so that the pyridine nucleotide could no longer be reduced by succinate (Chance and Hollunger, 1961). The respiration rate with the succinate was maximal (no stimulation by ADP) and continued until oxygen was exhausted. At this point a small increase of fluorescence was observed, indicating pyridine nucleotide reduction; however, the bulk of pyridine nucleotide could not be reduced even in anaerobiosis, despite the presence of both succinate and β -hydroxybutyrate. The inhibition of respiration with β -hydroxybutyrate in the presence of arsenite-2,3-dimercaptopropanol (before addition of succinate) is consistent with manometric data (Fluharty and Sanadi, 1960, 1961) which showed that the respiration could be restored only by the addition of DPN.

Similar experiments with cadmium ion are illustrated in Figure 2. The mitochondria in this case were suspended in a medium containing sucrose, phosphate, MgCl₂, and β -hydroxybutyrate. Thus, the respiration proceeded at a normal rate

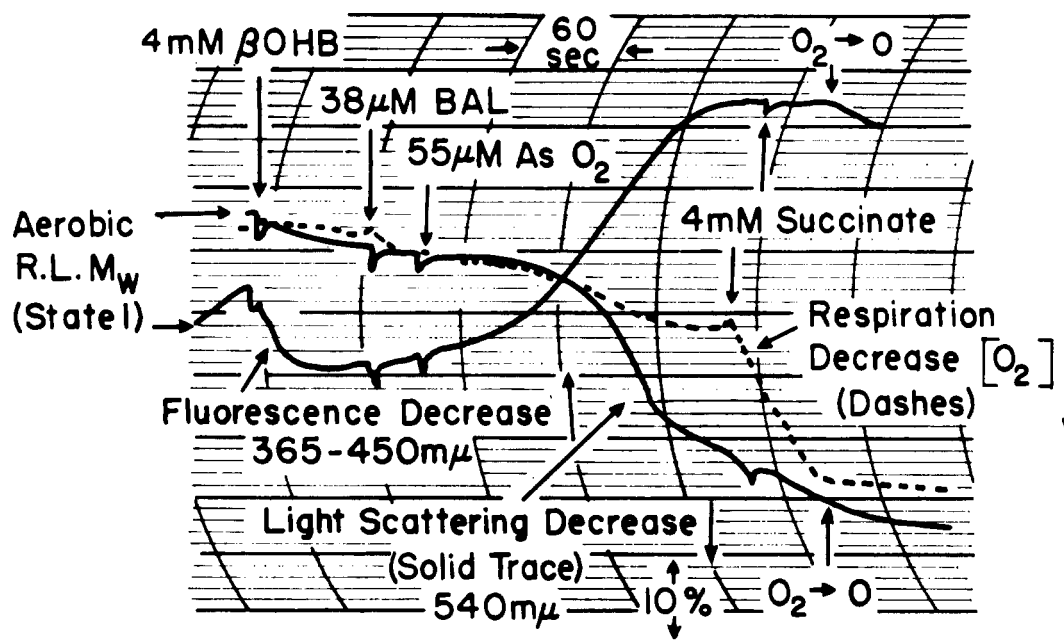


FIG. 1.—Simultaneous recordings of the effects of arsenite-2,3-dimercaptopropanol (BAL) on mitochondrial respiration, oxidation of reduced pyridine nucleotide, and light scattering. The mitochondria were suspended in an aerobic medium containing 0.25 M sucrose, 0.02 M phosphate, pH 7.2, and 7 mM MgCl₂. The various additions shown in the figure are described in the text.

