THE DEPENDENCE OF PIGEON BREAST SUCCINOXIDASE ACTIVITY ON CYTOCHROME *c* CONCENTRATION

by

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INTRODUCTION

In the course of experiments on the succinoxidase complex of pigeon breast muscle it was noted[1] that addition of the supernate from certain enzyme preparations caused a marked increase in the rate of oxidation of substrate. Subsequent work, to be reported here, has shown that the activating factor present in these supernates can be identified as cytochrome *c*. Previous investigators[2,3] have shown that, in general, homogenates give maximum activity only with added cytochrome *c*, whilst TSOU[4] has recently described in detail conditions under which it is possible to remove the greater part of the bound cytochrome *c* from the succinoxidase complex of heart muscle. Although the extracted, or exogenous cytochrome *c* had a much lower catalytic activity than the endogenous form (KEILIN AND HARTREE[5], SLATER[6,7]) it restored the activity of the cytochrome *c* deficient complex to near that of the enzyme complex which was not cytochrome *c* deficient if added in sufficient amount. Other workers have similarly reported the effects of electrolyte concentration on the cytochrome *c* requirement of rat liver preparations[3,8] and on the redistribution of cytochrome *c* in rat liver fractionation[3,9].

EXPERIMENTAL

Preparations

The standard enzyme preparation designated as R_2 was prepared by the method of ALBERTY AND GREEN[1]. One-half of the breast muscle of a pigeon (30 to 40 g wet weight) was blendorized for one minute in a Waring blendor with 450 ml of 0.9% potassium chloride or suspending media as indicated in Table I; all operations were carried out in the cold. The process was repeated with the other half of the pigeon breast muscle tissue. The pH was maintained near 7.0 during the blendorization by the addition of several drops of 6 *N* sodium hydroxide. The combined suspensions were centrifuged at 200 × *g* for 12 minutes while keeping the temperature between —4° and —6° C. The floating layer of solid fat and the sediment were rejected. The deep red supernatant was filtered through cheese cloth and then centrifuged at 2,000 × *g* for 15 minutes at 1°–2° C. All subsequent operations were carried out at 1°–2° C. The deep red cloudy supernatant designated as S_1 contains the activating "factor". The residue was washed with 200 ml of 0.9% potassium chloride at pH 7.0 and centrifuged at 2,000 × *g* for five minutes. The supernatant was rejected. The residue R_2, was

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suspended in neutral 0.9% potassium chloride to a final volume of 20 ml, then frozen and stored at −20° C. The enzyme complex, before use in a test system, was thawed and centrifuged for ten minutes at 18,000 × *g*. The supernatant was rejected and replaced, with an equal volume of 0.9% potassium chloride of pH 7.0. R_2 was then homogenized to a very fine suspension in a Potter-Elvehjem glass homogenizer. After this treatment equal volumes of R_2 suspension gave reproducible oxygen uptakes in succinate oxidation. The S_1 was cleared of endogenous enzymic activity by centrifugation at 18,000 × *g* for 30 minutes to produce a clear red solution. The S_1 is stable for several days if stored near freezing and the R_2 for a similar period when frozen and stored at −20° C.

Assay systems

1. Succinoxidase: 0.3 ml R_2; 0.1 ml *M* succinate; 1.0 ml 0.1 *M* phosphate buffer pH 7.3; 0.2 ml 6 *N* sodium hydroxide (centre well); S_1 and/or 0.9% potassium chloride to a final volume of 3 ml. The optimum succinoxidase substrate relationship of 100 micromoles of succinate for 0.3 ml of R_2 gave the best initial and sustained linear activity for at least one hour. Although lower concentrations of succinate gave the same initial activity the O_2 uptake fell off rapidly with time. Three hundred micromoles of succinate inhibited the activity by 50%.

2. Succinic dehydrogenase[6]: R_2 and succinate as above; 0.3 ml 0.01 *M* methylene blue; 0.3 ml 0.1 *M* potassium cyanide (freshly prepared and neutralised); S_1 and/or 0.1 *M* phosphate buffer pH 7.3 to a final volume of 3 ml.

3. Cytochrome oxidase: R_2 as above, 0.3 ml 5% ascorbic acid in the side arm (freshly prepared and neutralised); 1.0 ml 0.1 *M* phosphate buffer pH 7.3, 0.9% potassium chloride pH 7.0 to a final volume of 3 ml; cytochrome *c* or S_1. Oxygen uptake was measured at 30° C after ten minutes preincubation. Linear uptake of O_2 could be obtained with cytochrome *c* concentrations as low as $1.73 \cdot 10^{-6}$ *M*. At least three different concentrations of cytochrome *c* were used and in all cases a straight line was obtained when concentration of cytochrome *c* was plotted against O_2 uptake.

