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STUDIES ON A CYTOCHROME OXIDASE ANTIBODY

I. IMMUNOCHEMISTRY

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SUMMARY

1. The immunochemical properties of a highly purified beef heart cytochrome oxidase are described.

2. Immunodiffusion, quantitative precipitation, passive hemagglutination and immunoelectrophoresis studies have demonstrated that this enzyme is capable of eliciting an immunological response in rabbits.

INTRODUCTION

Cytochrome oxidase, the hemoprotein enzyme which catalyzes the aerobic oxidation of cytochrome *c*, appears to be intimately associated with the inner mitochondrial membrane[1–4]. Various particulate[5] and solubilized preparations[6–8] of the enzyme have been employed to examine the kinetics of its reaction with cytochrome *c*[9–14]. In the case of the studies with the solubilized oxidase the pertinent question may be raised as to what extent the kinetic information obtained from the relatively simple solubilized system may be applied to understanding the behavior of the oxidase in its natural milieu.

In this study we have employed a cytochrome oxidase antibody to compare the structure and reactivity of the solubilized oxidase to that located in the Keilin–Hartree heart muscle particles. The present paper presents the immunochemical evidence for the characterization of the antibody, as well as the reactivities of various preparations toward this antibody. The following paper[15] describes the inhibition kinetics of the antibody on both the soluble and particulate oxidase of the Keilin–Hartree particle. A preliminary account of this work has appeared previously[16].

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MATERIALS AND METHODS

Cytochrome oxidase

Cytochrome oxidase was prepared from beef heart according to the procedure of YONETANI[17]. Two preparations were utilized during these experiments and are designated as beef Prep. 1 and Prep. 2. They differed in final solubilizing step and purity index. Prep. 1 had a purity index ($A_{280\ m\mu}$ (oxidized)/$A_{445\ m\mu}$ (reduced)) of 3.26 and was solubilized in 0.1 M potassium phosphate buffer (pH 7.4) containing 1 % Emasol, whereas Prep. 2 had a purity index of 2.09 and was taken up in buffer containing 1 % Tween-80. In addition, this preparation was shown by cellulose acetate electrophoresis and analytical ultracentrifugation to be relatively devoid of contaminating protein. Type II pig heart cytochrome oxidase was prepared, by the procedure of SMITH AND STOTZ[7].

Cytochrome c

Horse heart cytochrome *c*, Type III, was obtained from the Sigma Chemical Co. Chromatography on Amberlite CG-50 indicated no significant contamination, and therefore, cytochrome *c* was used without further purification for the enzymatic assay. Ferrocytochrome *c* (90–95 % reduced) was prepared by the anaerobic gel-filtration method of YONETANI[17].

Additional reagents used were Tween-80 (Baker Chemical Co.), Triton X-100 (Sigma Chemical Co.), Emasol 4130 (Kao Soap Co., Japan), normal rabbit serum (Pel-Frez, Type II), noble agar (Difco), incomplete and complete Freund's adjuvant (Difco), Amido Schwartz 10B (Allied Chemical, National Stain Commission No. 20470) and *N*,*N*-dimethyl-*p*-phenylenediamine·HCl (Eastman Organic Chem.).

Immunization

Two New Zealand white rabbits were immunized with beef heart cytochrome oxidase Prep. 1 and one with Prep. 2 according to the following schedule. The first intradermal injection contained beef cytochrome oxidase (approx. 6 mg protein) emulsified with an equal volume of Freund's complete adjuvant. Injections at 14 days and monthly thereafter in Freund's incomplete adjuvant, contained increasing amounts of cytochrome oxidase (up to approx. 15 mg protein). The rabbits were bled from the marginal ear vein at 7–10-day intervals during the course of immunization. The serum was separated by centrifugation at 3000 × *g* for 40 min at 2° and stored frozen at —15°.

Double diffusion

Gel diffusion tests were performed in 0.5 % agar containing 0.05 M potassium phosphate buffer (pH 7.0). When dispersing reagent (*e.g.* 2 % (v/v) Triton X-100) was used, it was added to the cytochrome oxidase sample or incorporated directly into the agar prior to solidification.

