BBA 46036

ISOLATION OF NADH–CYTOCHROME B_5 OXIDOREDUCTASE FROM RAT LIVER MICROSOMES

J. R. SARGENT*, P. J. ST. LOUIS AND PATRICIA A. BLAIR
Department of Biochemistry, University of Aberdeen, Aberdeen (Great Britain)
(Received July 13th, 1970)

SUMMARY

1. NADH–cytochrome b_5 oxidoreductase is released from rat liver microsomes when the latter are incubated at 37° with a lysosomal fraction from rat liver.

2. Release is maximal when equal amounts of microsomes and the lysosomal fraction a on protein basis are incubated. The release is optimal at pH 5.5 and is unaffected by iodoacetamide.

3. A 3–4-fold activation of NADH–cytochrome b_5 oxidoreductase activity occurs during release from microsomes but NADH–ferricyanide oxidoreductase activity, although released, is not activated.

4. A 20-fold purified protease fraction from lysosomes, containing cathepsin D activity, is 20 times more active in releasing the oxidoreductase than is the original lysosomal fraction.

5. A simple procedure, involving ultrafiltration, molecular sieving and isoelectric focussing, is described for isolating the oxidoreductase as a single protein species.

INTRODUCTION

NADH–cytochrome b_5 oxidoreductase and cytochrome b_5 are constitutive proteins of liver microsomes that together constitute an electron transfer system capable of reducing added cytochrome *c* in the presence of NADH[1]. The oxidoreductase is a flavoprotein that was first isolated in a pure state by STRITTMATTER AND VELICK[1] from calf liver microsomes. In the original purification the enzyme was released from microsomes by digesting with snake venom at pH 5.9. Later work by TAKESUE AND OMURA[2–4], however, showed that the oxidoreductase is released from microsomes not by an active principle in venom, but by lysosomes contaminating the microsomes.

The present work was undertaken to investigate the nature of the lysosomal principle responsible for releasing the oxidoreductase from microsomes, and also to isolate the pure enzyme by simple methods. Evidence is presented that cathepsin D is involved in releasing the oxidoreductase from microsomes. Some aspects of this work have already been communicated[5].

* Present address: N.E.R.C. Fisheries Biochemical Research Unit, University of Aberdeen, Scotland.

EXPERIMENTAL

Animals

Male Wistar strain rats (200–250 g weight) were maintained on a normal laboratory diet and were starved for 16 h before being killed. The animals were anaesthetised with ether and the livers perfused *in situ* with chilled 0.9% NaCl *via* the portal vein. Livers were rapidly excised and transferred to cold 0.9% NaCl.

Preparation of cell-free fractions

All steps were carried out at 2–4°. Perfused livers were chopped finely and homogenised with 9 vol. of 0.25 M sucrose containing 1 mM EDTA (pH 7.4), in a glass-Teflon homogeniser with a clearance of 0.006–0.009 inch. The pestle was driven mechanically at about 1000 rev./min and three complete passes were used to effect cell rupture. The homogenate was centrifuged at $9.00 \cdot 10^3$ $g_{av} \cdot$min and the nuclear pellet discarded. The supernatant was centrifuged at $5.00 \cdot 10^4$ $g_{av} \cdot$min to yield a mitochondrial pellet and the supernatant further centrifuged at $8.00 \cdot 10^4$ $g_{av} \cdot$min to yield a lysosomal pellet. The supernatant was finally centrifuged at $6.30 \cdot 10^6$ $g_{av} \cdot$min to yield the microsomal fraction. This fractionation is essentially the same as that described by TAKESUE AND OMURA[3]. The microsomal and lysosomal fractions were normally suspended by homogenising in 0.1 M Tris–maleate (pH 5.7) to give a protein concentration each of about 20 mg/ml and stored at 0°.