Cytochrome *c* was estimated according to POTTER[10].

Electrophoretic analyses were carried out in a Tiselius apparatus equipped with a cylindrical lens schlieren optical system using the ALBERTY[11] modification of the LONGSWORTH[12] electrophoresis cell. The phases formed between the moving boundaries were removed with a mechanically manipulated syringe equipped with a long stainless steel needle.

RESULTS

The activation of the succinoxidase complex by the supernate S_1.

The effect of suspending media on the activities of the various R_2 complexes is summarised in Table I.

TABLE I

EFFECT OF SUSPENDING MEDIA ON THE ACTIVITY OF R_2 AND S_1

Solution used to blendorize muscle	*Activity of* R_2 *alone** $\mu l\ O_2/20'$	*Activity of* R_2 *in presence of various* S_1 *preparations* $\mu l\ O_2/20'$			
		S_1^A	S_1^B	S_1^C	S_1^D
A. 0.9% KCl, pH 7.0	59	263	205	92	104
B. 0.02 *M* phosphate pH 7.3	171	251	232	214	232
C. 0.001 *M* phosphate pH 7.3	94	159	141	90	85
D. Deionized water	74	144	126	86	85

* All the R_2's were made up in 0.02 *M* phosphate buffer pH 7.3 and frozen for 24 hours before use.

The relative stimulation of the succinoxidase complex R_2 is greater with the S_1 prepared at the highest salt concentration, *i.e.* 0.9% KCl, especially when this S_1 is used in conjunction with the R_2 prepared at the same salt concentration. For this reason the R_2 and S_1 prepared with 0.9% KCl have been used throughout. Activation of R_2 is linear with respect to S_1 concentration up to an added volume of about 0.8 ml (Fig. 1).

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The levelling off above this volume may be due either to saturation of R_2 by S_1 or to the presence of competitive inhibition in S_1 — in either case it appears to be an additional and important property of these systems. The maximum activation of R_2 by S_1 ranges from two to ten-fold depending on the oxygen uptake of R_2 alone. Despite its effect on the oxidation of succinate by R_2 the supernate S_1 appeared to neither inhibit nor activate the succinic dehydrogenase system or the cytochrome oxidase system.

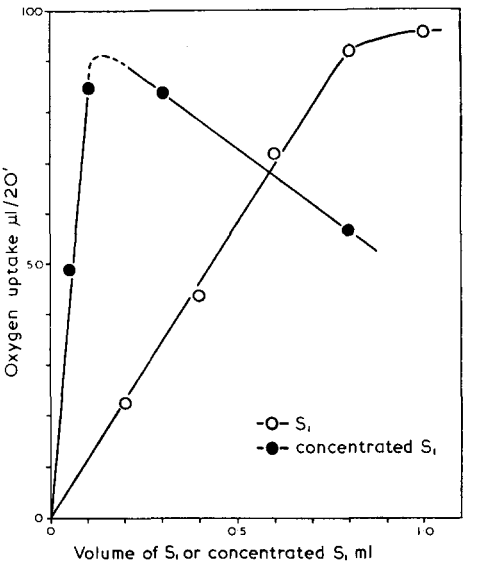

Fig. 1. Activation of R_2 with S_1 and the heated and concentrated S_1. Assay system for succinoxidase as indicated in the text. The endogenous activity of R_2 has been set at zero; its normal value varies from 30–50 μl $O_2/20'$.

Concentration of the active factor in S_1.

Since the supernate S_1 was obtained from unwashed muscle it was too complex to analyse directly in the spectrophotometer. Precipitation of S_1 with ammonium sulphate or with ethanol at —5° to —10° C proved unsatisfactory. However it was noted that the active factor withstood moderate heating. Considerable coagulation of protein in S_1 occurs between 60° and 65° C at pH 7.05, the distinctive red colour changes to orange after thirty minutes at 65° C and is almost lost on boiling. In many preparations no loss of the active factor was found when S_1 was heated to 65°C for five minutes (Table II). With longer intervals at 65°C or at higher temperatures loss of activation at the 1.0 ml level was noted.

TABLE II

EFFECT OF HEATING THE SUPERNATE S_1

0.3 ml R_2, 0.1 ml M succinate, 0.2 ml 6 N NaOH (centre well), 1.0 ml 0.1 M phosphate buffer pH 7.3, 0.9% KCl, S_1 or heated S_1 to a total volume of 3.0 ml. O_2 uptake in μl/20'.