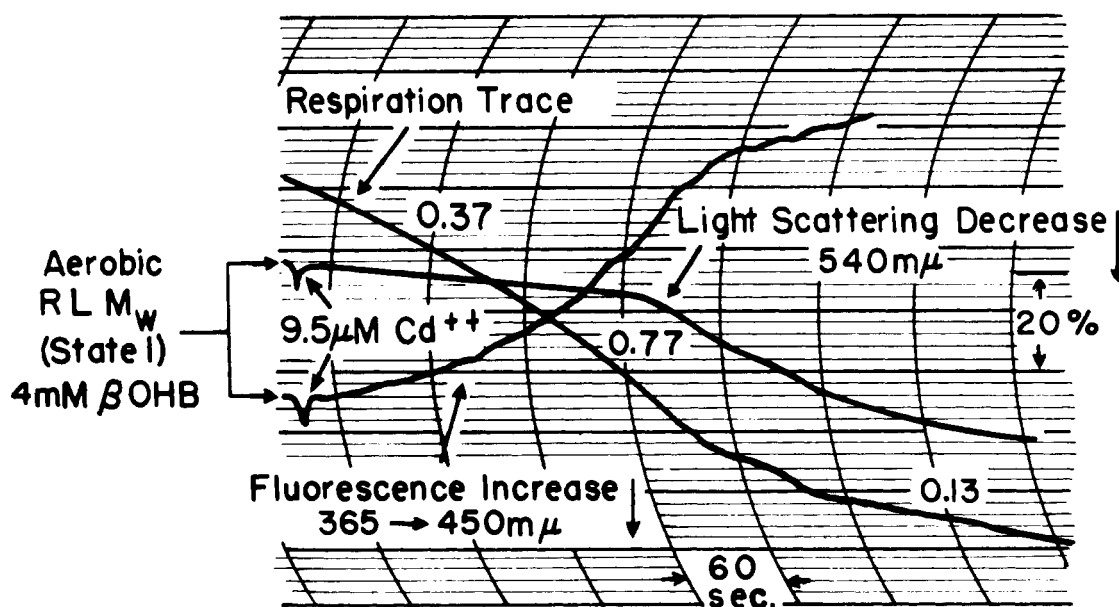


FIG. 2.—Simultaneous recordings of the effects of cadmium ion on mitochondrial respiration, oxidation of reduced pyridine nucleotide, and light scattering. The experiment was done as in Figure 1. The initial medium also contained β -hydroxybutyrate. The rates of change in oxygen concentration (μ mole per minute) at various times are shown opposite the respiration trace, and the addition is described in the text.

and the pyridine nucleotide was largely reduced. Addition of $9.5 \mu\text{M}$ cadmium chloride caused no immediate effect on fluorescence, light-scattering, or respiration. After a few seconds the fluorescence trace drifted upward, indicating oxidation of re-

duced pyridine nucleotide, a reaction which accelerated sharply at the end of 2 minutes and reached a maximal rate at the end of 3 minutes. At this point the respiration rate had doubled, and the light scattering began to decrease abruptly. There was nearly a 3-minute difference between the onset of fluorescence change and the onset of light-scattering change. As the fluorescence change reached a plateau, the decrease in light-scattering slowed and respiration became inhibited.

In Figure 3 the swelling effect of arsenite-2,3-dimercaptopropanol has been compared with that of phosphate under the experimental conditions used by Hunter *et al.* (1959). It is apparent that arsenite-2,3-dimercaptopropanol is a far more potent swelling agent than either arsenite or 2,3-dimercaptopropanol alone at these levels. Since the data in Figure 3 do not shed any light on the relationship between swelling and oxidative phosphorylation, the experimental conditions were altered to allow parallel measurement of both light scattering and phosphate esterification. Figure 4 shows that the rapid swelling of mitochondria observed in the presence of arsenite-2,3-dimercaptopropanol is largely prevented if excess 2,3-dimercaptopropanol is added while the mitochondrial suspension is still cold; *i.e.*, time zero. If the 2,3-dimercaptopropanol is added to the assay mixture after the oxidation and swelling have proceeded for 4 to 6 minutes, the swelling is not reversed. However, coupled phosphorylation is restored to a large extent, as shown by the P/O values of 1.20 and 1.30.

