Biochimica et Biophysica Acta, 423 (1976) 293–302

BBA 47031

IMMUNOLOGICAL SIMILARITY BETWEEN NADH-CYTOCHROME b_5 REDUCTASE OF ERYTHROCYTES AND LIVER MICROSOMES

RYOKO GOTO-TAMURA, YOSHIKI TAKESUE* and SACHIKO TAKESUE**

Research Institute for Environmental Medicine, Nagoya University, Chikusa-ku, Nagoya, Aichi 464, and Institute for Protein Research, Osaka University, Suita, Osaka 565 (Japan)

(Received September 1st, 1975)

SUMMARY

In a number of animal species soluble NADH-cytochrome b_5 reductase of erythrocytes was compared with membrane-bound NADH-cytochrome b_5 reductase of liver microsomes by using an antibody to purified NADH-cytochrome b_5 reductase from rat liver microsomes. The results obtained indicated clearly that they are immunologically very similar to each other. The data with erythrocyte ghosts suggested that cytochrome b_5 and NADH-cytochrome b_5 reductase are also present in the ghost.

INTRODUCTION

Cytochrome b_5 and NADH-cytochrome b_5 reductase (EC 1.6.2.2) have been detected in microsomes from many tissues and animals [1], and a system containing cytochrome b_5 and cytochrome b_5 reductase has been suggested to be involved in the mechanism of fatty acid desaturation by liver microsomes [2–4]. A cytochrome b_5 and NADH-cytochrome b_5 reductase have also been found in the erythrocyte [5, 6] but here they are in the soluble form. In human erythrocytes a system consisting of cytochrome b_5 and NADH-cytochrome b_5 reductase has been implicated in the mechanism of methemoglobin reduction [7]. About 15 years ago, Petragnani et al. [8] showed that liver microsomal cytochrome b_5 stimulated the reduction of methemoglobin catalyzed by liver microsomal NADH-cytochrome b_5 reductase. Recently, Passon et al. [9] purified a soluble cytochrome b_5 from human erythrocytes and showed that it was similar in many respects to cytochrome b_5 solubilized from liver microsomes. Reductases catalyzing the reduction of cytochrome b_5 by NADH have been purified from human erythrocytes and shown to be effective in methemoglobin reduction [10, 11]. However, they are apparently different from each other

Abbreviation: Cl_2-Ind, 2,6-dichlorophenolindophenol

* To whom reprint requests should be addressed.

** Present address: Biological Institute, Faculty of Science, Nagoya University, Chikusa-ku, Nagoya, Aichi 464

in some respects. For example, the reductase purified by Sugita et al. [10] containe no flavins, while the one by Passon and Hultquist [11] had FAD as a prostheti group. The main difficulty in purification of reductases from erythrocytes lies i their trace amounts, compared with a extremely large amount of hemoglobin, i the hemolysate, and furthermore, some modifications in the enzyme molecule woul have occurred during their purification [12].

NADH-cytochrome b_5 reductase from liver microsomes has been establishe to be a flavoprotein with FAD as the prosthetic group [13, 14]. A specific antibod to NADH-cytochrome b_5 reductase purified from rat liver microsomes has bee prepared in a rabbit [14], and successfully used to identify NADH-cytochrome b reductase in the outer membrane of mitochondria [15].

In the present work this antibody was used to compared NADH-cytochrom b_5 reductases of erythrocytes and liver microsomes. The results obtained indicat that they are immunologically very similar to each other. The preliminary report have been presented previously [16, 17]

EXPERIMENTAL PROCEDURE

Assay and analytical methods

The enzyme assays were carried out in a Cary model 14 or 15 recording spectrophotometer at 25 °C. NADH-cytochrome b_5 reductase activity of blood preparations was assayed by measuring the increase in absorption at 423 nm. The reaction mixture contained $1 \cdot 10^{-4}$ M NADH, $1.1 \cdot 10^{-6}$ M cytochrome b_5, and enzyme in 0.8 ml of 0.05 M Tris/acetate buffer (pH 8.5). As shown in Fig. 1, the NADH-ferricyanide reductase activity of rabbit erythrocytes gave a broad pH optimum over pH 6–9, while the NADH-cytochrome b_5 reductase activity exhibited an unusual