Sample	*Volume of S_1 or heated S_1 in assay*	
	0.3 ml	*1.0 ml*
Original S_1	93	190
Heated 65° C for 5'	109	186
Heated 65° C for 30'	—	166
Heated 75° C for 5'	105	155

After removal of heat coagulation protein by centrifugation the remaining solution of S_1 was freeze dried, the powder dissolved in 5 to 10 ml of water and dialysed exhaustively against the 0.9% KCl used in the original mincing in the Waring blendor. Aliquots of this concentrated S_1 diluted back to the concentration of the original S_1 were found to have identical oxygen uptake curves to the original S_1. Although maximum activation of R_2 was attained with smaller volumes of the concentrated S_1 than of the S_1 from which it was derived, the maximum activity appeared to be of the same order in

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both cases (Fig. 1). The ratio of the volumes of S_1 and concentrated S_1 required to achieve an oxygen uptake of 100 μl in twenty minutes (*i.e.* an oxygen uptake on the linear portion of both activity plots) was the same as the concentration factor for the S_1. It is evident therefore that the supernate S_1 can be concentrated by heating under specified conditions and freeze drying without loss of active factor. A striking feature of the activity curve of the concentrated S_1 is the rapid fall in activity at the higher levels of concentration. The method employed for concentrating the active factor would seem to apply equally to the inhibitors in S_1.

Electrophoretic examination of concentrated solutions of S_1.

The complexity of the heat treated, concentrated solutions of S_1 is revealed by the electrophoretic analysis given in Fig. 2.

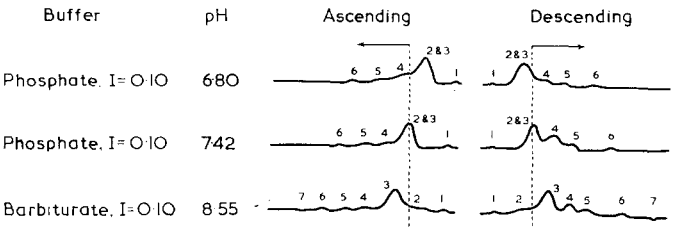

Fig. 2. Electrophoretic analysis of heat treated and concentrated S_1.

At least seven groups of proteins are detected in analyses from pH 6.80 to 8.55. The term group is used, rather than component, because considerable overlapping of the schlieren boundaries occurs and insufficient evidence has been collected to show whether all seven groups contain single protein components. However, groups 1 and 6 which migrate as single boundaries at the three pH's could probably be considered as single components. Group 7 is detected in barbiturate buffer at the higher pH and produces an inverted schlieren peak. The main light absorption takes place across the boundary due to group 3 which contains, therefore, the residual haem compounds (*e.g.* haemoglobin, chromogens) of the S_1. It was possible at the end of each electrophoretic experiment to remove separate samples of groups 1 and 6 or mixtures of these two groups with the remaining groups and to test these samples for activation of R_2.

The results of a series of experiments are given in Table III. It is evident that groups 2, 3, 4, 5, 6 and 7 cause no increase in the oxygen uptake of R_2 alone. Group 1 stimulates the succinoxidase complex R_2 and therefore contains the essential active factor.

In the experiment recorded in line (b) of Table III it is seen that the sample containing groups 1, 2 and 3 has an oxygen uptake equal to that of the S_1 before electrophoresis. Although the sample containing only group 1 caused considerable stimulation of R_2 its absolute oxygen uptake was lower than that of the S_1 or of the sample of groups 1, 2 and 3. This result is to be expected from the nature of electrophoretic sampling. The separation of the schlieren peaks due to group 1 and to groups 2 and 3 in phosphate buffer at pH 7.42 is not great (*i.e.* < 1 cm). In the sampling of group 1 the tip of the syringe needle is set in a position corresponding to the top of the schlieren peak of group 1 at the beginning of sampling. With both electrode vessels open the solution containing group 1 moves gradually up to the needle tip as the syringe is expanded. It is impossible with this procedure, when care is being taken not to conta-

