AGGREGATION OF MITOCHONDRIA, MITOCHONDRIAL FRAGMENTS, AND MICROSOMES BY CYTOCHROME *c*[*]

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KEILIN AND HARTREE[1] have noted that the activity of particulate enzyme systems derived from heart sarcosomes, such as succinoxidase, can be sedimented at relatively low centrifugal forces if the pH is adjusted to approximately 5.5. Considerably higher centrifugal fields are required to sediment the particles at pH 7.5. Presumably the enzymically active particles aggregate readily at pH 5.5 because electrostatic charge is a major determinant of the stability of such particulate dispersions. CLAUDE AND DOUNCE *et al.*[2,3] have reported additional information on the characteristic aggregation that occurs with both mitochondria and microsomes at pH 5.5.

Observations to be described in this report indicate that a similar agglutination phenomenon will occur at pH values up to 7.5 if cytochrome *c* is added at very low concentrations ($5 \cdot 10^{-6}$ *M*). This physico-chemical interaction between subcellular particles in the presence of cytochrome *c* has been studied in terms of the pH and ionic strength of the media and of the initial event in the reaction sequence, the binding of the heme protein to the particulate material. As is the case with the aggregation reaction occurring at pH 5.5 studied by KEILIN AND HARTREE, the aggregation induced at higher pH values by cytochrome *c* has been found to have relatively little effect upon the inherent enzymic activity of the sedimented particles.

METHODS

Mitrochondria and microsomes were prepared from rat liver homogenates in 0.25 *M* sucrose by conventional methods.[4] The term "mitochondrial fragments" as used in this report refers to the fraction from mechanically fragmented mitochondria[5] that sediments between 25,000 and 100,000 $\times g$.

A light-scattering microphotometer (American Instrument Co.) was found to be the most convenient apparatus for following the aggregation of mitochondrial fragments in the presence of cytochrome *c*. The measurements of scatter were made at an angle of 45° from the incident light path. A 546 mμ monochromatic filter was used with a mercury light source. In calibration experiments, in which increasing amounts of the cytochrome *c*-mitochondrial fragment complex were added to the cuvette, the increment of scattered light was found to exhibit close proportionality to the amount of material in suspension.

The binding of cytochrome *c* by the particles was determined by spectrophotometric measurement of cytochrome *c* at 406 mμ in solution before addition of the mitochondrial fragments and again after addition of the fragments and subsequent centrifugation to remove the fragment-cytochrome *c* complex.

Commercial horse-heart cytochrome *c* (Sigma Chemical Co.) was used in most of the experiments; purification of this material by the method of MARGOLIASH[6] produced no significant change in behavior.

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RESULTS

The aggregation reaction and the effect of pH

Initial observations of the aggregation reaction were made in studies of the cytochrome oxidase activity of particles obtained from mechanically fragmented rat liver mitochondria collected by sedimentation between 25,000 and 100,000 × g^5. It was noted that suspensions of these particles in 0.01 *M* histidine buffer (pH 6.5) became more turbid when cytochrome *c* was added and that the increase in turbidity could be prevented by the addition of ADP or ATP. The following studies describe these effects quantitatively.

The curves in Fig. 1 describe changes in light scattering which occurred when a suspension of mitochondrial fragments was added to 0.01 *M* histidine buffer at 3 different pH values. The pH of the suspension prior to addition to the buffers was 7.2. It is seen that light scattering increased when the particles were suspended in somewhat more acidic media, the increase being most rapid at pH 5.5 and negligible at pH 7.5. Addition of cytochrome *c* at 3 minutes induced additional increments of light scattering. The effect of cytochrome *c* was maximal at pH 6.5 and less dramatic at pH 7.5. The minimal effect at pH 5.5 is presumably a consequence of the spontaneous and near maximal aggregation that had already taken place at this pH prior to the addition of the cytochrome *c*.