Enzymatic activity of the precipitated antibody–oxidase complex in agar was assayed with the "nadi" reagent prepared as described by LAWRENCE *et al.*[18]. The agar plates were washed with several changes of 0.9 % NaCl at 4° for 2 days prior to flooding with the reagent. The plates were incubated at 25° for 1–2 h. Enzymatic activity was indicated by the appearance of blue lines. The plate was immediately

washed in distilled water and the results recorded photographically. Prolonged incubation resulted in a non-specific irreversible staining of the entire agar plate.

Immunoelectrophoresis

Electrophoresis and diffusion were performed in 0.5 % agar containing sodium barbital–acetate buffer (pH 8.6), ionic strength 0.05. Electrophoresis was carried out at 150 V for 75 min at 25° with a distance between the wicks of 24 cm. The trough was 75 mm × 2 mm and the diameter of the well was 2 mm. Diffusion was complete within 24 h. The slides were then washed as described above for agar plates and stained with either amido schwartz 10B or nadi reagent.

Quantitative precipitation

Quantitative precipitation was performed according to the method of KABAT AND MAYER[19]. Appropriate dilutions of cytochrome oxidase in 0.05 M potassium phosphate buffer (pH 7.0) were mixed with a constant volume of rabbit antiserum or normal rabbit serum, which had been clarified by centrifugation at 18000 × *g* (2°) for 30 min. The various enzyme–serum mixtures were incubated for 1 h at room temperature and then overnight at 0°. The precipitate was separated by centrifugation at 3000 × *g* for 30 min at 4° (International clinical centrifuge) and washed twice with 0.9 % NaCl (0°). The washed precipitate was resuspended in 0.1 M potassium phosphate buffer (pH 7.0) + 1 % Tween-80 (with a Vortex Jr. mixer) for spectral analysis using a Cary-11 recording spectrophotometer (Cary Instruments). Scattering was compensated for by using an appropriate dilution of milk. Aliquots were set aside for protein determination using a modified technique of LOWRY *et al.*[20] incorporating an equivalent concentration of Tween-80 in the bovine serum albumin standards.

The supernatants, after the antibody–antigen precipitated, as well as the control set containing normal rabbit serum, were assayed spectrophotometrically for cytochrome oxidase activity remaining, by following the rate of ferrocytochrome *c* oxidation at 550 mμ (ref. 10). No appreciable autoxidation of ferrocytochrome *c* was detected. The reaction was initiated by addition of 2–50-μl quantities of the solutions to be analyzed to a 1 ml cuvette containing 20 μM ferrocytochrome *c* in 0.05 M potassium phosphate buffer (pH 7.0) at 25°. The initial velocities were obtained from the slope of a tangent drawn on the extended time-course curve of the reaction at zero time. Percent activity relative to the control was calculated from the difference in initial velocity (v) between fractions treated with antibody serum and those treated with control rabbit serum according to the following equation:

$$\text{Percent activity} = 100 \times \frac{v \text{ (antibody serum)}}{v \text{ (normal rabbit serum)}}$$

Hemagglutination

Passive hemagglutination was performed by a slight modification of the method of KABAT AND MAYER[19]. Cytochrome oxidase was diluted with 0.075 M potassium phosphate buffer (pH 7.4) containing 0.025 M NaCl and 0.1 % Tween-80. An equal volume of diluted cytochrome oxidase was mixed with a 3 % suspension of tanned erythrocytes. The cells were precipitated and washed 3 times. The cells were then washed in the diluent buffer, 0.05 M sodium potassium phosphate *plus* 0.1 M NaCl and 1 % normal rabbit serum.

Serial dilutions of the antiserum and control serum were made in the diluent buffer. A constant volume of antigen-coated erythrocytes was added to each tube. The tubes were then incubated 3 h at room temperature, read, suspended, left overnight at 4°, and read again. The results were recorded as +, agglutination; ±, partial agglutination; —, no agglutination.

RESULTS

Some properties of the purified cytochrome oxidases

Fig. 1 illustrates the spectra of three different cytochrome oxidases used in this study. Preps. 1 and 2 (beef), as well as pig oxidase, were reduced with sodium dithionite, reaching full reduction within 10 min. As can be seen, these preparations are spectrally devoid of cytochrome c_1 or b. The β- and α-peaks are at 445 and 605 mμ, respectively. Prep. 1 (beef) and pig oxidase displayed shoulders at 424 mμ as time of storage increased, indicating the presence of small amounts of an inactive form of the oxidase[21]. Electrophoresis on cellulose acetate of Prep. 2 and pig oxidase in a Tris–HCl buffer (pH 8.1) yielded one band migrating toward the anode. However, an additional band appeared toward the cathode in Prep. 1. This basic non-heme protein was sometimes observed in other cytochrome oxidase preparations.