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TABLE III

ACTIVITY OF SAMPLES FROM THE ELECTROPHORESIS OF HEATED AND CONCENTRATED S_1

1	2	3	4	5	6
Buffer and pH	*Protein groups in Electrophoretic sample**	*Oxygen uptake in µl/20'*			*Vol. of S_1 or Sample under Test ml*
		R_2	$R_2 + S_1$ *Prior to electrophoresis*	R_2 + *Sample as in Col. 2*	
a. Phosphate pH 6.80	1, 2, 3, (4)	27	160	203	0.3
	(2), (3), 4, 5, 6	27	160	31	0.3
b. Phosphate pH 7.4	2, 3, 4, 5, 6	71	135	76	0.05
		71	204	78	0.10
	1, 2, 3, (4)	71	135	140	0.05
		71	204	203	0.10
	1	71	135	120	0.05
		71	204	136	0.10
c. Phosphate pH 7.33	2, 3, 4, 5, 6	35	75	38	0.05
		35	123	32	0.10
		35	210	39	0.30
	1	35	75	72	0.05
		35	123	92	0.10
		35	210	172	0.30
d. Barbiturate pH 8.55	1, 2, (3)	12	83	131	0.30
	(3), 4, 5, 6, 7	12	83	12	0.30

* The numbers without brackets are the main groups in the sample. However, because of boundary overlap, small amounts of neighbouring groups are sampled at the same time; the latter groups are indicated by brackets.

minate the solution with groups 2 and 3, to avoid diluting the sample of group 1 with supernatant buffer—the extent of dilution may be to reduce the concentration of group 1 to one half or one third of its concentration in the S_1. It is to be expected therefore that its activation of R_2 would be less than the activation due to the S_1. On the other hand when sampling groups 1, 2 and 3 the syringe needle is far below the boundary of group 1 and its concentration in the sample is the same as in the S_1, correspondingly its activation of R_2 is also the same as with S_1. In two instances (lines a and d in Table III) certain electrophoretic samples give a greater stimulation of the activity of R_2 than did the S_1 before electrophoresis. It is possible that such groups do not contain the inhibitor present in the original S_1.

Identification of the electrophoretic group 1 with cytochrome c.

The migration of group 1 is towards the cathode up to and above pH 8.55; its isoelectric point is, therefore, at a pH higher than 8.55. Samples of group 1 show sharp maxima in their reduced spectra at 415, 520 and 550 mµ when tested according to POTTER[10]. These maxima are given by cytochrome *c*[13]. In addition the ratio of intensities at the 550 and 520 mµ maxima to the 535 mµ minimum correspond with the ratios found for purified samples of cytochrome *c* (Table IV).

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TABLE IV

RATIOS OF ABSORPTION MAXIMA FOR CYTOCHROME *c* AND THE ELECTROPHORETIC GROUP 1

pH of test solution 7.3.

Ratio of absorption maxima	*Group 1 line b Table III*	*Group 1 line c Table III*	*Published value*
$\varepsilon_{550}/\varepsilon_{535}$	3.8	3.3	3.44 at pH 4.1[14] 3.90 at pH 13[14] 3.76–3.82 at pH 6.0[15]
$\varepsilon_{520}/\varepsilon_{535}$	2.1	2.0	2.02 at pH 4.1 and pH 13[14]

Again the addition of Sigma cytochrome *c* to a solution of heated and concentrated S_1 at pH 7.42 to a final concentration of 0.2% causes a considerable increase in the area of the schlieren peak due to group 1. Thus the electrochemical characteristics of Sigma cytochrome *c* and group 1 are identical under these conditions. Small increases in the area of the schlieren peaks due to groups 2, 3, 4 and 5 also occur on the addition of the Sigma product. This was shown to be due to other components present in Sigma cytochrome *c* which had mobilities equal to those of the above groups and not group 1. These additional components, which account for some 30% of the Sigma product, show the maxima and minima of cytochrome *c* but do not give the same ratios of the absorption at the 550 mμ and 520 mμ maxima to the 535 mμ minimum quoted in Table IV. They also showed no activity in the R_2 succinoxidase test system.

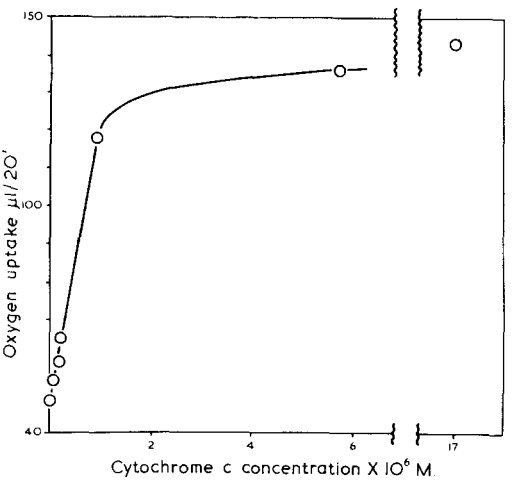

Fig. 3. Activation of R_2 by commercial cytochrome *c* (Sigma). Assay system for succinoxidase as indicated in the text.