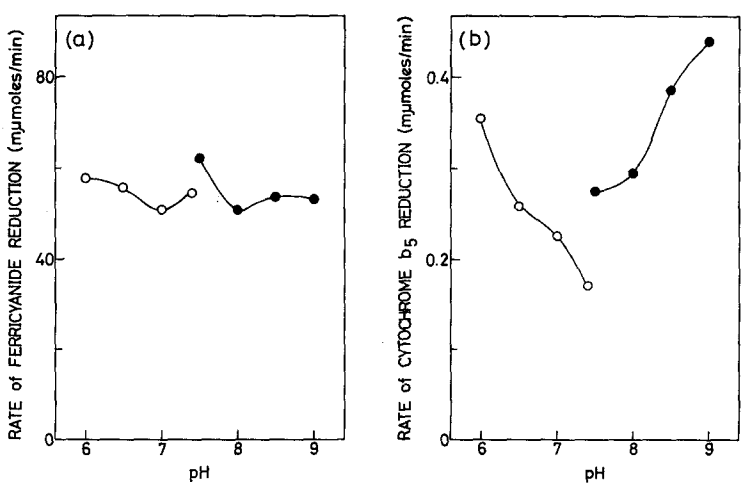

Fig. 1. pH-dependence of ferricyanide and cytochrome b_5 reduction by soluble erythrocyte NADH-cytochrome b_5 reductase. Soluble NADH-cytochrome b_5 reductase partially purified from rabbit erythrocytes was reacted with substrate, ferricyanide (a) or cytochrome b_5 (b), in buffers of indicated pH. ○, 0.05 M sodium phosphate buffer; ●, 0.05 M Tris-acetate buffer.

pH activity curve similar to that observed with human erythrocyte cytochrome b_5 reductase [11]. Since the concentration of cytochrome b_5 used was below the K_m value for the erythrocyte reductase (about $1 \cdot 10^{-5}$ M for rabbit reductase, Goto-Tamura and Takesue, unpublished; see also ref. 10), the initial rate of reduction was used in calculating the reductase activity as in the case of the microsomal enzyme [18]. NADH-cytochrome *c*, -ferricyanide, -2,6-dichlorophenolindophenol (Cl_2-Ind) and -neotetrazolium reductase activities were assayed as described previously [14, 18]. One unit of enzyme was defined as the amount of enzyme reducing one μmol of electron acceptor per min under the assay conditions.

Hemoglobin was determined by the difference in absorption between 420 and 450 nm of dithionite-reduced samples which were saturated with carbon monoxide. Protein was determined by the method of Lowry et al. [19] using bovine serum albumin as standard.

Determination of inhibition of reductase activities by antibody

The effect of antibody on reductase activities was determined as follows. Enzyme was preincubated with varying amounts of antibody in the complete mixture without NADH at 25 °C for 10 min (microsomes) or 20 min (erythrocyte preparations), and then the reaction was started by the addition of NADH. The inhibition of reductase activities by antibody was dependent on the time of preincubation of of enzyme with antibody, and the 10-min preincubation caused more than 90 % of the maximum inhibition attained at the concentration of antibody used. The measured activity was expressed as a percentage of control activity obtained without antibody. Preincubation with normal γ-globulin resulted in little, if any, effect on any of the reductase activities.

Fractionation of blood

In experiments to investigate the distribution of NADH-cytochrome b_5 reductase and other NADH-linked reductase activities in blood, rabbit blood was fractionated as follows. About 3 ml of rabbit blood, obtained on heparin from the ear vein, was spun at $1000 \times g$ for 10 min. The plasma was removed together with buffy coat. Packed erythrocytes were gently washed three times each with 3 vol. of isotonic saline. The washings were combined with the plasma fraction. Washed erythrocytes were hemolysed in 9 vol. of 5 mM sodium phosphate (pH 7.5) for 1–2 h [20]. The hemolysate was centrifuged at $10\,000 \times g$ for 40 min, and the supernatant was removed by decantation. The precipitate was washed repeatedly, usually 7 times, each with 6 vol. of the same buffer until it became colorless. The final precipitate was suspended in 6 ml of the buffer and used as erythrocyte ghosts. All the washings were combined together with the first supernatant and used as erythrocyte cytosol fraction.

