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SOLUBLE NADH-CYTOCHROME b_5 REDUCTASE FROM RABBIT LIVER CYTOSOL: PARTIAL PURIFICATION AND CHARACTERIZATION

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Summary

A soluble form of NADH-cytochrome b_5 reductase (NADH:ferricytochrome b_5 oxidoreductase, EC 1.6.2.2) was found in the cytosolic fraction of rabbit liver. The partially purified enzyme was strictly specific for NADH. It catalyzed the reduction of several substrates such as the methemoglobin · ferrocyanide complex (Hegesh, E. and Avron, M. (1967) Biochim. Biophys. Acta 146, 91—101) (apparent K_m: 8 μM), potassium ferricyanide (apparent K_m: 10 μM) and ferricytochrome b_5 (apparent K_m: 15 μM). Upon acrylamide gel isoelectrofocusing followed by specific staining, the enzyme was resolved into four bands (isoelectric pH: 7.05, 6.70, 6.50 and 6.30). The optimum pH of activity with ferricytochrome b_5 as a substrate was 6.5. The estimated molecular weight was 25 000—30 000. The enzyme was unsensitive to cyanide. It was strongly inhibited by *p*-hydroxymercuribenzoate. The cytosolic liver cytochrome b_5 reductase was immunologically related to the soluble cytochrome b_5 reductase from human and rabbit red-cells, and to the microsomal cytochrome b_5 reductase from rabbit liver.

Introduction

It was recently demonstrated in man [1,2] and other animals [3,4] that the enzyme which promotes the reduction of methemoglobin in the erythrocytes: methemoglobin reductase, or NADH-diaphorase [5] is actually a soluble NADH-cytochrome b_5 reductase (NADH:ferricytochrome b_5 oxidoreductase, EC 1.6.2.2). It was also shown that the soluble cytochrome b_5 reductase isolated from human and animal red cells and human placenta cytosol was immunologically related to the enzyme isolated from animal liver microsomes and human placenta microsomes [2—4].

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Therefore there are at least two subcellular localizations of NADH-cytochrome b_5 reductase: one is microsomal [6], the other is soluble and present in the cytosol. Both forms are ubiquitous [7] and coded by a single gene [8—10]. In the present study we report on the isolation and characterization of the soluble NADH-cytochrome b_5 reductase in the cytosolic fraction of rabbit liver.

Materials

Phenylmethyl sulfonyl fluoride, NADH, NADPH, 2,6-dichlorophenol indophenol, 3-(4,5-dimethyl thiazolyl-2)-2,5 diphenyl tetrazolium bromide, Triton X-100, dicumarol, *para*-hydroxymercuribenzoate were purchased from Sigma.

DEAE-cellulose 52, Biogel P 60 and Hypatite C were respectively obtained from Whatman, Biorad and Clarkson Chemical Company.

Methods

Preparation of cytochrome b_5. Cytochrome b_5 was purified to homogeneity from rabbit liver microsomes after solubilization by trypsin according to the method of Omura and Takesue [11].

Enzyme assays. NADH-methemoglobin · ferrocyanide reductase activity was assayed according to the method of Hegesh et al. [12] using the methemoglobin · ferrocyanide complex as electron acceptor [13]. NADH-diaphorase activity was measured according to the method of Scott and McGraw [5], using 2,6-dichlorophenol indophenol as a final electron acceptor. NADH-ferricyanide reductase activity was assayed according to the method of Zamudio and Canessa [14], using potassium ferricyanide as electron acceptor. NADH-cytochrome b_5 reductase activity was assayed according to Leroux et al. [2]. The velocity was defined as the apparent first order rate constant k (min^{-1}). Results are expressed as k · mg $protein^{-1}$.

DT diaphorase was assayed according to the method of Ernster [15], based on the complete inhibition of the enzyme by 1 μM dicumarol. NADPH-cytochrome *c* reductase was assayed according to Yasukochi and Masters [16].