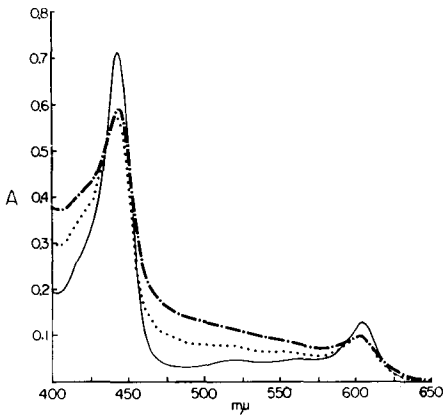

Fig. 1. Visible absorption spectra of reduced beef Preps. 1 and 2 and pig cytochrome oxidase. Beef Prep. 1 (· · · · · ·), Prep. 2 (———) and pig (–·—·–) cytochrome oxidase in 0.05 M potassium phosphate buffer (pH 7.0), were reduced by the addition of sodium dithionite and the spectra were recorded approx. 10 min after the addition. Yonetani-type beef Preps. 1 and 2 were initially solubilized in 1 % Emasol 4130 and 1 % Tween-80, respectively. Pig cytochrome oxidase, Smith–Stotz Type II was solubilized in 0.25 % $(NH_4)_2SO_4$ and 0.5 % sodium cholate.

Ouchterlony reaction. A typical double diffusion reaction of the various oxidase preparations in 0.5 % agar with antiserum prepared against Prep. 1 is illustrated in Fig. 2A. Because of the high molecular weight and lipoprotein nature of the oxidase, as well as its tendency to aggregate, 0.5 % agar was found most suitable for the immunodiffusion experiments. The plate was treated with the nadi reagent which is specific for the oxidase. The positive nadi reaction clearly demonstrated that the inner precipitin line belongs to the oxidase. The second precipitin line opposite Well 3 with Prep. 1 was unreactive toward the nadi stain. This line was identified as the positive

protein observed electrophoretically in this preparation. Inspection of the inner precipitin lines, identified as those belonging to the oxidase, revealed that the lines completely fused, indicating immunological identity between Prep. 1, Prep. 2 and pig oxidase. This diffusion experiment also demonstrates the immunological purity of Prep. 2 and pig oxidase since they fail to show a second precipitin line corresponding to the positive non-heme containing protein contaminant. Neither beef or horse heart cytochrome *c* reacted with the antibody.

Immunoelectrophoresis. Fig. 3 illustrates the results of immunoelectrophoresis. The electrophoretic pattern of the oxidase antiserum prepared against Prep. 2, after

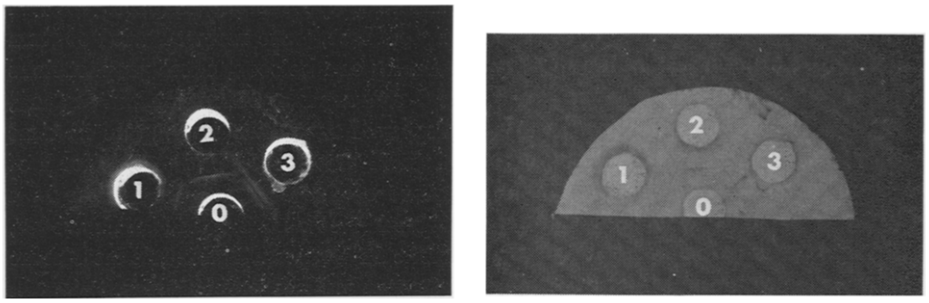

Fig. 2. Double diffusion of cytochrome oxidase in agar. A (left). Double diffusion pattern for rabbit anti-cytochrome oxidase in 0.5% agar containing 0.05 M potassium phosphate buffer (pH 7.0). The outer wells contained the following: 1, 37.5 μg pig heart cytochrome oxidase; 2, 45 μg beef heart cytochrome oxidase Prep. 2; 3, 45 μg beef heart cytochrome oxidase Prep. 1. The center well (0) contained antiserum prepared against beef heart cytochrome oxidase Preps. 1 and 2. B (right). Part A after reaction with nadi reagent for 30 min.