Isolation of NADH–cytochrome b_5 oxidoreductase

Microsomal and lysosomal fractions in 0.1 M Tris–maleate buffer (pH 5.7) were mixed to give equal concentrations of microsomal and lysosomal protein (10 mg/ml) unless stated otherwise. The mixture was incubated at 37° in air for 120 min with shaking unless otherwise stated. The suspension was cooled and centrifuged at $12.60 \cdot 10^6$ $g_{av} \cdot$min to yield a slightly yellow, clear supernatant. All further steps were carried out at 2–4°. The supernatant was concentrated on a Diaflo XM-50 ultrafilter (Amicon N.V., Oosterhout, N.B., Holland) and the concentrated solution fractionated on a Sephadex G-100 column equilibrated and developed with 0.1 M sodium phosphate buffer (pH 7.5). The enzymically active fraction from the column was concentrated on a Diaflo UM-10 ultrafilter and the concentrate dialysed against distilled water for 16 h. The solution was reconcentrated where necessary and subjected to isoelectric fractionation (1% (w/v) Ampholine, pH 3–10) at 2° for 48 h (LKB-Produkter, Sweden). The enzymically active fraction from the column was passed through a column of Sephadex G-100 equilibrated with 0.1 M sodium phosphate (pH 7.5) to remove Ampholine and the enzymically active fraction stored at 0 or −15°. The enzyme was stable at 0° for several weeks at least and at −15° for several months.

Partial purification of protease activity from lysosomes

All steps were carried out at 2–4°. Aqueous Triton X-100 (10%, w/v) was added to a suspension of the lysosomal fraction (10 mg of protein per ml) in 0.1 M Tris–maleate buffer (pH 5.7) to give a final concentration of 1% (w/v). After 5 min the clear solution was centrifuged at $6.30 \cdot 10^6$ $g_{av} \cdot$min to yield a clear supernatant which was concentrated on a Diaflo XM-50 ultrafilter. The concentrate was stirred mechanically at 0° for 15 min with an equal volume of redistilled *n*-pentanol, and the milky

suspension centrifuged at $1.05 \cdot 10^6$ $g_{av} \cdot$ min. The aqueous phase was carefully removed and dialysed against 0.1 M Tris–maleate (pH 5.7) before being fractionated on a column of Sephadex G-200 equilibrated with 0.1 M Tris–maleate buffer (pH 5.7). Fractions emerging from the column were detected with ninhydrin and assayed for protease activity. The protease fraction with highest specific activity was that emerging with the void volume of the column.

Enzyme assays

NADH–cytochrome b_5 oxidoreductase activity was assayed essentially as described by STRITTMATTER AND VELICK[1] using cytochrome b_5 as electron acceptor. The reaction and control cells contained 0.38 ml of a solution of cytochrome b_5 (10 nmoles) and 0.02 ml of a solution of NADH (50 nmoles), both in 0.1 M Tris–acetate buffer containing 1 mM EDTA (pH 8.1). The reaction was started by adding 1–10 μl of enzyme solution to the reaction cell and the increase in absorbance at 423 nm followed in a Unicam SP-800 spectrophotometer at 20°. The initial linear slope was used to determine the rate of the reaction and enzyme units present which were expressed as nmoles of cytochrome b_5 reduced per min per ml of assay solution. An extinction coefficient of 100 $mM^{-1} \cdot cm^{-1}$ was used for reduced *minus* oxidised cytochrome b_5 at 423 nm (ref. 6).

NADH–ferricyanide oxidoreductase activity was also assayed essentially as detailed by STRITTMATTER AND VELICK[1]. The reaction cell contained 0.38 ml of potassium ferricyanide (100 nmoles) and 0.02 ml of NADH (50 nmoles) in 0.1 M Tris–acetate buffer containing 1 mM EDTA (pH 8.1). The control cell contained buffer alone. The reaction was started by adding enzyme solution (1–10 μl) and followed as the decrease in absorbance at 420 nm at 20°. Enzyme units were expressed in this case as the change in absorbance at 420 nm per ml per unit time.