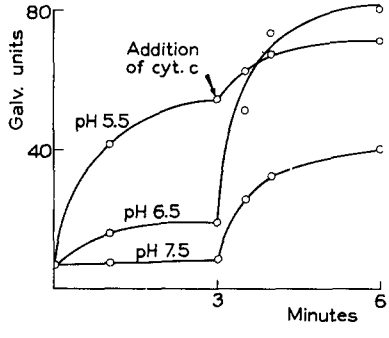

Fig. 1

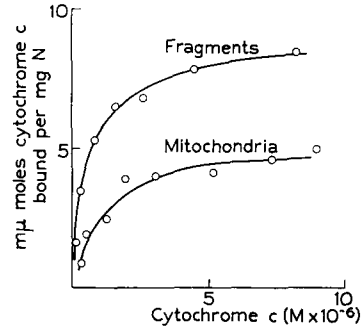

Fig. 2

Fig. 1. Light-scattering changes of suspensions of mitochondrial fragments at various pH values. Mitochondrial fragments (0.1 mg nitrogen) were added to 6 ml of 0.01 *M* histidine buffer at the indicated pH. Cytochrome *c* was added at 3 minutes to give a concentration of $4 \cdot 10^{-6}$ *M*. The galvanometer units record the intensity of the scattered light.

Fig. 2. The relation between the concentration of cytochrome *c* and its binding to mitochondria and mitochondrial fragments. Particulate material containing equivalent amounts of nitrogen (0.8 mg) were incubated in 3 ml of histidine buffer 0.01 *M* pH 6.5, for 30 minutes. The bound cytochrome *c* was measured by the method described in the text.

Aggregation and complex formation

The increase in light scattering appears to be associated with aggregation of the suspended particles. The addition of cytochrome *c* to suspensions of intact mitochondria in dilute buffer at pH 6.5 produces visible clumping and rapid precipitation. Mitochondrial fragments which initially required 50,000 × *g* for sedimentation can be deposited at 500 × *g* after reacting with cytochrome *c*, suggesting a very large increase in particle size following addition of the latter.

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The first step in the sequence of events leading to the agglutination appears to involve complex formation between the cytochrome *c* and the particle. Certain characteristics of the binding of cytochrome *c* to intact mitochondria and mitochondria fragments are shown in Fig. 2. In these studies the particulate material was allowed to react with varying concentrations of cytochrome *c*. The particulate material was then removed from the reaction medium by centrifugation and the disappearance of the cytochrome *c* from the supernatant medium was determined spectrophotometrically. It is seen that maximum binding of cytochrome *c* by intact mitochondria was approached at $5 \cdot 10^{-6}$ *M* cytochrome *c*; at this concentration the mitochondria bound approximately 4 mμmoles of cytochrome *c* per mg total N. The mitochondrial fragments were able to bind almost twice as much cytochrome *c* per mg total N as intact mitochondria. The apparent dissociation constant of the complex (the concentration of cytochrome *c* at which binding is half maximal) would appear to be approximately 10^{-6} *M* for both the larger and the smaller particle. This concentration range was found to be critical also in light-scattering studies in which more dilute suspensions of mitochondrial fragments (5–20 γ nitrogen per ml) were used.

Prevention and reversal of aggregation

The aggregation reaction induced by cytochrome *c* does not occur in solutions containing low concentrations of neutral salts. In the studies summarized in Table I various neutral salts were added in increasing amounts to suspensions of mitochondrial fragments until a concentration was found which effected at 50% reduction of the cytochrome *c*-induced increment of light scattering. As shown, salts supplying polyvalent anions are the most efficient in preventing aggregation, but this effectiveness cannot be correlated quantitatively with the calculated ionic strength. The decrease of the light-scattering reaction in the presence of the non-electrolyte sucrose clearly demonstrates that factors other than ionic strength determine the extent of the aggregation reaction. It is especially significant that adenine nucleotides, $MgCl_2$, and KCl in concentrations such as exist intracellularly have pronounced effects.

TABLE I

PREVENTION OF AGGREGATION BY NEUTRAL SALTS

The aggregation of mitochondrial fragments (10 γ N/ml) was followed with the light-scattering photometer. The reagents listed below were added in increasing amounts until that concentration was found which effected a 50% reduction of the cytochrome *c*-induced increment of light scattering. The reactions were carried out at pH 6.5 in histidine buffer (0.01 *M*, $\Gamma/2 = 0.007$)

Reagent	*for 50% reduction concentration moles per liter*	*Ionic strength* $\Gamma/2$
ATP	0.0002	0.0016
ADP	0.0008	0.0039
AMP	0.0025	0.0061
Tripolyphosphate	0.0006	
Pyrophosphate	0.0010	0.0045
Orthophosphate	0.0060	0.0091
Ethylenediaminetetraacetate	0.0008	0.0036
$MgCl_2$	0.0040	0.0120
NaCl, or KCl	0.0080	0.0080
Sucrose	0.5000	0.0000

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The ability of adenine nucleotides to reverse the aggregation reaction induced by cytochrome *c* is demonstrated by the experiments summarized in Fig. 2. The reversal is seen to be large and almost complete with ADP* and ATP but only slight with AMP. Complementary spectrophotometric studies indicated that the ATP and ADP brought about almost complete dissociation of the cytochrome *c* from the particle.