Polyacrylamide gel electrophoresis. This was carried out according to the method of Ornstein [17], using a 7.5% acrylamide gel with 2 mA per tube for 2 h at 4°C. The cathode buffer contained 0.05 M Tris/glycine buffer (pH 9.2) and the anode buffer contained 0.1 M Tris/HCl buffer (9.2). The NADH-diaphorase was located by specific staining according to the method of Kaplan and Beutler [18] using a mixture composed of 1.2 mM 3-(4,5-dimethyl thiazoyl-2)-2,5-diphenyl tetrazolium-bromide, 0.06 mM 2,6-dichlorophenol indophenol and 1.3 mM NADH in a 0.25 M Tris · HCl buffer pH 8.4. Proteins were stained with Coomassie Blue.

Isoelectric focusing. This was carried out according to the method of Drysdale et al. [19]. The electrophoresis was performed at 4°C for 4 h with a constant voltage of 400 V using 2% ampholine carrier in the 3.5—10 pH range. The gels were stained for NADH-diaphorase activity as described above.

Immunological methods. Chicken anti-human-red-cell NADH-methemoglobin reductase was prepared as described previously [7]. Baseline activities of the enzymes were made equal before incubation with antiserum. The samples

were preincubated with varying amounts of antibody in the incubation mixture (the final volume brought to 60 μl with saline) for 30 min at 37°C and 12 h at 4°C. Residual activities were assayed according to the method of Hegesh et al. [12]. In control experiments, antiserum was replaced by serum from a non-immunized chicken.

Molecular weight determination. Two methods were employed to estimate the molecular weight of the cytosolic enzyme. The purified enzyme was subjected to Biogel P-60 chromatography on a column (93 × 3 cm) equilibrated with 10 mM potassium phosphate buffer pH 6.4. Hemoglobin (mol. wt. 64 000), ovalbumin (mol. wt. 45 000), cytochrome *c* (mol. wt. 12 500) were used as reference compounds. The void volume was determined with Blue Dextran. Linear plots of V_e/V_0 vs. log mol. wt. were obtained. The molecular weight of the enzyme was also determined by sucrose density centrifugation, as described by Martin and Ames [20] in a linear 5—20% sucrose gradient. Glucose oxidase (mol. wt 153 000), hemoglobin and cytochrome *c* were used as marker proteins.

Partial purification of soluble NADH-cytochrome b_5 reductase. Livers from overnight fasted rabbits were perfused with 0.9% NaCl containing 0.25 mM phenylmethyl sulfonyl fluoride. About 400 g liver were homogenized in 0.25 M saccharose in a 1 mM Tris · HCl buffer (pH 7.0)/1 mM EDTA/1 mM phenylmethyl sulfonyl fluoride, and centrifuged at 800 × *g* to discard nuclei and cellular debris. The supernatant was subsequently centrifuged at 8000 × *g* for 15 min and the pellet was discarded. The supernatant was then submitted to centrifugation at 105 000 × *g* for 90 min. All experiments were carried out at 4°C. The supernatant, devoid of NADPH-cytochrome *c* reductase activity, was used as starting material for the purification procedure which comprised the following steps:

$(NH_4)_2SO_4$ was added to the cytosol to 40% saturation (240 g/l); the mixture was stirred for 60 min, then centrifuged for 20 min at 30 000 × *g*. The supernatant was collected and $(NH_4)_2SO_4$ was added to 75% saturation (245 g/l). The mixture was stirred overnight; the precipitate collected by centrifugation was suspended in a minimal volume of buffer and extensively dialysed against 10 mM potassium phosphate buffer (pH 6.4).

The preparation (3020 mg protein) was layered onto a DEAE-cellulose 52 column (93 × 3 cm) equilibrated with 10 mM phosphate buffer (pH 6.4). The column was washed with the same buffer at a flow rate of 38 ml/h. The enzyme was eluted with a linear 10—300 mM gradient of phosphate buffer (pH 6.4). Active fractions were collected and concentrated by ultrafiltration.

The preparation (464 mg protein) was applied to a Biogel P-60 column (90 × 3 cm) equilibrated with 10 mM phosphate buffer (pH 6.4). The active fractions were pooled and concentrated.

The enzyme preparation was layered on a Hypatite C column (15 × 1.3 cm) equilibrated with the same buffer. The column was washed, and the enzyme was eluted stepwise with a 40, 70, 100 mM gradient. The active fraction was in the 70 mM buffer.