Lysosomal protease activity was assayed essentially as described by COFFEY AND DE DUVE[7]. Globin was prepared from crystalline bovine haemoglobin by treatment with methyl ethyl ketone at pH 2.0 (ref. 8). Fractions containing protease activity were incubated with 5 mg of globin in a total volume of 1.0 ml of 0.1 M Tris–maleate buffer (pH 5.7) at 37° for 4 h. The reaction was stopped by adding 1.0 ml of 10% trichloroacetic acid and 0.1 ml of the resulting supernatant taken for analysis with ninhydrin. Values so obtained in the presence of globin and protease fractions were corrected by subtracting the ninhydrin values obtained in the presence of protease fractions alone. Results were expressed as μmoles equivalent of leucine formed per ml of assay per 4 h. Under the conditions used here protease activity was proportional to the amount of enzyme added and was linear with time for at least 10 h. The optimal pH for protease activity was 4.5 and the optimal temperature was 45°. The activity at pH 5.7 was 70% of the activity at pH 4.5.

Analytical methods

Total protein was measured by the method of LOWRY *et al.*[9] using bovine serum albumin as standard. Ninhydrin analyses were carried by the method of Moore and Stein as described by SPIES[10].

Materials

Cytochrome b_5 was isolated in a pure state from rat liver microsomes as describ-

ed by SARGENT AND VADLAMUDI[11]. Crystallised bovine haemoglobin and NADH were purchased from Sigma London Chemical Co., Ltd.

RESULTS AND DISCUSSION

One objective of the present work was to isolate NADH–cytochrome b_5 oxidoreductase from small amounts of liver microsomes as part of a programme on the biosynthesis of membrane-bound proteins of microsomes. Accordingly, microsomes were isolated conventionally as that fraction sedimenting from a liver homogenate between $1.00 \cdot 10^5$ and $6.30 \cdot 10^6$ $g_{av} \cdot$min. When these microsomes were treated with snake venom at pH 5.9 as detailed by STRITTMATTER AND VELICK[1] release of NADH–cytochrome b_5 oxidoreductase into the high-speed supernatant phase of the digest was negligible. Trypsin, chymotrypsin, steapsin and snake venom at pH 8.0 and pepsin at pH 3.0 likewise were inactive in releasing active enzyme from microsomes.

While this work was in progress TAKESUE AND OMURA[2] reported that, in the original work of STRITTMATTER AND VELICK[1], the oxidoreductase was released from microsomes not by snake venom but by lysosomes contaminating the microsomes. In the original procedure the microsomes had been prepared by steps including an acid precipitation. The data in Fig. 1 show that microsomes prepared under the present conditions can be treated with a lysosomal fraction of liver at pH 5.7 causing release of active oxidoreductase into the high-speed supernatant phase. Release

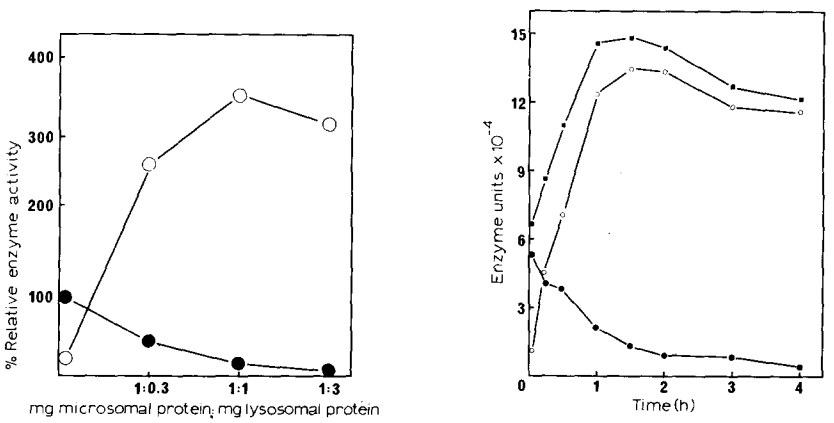

Fig. 1. Release of NADH–cytochrome b_5 oxidoreductase from microsomes digested with lysosomes. Microsomes (100 mg of protein) were digested in the absence and in the presence of lysosomes (33, 100 and 300 mg of protein) for 2 h in 20 ml of 0.1 M Tris–maleate buffer (pH 5.7). The digests were centrifuged at 105000 × g_{av} for 120 min, the supernatants decanted and the pellets suspended in 0.1 M Tris–maleate (pH 5.7). Both the supernatants and pellets were assayed for oxidoreductase activity at pH 8.1 as detailed in EXPERIMENTAL. ○—○, activity in supernatant phases; ●—●, activity in pellets.