The activation of R_2 by commercial cytochrome *c* is shown in Fig. 3.

The same maximum activity of the succinoxidase is achieved with cytochrome *c* as with either S_1, heated S_1 or heated and concentrated S_1. Excess cytochrome *c* does not however produce the same inhibitory effect noticed with the heated and concentrated S_1.

DISCUSSION

The supernate S_1 obtained from the unwashed muscle contained a relatively high concentration of red pigments (haem compounds *etc.*) and it was not therefore possible to estimate the cytochrome *c* concentration of S_1 directly. However, in the case of a number of electrophoretically separated samples of cytochrome *c* (group 1), the actual cytochrome *c* content was known. A comparison of the amounts of S_1 and commercial cytochrome *c* required to produce an equal oxygen uptake in the R_2 system—the par-

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ticular level being well below the saturation value and therefore on the linear part of each activity plot—showed that the S_1 cytochrome *c* was 1.6 to 1.9 times as active as the commercial preparation. This result may may not be particularly significant for the commercial sample contained a considerable amount of inactive material which absorbed at 550 mμ.

The succinoxidase preparation from pigeon breast muscle described here is clearly similar to the cytochrome *c* deficient preparations of POTTER[2] from rat liver and of TSOU from heart muscle. As with the heart muscle succinoxidase the higher electrolyte concentrations were most effective in extracting cytochrome *c* from the pigeon muscle complex (Table I). It is known that 0.001 *M* phosphate and water extract little or no cytochrome *c* from such preparations and therefore the increased activity of the 0.9% KCl R_2 and the 0.02 *M* phosphate R_2 in the presence of supernates made with these solvents must be attributed to other effects.

If it is assumed that the cytochrome *c* content of the electrophoretic samples is from one half to one third of that in the original solution used for the electrophoretic examination then it follows that the concentration of cytochrome *c* in S_1 is of the order of $2 \cdot 10^{-6} M$. The maximum amount of cytochrome *c* in the form of S_1 added to the cytochrome oxidase system was $2 \cdot 10^{-3}$ μmoles per flask; this addition caused no marked increase in activity. However, the addition of $2 \cdot 10^{-3}$ μmoles per flask of Sigma cytochrome *c* also gave no increase in cytochrome oxidase activity. On the other hand $2 \cdot 10^{-2}$ μmoles of Sigma cytochrome *c* per flask increased the oxygen uptake eight-fold whilst the same amount added in the form of the heated and concentrated S_1 gave only two-fold stimulation. The addition of this amount of heated and concentrated S_1 (1.0 ml in the units of Fig. 1) to the succinoxidase system gave an activity well below the saturation value. It is probable therefore that the site of the inhibitory effect of S_1 is on the cytochrome oxidase.

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SUMMARY

Particulate preparations from pigeon breast muscle have been found to require the addition of a factor for maximum activity in the oxidation of succinate. This factor is found in the supernate from the enzyme preparation and has been identified after electrophoretic separation as cytochrome *c*. This method of identification is particularly useful when other coloured compounds are present in the supernate. Unlike cytochrome *c* addition of excess of the supernate S_1 inhibits the succinoxidase system.

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RÉSUMÉ

Des préparations de particules de muscle pectoral de pigeon nécessitent l'addition d'un certain facteur pour que l'oxydation du succinate y ait lieu avec l'activité maximum. Ce facteur se trouve dans le surnageant des préparations enzymatiques et a été identifié, après séparation par électrophorèse, avec le cytochrome *c*. Cette méthode d'identification est particulièrement utile lorsque d'autres produits colorés sont présents dans le surnageant. A la différence du cytochrome *c*, l'addition d'un excès du surnageant S_1 inhibe le système succinoxydasique.

ZUSAMMENFASSUNG

Es wurde beobachtet, dass Partikeln aus Taubenbrustmuskeln die Hinzufügung eines Faktors für eine Höchstaktivität in der Oxydation von Succinat benötigen. Dieser Faktor wird in der überstehenden Flüssigkeit des Enzympräparates gefunden und konnte, nach elektrophoretischer Separation, als Cytochrom *c* identifiziert werden. Diese Identifikationsmethode ist besonders nützlich, wenn andere farbige Körper in der überstehenden Flüssigkeit zugegen sind. Im Gegensatz zu Cytochrom *c* hemmt die Hinzufügung eines Überschusses an überstehender Flüssigkeit S_1 das Succinoxydasesystem.

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