Throughout the whole procedure, the presence of enzyme was monitored by the NADH-methemoglobin · ferrocyanide reductase assay [12].

Other methods. Microsomal cytochrome b_5 reductase was studied after solu-

bilization of rabbit liver 105 000 $\times g$ pellet with Triton X-100 (0.1% final). The soluble cytochrome b_5 reductase from rabbit erythrocytes was studied in a destromatized 1 : 10 hemolysate.

The protein concentration was determined by the method of Lowry using bovine serum albumin as a standard [21].

Results

1. Purification of soluble NADH-cytochrome b_5 reductase

In the final preparation, the specific activity of the cytosolic liver enzyme was 1.69 units/mg protein with the methemoglobin · ferrocyanide reductase assay and 46.0 (k) per mg protein with the cytochrome b_5 reductase assay (Tables I and II). Only slight contamination by other proteins was found after disc electrophoresis in 7.5% polyacrylamide gel. The final enzymatic preparation was devoid of DT Diaphorase (Table I), a dicumarol-sensitive enzyme which represented about half of the total NADH-diaphorase activity in the starting cytosol.

The purified enzyme could be kept frozen at —20°C for several months without any loss of activity.

Properties

(a) Substrate specificity and kinetic data. As shown in Table II, purified cytochrome b_5 reductase catalyses the reduction of the methemoglobin · ferrocyanide complex of Hegesh et al. [13], ferricyanide, 2,6-dichlorophenol indophenol and cytochrome b_5. The enzyme exhibited strict specificity for NADH.

The apparent K_m for the methemoglobin · ferrocyanide complex of Hegesh, potassium ferricyanide and cytochrome b_5 were 8, 10 and 15 μM respectively (Table III).

(b) Effect of inhibitors. The effect of several inhibitors on the cytosolic cytochrome b_5 reductase was examined (Table IV). The enzyme was inhibited by *para*-hydroxymercuribenzoate. Potassium cyanide and atebrine had no effect on the rate of reduction of the methemoglobin · ferrocyanide complex or of 2,6-dichlorophenol indophenol.

(c) Molecular weight estimation. The molecular weight of the cytosolic reductase was estimated to about 30 000 by Biogel P-60 chromatography and 25 000 on a linear (5—20%) sucrose density gradient.

(d) pH curve. The pH activity dependence was studied in three different buffers (Fig. 1): 50 mM triethanolamine hydrochloride/NaOH, 50 mM Tris/bis Tris · HCl, and 50 mM potassium phosphate. The pH curve of the reductase with cytochrome b_5 as a substrate showed an optimal pH at 6.5 with Tris/bis Tris buffer and phosphate buffer. With triethanolamine the maximum was shifted to pH 7.0.

(e) Electrophoretic studies. Fig. 2 shows the electrophoresis pattern of cytosolic and microsomal cytochrome b_5 reductase from liver and erythrocyte soluble enzyme stained as NADH-diaphorase. The two soluble enzymes exhibited two common bands (A and B). An additional more cathodic band (C) was found for the erythrocyte enzyme. A single slow-moving band (D) was observed for the microsomal enzyme solubilized by Triton X-100 which hardly

TABLE I

STEPS OF THE PURIFICATION PROCEDURE

Step	Volume (ml)	Total protein (mg)	Total activity (units) *	Specific activity (units · mg^{-1} · protein)	DT Diaphorase % inhibition with dicumarol		Recovery	Purification **
					NADH	NADPH		
Crude cytosol	665	9243	102	0.011	54	83	100	1
Ammonium sulfate precipitation	80	3023	39	0.013	68	78	39	1.2
DEAE-cellulose	13	464	31	0.067	23	8	31	6
Bio-Gel P-10	3.3	89	13.9	0.157	0	0	14	14
Hypatite C	0.5	4.4	7.4	1.69	0	0	7	154

* NADH methemglobin-ferrocyanide reductase activity assayed according to the method of Hegesh et al. [12]; units are μmol of substrate reduced per min.
** These figures are minimized by the presence in the crude cytosol of several interfering enzymes (such as DT Diaphorase).