Effect of aggregation upon enzymic activities

Table II summarizes the results of experiments designed to show the effect of aggregation by cytochrome *c* upon the enzymic activities of the intact mitochondria and mitochondrial fragments. The measurements of oxygen uptake were conducted in buffers of low and high ionic strength (*i.e.* 0.002 *M* and 0.03 *M* phosphate). In the dilute buffer the addition of the cytochrome *c* effected obvious clumping in the vessels containing the mitochondrial fragments. The rate of oxidation of succinate or β-hydroxybutyrate in this test system was relatively unaffected by the obvious change in the physical state of the particulate enzymes induced by cytochrome *c*. The assays carried out at higher buffer concentrations, in which aggregation did not occur, served as controls to show that addition of cytochrome *c* was not required under the test conditions for maximum rates of oxygen uptake with succinate or β-hydroxybutyrate as substrates. The cytochrome oxidase reaction, in which ascorbic acid was used as reductant of cytochrome *c*, requires cytochrome *c* as a cofactor. Again it is seen that the aggregation occurring in the dilute buffer apparently has little effect *per se* upon the rate of oxidation of ascorbate.

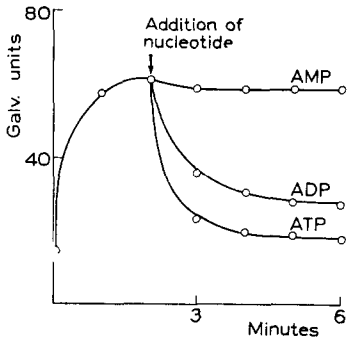

Fig. 3. Reversal of the cytochrome *c*-induced aggregation by adenine nucleotides as reflected by changes in light scattering. The reaction media contained mitochondrial fragments (0.1 mg nitrogen) in 6 ml of histidine buffer, 0.1 *M* pH 6.5. Cytochrome *c* was added at zero time to give a final concentration of $4 \cdot 10^{-6}$ *M*. Nucleotides were added at two minutes to give a final concentration of $4 \cdot 10^{-3}$ *M*. The galvanometer units record the intensity of the scattered light.

In other studies microsomes and mitochondria were allowed to react with cytocytochrome *c* at pH 6.5 in 0.01 *M* histidine, sedimented at low centrifugal forces, and then resuspended. The microsomes prepared in this way were shown to retain glucose-6-phosphatase, and DPNH-cytochrome *c* reductase activity. Mitochondria sedimented by aggregation at low speeds after addition of cytochrome *c* were shown to be capable of carrying out oxidative phosphorylation, with BOH as substrate, with a P:O ratio of 2.5.

In summary, the aggregation reaction induced by cytochrome *c* appears to have relatively little effect upon the activity of the particulate enzyme systems.

Comparisons with the protamine reaction

Since cytochrome *c* is a basic protein with its isoelectric point at pH 10.1[7], its action was compared to that of protamine (I.E.P. 12). Protamine will also agglutinate mitochondria, mitochondrial fragments, and microsomes. In the case of protamine, the reaction is a fast one, being complete in a matter of seconds; and it is effective in con-

* Abbreviations, ATP, ADP and AMP for adenosine tri-, di- and monophosphates respectively; DPNH, reduced diphosphopyridine nucleotide; BOH, β-hydroxybutyrate; N, nitrogen.

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TABLE II

THE EFFECT OF AGGREGATION INDUCED BY CYTOCHROME *c* ON RESPIRATORY ACTIVITY

Oxygen uptake was measured manometrically at 25° in 0.25 *M* sucrose with phosphate buffer, pH 6.5, at the indicated molarity. The total volume was 1.0 ml. Reactions were started by tipping in 10 μmoles of substrate. DPN (10^{-3} *M*) was present in the vessels containing β-hydroxybutyrate. The cytochrome *c* concentration when added was $5 \cdot 10^{-5}$ *M*. Activity is expressed as μmoles oxygen uptake per hour per mg nitrogen.