Figure 5 demonstrates that cadmium ion produces similar swelling in mitochondria which is prevented but not reversed by 2,3-dimercaptopro-

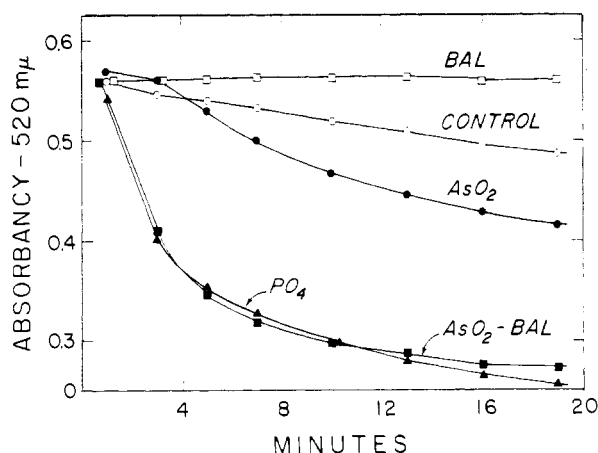


FIG. 3.—Light-scattering changes in mitochondria under the influence of various compounds. The reaction medium contained 0.3 M sucrose, 0.025 M tris(hydroxymethyl)amino-methane (pH 7.5), and 0.6 mg. mitochondria in 3 ml. The first measurement at $540 \text{ m}\mu$ in a Beckman DU spectrophotometer (slit width of 0.02 mm) was made 1 minute after the addition of mitochondria. The compound under test was present in the medium prior to the addition of mitochondria except with arsenite-2,3-dimercaptopropanol (BAL). In this case, arsenite was in the medium, and mitochondria and 2,3-dimercaptopropanol were added in quick succession. $\circ$ — $\circ$ control; $\blacktriangle$ — $\blacktriangle$ 2.5 mM phosphate; $\bullet$ — $\bullet$ 0.17 mM arsenite; $\square$ — $\square$ 0.19 mM 2,3-dimercaptopropanol; $\blacksquare$ — $\blacksquare$ 0.17 mM arsenite plus 0.19 mM 2,3-dimercaptopropanol.

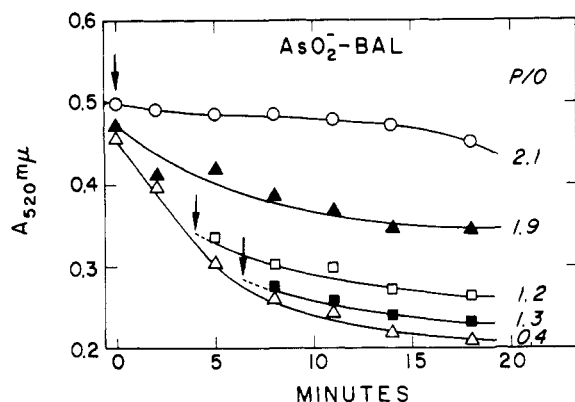


FIG. 4.—Effect of excess 2,3-dimercaptopropanol (BAL) on arsenite-2,3-dimercaptopropanol uncoupled mitochondria. Arsenite (0.3 mM) and 2,3-dimercaptopropanol (0.35 mM) were mixed with rat liver mitochondria in 0.3 M sucrose at 0°. One ml of the suspension containing 6 mg mitochondrial protein was added to Warburg flasks containing the reaction mixture for the assay of oxidative phosphorylation, and the flasks were then attached to manometers and transferred to a bath at 30° for measurement of P/O. Excess 2,3-dimercaptopropanol (0.3 ml of 11.8 mM) was tipped into the flask from the side-arm at intervals (0, 4, and 6 minutes indicated by arrows), and the measurements were continued. At the same time, another 1 ml of the mitochondria plus arsenite-2,3-dimercaptopropanol suspension was added to 25-ml Erlenmeyer flasks containing a reaction mixture identical to that in the Warburg flasks, and incubated at 30° with constant agitation. At intervals (0, 4, and 6 minutes) excess 2,3-dimercaptopropanol was added to these flasks as in the above series. Aliquots of 0.2 ml were transferred at intervals to cuvettes containing 2.8 ml of the complete oxidative phosphorylation reaction mixture and the absorbancy at 520 mμ was determined. The P/O values are given at the end of the curves which show the light-scattering changes. The final reaction mixture for these measurements contained 10 mM phosphate, 2 mM ATP, 6.7 mM MgCl₂, 6.7 mM glucose, 0.3 M sucrose, 6.7 mM succinate, and 0.5 mg hexokinase in 3 ml at pH 7.4. ○—○ control with no arsenite-2,3-dimercaptopropanol; △—△ arsenite-2,3-dimercaptopropanol. Others received excess 2,3-dimercaptopropanol at the times indicated by the arrows: ▲—▲ 0 min.; □—□ 4 min.; ■—■ 6 min.

panol. Again coupled phosphorylation is restored in the swollen mitochondria after the addition of 2,3-dimercaptopropanol.