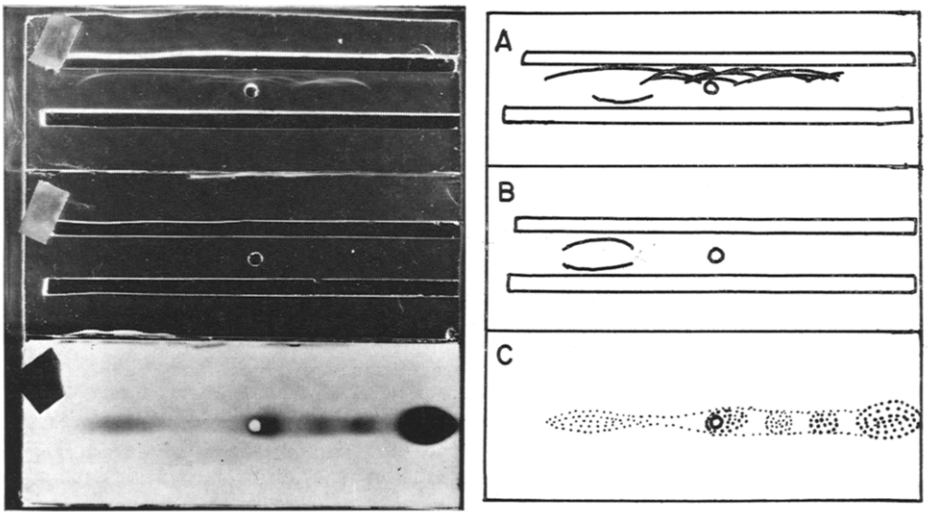

Fig. 3. Electrophoresis and diffusion of antisera to cytochrome oxidase. Electrophoresis of the antiserum to cytochrome oxidase prepared against Preps. 1 and 2 was in 0.5% agar in sodium barbitone–acetate buffer (I = 0.05; pH 8.6) at 150 V, 75 min at 25°. Diffusion occurred for 20 h at 25°. C. The lower trough contained 0.075 mg/ml (0.59 μM heme *a*), and the upper trough contained anti-rabbit serum prepared in goat. B. The lower trough contained 0.075 mg/ml (0.59 μM heme *a*) beef cytochrome oxidase preparation; the upper trough contained 0.15 mg/ml (1.18 μM heme *a*) preparation. A. No diffusion with cytochrome oxidase; the slide was stained immediately after electrophoresis with amido schwartz 10B.

staining with amido schwartz 10B, is shown in Fig. 3C. The pattern observed is typical of the distribution of serum proteins subjected to agar electrophoresis.

Diffusion of anti-rabbit serum prepared against whole rabbit serum in goats is illustrated in the upper trough of Fig. 3A. A normal precipitin line for the IGg immunoglobulin is apparent.

Cytochrome oxidase Prep. 2 (in which no significant protein contaminant was detected) was diluted in 0.05 M potassium phosphate buffer (pH 7.0) with 1 % Tween-80 and allowed to diffuse against the separated serum proteins (Fig. 3). The lower trough of slide A as well as both troughs of slide B contained cytochrome oxidase. It is evident from the correlation of the normal electrophoretic and immunodiffusion pattern of the oxidase antiserum with anti-rabbit serum, that the precipitin lines developed with cytochrome oxidase appear in the IGg class of γ-globulin. Pig oxidase showed a similar pattern. The precipitin lines that developed with cytochrome oxidase were active toward the nadi stain, indicating the presence of the oxidase in the precipitin line. No electrophoretic migration of cytochrome oxidase was demonstrable in agar preventing inverse immunoelectrophoresis.

Passive hemagglutination. The precipitating antibody was also detected by passive hemagglutination.

The results of a typical hemagglutination experiment are listed in Table I. The end point titer for the serum sample used was 1:21 879 dilution of antiserum at a coating level of 12.5 μg oxidase protein per ml using Prep. 2. At high coating levels of the oxidase, a non-specific agglutination occurred as evidence by the positive results using normal rabbit serum (at 19 μg oxidase protein per ml) and the normal rabbit serum control becomes negative. At a coating level of 12.5 μg oxidase protein per ml, the titration reached a clear end point while controls were negative. This ndicates a normal (+) reaction to a point where the antibody becomes limiting (±).

