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Activity of the Cytochrome System in Heart Muscle Preparations

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The best material for the study of the cytochrome system *in vitro* is the heart muscle preparation which we have previously described (Keilin & Hartree, 1938, 1940). The preparation consists of a uniform colloidal suspension of highly active material completely devoid of haemoglobin and suitable both for manometric and spectroscopic studies. In addition to the complete cytochrome system this preparation contains a powerful succinic dehydrogenase, α -glycerophosphoric dehydrogenase, fumarase and variable amounts of diaphorase, catalase and certain other enzymes, all bound to a protein the nature of which is not yet fully determined. This preparation is often referred to as cytochrome oxidase or as succinic dehydrogenase according to the nature of the substrate under investigation. The cytochrome oxidase activity of this preparation is determined by the rate of the catalyzed O_2 uptake in presence of an excess of cytochrome *c* and of a substance such as *p*-phenylenediamine, hydroquinone, adrenaline or ascorbic acid which, by reducing cytochrome *c*, ensure a constant supply of substrate for the oxidase. The activity of our preparation estimated in this way and expressed as Q_{O_2} , i.e. $\mu l. O_2/mg.$ dry weight/hr. at 39° is about 1400 (Keilin & Hartree, 1940). Although this activity is well below that of several completely purified oxidizing enzymes all attempts to raise it have so far failed. More recently, by introducing a new method of preparation, Haas (1943) claimed to have extracted material containing 15 times more enzyme and being six times more active than the preparation obtained by our method. However, this claim is not supported by his own

observations since the Q_{O_2} of his enzyme preparation was only 203 at 17° and was calculated by him to be 812 at 39° assuming that the activity of the preparation increases fourfold with this rise in temperature. The calculated activity of Haas's material is therefore not six times higher but 40 % lower than that of our preparation.

Since heart muscle preparations are often used for the study of cytochrome in relation to other intracellular catalytic systems we propose to compare the main properties of our preparation with that of Haas. We shall also examine the claims put forward by Haas (1943, 1944) (a) that the use of ultrasonic vibrations has led to the production of a soluble cytochrome oxidase and (b) that the oxidase may be separated into two protein components.

EXPERIMENTAL

Methods

O_2 uptakes. These were measured in Barcroft differential manometers at 39° . The oxidase sample was diluted to 3 ml. with 0.3 ml. cytochrome *c* (Keilin & Hartree, 1945) and phosphate buffer (0.1M, pH 7.3). The substrate (*p*-phenylenediamine or hydroquinone) was added as 0.3 ml. aqueous solution from dangling tubes.

Enzyme units. In order to work out a balance sheet of the enzyme content at different stages of our preparation, samples of successive fractions were withdrawn, ground with sand and diluted with phosphate buffer (0.1M, pH 7.3) to a known volume. The enzyme content was expressed in arbitrary units corresponding to the quantity of enzyme which catalyzes an O_2 uptake of 100 ml./hr. under the above standard conditions.

Cytochrome oxidase

This was prepared essentially as previously described. Finely minced horse heart muscle (500 g.) (A) was washed eight times with 5 l. tap water, the mince being squeezed out through muslin after each washing. The residual mince, 390 g. (B), was ground 1.5 hr. in a mechanical mortar with 100 g. sand

buffer pH 10.4 and centrifuged. The red fluid is then precipitated in the manner described above and resuspended in buffer pH 9.5 giving 742 ml. reddish opalescent fluid containing 11.4 mg. dry weight/ml. The activity of this preparation estimated with hydroquinone at 39°, as shown in Table 2, is higher than that calculated by Haas and is only slightly lower than that of our fraction *E* estimated under

Table 1. *Activities and yields of cytochrome oxidase in different fractions of heart muscle preparation*

(Samples tested in differential manometers at 39° with 0.3 ml. 1% cytochrome *c* and 20 mg. *p*-phenylenediamine; volumes made up to 3.3 ml. with 0.1M-phosphate buffer pH 7.3. Dry weight determined after removal of buffer by dialysis.)