Although the data in Figures 4 and 5 supported the conclusion that excess 2,3-dimercaptopropanol reverses the uncoupling, there was still a possibility that the 2,3-dimercaptopropanol merely stabilizes the phosphorylation activity existing at the moment of its addition. In order to examine this possibility, the rate of phosphate esterification was measured before and after the addition of excess 2,3-dimercaptopropanol (Table I). In experiment 1, arsenite-2,3-dimercaptopropanol reduced the rate of phosphorylation from 1.7 to 1.3 μmole/minute, which corresponded to a decrease in P/O of 0.5 to 0.7 unit over the 12-minute period. When excess 2,3-dimercaptopropanol was added to mitochondria which had respired at this partially uncoupled rate for 3 minutes, the phosphorylation rate during the subsequent 3 to 9 minutes was increased to a level even higher than the control rate. However,

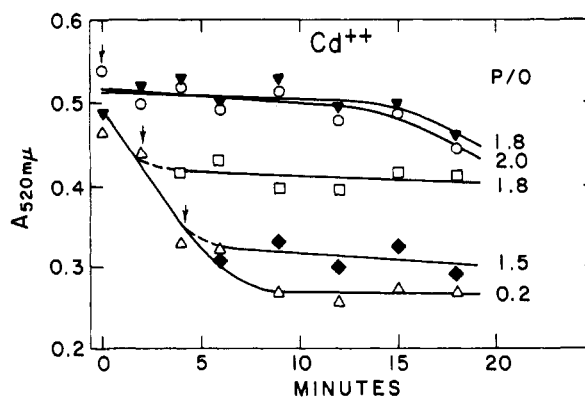


FIG. 5.—Effect of excess 2,3-dimercaptopropanol (BAL) on cadmium ion-uncoupled mitochondria. The experimental conditions were as in Figure 4 except that 0.015 mM cadmium chloride replaced arsenite-2,3-dimercaptopropanol. ○—○, control with no cadmium; △—△, no excess 2,3-dimercaptopropanol. Others received excess 2,3-dimercaptopropanol at the times indicated by the arrows: ▼—▼, 0 min.; □—□, 2 min.; ◆—◆, 4 min.

this increase over the control rate did not result in a net increase in P/O value but merely reflected the slight stimulation in oxygen uptake observed in the presence of excess 2,3-dimercaptopropanol (Fluharty and Sanadi, 1960, 1961). A similar increase in phosphorylation rate after addition of excess 2,3-dimercaptopropanol was seen also in experiment 2, which represents an example where more extensive uncoupling occurred. These two experiments were selected for presentation to emphasize that the degree of uncoupling by a given level of arsenite-2,3-dimercaptopropanol varied to some extent from preparation to preparation and that reversal by excess 2,3-dimercaptopropanol was more complete when the uncoupling was less effective. If excess 2,3-dimercaptopropanol merely stabilized the phosphorylation rate at the instant of its addition, no increase in phosphorylation rate should have been observed. The results favor the interpretation that 2,3-dimercaptopropanol causes a true reversal of the uncoupling produced by arsenite-2,3-dimercaptopropanol. In experiment 3, the phosphorylation rate in the presence of cadmium ion, averaged over a 12-minute period, is very low compared to the control rate. Even if it is assumed that the entire phosphate esterification occurred in the first 4 minutes prior to the addition of excess 2,3-dimercaptopropanol, the maximum possible rate at the time of addition of 2,3-dimercaptopropanol would have been 0.35 μmole/minute. This is considerably lower than the rate (2.0 μmole/minute) observed over the 4 to 12 minute period subsequent to the addition of 2,3-dimercaptopropanol. In this instance, there is no doubt that the phosphate uptake observed after addition of 2,3-dimercaptopropanol could not have been due to the maintenance of a phosphorylation rate present at the time of addition of 2,3-dimercaptopropanol.