TABLE II

SPECIFIC ACTIVITY OF PURIFIED ENZYME FOR ELECTRON DONORS AND ACCEPTORS

Conditions were those described in Methods. Each assay system contained 8 μg of purified enzyme in a final volume of 1 ml.

Acceptor	Concentration (mM)	Donor	Concentration (mM)	Specific activity (μmol substrate/min per mg protein)
Methemoglobin · ferrocyanide complex	0.018	NADH	0.15	1.69
		NADPH	0.15	0
Ferricyanide	0.33	NADH	0.20	0.11
		NADPH	0.20	0
2,6 dichlorophenol indophenol	0.10	NADH	0.10	0.73
		NADPH	0.10	0
Cytochrome b_5	0.01	NADH	0.10	46.0 *
		NADPH	0.10	0

* Activity expressed as first rate order constant (k) per mg protein.

TABLE III

K_m DETERMINATION

Assay conditions were described in Methods. The amount of enzyme was 8 μg in 1 ml final volume. Apparent K_m was obtained from Lineweaver-Burk plots, with methemoglobin · ferrocyanide complex and potassium ferricyanide as electron acceptor; the concentration range was from 0.007 to 0.030 mM with the methemoglobin · ferrocyanide complex and from 0.05 to 0.60 mM with potassium ferricyanide. The concentration of cytochrome b_5 was 10 μM and the K_m value was calculated using the integrated form of the Michaelis equation.

Electron acceptor	Electron donor NADH (mM)	Apparent K_m for acceptor M
Methemoglobin · ferrocyanide complex	0.15	$8 \cdot 10^{-6}$
Potassium ferricyanide	0.20	$10 \cdot 10^{-6}$
Cytochrome b_5	0.10	$1.5 \cdot 10^{-5}$

TABLE IV

EFFECTS OF INHIBITORS ON LIVER CYTOSOLIC CYTOCHROME b_5 REDUCTASE

Incubation of the enzyme with each of the compounds listed was carried out for 15 min in the standard assay mixture. The amount of enzyme added was 8 μg in a final volume of 1 ml. The effect of cyanide could not be investigated with the methemoglobin · ferrocyanide complex as a substrate because of the formation of cyanmethemoglobin.

Inhibitors	Concentration (mM)	Acceptors	
		Methemoglobin · ferrocyanide complex	2,6 dichlorophenol indophenol
None		100	100
p-hydroxymercuribenzoate	0.1	0	0
	0.01	38	25
Potassium cyanide	1	—	97
Atebrine (quinacrine)	1	98	100

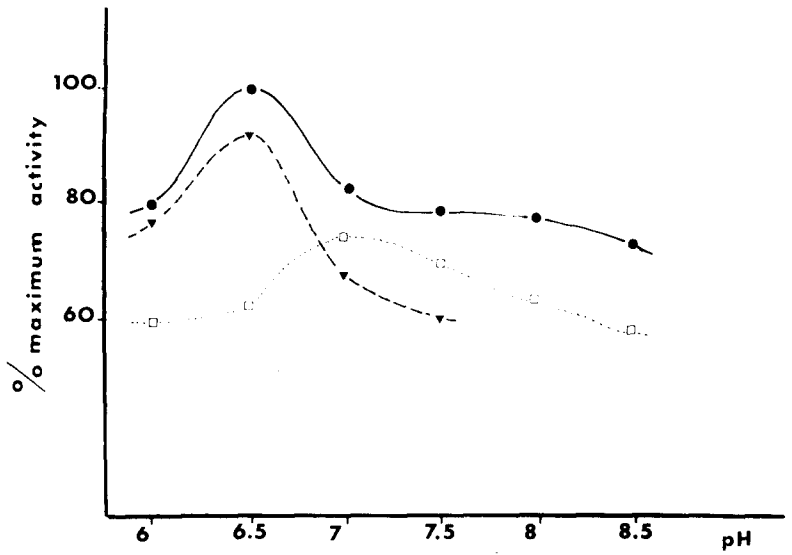

Fig. 1. pH vs. activity curve of rabbit liver cytosolic cytochrome b_5 reductase with cytochrome b_5 as a substrate (2). ●——●, 50 mM Tris/bis Tris · HCl buffer; ▼——▼, 50 mM potassium phosphate buffer; □- - - - -□, 50 mM triethanolamine hydrochloride/NaOH buffer.

penetrated the gel. This may be explained by the tendency of the detergent-treated enzyme to form aggregates.