TABLE I

PASSIVE HEMAGGLUTINATION WITH SOLUBLE CYTOCHROME OXIDASE

Cytochrome oxidase (Prep. 2) was diluted in 0.075 M potassium phosphate buffer (pH 7.0) + 0.025 M NaCl + 0.1 % Tween-80 buffer. The first wash of the tanned-oxidase coated red blood cells was in the same buffer. The results are recorded as +, hemagglutination; ±, partial hemagglutination; —, no hemagglutination. Antiserum, No. 127-i; normal rabbit serum, NRS.

Dilution of serum	*Coating level (μg protein oxidase per ml)*											
	25		*19*		*15*		*12.5*		*9.5*		*7.5*	
	127-i	*NRS*	*127-i*	*NRS*	*127-i*	*NRS*	*127-i*	*NRS*	*127-i*	*NRS*	*127-i*	*NRS*
1:10	+	+	+	—	+	—	+	—	—	—	—	—
1:30	+	+	+	—	+	—	+	—	—	—	—	—
1:90	+	—	+	—	+	—	+	—	—	—	—	—
1:270	+	—	+	—	+	—	+	—	—	—	—	—
1:810	+	—	+	—	+	—	+	—	±	—	—	—
1:2 430	+	—	+	—	+	—	+	—	±	—	—	—
1:7 290	+	—	+	—	+	—	+	—	±	—	—	—
1:21 870	+	—	+	—	+	—	+	—	—	—	—	—
1:65 610	+	—	+	—	+	—	±	—	—	—	—	—
1:196 830	+	—	+	—	±	—	±	—	—	—	—	—
1:590 490	+	—	—	—	—	—	—	—	—	—	—	—
1:1 771 570	+	—	—	—	—	—	—	—	—	—	—	—

Further dilutions of the oxidase for coating yielded negative results, indicating that antigen concentration was becoming the limiting factor.

Quantitative precipitation. The results of a typical quantitative precipitation reaction are graphically represented in Fig. 4. The data was obtained as described in

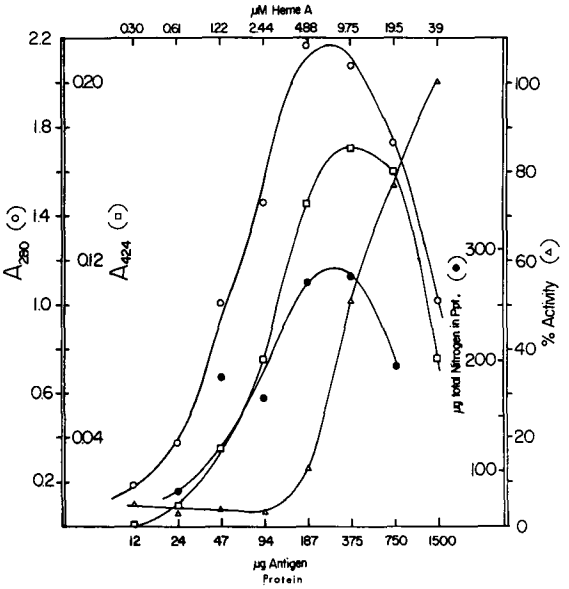

Fig. 4. Quantitative precipitation reaction for rabbit antisera to beef cytochrome oxidase. Various concentrations of beef heart cytochrome oxidase Prep. 2 were diluted to a final volume of 0.1 ml in 0.05 M potassium phosphate buffer (pH 7.0), 0.2 ml of rabbit anti-cytochrome oxidase serum, prepared against Prep. 2, was then added, and the mixture incubated for 24 h at 2°. The supernatants and precipitates were then separated. The supernatants were assayed with ferrocytochrome *c* by following the change in absorbance at 550 mμ. The percent activity remaining is determined from the initial velocity obtained with the fraction containing antiserum, compared with the fraction containing normal rabbit serum. A plot of velocity against cytochrome oxidase concentration was linear in the range assayed. Total protein was assayed using a modified Lowry method and the absorbance was read at 424 mμ. ●—●, μg total protein in precipitate; ○—○, absorbance at 280 mμ of solubilized precipitate; □—□, $A_{424\ m\mu}$ of solubilized precipitate; △—△, percent activity in supernatant.