TABLE I
RESTORATION OF PHOSPHORYLATION BY EXCESS 2,3-DI-MERCAPTOPROPANOL (BAL)

Ex- peri- ment	Addition	Period of Measurement (min.)		Rate of Phosphoryla- tion (μ mole/min.)
		Start	End	
1	None	0	12	1.7
	AsO ₂ -BAL (0.12 mM)	0	12	1.3
	AsO ₂ -BAL (0.12 mM)	1	3	1.3
	AsO ₂ -BAL, excess BAL (2 mM)	3	6	1.9
		6	9	1.8
2	None	0	18	1.0
	AsO ₂ -BAL (0.12 mM)	0	18	0.32
	AsO ₂ -BAL (0.12 mM)	1	4	0.32
	AsO ₂ -BAL, excess BAL (2 mM)	4	10	0.54
3	None	0	12	1.6
	Cd ⁺⁺ (0.005 mM)	0	12	0.12
	Cd ⁺⁺ (0.005 mM)	0	4	<0.35
	Cd ⁺⁺ , excess BAL (0.067 mM)	4	12	2.0

All reactions were carried out employing the standard oxidative phosphorylation assay system. For experiment 1 individual flasks containing 10 mg mitochondrial protein in 3 ml reaction medium were employed for each time point, the reaction being stopped with perchloric acid at the desired time. Excess BAL was added 3 minutes after start of reaction where indicated. Rates of phosphorylation were determined by the change in inorganic phosphate over the specific interval. For experiment 2 three flasks were allowed to incubate with agitation in a bath at 30°, two containing arsenite-BAL and one serving as the control. To one of the experimental flasks, excess BAL was added after the reaction had proceeded for 4 minutes. Aliquots were removed at various times for the analysis of inorganic phosphate. Rate of phosphorylation was calculated from the difference in inorganic phosphate at the start and end of the indicated time period in the same reaction flask. The protein was 6 mg per 3 ml reaction mixture. The data of experiment 3 were obtained from the same experiment as that described in Figure 5. The 0 to 4 minutes phosphorylation rate (line 3) is a maximum value calculated on the assumption that the total inorganic phosphate that disappeared in 12 minutes (line 2), amounting to 1.4 μ moles, was esterified during the first 4 minutes. The rate over the 4 to 12 minutes (line 4) was obtained by again assuming that the phosphate esterified in the first 4 minutes was 1.4 μ moles and subtracting this from the total esterification in the sample over the 0 to 12 minutes.

DISCUSSION

The addition of either arsenite-2,3-dimercaptopropanol or cadmium ion to a suspension of mitochondria in the presence of β -hydroxybutyrate but no ADP produces first the oxidation of the endogenous DPNH accompanied by a temporary increase in respiration. These changes can best be ascribed to loss of respiratory control due to uncoupling of the phosphorylation. When the oxidation of pyridine nucleotide reached completion, respiration was sharply reduced. On addition of succinate to the system treated with arsenite-2,3-dimercaptopropanol, the rapid oxidation characteristic of uncoupled mitochondria with this substrate could be observed. With both uncouplers employed in this study, mitochondrial swelling also took place as evidenced by a decrease in the light-scattering ability of the mitochondrial suspensions. The swelling commenced subsequent to the onset of uncoupled oxidation of the reduced pyridine nucleotides. In the case where cadmium

ion was employed and where a clearer distinction between the time course of events could be observed, the decrease in light scattering was seen to accelerate well after the reduced pyridine nucleotide fluorescence had dropped and respiratory control had been lost.

A further demonstration that the uncoupling action of either arsenite-2,3-dimercaptopropanol or cadmium ion was not due to its effectiveness as a swelling agent was accomplished by measuring oxidative phosphorylation and light-scattering changes under exactly parallel conditions. Phosphate esterification activity could be restored in mitochondria which had been allowed to respire for several minutes in an uncoupled condition and which had undergone very extensive swelling. These experiments also established that excess 2,3-dimercaptopropanol brought about a true reversal of the uncoupling caused by either arsenite-2,3-dimercaptopropanol or cadmium ion. In previous experiments concerning this reversal the effect of excess 2,3-dimercaptopropanol could have been interpreted as being due to the prevention of uncoupling which expressed itself only after the reaction mixtures had been taken to assay temperature. The swelling did appear to occur only after the suspension was brought to assay temperature.