Preparation of liver microsomes

Microsomes were prepared from livers of the rat, mouse, guinea pig, rabbit, pig and beef. Liver was homogenized in 9 vol. of 0.25 M sucrose using a Potter glass homogenizer equipped with a Teflon pestle. The homogenate was centrifuged at $12\,000 \times g$ for 20 min. The precipitate was discarded, and the supernatant was centrifuged at $105\,000 \times g$ for 90 min to sediment microsomes, which were washed

once with 0.15 M KCl containing 1 mM EDTA (pH 7.5) and finally suspended i 0.01 M potassium phosphate (pH 7.5). Preliminary experiments indicated that th microsomes obtained in this way were contaminated less than 2 % with mitochondri as judged by the activity of succinate-cytochrome *c* reductase.

Crude preparation of soluble erythrocyte NADH-cytochrome b_5 reductase

In preliminary experiments the NADH-ferricyanide reductase activity in th supernatant obtained by centrifugation at 10 000 × *g* for 40 min from hemolysat of rabbit erythrocytes was found to be proportional to the amount of the super natant over a limited range (Fig. 2); dialysis of the supernatant against 0.01 M phosphate buffer (pH 7.5) did not result in any improvement as can be seen fron Fig. 2. Upon ammonium sulfate fractionation of the supernatant half the activity was recovered in the fraction precipitated at 65 % saturation of the salt; higher satura tion of the salt could bring about more activity but together with a greater amoun of hemoglobin. When this fraction was used as an enzyme preparation, the ferri cyanide reductase activity became proportional to the amount of enzyme over a much wider range (Fig. 2). The cytochrome b_5 reductase activity nearly parallelec the ferricyanide reductase activity in the ammonium sulfate fractionation. In orde to obtain erythrocyte enzyme preparations for the inhibition experiment, therefore, the supernatants from hemolysates were fractionated with ammonium sulfate.

Fresh blood was obtained by bleeding rabbits and guinea pigs from the ear vein and by heart puncture, respectively. Rats and mice were bled from the abdominal

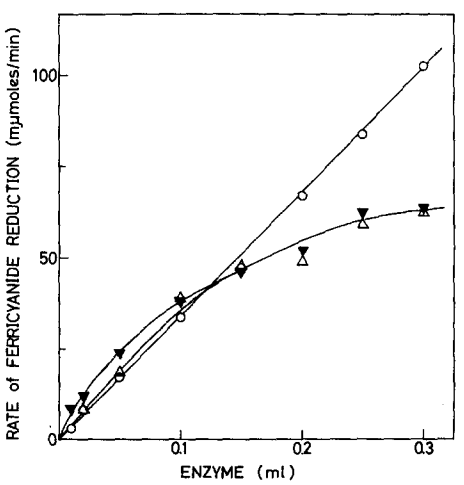

Fig. 2. Dependence of the rate of ferricyanide reduction on the amount of NADH-cytochrome b_5 reductase from erythrocytes. Hemolysate prepared from rabbit erythrocytes as described in the text was centrifuged at 10 000 × *g* for 40 min, and the resulting supernatant was dialyzed against 0.05 M sodium phosphate (pH 7.5) at 4 °C overnight. Solid ammonium sulfate was added to the dialyzed supernatant to 65 % saturation, and the formed precipitate was dissolved in 0.05 M sodium phosphate (pH 7.5) of half the volume of the original supernatant, followed by dialysis against the buffer at 4 °C overnight. Different enzyme preparations obtained as above were assayed for the ferricyanide reductase activity under the conditions described in the text. △, original supernatant from hemolysate; ▼, dialyzed supernatant; ○, ammonium sulfate fraction.

vein under ether anesthesis. Fresh pig and beef blood was obtained from a slaughterhouse. Erythrocytes were collected, washed and lysed in the same way as described above. Solid ammonium sulfate was added to the hemolysate, and the precipitate formed between 45 and 65 % saturation of the salt was dissolved in 5 mM Tris/acetate (pH 8.5), followed by dialysis against the buffer at 4 °C overnight. The preparation was centrifuged at 10 000 $\times g$ for 20 min, and the supernatant was used as a crude preparation of soluble erythrocyte NADH-cytochrome b_5 reductase in the inhibition experiment.