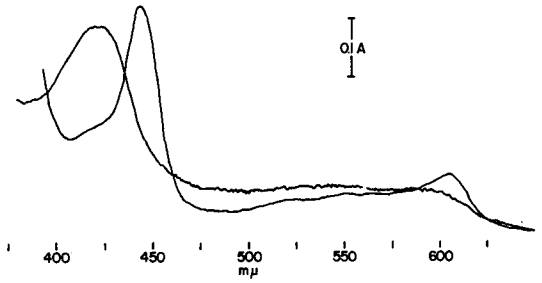

Fig. 5. Visible spectra of cytochrome oxidase–antibody complex. The absolute spectra of the precipitated antibody–oxidase complex, precipitated in antibody excess region (Fig. 4 and Table II) is shown. The scattering due to the aggregated complex was compensated for by using an appropriate dilution of pasteurized milk in the reference cuvette. The complex was reduced with sodium dithionite and the spectrum obtained after 10 min.

MATERIALS AND METHODS using Prep. 2. Spectra were taken using a Cary-11 recording spectrophotometer of the oxidized and reduced oxidase–antibody complex of the resuspended precipitate, and showed maximum absorption peaks in the same region as the uncombined oxidase (Fig. 5). This suggests that no extensive structural changes occurred upon combination and precipitation with the antibody.

The percent activity of the supernatant was calculated as discussed under MATERIALS AND METHODS. The logarithm of the initial velocities of the control normal rabbit serum were linear with respect to volume of supernatant measured over the range of oxidase dilutions examined. The percent activity is a function of both the quantity of oxidase removed from the supernatant and any inhibitory effect by the antibody. In separate experiments to be discussed in the following paper, measurement of the activity of the oxidase in the antibody excess region, showed that the antibody was an effective inhibitor of the cytochrome *c*–cytochrome oxidase reaction.

The molecular and molar compositions of cytochrome oxidase and antibody in the various dilutions of the supernatant and precipitate are listed in Table II. The antibody excess zone corresponds from approx. 12–94 μg oxidase protein added. In this region nearly all of the oxidase is precipitated, as indicated from the low (approx. 3 %) activity remaining in the supernatant and from an inspection of the number of moles of antibody:oxidase precipitated. The equivalence zone extends from 94 to 187 μg oxidase protein added where the molecular composition is antibody$_2$–oxidase and the reaction is partly inhibited by excess antigen. This is additionally evident from the rise in percent activity remaining in the supernatant and furthermore indicates that the equivalence zone is occurring on the antibody excess side of the precipitin curve. In the antigen excess region, the precipitation reaction is partly inhibited and a steady rise in percent activity is observed, indicating an increase in the oxidase remaining in ~~the~~ supernatant. The molecular composition becomes antibody–oxidase$_2$. This is in agreement with reported ratios in other systems[19].

TABLE II

QUANTITATIVE PRECIPITATION WITH CYTOCHROME OXIDASE

Quantitative precipitation was performed as in Fig. 8. The concentration of cytochrome oxidase in the precipitate was determined from the absorbance at 424 mμ (ε, 575 (g protein per ml)$^{-1}\cdot$cm^{-1}; YONETANI[26]). The number of moles of antibody (Ab) precipitated was calculated from the difference between the total protein assayed and that calculated from the absorbance for cytochrome oxidase (Ox).

Oxidase added (μg)	*Oxidase added** (*nmoles*)	*Oxidase precipitated* (*nmole*)	*Antibody precipitated*** (*nmole*)	*Ratio antibody/oxidase* (*moles/mole*)	*Molecular composition*
1500	6.0	0.26	0.91	3.5	Ab_4Ox
750	3.0	0.56	0.35	0.6	$AbOx_2$
375	1.5	0.59	0.79	1.3	AbOx
187	0.75	0.50	0.90	1.8	Ab_2Ox
94	0.375	0.26	0.62	2.4	Ab_5Ox_2
47	0.188	0.12	0.94	7.8	Ab_8Ox
24	0.096	0.06	0.41	6.5	Ab_7Ox

* Mol. wt. oxidase = 260000 (estimated on the basis of heme content per mg protein; ref. 27).
** Mol. wt. antibody = 160000 (ref. 19).