Isoelectric focusing of the liver cytosolic purified enzyme showed four bands with an isoelectric pH of 7.05, 6.7, 6.5, 6.3; the major band had an isoelectric pH of 6.7. With the erythrocyte extract, the same bands were observed and an

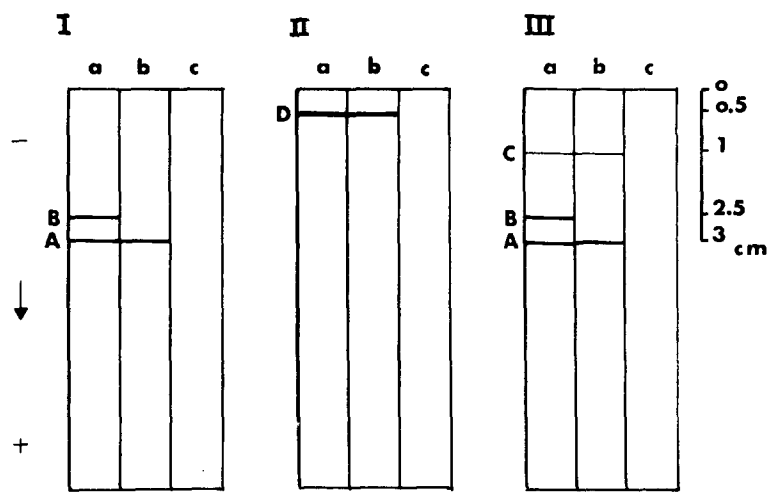

Fig. 2. Polyacrylamide gel electrophoresis of semi-purified cytosolic cytochrome b_5 reductase (I), detergent-solubilized microsomal cytochrome b_5 reductase (II) from rabbit liver, and rabbit red-cell hemolysate (III). The enzyme was stained as NADH diaphorase [18]. Prior to electrophoresis, each enzyme preparation was preincubated with: (a) saline, (b) normal chicken serum, (c) chicken antiserum directed against human red-cell methemoglobin reductase. Bands A, C, D are specifically extinguished by preincubation with the antiserum. The significance of band B is not clear since it was also inhibited by normal chicken serum.

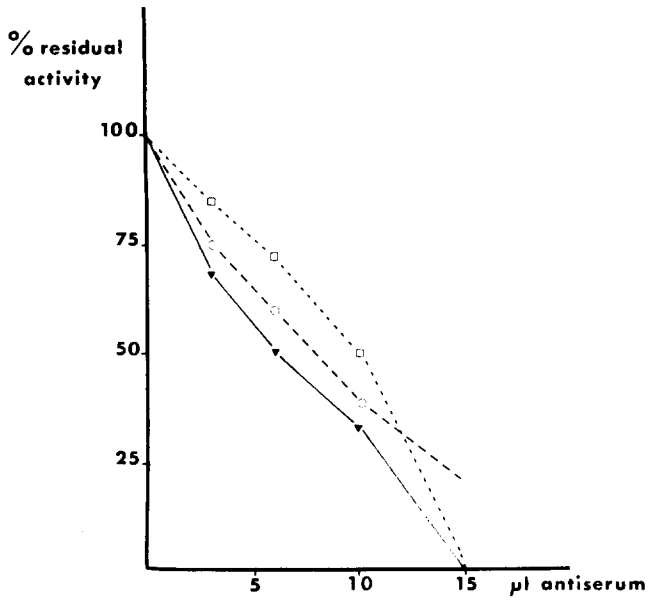

Fig. 3. Inactivation of cytosolic, microsomal and red-cell enzymes by chicken antiserum prepared against human red-cell methemoglobin reductase. Assay conditions are described in Methods. The results are expressed as percentage of the activity measured in control experiments. ▼———▼, cytosolic cytochrome b_5 reductase from rabbit liver; □- - - - -□, detergent-solubilized microsomal cytochrome b_5 reductase from rabbit liver; ○- - - - -○, rabbit red-cell cytochrome b_5 reductase.

additional band with isoelectric pH 8.0 was found. The microsomal enzyme exhibited isoelectric pH of 8.6 and traces at 6.7, 6.5 and 6.3.