Preparation	*Substrate*	*Phosphate buffer concentration*			
		0.002 M		*0.03 M*	
		No cyt. c	*Cyt. c*	*No cyt. c*	*Cyt. c*
Intact mitochondria		*activity*	*activity*	*activity*	*activity*
	Succinate	29	34	40	40
	β-hydroxybutyrate	29	28	30	34
	Ascorbate	—	42	—	38
Mitochondrial fragments	Succinate	28	25	30	32
	β-hydroxybutyrate	40	42	36	32
	Ascorbate	—	99	—	52
Degree of aggregation		0	+++	0	0

centrations as low as 10^{-7} *M*. The protamine reaction can also be reversed by nucleotides and the effect of pH is qualitatively similar to that obtained with cytochrome *c* (Fig. 1).

Of particular interest is the finding that of the materials resulting from disintegrated mitochondria, namely fragments and soluble proteins, aggregation by cytochrome *c* occurs only with the former. Reaction of cytochrome *c* with the soluble protein fraction from mechanically fragmented mitochondria[5] produced only a trace of turbidity. Protamine addition, on the other hand, brought about a dense flocculation. In contrast to the properties of protamine, cytochrome *c* evoked no precipitation when added to a solution of yeast nucleic acid (Schwartz). Definite turbidity was produced, however, when cytochrome *c* was added to a solution of egg lecithin (Nutritional Biochemicals).

Reactivity of cytochrome c with bacteria and virus

Cytochrome *c* produced agglutination and precipitation when added to suspensions of two bacteria, *H. influenza* and *E. coli* and of a virus, *E. coli* bacteriophage T2R1. A medium of 0.01 *M* histidine, pH 6.5, was used for these observations.

DISCUSSION

Complex formation between cytochrome *c* and subcellular particles has had previous consideration in the literature. BEINERT[8] studied the adsorption of labeled cytochrome *c* to mitochondria, microsomes and nuclei. He observed that the binding did not occur or was diminished in solutions containing isotonic sodium chloride or sucrose. Other workers have considered the reaction in terms of environmental conditions which produce net charges of the opposite sign on proteins. TINT AND REISS[9] have described a specific interaction between cytochrome *c* and a polypeptide, bacillomycin *b*, with the formation of a tacky red precipitate. These authors emphasized the importance of pH and the ionic strength of the media. BURGER AND STAHMAN[10] showed that a synthetic lysine polypeptide will combine with tobacco mosaic virus. With reference to this

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latter finding, it is noted that a molecule of cytochrome *c* contains 22 lysine residues[7].

MICHELAZZI[11] observed formation of turbidity when a lipid extract of yeast was mixed with solutions of cytochrome *c*. He fractionated the extract and found that the lecithin-containing fractions evinced the most dramatic reactions. Lecithins obtained from rat and rabbit livers were also reactive. Under the conditions used in the present study, cytochrome *c* did not produce turbidity when added to solutions containing nucleic acids or a soluble protein fraction obtained from mechanically fragmented mitochondria. These considerations emphasize the possibility that it is the phospholipid that is responsible for the reactivity of the subcellular particles. On the other hand, the work with bacillomycin[9] indicates that cytochrome *c* can combine with and cause precipitation of certain specific types of polypeptide or protein.

In this laboratory[12] studies have been carried out on the mitochondrial swelling phenomenon[13]. It is noted that sucrose, Mg^{++}, versene, and the polyphosphate compounds, all of which inhibit the cytochrome *c*-induced aggregation of mitochondria and mitochondrial fragments described in this report, also protect intact mitochondria against passive swelling. This is not a general correlation, however. Orthophosphate, which promotes swelling of mitochondria was found to prevent cytochrome-induced aggregation. Furthermore, interaction between cytochrome *c* and mitochondria did not produce a measurable increase in the water content as determined by wet weight measurements.

Since the effects of cytochrome *c*, ATP, Mg^{++}, etc. on the aggregation reaction occur at concentrations existing intracellularly, it is conceivable that these effects could determine in part the intracellular physical state of structure such as mitochondria and the endoplasmic reticulum, from which microsomes are derived[14].

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SUMMARY

Cytochrome *c* at a concentration of $5 \cdot 10^{-6}$ *M* in solutions of low ionic strength at pH 6.5 will complex with mitochondria, mitochondrial fragments, and microsomes, causing these particulate structures to aggregate. This effect can be reversed by the addition of neutral salts to the medium, although the reversal is not strictly a function of ionic strength alone. ATP and ADP are particularly effective in physiological concentrations. This cytochrome *c*-induced aggregation reaction has been found to have no significant effect on the enzymic activities of these particles.

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