Fig. 2. Release of NADH–cytochrome b_5 oxidoreductase from microsomes digested with lysosomes as a function of time. Microsomes (100 mg of protein) were digested with lysosomes (100 mg of protein) for various periods of time in 20 ml of 0.1 M Tris–maleate (pH 5.7) at 37°. The reaction was stopped by rapid chilling in ice, a sample of the digest was retained and the remainder centrifuged at 105000 × g_{av} for 120 min to yield a supernatant phase and pellet. Total digests, supernatant phases and pellets were assayed for NADH–cytochrome b_5 oxidoreductase activity at pH 8.1 as in EXPERIMENTAL. ■—■, enzyme units × 10^{-4} present in total digests; ●—●, enzyme units × 10^{-4} present in pellets; ○—○, enzyme units × 10^{-4} present in supernatant phases.

of the enzyme is maximal when equal amounts of microsomes and the lysosomal fractions are incubated on a protein basis, and an approximately 4-fold activation of the oxidoreductase occurs during the release process. These results are completely in accord with those of TAKESUE AND OMURA[2-4]. The levels of cytochrome b_5 released from microsomes under the conditions used in Fig. 1 were not measurable.

Fig. 2 shows that release of the oxidoreductase from microsomes is maximal after digesting microsomes for 120 min. Thereafter the enzyme appears to undergo slow inactivation. In all subsequent work the digestion of microsomes was terminated at 120 min. The apparent pH optimum of the releasing activity is approx. 5.5 (Fig. 3). Unfortunately it cannot be stated that the releasing factor is indeed optimally active at this pH, since marked inactivation of the oxidoreductase occurred during digestion at pH values less than 5.

It is well known from previous work that alkaline proteases are effective in releasing certain membrane-bound enzymes from microsomes, in particular cytochrome b_5 and NADPH–cytochrome *c* oxidoreductase [11-13]. In contrast cytochrome P450 is released from microsomes, not by proteases, but by alkaline phospholipase activity present in snake venoms[12,11]. It was decided, therefore, to purify protease activity from lysosomes in order to assess whether such activity was involved in

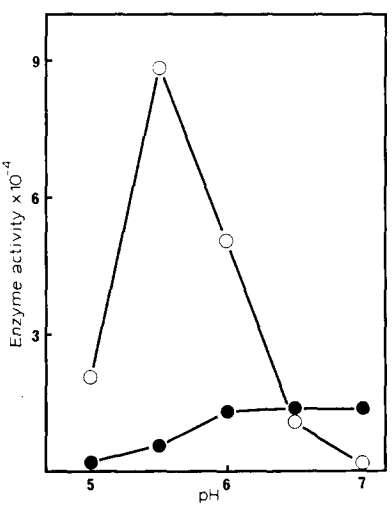

Fig. 3. pH optimum for the release of NADH–cytochrome b_5 oxidoreductase from microsomes digested with lysosomes. Microsomes (100 mg protein) were digested with lysosomes (100 mg protein) in 20 ml of 0.1 M Tris–maleate buffers of varying pH's at 37° for 2 h. The digests were centrifuged at 105000 × g_{av} for 120 min and the supernatant phases and pellets assayed for oxidoreductase activity at pH 8.1 as in Experimental. ○—○, enzyme activity in supernatant phases; ●—●, enzyme activity in pellets.

releasing the oxidoreductase from microsomes. Table I summarises a procedure that resulted in the purification of protease activity from lysosomes by a factor of approx. 20. Protease activity in this procedure refers to the ability of lysosomal fractions to hydrolyse globin as detailed in EXPERIMENTAL, a conventional assay for cathepsin D (ref. 14). The pH optimum of such protease activity was 4.5, the activity at pH 5.7 being 70% of the optimal activity. Attempts to purify the protease activity further were unsuccessful, and although the final preparation is 20 times enriched in cathepsin

TABLE I

PARTIAL PURIFICATION OF PROTEASE ACTIVITY FROM A LYSOSOMAL FRACTION OF LIVER

Protease activity refers to the ability of the various fractions to hydrolyse globin to material soluble in 5% trichloroacetic acid. Details of the assay for protease activity and the purification procedure are described in EXPERIMENTAL.