(f) Immunological studies. The effect of antihuman red-cell methemoglobin reductase antiserum on cytosolic, microsomal and red-cell enzymes was determined. Fig. 3 shows that the inactivation curve obtained was nearly identical for these three enzymes.

Upon polyacrylamide gel electrophoresis (Fig. 2), no bands with NADH diaphorase activity were seen when the samples were preincubated with the antiserum.

Discussion

Preliminary studies from our laboratory had suggested, on electrophoretic and immunologic grounds, that the NADH-dependent enzyme which promotes methemoglobin reduction in the red cells (so-called methemoglobin reductase or NADH diaphorase) was actually ubiquitously distributed [7]. Since the red cell enzyme was identified by Passon and Hultquist [1] as a soluble form of NADH-cytochrome b_5 reductase, it became evident that the actual nature of this ubiquitous soluble enzyme was that of a soluble cytochrome b_5 reductase.

This was indeed proved by subsequent studies showing that the cytosolic fraction of placenta contained a soluble enzyme which was indistinguishable from the red cell enzyme, and very similar to the microsomal cytochrome b_5 reductase from placenta [2]. These studies were conducted in human tissues. It was interesting to know whether in animals the soluble enzyme could also be found.

Cytochrome b_5 and cytochrome b_5 reductase have already been described in different cellular compartments such as endoplasmic reticulum, outer membrane of mitochondria, Golgi apparatus and nuclear membranes [22]. In a previous report [23], we found evidence that rat liver cytosol does contain soluble cytochrome b_5 reductase. This enzyme was now partially purified and characterized in rabbit liver cytosol. That this entity is not a mere artifact is indicated by the following results:

(i) the cytosolic fraction was not substantially contaminated by microsomes since it was devoid of NADPH-cytochrome *c* reductase activity;

(ii) repeated washings of the microsomal pellet did not release the microsomal cytochrome b_5 reductase which is known to be strongly attached to the endoplasmic reticulum;

(iii) since it had been shown that microsomal cytochrome b_5 reductase is selectively released by lysosomal cathepsin [24—26], it was also imperative to check whether this phenomenon could have occurred during the preparation of our cytosolic fraction. In control experiments we incubated equal weights of microsomes and lysosomes (on a protein basis) in the same medium as that used for subcellular fractionation (see Materials and Methods). After 1 h of incubation at 4°C there was no detectable release of cytochrome b_5 reductase in the 105 000 $\times g$ supernatant. This indicates that the soluble cytochrome b_5 reductase which we isolated from liver cytosol could not result from an in vitro accidental lysosomal digestion.

That this entity is not DT Diaphorase, a soluble enzyme described by Ernster in liver cytosol [15], is indicated by its inability to accept electrons from NADPH, and its lack of sensitivity towards dicumarol. Conversely DT Diaphorase is devoid of cytochrome b_5 reductase activity [15].

This entity might be identical to the enzyme from rabbit liver briefly reported by Zinsmeyer et al. [27].

Its characteristics were strikingly similar to the rabbit and human red cell enzyme and to the rabbit liver microsomal cytochrome b_5 reductase. It is likely that the soluble cytosolic cytochrome b_5 reductase derives from the microsomal enzyme. The in vitro proteolytic processing of this amphipathic molecule could result in a hydrophilic protein, with a mol. wt of about 30 000, similar to the soluble protein obtained by proteolytic digestion of the microsomal enzyme [28—30].

The hypothesis that the cytosolic soluble cytochrome b_5 reductase is a post-translational product of the microsomal enzyme is also the only explanation of our finding of a concomitant defect of both entities in congenital methemoglobinemia type II, with severe encephalopathy [8,9]. Both entities are coded for by a single gene, DIA_1, which was recently localized on human chromosome 22 [10,31]. We assume that the soluble cytochrome b_5 reductase which we described here is identical to the DIA_1 gene product (soluble NADH-diaphorase) which has already been described in the erythrocytes and several different human tissues including fibroblasts [1,2,7,10,31,32]. Further studies are needed to provide insight on the mechanism of post-translational maturation of the primary gene product, i.e. the microsomal cytochrome b_5 reductase, into its soluble hydrophilic derivative. Another question, still unsolved, is the role of such an enzyme in the cytosolic compartment of liver.