Antibody and other materials

Preparation of rabbit antisera against purified NADH-cytochrome b_5 reductase from rat liver microsomes was described in a previous paper [14]. The antisera were fractionated with ammonium sulfate, and the fraction which precipitated between 20 and 33 % saturation of the salt was used as anti-reductase immunoglobulin [15]. Normal γ-globulin was prepared from serum of a non-immunized rabbit. Cytochrome b_5 was purified from rat and rabbit liver microsomes according to the method of Omura and Takesue [21]; rat cytochrome b_5 was used in the assay for liver microsomal activities and rabbit cytochrome b_5 in the assay for blood activities. Yeast cytochrome *c* was a gift from Sankyo Co. Ltd., and purified before use [22]. NADH was purchased from Sigma Chemical Co..

RESULTS AND DISCUSSION

Table I summarizes the results on the NADH-cytochrome b_5 reductase and other reductase activities in the three fractions from rabbit blood. The absolute values of the activities in whole blood varied with the individual, but their relative distribution among the fractions were fairly constant. The reductase activities in

TABLE I

HEMOGLOBIN AND NADH-LINKED REDUCTASE ACTIVITIES IN RABBIT BLOOD AND ITS FRACTIONS

The methods of fractionating blood into the three fractions and of determining hemoglobin and NADH-linked reductase activities are described in the text. The results are from four experiments. Total content is the sum of the contents in the three fractions and expressed as the difference in absorption at between 420 and 450 nm for hemoglobin and μmol of electron acceptor reduced/min/ml of blood for enzyme activities. The values quoted are averages of four experiments, the values in parentheses indicating the ranges. Percentage (mean $\pm$ s.e.) represents the distribution of hemoglobin and reductase activities recovered in each fraction based on the total content in blood.

Component or Enzyme activity	Total content in blood	Percentage		
		Plasma fraction	Erythrocyte cytosol	Erythrocyte ghosts
Hemoglobin	1.22 (0.59 – 2.01)	0.04 $\pm$ 0.07	99.8 $\pm$ 0.09	0.05 $\pm$ 0.03
Cytochrome b_5 reductase	1.43 (0.75 – 2.03)	0.02 $\pm$ 0.01	99.6 $\pm$ 0.35	0.61 $\pm$ 0.53
Cytochrome *c* reductase	0.082 (0.032 – 0.178)	0.05 $\pm$ 0.09	75.8 $\pm$ 5.91	24.1 $\pm$ 6.03
Ferricyanide reductase	27.1 (8.7 – 42.7)	0.24 $\pm$ 0.22	98.1 $\pm$ 0.62	1.57 $\pm$ 0.42
Cl_2-Ind reductase	0.66 (0.30 – 0.82)	0.00 $\pm$ 0.00	96.8 $\pm$ 2.36	3.20 $\pm$ 2.38

the plasma fraction were negligibly small and might be due to hemolysis durir preparation and/or lymphocytes [23] recovered in the fraction. The NADH-cyt chrome b_5 reductase activity was almost exclusively localized in the cytosol, qui similar to hemoglobin. Similarly, more than 97 % of the NADH-ferricyanide an Cl_2-Ind reductase activities were recovered in the cytosol. In contrast to these activ ties, the NADH-cytochrome *c* reductase activity was recovered in the ghosts by ε much as 24 %.