Reaction of antibody with detergent-treated oxidase

In view of the lipoprotein nature of the oxidase and consequently the necessity of detergents for solubilization and the reported modification of the aggregation state of cytochrome oxidase[22,23] by detergents, the effect of antibody on the detergent-modified oxidase was examined.

YONETANI[24] has shown that surfactants such as Tween-80 and Emasol 4130 had no effect on the spectral properties of the oxidase. Similarly, JACOBS *et al.*[25] observed only slight changes in the oxidase spectrum upon treatment with Triton X-100. Thus, from these studies, it appears that these detergents cause no large changes in the protein–heme interaction of the oxidase. However, Triton X-100 does appear to have a "dispersing effect" on the oxidase since it can apparently give rise to a low-molecular-weight form of the oxidase[22,25].

Location of the precipitin lines of the antibody–Triton X-100-treated oxidase is illustrated in Fig. 6. Cytochrome oxidase (0.75 mg protein) was mixed with 1% Triton X-100 or with 5 mM NaN_3 for comparative purposes. Immunodiffusion shows that Triton X-100 induces a new immunological species (Well 1) which gave a precipitin line closer to the center well indicating that this species is in a lower aggregation state. A faint line is also observed which formed an apparent line of identity with the precipitin line of the untreated oxidase (Well 3) as traced through the precipitin line of Well 2 containing NaN_3. No change was noted for the precipitin line in the presence of NaN_3 (Well 2). Treatment of the agar plate with the nadi reagent indicated that the new precipitin line in Well 1 was reactive towards the reagent (Fig. 6B). From these studies it is clear that the antibody can react with an active form of the Triton X-100-modified oxidase.

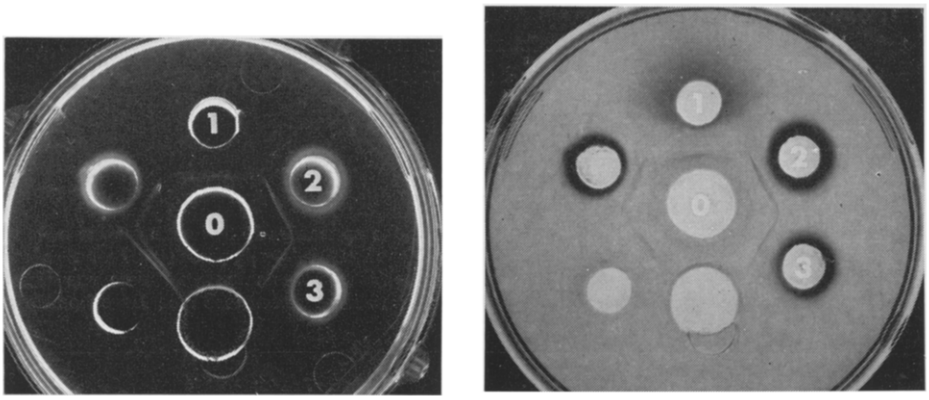

Fig. 6. Antibody interaction with Triton X-100-treated cytochrome oxidase. A (left). Antiserum and 750 μg cytochrome oxidase protein (Prep. 2) were diffused in 0.5% agar containing 0.05 M potassium phosphate buffer (pH 7.0). Well 1 contained cytochrome oxidase in 2% Triton X-100; Well 2 untreated cytochrome oxidase + 5 mM NaN_3; Well 3, untreated cytochrome oxidase. The center well (0) contained undiluted antiserum. B (right). Part A after reaction with the nadi reagent.

DISCUSSION

On the basis of agar diffusion, quantitative precipitation and passive hemagglutination, the present study has shown unequivocally that purified mammalian cytochrome oxidase, prepared by the method of YONETANI[17] is capable of eliciting

an immunological response in rabbits. These studies have further revealed that the antibody is of the precipitating variety. Furthermore, the immunoelectrophoresis studies have led to a tentative identification of the antibody as one of the class of IGg immunoglobulins.

As anticipated, the antibody was highly specific for cytochrome oxidase, since cytochrome *c*, either beef or horse, did not cross-react with the antibody as judged by immunodiffusion. However, the antibody was reactive towards cytochrome oxidase of the Keilin–Hartree heart muscle particles (beef), and neutralized (*i.e.* inhibited) the reaction of either heart muscle particles or soluble oxidase with ferrocytochrome *c* (see following paper, ref. 15). In addition, the antibody was also reactive towards the Triton X-100-modified oxidase.

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