Acknowledgments

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References

1 Passon, P.G. and Hultquist, D.E. (1972) Biochim. Biophys. Acta 275, 62—73
2 Leroux, A., Torlinski, L. and Kaplan, J.C. (1977) Biochim. Biophys. Acta 481, 50—62
3 Goto-Tamura, R., Takesue, Y. and Takesue, S. (1976) Biochim. Biophys. Acta 423, 293—302
4 Kuma, F., Prough, R.A. and Masters, B.S.S. (1976) Arch. Biochem. Biophys. 172, 600—607
5 Scott, E.M. and McGraw, J.C. (1962) J. Biol. Chem. 237, 249—252
6 Strittmatter, P. (1963) in The Enzymes (Boyer, P., Lardy, M. and Myrbäck, K., eds.), Vol. 8, pp. 113—145, Academic Press, New York
7 Leroux, A. and Kaplan, J.C. (1972) Biochem. Biophys. Res. Commun. 49, 945—950
8 Leroux, A., Junien, C., Kaplan, J.C. and Bamberger, J. (1975) Nature 258, 619—620
9 Kaplan, J.C., Leroux, A. and Beauvais, P. (1977) Hum. Hered. 27, 188
10 Junien, C., Vibert, M., Weil, D., Nguyen Van Cong and Kaplan, J.C. (1978) Hum. Genet., 42, 233—239
11 Omura, T. and Takesue, S. (1970) J. Biochem. (Tokyo) 67, 249—257
12 Hegesh, E., Calmanovici, N. and Avron, M. (1968) J. Lab. Clin. Med. 72, 339—344
13 Hegesh, E. and Avron, M. (1967) Biochim. Biophys. Acta 146, 91—101
14 Zamudio, I. and Canessa, M. (1966) Biochim. Biophys. Acta 120, 165—169
15 Ernster, L. (1967) Methods Enzymol. 10, 309—317
16 Yasukochi, Y. and Masters, B.S.S. (1976) J. Biol. Chem. 251, 5337—5344
17 Ornstein, L. (1964) Ann. N.Y. Acad. Sci. 121, 151—163
18 Kaplan, J.C. and Beutler, E. (1967) Biochem. Biophys. Res. Commun. 29, 605—610
19 Drysdale, J.W., Righetti, P. and Bunn, H.F. (1971) Biochim. Biophys. Acta 229, 42—50
20 Martin, R. and Ames, B. (1961) J. Biol. Chem. 236, 1372—1379
21 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
22 Enoch, H.G., Fleming, P.J. and Strittmatter, P. (1977) J. Biol. Chem. 252, 5656—5660
23 Vieira de Barros, A., Kaplan, J.C., Duvaldestin, P. and Berthelot, P. (1977) Biochem. Pharmacol. 27, 367—368
24 Takesue, S. and Omura, T. (1970) J. Biochem. (Tokyo) 67, 259—266
25 Sargent, J.R., St. Louis, P.J. and Blair, P.A. (1970) Biochim. Biophys. Acta 223, 339—348
26 Ito, A. (1974) J. Biochem. (Tokyo) 75, 787—793
27 Zinsmeyer, J., Schönfeld, W. and Wagenknecht, C. (1972) Acta Biol. Med. Germ. 28, 63—71
28 Takesue, S. and Omura, T. (1970) J. Biochem. (Tokyo) 67, 267—276
29 Spatz, L. and Strittmatter, P. (1973) J. Biol. Chem. 248, 793—799
30 Mihara, K. and Sato, R. (1975) J. Biochem. (Tokyo) 78, 1057—1073
31 Fisher, R.A., Povey, S., Bobrow, M., Solomon, E., Boyd, Y. and Carrits, B. (1977) Ann. Hum. Genet. 41, 151—155
32 Fisher, R.A., Edwards, Y.H., Putt, W., Potter, J. and Hopkinson, D.A. (1977) Ann. Hum. Genet. 41, 139—149