Some comments should be made on this apparent high percentage of th cytochrome *c* reductase activity in ghosts. The mean values of reductase activity i ghosts were 8.5, 20, 425 and 21 milliunit per ml of blood with cytochrome b_5, cyt chrome c, ferricyanide and Cl_2-Ind, respectively, as electron acceptor. In view c their exhaustive washing these activities seem to be intrinsic to the ghosts. Additio of cytochrome b_5 to the assay mixture for NADH-cytochrome *c* reductase stimu lated the reduction of cytochrome *c* not only when the cytosol was used as enzym but also when the ghosts were used. This effect is most probably the same as the well known phenomenon that cytochrome b_5 stimulates the reduction of cytochrome by microsomal NADH-cytochrome b_5 reductase [14, 24]. Passon and Hultquist [11 utilized this phenomenon in the assay for soluble erythrocyte NADH-cytochrome b_5 reductase. In other words the finding that the above-mentioned effect was observe with ghosts suggests the presence of cytochrome b_5 and NADH-cytochrome b_5 reductase in ghosts as well. If so, disproportionally high percentage in ghosts of th NADH-cytochrome *c* reductase activity compared with that of the NADH-cyto chrome b_5 reductase activity can be explained by assuming that membrane-bounc cytochrome b_5 reductase reduces endogenous membrane-bound cytochrome b_5 much more rapidly than externally added cytochrome b_5 and that reduced bounc cytochrome b_5, in turn, reduces cytochrome *c* non-enzymatically very rapidly, as in microsomes [25]. The presence of NADH-cytochrome *c* reductase in erythrocyte ghosts has been reported [26], and Passon and Hultquist [11] said that it is distinguished from soluble NADH-cytochrome b_5 reductase. As described later, however the NADH-cytochrome b_5 reductase and NADH-cytochrome *c* reductase activities in ghosts were inhibited by antibody in the same manner as that of the NADH-cytochrome b_5 reductase activity in the cytosol, supporting our suggestion mentioned above.

Liver microsomes have two distinct flavin enzymes, NADH-cytochrome b_5 reductase and NADPH-cytochrome *c* reductase (EC 1.6.2.4) [27]. The NADH-cytochrome b_5 and NADH-cytochrome *c* reductase activities in rat liver microsomes are inhibited more than 95 % by anti-reductase immunoglobulin, the γ-globulin fraction from antisera to purified NADH-cytochrome b_5 reductase from rat liver microsomes, but the NADPH-cytochrome *c* reductase activity is not inhibited by the antibody at all [15, 28, 29]. Mitochondrial rotenone-sensitive NADH-cytochrome *c* reductase activity is also not inhibited by the antibody [15]. These facts indicate that the antibody is highly specific for NADH-cytochrome b_5 reductase. Solubilization of NADH-cytochrome b_5 reductase from microsomes by lysosomal digestion seems to bring about little, if any, modification in the immunological properties of the reductase, because purified NADH-cytochrome b_5 reductase from rat liver microsomes is inhibited by the antibody in a manner quite similar to that of microsome-bound reductase [28].

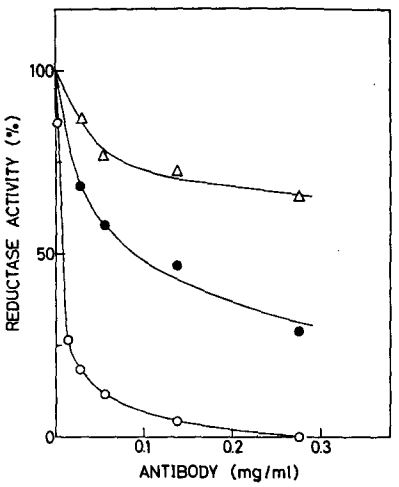

Fig. 3. Effect of anti-reductase immunoglobulin on soluble NADH-cytochrome b_5 reductase from rat erythrocytes. Soluble NADH-cytochrome b_5 reductase from rat erythrocytes was preincubated with varying amounts of anti-reductase immunoglobulin in the complete reaction mixture without NADH for 20 min at 25 °C, and then the reaction was started by the addition of NADH. The amounts of reductase used were 7.7, 231 and 77 μg of protein for cytochrome b_5 (○), ferricyanide (●) and Cl_2-Ind (△), respectively. The data were expressed as per cent of control activities obtained without antibody; the control activities were 0.7, 83 and 4.3 mμmol acceptor reduced/min for cytochrome b_5, ferricyanide and Cl_2-Ind reduction, respectively.