Source of enzyme	Dry weight (mg./ml.)	O ₂ uptake (μl. O ₂ /30 min.)	Q _{O₂}	Total enzyme units/fraction
0.5 ml. <i>A'</i>	2.21	167	150	167,000
0.5 ml. <i>B'</i>	1.87	190	204	190,000
0.05 ml. <i>C</i>	14.1	333	940	94,000
0.05 ml. <i>D</i>	31.8	177	221	51,000
0.02 ml. <i>E</i>	23.0	275	1190	72,500

and 500 ml. 0.02M-phosphate buffer pH 7.3. The mixture was separated in a centrifuge into 690 ml. supernatant fluid (*C*) and a solid residue (above the layer of sand) which was resuspended in water to 690 ml. (*D*). The supernatant fluid was cooled by the addition of 100 g. crushed ice and the pH adjusted to 5.5 with *N*-acetic acid. The mixture was immediately centrifuged and the solid resuspended in 0.1M-phosphate buffer pH 7.3 giving 247 ml. of the final preparation (*E*) containing the complete cytochrome system and a powerful succinic dehydrogenase. During the fractionation 20 g. of mince *A* was removed, ground with sand, diluted to 1 l. with 0.1M-phosphate buffer pH 7.3, centrifuged to remove the sand, giving the fraction *A'*. 15.6 g. washed mince *B* treated in the same way gave fraction *B'*.

The activities of different fractions were estimated manometrically over a period of 30 min. as with some of the cruder preparations there was a slight lag at the beginning of the experiment which precluded the use of initial rates of oxygen uptake in estimating enzyme activity. Table 1, which gives the enzyme activities and enzyme contents of different fractions, shows that the final fraction of Q_{O₂} 1190 contains 42% of the activity of the unwashed mince. The Q_{O₂} of this fraction estimated from the initial O₂ uptake (5 min.) is about 1420 (Table 2). The apparent slight rise in enzyme content of minced muscle after washing may be due to the removal of some inhibitors.

Cytochrome oxidase according to Haas

Minced horse heart (500 g.) was used instead of the pig heart as originally specified. The method consists in grinding the unwashed mince with sand and toluene for 12 hr. and storing it overnight at 0°. Next day the mixture is ground with ammonia

similar conditions. The yield by Haas' method is only 30% higher than by our method: thus the Haas preparation contains 109,000 units whereas our fraction *E* contains 72,500 units. However, this slightly higher yield has no practical advantage since no methods are so far available for further purification of this fraction and the quantity of material

Table 2. *Activities of two heart muscle preparations, E (this paper) and Haas' oxidase*

(Activities tested manometrically at 39° with 0.3 ml. 1% cytochrome *c* and 18 mg. hydroquinone; volumes made up to 3.3 ml. with 0.1M-phosphate buffer pH 7.3.)

Source of enzyme	O ₂ uptake (μl. O ₂ /5 min.)	Q _{O₂}
0.025 ml. <i>E</i>	67	1420
0.05 ml. Haas' prep.	61	1280

produced in one operation is always far in excess of that which can be used in the most extensive experiments. On the other hand the chief disadvantages of the Haas preparation are that it cannot be used for spectroscopic studies of cytochrome owing to the presence of denatured myoglobin and a partial destruction of component *b*, and that furthermore the preparation is useless for the study of succinic dehydrogenase which is rapidly destroyed at pH values above 9 (Keilin & Hartree, 1940).

Effect of ultrasonic vibrations

Haas has described the use of ultrasonic waves as a means of breaking down the aggregates which constitute oxidase preparations of the above type. This treatment reduces the proportion of the total solids which can be centrifuged down in a gravitational field of 10,000 *g* though the clear fluids so obtained show a marked Tyndall effect which demonstrates

that the particles are still much larger than the usual molecular size of purified proteins.