Fraction	*Total units*	*Specific activity (units/mg)*	*Purification factor*
Lysosomal fraction	3700	6	1.0
Material soluble in 1% Triton X-100	6950	27	4.5
Aqueous phase after *n*-pentanol treatment	4100	45	7.5
Sephadex G-200 void volume	608	117	19.5

TABLE II

RELEASE OF NADH–CYTOCHROME b_5 OXIDOREDUCTASE FROM MICROSOMES BY A PARTIALLY PURIFIED PROTEASE FRACTION

Microsomes (100 mg of protein) were digested with the partially purified protease fraction described in Table I (5 mg of protein) in 10 ml of 0.1 M Tris–maleate buffer (pH 5.7) at 37° for 2 h. The digest was centrifuged at 105000 × g_{av} for 120 min and the supernatant phase and pellet assayed for NADH–cytochrome b_5 oxidoreductase at pH 8.1 and total protein as detailed in EXPERIMENTAL.

		0 h	*2 h*
Total protein (mg)	Pellet	82	80
	Supernatant	18	20
Total Units	Pellet	6400	1700
	Supernatant	2400	39000
Specific units (units/mg)	Pellet	70	20
	Supernatant	120	1950

D activity, it is probable that other proteases are also present. Indeed the preparation hydrolysed carbobenzoxy-L-glutamylphenylalanine and glycyltyrosyl amide at approximately one-half the rate of globin, indicating the presence of cathepsins A and C (ref. 15). It is known that lysosomes contain acid phospholipase A activity[16]. When assayed for acid phospholipase activity by the method of GATT[17] the original lysosomal fraction used here contained significant activity. However, no detectable acid phospholipase activity was present in the final preparation in Table I. It may be noteworthy that the final preparation in Table I was obtained in the void volume of the Sephadex G-200 column. The preparation, therefore, has a molecular weight in excess of 200000 and may represent small fragments of the lysosomal membrane.

Table II shows that the partially purified protease fraction is active in releasing the oxidoreductase from microsomes. Release of the enzymes is quantitative with a ratio of microsomes to purified protease of 20:1 on a protein basis. This result together with the data in Fig. 1 shows that enrichment of cathepsin D activity is accompanied by an enrichment in activity with respect to releasing the oxidoreductase from microsomes. These results show that protease activity, probably cathepsin D activity, and not acid phospholipase A activity is responsible for releasing NADH–cytochrome b_5 oxidoreductase from microsomes.

TABLE III

EFFECT OF TREATING LYSOSOMES WITH IODOACETAMIDE ON THE RELEASE OF NADH–CYTOCHROME b_5 AND NADH–FERRICYANIDE OXIDOREDUCTASE ACTIVITIES FROM MICROSOMES

The lysosomal fraction was incubated with 1 mM iodoacetamide in 0.1 M Tris–HCl (pH 7.4) at 37° for 60 min, then dialysed successively against 0.1 M Tris–HCl (pH 7.4), water and 0.1 M Tris–maleate (pH 5.7) at 0° over a period of 18 h. Control lysosomes were treated in the same way without iodoacetamide. Fractions were incubated with microsomes in the proportions shown in 10 ml of 0.1 M Tris–maleate (pH 5.7) for 2 h at 37° and centrifuged at 105000 × g_{av} for 120 min to yield supernatant phases. The latter and samples of the original mixtures stored on ice were assayed for oxidoreductase activities as detailed in EXPERIMENTAL. Results are presented as % of the activity per ml in supernatants: activity per ml in the original mixtures.