Fig. 3 shows the effect of anti-reductase immunoglobulin on NADH-linked reductase activities of soluble NADH-cytochrome b_5 reductase from rat erythrocytes. The cytochrome b_5 reductase activity was inhibited by the antibody, and the complete inhibition was obtained at a high concentration of the antibody. Although not shown in the figure, the cytochrome *c* reductase activity was also similarly inhibited, indicating that this activity was also mediated by NADH-cytochrome b_5 reductase as the microsomal activity [14, 24]. The antibody also inhibited ferricyanide and Cl_2-Ind reductase activities but to a lesser extent, the extent of inhibition being 70 and 35 %, respectively, at the highest concentration of antibody used. The amount of soluble cytochrome b_5 reductase used was 30 and 10 times higher in the ferricyanide and Cl_2-Ind reductase assays, respectively, than in the cytochrome b_5 reductase assay. Therefore, the greater amount of antibody would have caused more inhibition, especially in the ferricyanide reductase activity. The reduction of ferricyanide and Cl_2-Ind by purified microsomal cytochrome b_5 reductase has been found to be inhibited similarly to each other [28], but in the case of the erythrocyte preparation Cl_2-Ind, reduction was inhibited to a much lesser extent compared to ferricyanide reduction at the same concentration of antibody per enzyme protein. This may be ascribed to the presence of NADH diaphorases other than the reductase sensitive to the antibody. In fact, at least two kinds of NADH diaphorases have been reported in human erythrocytes [30, 31], and one of them is believed to function in methemoglobin reduction [32]. Soluble cytochrome b_5 reductase from rat erythrocytes could not catalyze the reduction of neotetrazolium by NADH. This finding is interesting, since purified cytochrome b_5 reductase from rat liver microsomes

also does not reduce neotetrazolium by NADH [14]. On the other hand, microsome catalyze the reaction, which is inhibited by anti-reductase immunoglobulin [28

Thus soluble NADH-cytochrome b_5 reductase from rat erythrocytes wa found to be inhibited by antibody in a manner very similar to that of microsoma NADH-cytochrome b_5 reductase from rat liver, either membrane-bound or solubi lized. Next it was examined whether similar results were observed with cytochrom b_5 reductases of erythrocytes and liver microsomes of other animal species. In th following experiments NADH-cytochrome b_5 reductase activity of erythrocyte was compared with NADH-cytochrome *c* reductase activity of liver microsomes because the effect of antibody on microsomal NADH-cytochrome b_5 reductas activity can be correctly followed by measuring its effect on the cytochrome *c* reduc tase activity [15, 28, 29].

Figs. 4 shows the effects of anti-reductase immunoglobulin on the NADH cytochrome *c* reductase activity of liver microsomes from various animals. The NADH-cytochrome *c* reductase activity of mouse liver microsomes was inhibited by the antibody. Its maximum inhibition attained was the same as that of rat liver activity, though the extent of inhibition at various concentrations of antibody per reductase was less with mouse reductase than with rat reductase. The cytochrome *c* reductase activities of beef, pig and guinea pig liver microsomes were also inhibited