It thus appears that the primary action of both arsenite-2,3-dimercaptopropanol and cadmium ion involves the uncoupling of phosphorylation from oxidation. The swelling is clearly a secondary effect, although it is not clear from the data whether swelling is or is not a result of the uncoupling. It is apparent, however, that uncoupling is not an indirect result of swelling under these conditions.

These results are consistent with the recent findings (Fletcher *et al.*, 1962) that oxidative phosphorylation in fragments derived from the sonic disruption of rat liver and beef heart mitochondria was uncoupled by arsenite-2,3-dimercaptopropanol (but not by arsenite alone) and by cadmium ion.

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The Electron Transmitter System of Brown Adipose Tissue*

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The components of the electron transmitter system of interscapular brown adipose tissue of male rats have been examined. The presence of an abundance of cytochromes $a + a_3$, b , c , and c_1 was demonstrated spectrophotometrically in particulate matter derived from this tissue. The cytochrome c of the tissue was isolated chromatographically and quantitatively determined, and a content of 2.30 ± 0.22 mg per g of lipid-free dry weight of tissue was found, a value apparently as high as in any other tissue previously described in the rat. The particulate matter derived from the tissue was found to have a content of ubiquinone (coenzyme Q) equivalent on the average to 6 moles per mole of cytochrome c . An additional and approximately equal amount of ubiquinone was found to be present in the nearly colorless fat layer which separates during preparation of the particulate fraction. The ubiquinone was shown to be predominantly the form having a side-chain with 45 carbon atoms (coenzyme Q_9). The high cytochrome content of the tissue is discussed in relation to the tissue's characteristic color, high mitochondrial content, and unusually high rate of oxygen consumption under hormone stimulation.

Brown adipose tissue is most abundant in hibernating animals but also occurs in other animals. It is distinguished, as its name indicates, from the more plentiful white adipose tissue by its color. The nature of the pigmentation as well as the physiologic function of the tissue has been the subject of much speculation, especially in relation to its possible role in the hibernating animal (*cf.* Johansson, 1959). More recently interest in this tissue has been aroused by reports of its preferential invasion by a variety of viruses and the fact that the tissue may serve as a storage and multiplication site for such viruses (*cf.* Sulkin *et al.*, 1959). Data are presented herein which indicate that brown adipose tissue is extremely rich in the cytochromes and ubiquinone and that the cytochromes contribute importantly to the characteristic color of this tissue.

EXPERIMENTAL PROCEDURES

Determination of Cytochrome Pattern.—Male Holtzman rats of weights to be specified were maintained on an *ad libitum* diet of Purina laboratory chow. The animals were sacrificed by decapitation. The interscapular brown adipose tis-

sue was removed immediately; trimmed free of adhering muscle, connective tissue, and white adipose tissue; weighed on a torsion balance; and placed into an ice-cold isotonic sodium chloride solution. This process required approximately 10 minutes for each rat, and sufficient tissue (4 to 12 rats) for each experiment was accumulated in this manner before proceeding to the next step. One piece which was judged to be the nearest to the group average with respect to tissue weight, animal weight, and intensity of tissue color was set aside for determination of its total lipid content and lipid-free dry weight. The values obtained upon this piece of tissue were then used as representative of the total batch for later calculations. The remaining tissues were combined and homogenized in 10 to 15 ml water per g of fresh tissue. This and all subsequent steps in the preparation of the tissue for spectral studies were carried out either in a cold room at 5° or in a refrigerated centrifuge at 2°. Glass homogenizers of the Potter-Elvehjem type were used throughout. The resulting crude homogenate was centrifuged for 1 hour at $25,000 \times g$, to yield a brown pellet and a slightly turbid supernatant fluid which had a nearly white layer of fat on the surface. The fat layer was removed and in some instances saved for determination of its ubiquinone content. The supernatant fluid was discarded. It showed absorption bands with the hand spectroscope in the regions of 542 and 578 m μ , characteristic of oxygenated hemoglobin (*e.g.*, Drabkin, 1946). Addition of

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