Moreover the experimental distinction between colloidal and true solution in these cases is purely arbitrary as the gradation of particle size is continuous. In true solution a protein must be present as a separate entity and not as a component of a large heterogeneous aggregate. A centrifugal field of 10,000 *g* which was used by Haas to remove the insoluble fraction of his preparation will leave in suspension all particles with a 'molecular weight' up to 10^8 which is considerably higher than the probable size of a single enzyme molecule. According to Haas the activity of his oxidase preparation was not raised by exposure to ultrasonic radiation: however, had the oxidase been brought into true solution it should have been possible to devise a suitable method for further purification. So far, no data have been brought forward to indicate that the irradiation was helpful in this respect.

*Alleged separation of cytochrome oxidase
into two components*

Haas (1944) found that on ultracentrifuging his oxidase preparation, suitably diluted, a solid residue is obtained (component I) which on resuspension shows a much decreased activity in the oxidation of hydroquinone in presence of cytochrome *c*. By heating the undiluted oxidase preparation at 100° for 3 min. and centrifuging off the heat-denatured protein a thermostable yellowish solution containing component II, a protein, is obtained. The addition of the latter to the component I largely restores its catalytic activity, in other words two components are required for the oxidation of cytochrome *c* by molecular O_2 . This suggests, according to Haas, 'that Warburg's oxygen-transferring enzyme and cytochrome oxidase are not identical but represent two individual enzymes'. We repeated Haas' experiment using *p*-phenylenediamine in place of hydroquinone and have confirmed his results (Table 3). However, the method of preparation of component II suggested to us the possibility of replacing it by some other native or denatured proteins. In fact, we found that component II can be successfully replaced by plasma proteins or by a very low concentration of haematin-free denatured globin obtained from recrystallized haemoglobin (Table 3). It is therefore impossible to regard the component II

Table 3. *Fractionation of Haas' cytochrome oxidase*

(Activities of fraction I and II of the oxidase compared with the activities of fraction I in presence of plasma or denatured globin. Samples tested manometrically at 39° with 0.3 ml. cytochrome *c* and 20 mg. *p*-phenylenediamine. Volumes made up to 3.3 ml. with 0.1M-phosphate buffer pH 7.3. Fraction I equivalent to 0.1 ml. and fraction II to 1.0 ml. oxidase.)

Enzyme fractions	O_2 uptake (μ l. O_2 /25 min.)
0.1 ml. original oxidase	155
Fraction I	61
Fraction II	0
Fraction I + fraction II	129
Fraction I + 1% plasma proteins	136
Fraction I + 0.1% denatured globin	116

as an enzyme or as an essential cell catalyst. It affects only the colloidal structure of a preparation previously modified by ultracentrifuging and thus enhances the mutual accessibility of the components of the reaction.

SUMMARY

1. The heart muscle preparation obtained by our method is a colloidal suspension of active material free from haemoglobin and containing a complete cytochrome system, succinic dehydrogenase and certain other enzymes.

2. The estimation of the cytochrome oxidase activity of this preparation in presence of an excess of cytochrome *c* and substances like *p*-phenylenediamine or hydroquinone at 39° gives a Q_{O_2} of about 1400.

3. Haas' preparation of cytochrome oxidase is less active and, owing to the presence of haemoglobin derivatives and to the high pH (> 9) reached during purification, cannot be used for the spectroscopic study of cytochrome or for the study of succinic dehydrogenase.

4. The treatment with ultrasonic waves as applied by Haas does not bring cytochrome oxidase into solution, or raise the activity of the preparation and so far has not assisted the further purification of the enzyme.

5. Haas' claim that cytochrome oxidase may be split into two component enzymes (I and II) is not justified since his enzyme II has been successfully replaced in our experiments by plasma proteins or by small amounts of denatured globin.

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