Microsomes (mg)	*Control lysosomes (mg)*	*Treated lysosomes (mg)*	*% Enzyme released*	
			NADH–cytochrome b_5 oxidoreductase	*NADH–ferricyanide oxidoreductase*
7.5	1.5	—	247	69
7.5	3.0	—	339	83
7.5	—	1.5	262	58
7.5	—	3.0	307	69
7.5	0.75	0.75	227	61
7.5	1.5	1.5	363	71

Of the cathepsins present in rat liver lysosomes cathepsins B and C are sensitive to iodoacetamide whereas cathepsins A and D are not[15]. Accordingly we investigated the effects of iodoacetamide on the release of the oxidoreductase catalysed by the lysosomal fraction. NADH–cytochrome b_5 oxidoreductase has been reported to contain an essential thiol group[1]. Although the oxidoreductase was shown in the present work to be uninhibited by exposure to 1 mM iodoacetamide for 5 min at room temperature, the precaution was taken of first treating the lysosomal fractions with 1 mM iodoacetamide at 37° for 60 min, then removing excess iodoacetamide by dialysis before assaying release of the oxidoreductase from microsomes. Table III shows that the lysosomal fraction treated in this way is as effective as a control lysosomal fraction treated in the same way but in the absence of iodoacetamide. The data demonstrate that only very small amounts of microsomal protein are released, suggesting that under the conditions used here the protease activity is highly selective with respect to digesting microsomal proteins in general. It may be concluded from these results that cathepsins B and C are very unlikely to be involved in the release process. Since cathepsin A is considered to be an exopeptidase and cathepsin D is known to be an endopeptidase[15], it is probable that cathepsin D is the major if not the only enzyme involved in releasing the oxidoreductase. At the same time the possibility cannot be excluded that both cathepsins are active under the present conditions.

The reduction of cytochrome b_5 and ferricyanide is catalysed by a single protein species[1]. Nevertheless cytochrome b_5 reductase activity is considerably enhanced when the enzyme is released from microsomes whereas the ferricyanide reductase activity is not (Fig. 1, Table III). It may be inferred that the microsomal enzyme donates electrons to exogenous cytochrome b_5 at a slower rate than the soluble enzyme. Ferricyanide is reduced at the same rate by the enzyme in both states. Since it is certain that in microsomes the flavoprotein is in close juxtaposition to the cytochrome b_5, it seems likely that the flavin moiety of the flavoprotein is readily

TABLE IV

PURIFICATION OF NADH–CYTOCHROME b_5 OXIDOREDUCTASE FROM MICROSOMES

The oxidoreducatase was purified from microsomes isolated from approx. 100 g of liver. Individual steps are described in detail in EXPERIMENTAL.

Fraction	*Total units*	*Specific activity (units/mg)*	*Purification factor*
Original microsomal *plus* lysosomal fractions	49 000	250	1
Supernatant after high-speed centrifugation	2 100 000	2 800	11
Material retained on XM-50 ultrafilter	1 750 000	4 380	18
Fraction from Sephadex G-100	655 000	15 400	62
Fraction from isoelectric focussing	77 000	27 000	110

accessible to the small ferricyanide molecule, but considerably less accessible to the relatively large, exogenous cytochrome b_5 molecule.

Once the oxidoreductase has been released from microsomes it can readily be isolated in a pure state. Table IV summarises a procedure developed for purifying the enzyme. This procedure is essentially identical to that described recently by TAKESUE AND OMURA[4] except that we have replaced the $(NH_4)_2SO_4$ fractionation used by these authors by a more efficient fractionation based on ultrafiltration. Fig. 4 shows the elution profile of the enzyme from Sephadex G-100, from which