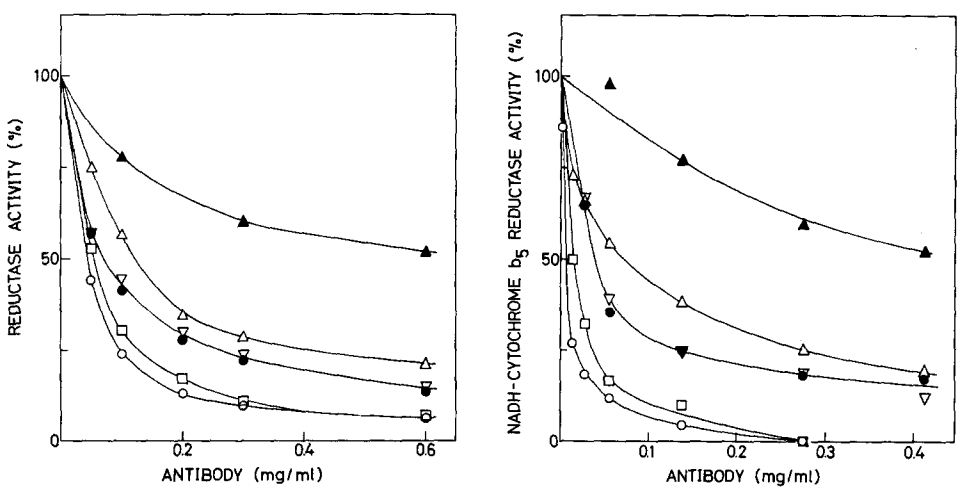

Fig. 4. Effect of anti-reductase immunoglobulin on NADH-cytochrome *c* reductase activities of liver microsomes from various animals. Liver microsomes (0.04 unit of NADH-cytochrome *c* reductase activity except for rat microsomes for which it was 0.08 unit) were preincubated with varying amounts of anti-reductase immunoglobulin in the complete reaction mixture without NADH for 10 min at 25 °C, and then the NADH-cytochrome *c* reductase activity was determined. The data were expressed as in Fig. 3. ○, rat; □, mouse; ●, beef; ▽, pig; △, guinea pig; ▲, rabbit.

Fig. 5. Effect of anti-reductase immunoglobulin on soluble NADH-cytochrome b_5 reductase activities of erythrocytes from various animals. Soluble NADH-cytochrome b_5 reductase (0.7 milliunit) of erythrocytes from various animals was preincubated with varying amounts of anti-reductase immunoglobulin in the complete reaction mixture without NADH for 20 min at 25 °C, and then the NADH-cytochrome b_5 reductase activity was determined. The data were expressed as in Fig. 3 and symbols as shown for Fig. 4.

by the antibody but to lesser extents than in the case of rat liver microsomes. It is interesting that cytochrome *c* reductase activity of rabbit liver microsomes was inhibited by the antibody, though to a much less extent; the inhibition was about 50 % at the highest concentration of the antibody used. Since the antibody was raised in a rabbit and the microsomes were obtained from another rabbit, it appears that the antibody preparation contained an isoantibody to the NADH-cytochrome b_5 reductase.

Fig. 5 shows the effects of anti-reductase immunoglobulin on the NADH-cytochrome b_5 reductase activity of erythrocyte cytosols from various animals. In each animal species the inhibition curve of cytochrome b_5 reductase activity of erythrocyte cytosol was very similar to that of cytochrome *c* reductase activity of liver microsomes. The cytochrome b_5 reductase activity of mouse erythrocytes as well as of rat erythrocytes was completely inhibited. The NADH-cytochrome b_5 reductase activities of beef, pig and guinea pig erythrocytes were also inhibited to the same extents as in the case of the NADH-cytochrome *c* reductase activities of of their liver microsomes. For erythrocyte NADH-cytochrome b_5 reductase and the rabbit reductase activity was inhibited by the antibody about 50 %, this value being same as that for the NADH-cytochrome c reductase activity of rabbit liver microsomes.

As shown in Table I, exhaustively washed ghosts from rabbit erythrocytes showed significant, though little, amount of NADH-cytochrome b_5 reductase and NADH-cytochrome *c* reductase activities. These activities were also inhibited by the antibody to the same extent as those of soluble erythrocyte activities of the same animal, rabbit. These findings suggest the presence of cytochrome b_5 and NADH-cytochrome b_5 reductase in ghosts.

The results in the present work indicate clearly that soluble NADH-cytochrome b_5 reductase in erythrocytes is immunologically very similar to NADH-cytochrome b_5 reductase in liver microsomes in a number of animal species. Strictly speaking the immunological similarity may be valid for the protein moiety of the reductases, since it has been considered that the antibody used is not directed against the flavin moiety itself of the antigen reductase [28]. The presence of flavin as a prosthetic group in NADH-cytochrome b_5 reductase of erythrocytes is possible.

The present work suggests that cytochrome b_5 and NADH-cytochrome b_5 reductase are present in erythrocyte ghosts as well. Soluble cytochrome b_5 and NADH-cytochrome b_5 reductase in erythrocytes may have been originally bound to membraneous structures in precursors of the blood cell, as suggested by Passon and Hultquist [11]. They may have been brought into the soluble form, e.g. by lysosomal enzymes as in the solubilization of cytochrome b_5 reductase from liver microsomes [14], in parallel with the disappearance of intracellular organelles during development of the blood cell. Cytochrome b_5 and NADH-cytochrome b_5 reductase in ghosts may be their remains.