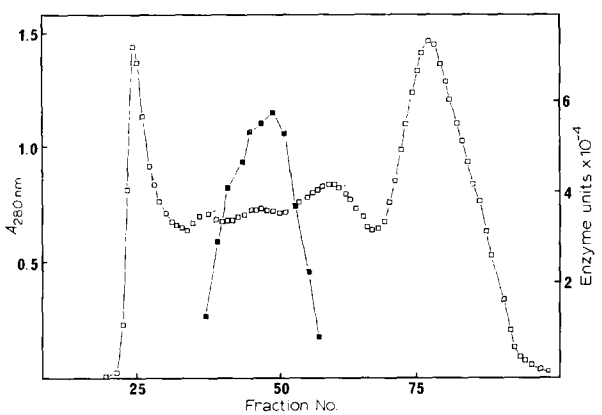

Fig. 4. Elution profile of NADH–cytochrome b_5 oxidoreductase activity from Sephadex G-100. The supernatant phase from a digest of microsomes and lysosomes was concentrated on a Diaflo XM-50 ultrafilter. The material retained on the filter was applied to a column of Sephadex G-100 and the column developed in 0.1 M sodium phosphate buffer (pH 7.5). □—□, $A_{280\,nm}$; ■—■, enzyme units × 10^{-4}.

a molecular weight af about 50000 can be deduced for the enzyme, and Fig. 5 shows the profile resulting from isoelectric fractionation of the active fraction from the Sephadex G-100 column. It is noteworthy that only a single peak of active enzyme with an isoelectric point of 6.5 is obtained. Fractionation could be carried out with equal efficiency in the pH ranges 5–8 or 3–10, although recovery of the enzyme after isoelectric fractionation was never more than 50%. It is possible that this poor recovery

reflects partial inhibition of the enzyme by the Ampholine used in the isoelectric fractionation. The latter is a metal-chelating agent and NADH–cytochrome b_5 oxidoreductase is known to be partly inhibited by high concentrations of EDTA[1].

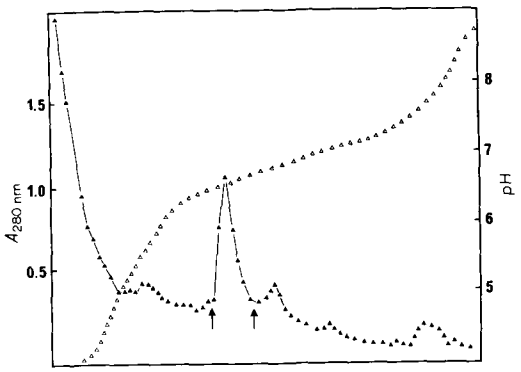

Fig. 5. Isoelectric fractionation of NADH–cytochrome b_5 oxidoreductase activity. The enzymically active fraction from the Sephadex G-100 column (Fig. 4) was concentrated on a Diaflo UM-10 ultrafilter, dialysed against water and applied as the middle zone of a stepwise sucrose gradient (0–50%, w/v) containing 1% (w/v) Ampholine (pH range 5–8) in an isoelectric focussing apparatus. Fractionation was carried out at 2° for 48 h. The anode was the dense solution at the bottom of the column corresponding to the low pH values at the left of the figure. Enzyme activity was confined to those fractions between the arrows and followed the $A_{280\ nm}$ profile within this region, ▲ ▲, $A_{280\ nm}$; △ △, pH values.

The final preparation showed a single peak when examined by electrophoresis on polyacrylamide gels at pH 8.6. TAKESUE AND OMURA[4] found that two enzymically active components are obtained after digesting microsomes with lysosomes for 5 h at pH 5.7. In the present results the digestion was terminated at 2 h because significant inactivation of the oxidoreductase occurred after this time (Fig. 2). It is probable that such inactivation represents partial hydrolysis of the oxidoreductase by lysosomal proteases causing the formation of more than one species of active enzyme. It is well known that more than one species of cytochrome b_5 may result from protease digestion of microsomes[11,18].

The present work strongly suggests that cathepsin D activity is involved in selectively releasing NADH–cytochrome b_5 oxidoreductase from microsomes. It is now well established that other hydrolytic enzymes including trypsin, chymotrypsin, pronase and phospholipase A will also selectively release electron transfer enzymes from microsomes including cytochrome b_5, NADPH–cytochrome *c* oxidoreductase and cytochrome P450(refs. 6, 11–13). An alternative approach to purifying electron transfer enzymes from microsomes is to use organic solvents and detergents under conditions where hydrolytic reactions are unlikely to occur. The latter approach yields preparations of phospholipoproteins containing NADH–cytochrome b_5 oxidoreductase, cytochrome b_5 and phospholipid as well as nonhaem iron (refs. 19, 20; and J. R. SARGENT AND P. ST. LOUIS, unpublished data)

It may be of interest to consider the release of electron transfer enzymes from microsomes by hydrolytic enzymes in terms of such phospholipoprotein complexes. A protease may release a protein from such a structural complex by cleaving the protein itself, in such a way that an enzymatically active core is released into solution.