ACKNOWLEDGEMENT

One of the authors (S. T.) wishes to express her thanks to Dr. T. Omura of Kyushyu University for his encouragement.

REFERENCES

1 Strittmatter, P. (1963) The Enzymes (Boyer, P. D., Lardy, H. and Myrbäck, K., eds), Vol. pp. 113–145, Academic Press, New York
2 Oshino, N., Imai, Y. and Sato, R. (1966) Biochim. Biophys. Acta 128, 13–28
3 Oshino, N., Imai, Y. and Sato, R. (1971) J. Biochem. (Tokyo) 69, 155–167
4 Holloway, P. W. and Wakil, S. J. (1970) J. Biol. Chem. 245, 1862–1865
5 Hultquist, D. E., Reed, D. W. and Passon, P. G. (1969) Fed. Proc. 28, 862
6 Goto, R. and Takesue, Y. (1969) Ann. Reports Res. Inst. Environment. Med., Nagoya Univ. 2 194–197
7 Hultquist, D. E. and Passon, P. G. (1971) Nat. New Biol. 229, 252–254
8 Petragnani, N., Nogueira, O. C. and Raw, I. (1959) Nature 184, 1651
9 Passon, P. G., Reed, D. W. and Hultquist, D. E. (1972) Biochim. Biophys. Acta 275, 51–61
10 Sugita, Y., Nomura, S. and Yoneyama, Y. (1971) J. Biol. Chem. 246, 6072–6078
11 Passon, P. G. and Hultquist, D. E. (1972) Biochim. Biophys. Acta 275, 62–73
12 Kuma, F. and Inomata, H. (1972) J. Biol. Chem. 247, 556–560
13 Strittmatter, P. and Velick, S. F. (1957) J. Biol. Chem. 228, 785–799
14 Takesue, S. and Omura, T. (1970) J. Biochem. (Tokyo) 67, 267–276
15 Takesue, S. and Omura, T. (1970) Biochem. Biophys. Res. Commun. 40, 396–401
16 Goto, R. and Takesue, Y. (1971) Seikagaku 43, 642
17 Takesue, S. and Omura, T. (1969) Seikagaku 41, 561
18 Takesue, S. and Omura, T. (1970) J. Biochem. (Tokyo) 67, 259–266
19 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265 275
20 Dodge, J. T., Mitchell, C. and Hanahan, D. J. (1963) Arch. Biochem. Biophys. 100, 119–130
21 Omura, T. and Takesue, S. (1970) J. Biochem. (Tokyo) 67, 249–257
22 Hagihara, B., Morikawa, I., Takawa, K. and Okunuki, K. (1958) Biochemical Preparation (Vestling, C. S., ed.), Vol. 6, pp 1–7, J. Wiley and Sons, Inc.
23 Cited in Ref. 11: Kaplan, J. C., Hanzlickova-Leroux, A. and Dreyfus, J. C. (1970) C. R. Acad Sci. Paris 270, Ser. D, 2223
24 Strittmatter, P. and Velick, S. F. (1956) J. Biol. Chem. 221, 277–286
25 Hirota, K., Omura, T., Nishibayashi, H. and Sato, R. (1963) Koso Kagaku Shimpojiumu 15, 55 57
26 Zamudio, I. and Canessa, M. (1966) Biochim. Biophys. Acta 120, 165–169
27 Siekevitz, P. (1965) Fed. Proc. 24, 1153–1155
28 Takesue, S. (1969) Studies on NADH-Cytochrome *c* Reductase System in Rat Liver Microsomes, Thesis, Osaka University, Osaka, Japan
29 Omura, T., Kuriyama, Y., Takesue, S. and Oshino, N. (1970) Abstracts of 8th Internationa Congr. Biochem. p 102
30 Scott, E. M., Duncan, I. W. and Ekstrand, V. (1965) J. Biol. Chem. 240, 481–485
31 Kuma, F., Ishizawa, S., Hirayama, K. and Nakajima, H. (1972) J. Biol. Chem. 247, 550–555
32 Scott, E. M. (1960) J. Clin. Invest. 39, 1176–1179