Such a core is unlikely to contain all the sites whereby the original protein was attached to the membrane complex and may, therefore, be of limited use in assessing the manner in which macromolecules are arranged to form the complex. It may also be possible to release a given protein by hydrolysing a second molecule, either enzymic or structural protein or lipid, to which the released protein was originally attached in the structure. A protein released in this way is likely to be representative of the protein in its original particulate state. At present it is know that cytochrome b_5 released from microsomes by trypsin comprises approximately one-half of the molecule as it exists in microsomes[21]. In this case release of the cytochrome by the former of the two aforementioned mechanisms is indicated. The manner in which NADH–cytochrome b_5 oxidoreductase is released from microsomes cannot be deduced from the present data alone. Such a problem, however, is particularly worthy of further study, since it may not only shed light on the way in which specific enzymes are integrated into membrane structures, but it may also reveal aspects of the relationship of lysosomes to microsomes in terms of membrane turnover within the cell.

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge the interest and encouragement shown by Emeritus Professor W. O. Kermack, F. R. S., during the course of this work. We are also grateful to Mr. J. Merchant for technical assistance. The work was supported in part by a grant from the Medical Research Council of the United Kingdom.

REFERENCES

1 P. STRITTMATTER AND S. F. VELICK, *J. Biol. Chem.*, 228 (1957) 785.
2 S. TAKESUE AND T. OMURA, *Biochem. Biophys. Res. Commun.*, 30 (1968) 723.
3 S. TAKESUE AND T. OMURA, *J. Biochem.*, 67 (1970) 259.
4 S. TAKESUE AND T. OMURA, *J. Biochem.*, 67 (1970) 267.
5 J. R. SARGENT, P. ST. LOUIS AND P. A. BLAIR, *Biochem. J.*, 118 (1970) 21P.
6 P. STRITTMATTER AND S. F. VELICK, *J. Biol. Chem.*, 221 (1956) 253.
7 J. W. COFFEY AND C. DE DUVE, *J. Biol. Chem.*, 243 (1969) 3253.
8 F. W. J. TEALE, *Biochim. Biophys. Acta.*, 35 (1959) 543.
9 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. F. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
10 J. R. SPIES, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. III, Academic Press, New York, 1957, p. 467.
11 J. R. SARGENT AND B. P. VADLAMUDI, *Biochem. J.*, 107 (1968) 839.
12 T. OMURA AND R. SATO, *J. Biol. Chem.*, 239 (1964) 2379.
13 A. H. PHILLIPS AND R. G. LANGDON, *J. Biol. Chem.*, 237 (1962) 2652.
14 L. F. KRESS, R. J. PEANASKY AND H. M. KLITGAARD, *Biochim. Biophys. Acta*, 113 (1966) 375.
15 A. J. BARRETT, in J. T. DINGLE AND H. B. FELL, *Lysosomes in Biology and Pathology*, Vol. 2, North Holland Publishing Co., Amsterdam, 1969, p. 245.
16 A. MELLORS AND A. L. TAPPEL, *J. Lipid Res.*, 8 (1967) 479.
17 S. GATT, *Biochim. Biophys. Acta*, 159 (1968) 304.
18 P. STRITTMATTER AND J. OZOLS, *J. Biol. Chem.*, 241 (1966) 4787.
19 D. H. MACLENNAN, A. TZAGOLOFF AND D. G. MCCONNELL, *Biochim. Biophys. Acta*, 131 (1967) 59.
20 B. MACKLER, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymologie*, Vol. X, Academic Press, New York, 1967, p. 551.
21 A. ITO AND R. SATO, *J. Biol. Chem.*, 243 (1968) 4922.