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Abstract

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LIPOYL DEHYDROGENASE ACTIVITY OF ERYTHROCYTES IN SPIELMEYER-VOGT-BATTEN'S DISEASE

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Abstract. Using the method for the determination of the lipoyl dehydrogenase activity in intact erythrocytes described by Seet and Lee (1975), it was demonstrated that in patients with Spielmeyer-Vogt-Batten's disease, this activity was around the lower limit of normal. In these patients, the enzymatic activity is significantly reduced to such an extent that it may affect the function and metabolism of the erythrocytes.

The Pd(II)-1,3 bis (2′-pyridyl)-1,2-diaza-prop-2-ene method for the determination of lipoyl dehydrogenase was first described by Seet and Lee (1). These authors used the method for the determination of lipoyl dehydrogenase in intact erythrocytes in normal adults and children, and in newborn infants with and without icterus neonatorum.

Pd(I) reacts with 1,3-bis(2′-pyridyl)-1,2-diaza-prop-2-ene (PAPHY) resulting in the formation of a red neutral 1 : 1 complex, and the method has been used for spectrophotometric determination of Pd(II) (Seet and Lee (3)).

Studies by Seet and Lee (2) have shown that reduced lipoic acid ($Lip(SH)_2$) releases PAPHY from the Pd-PAPHY complex, and this principle is applied in the method employed in the study reported below.

This study is concerned with the determination of the lipoyl dehydrogenase activity of intact erythrocytes in normal subjects, in patients with epilepsy, and in a small group of patients with Spielmeyer-Vogt-Batten's disease. (In this disease, there are genetic disturbances of lipid metabolism, loss of vision, generalized convulsive seizures, ataxia, mental retardation and other neurological symptoms. The patient's age is usually between seven and eighteen).

MATERIALS AND METHODS

Reagents. (a) 0.5 M phosphate buffer, pH 5.9, containing 10^{-3} M EDTA, (b) 1.0 M NaCl, (c) 0.3 mM NAD, (d) 3.0 mM NADH, (e) 0.03 M lipoic acid, (f) 0.5 mM Pd(II) in 90% ethanol; prepared by diluting 5 ml of 10 mM Pd(II) in 0.05 N HCl with 90% ethanol (g) 1.0 mM PAPHY (39.6 mg PAPHY dissolved in 200 ml of 90% ethanol) to give final concentration of 90% ethanol, (h) Pd-PAPHY complex, *i.e.* 4 ml of reagent (f) mixed with 6 ml of reagent (g) and

allowed to stand for 10 min before use (i) Straub's diaphorase with an activity corresponding to 9.6 units per mg protein diluted immediately before use to 1 : 600 with 40 mg/ml of bovine serum albumin.

Control analyses. The effect of $Lip(SH)_2$ on the Pd-PAPHY complex was studied in solutions in which the PAPHY concentration was kept constant at 0.6 μM while the Pd(II) concentration was varied. The analyses were performed with a final volume of 3 ml; dilution with 90% ethanol.

It appears from Fig. 1 that there was a linear relation between the concentration of Pd(II) used and the absorption measured at 540 nm in 1-cm cuvettes.

The action of $Lip(SH)_2$ on the Pd-PAPHY complex was studied with the Pd-PAPHY concentration kept constant at 0.2 μM, while the $Lip(SH)_2$ concentration was varied. Here, too, there was a usable relation between the absorption measured and the amount of $Lip(SH)_2$ used (Fig. 2).

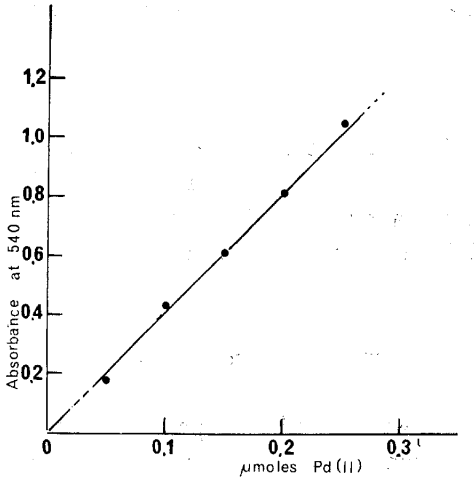

Fig. 1

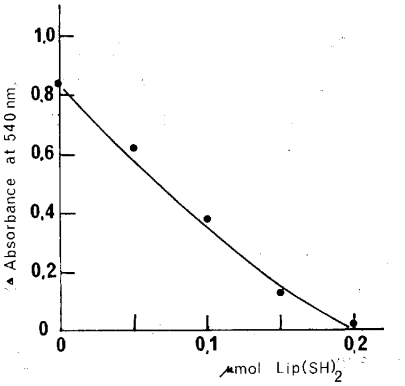

Fig. 2

Fig. 1. Pd-PAPHY standard curve. The PAPHY concentration was kept constant at 0.6 μmole, while the Pd(II) concentration was varied.

Fig. 2. The influence of Lip(Lip$(SH)_2$ on the Pd-PAPHY complex. The Pd-PAPHY concentration was kept constant at 0.2 μmole, while the $Lip(SH)_2$ concentration was varied.

The analyses were performed as tabulated below:

	Sample (ml)	Blank (ml)
Reagent (a)	0.1	0.1
Reagent (b)	0.05	0.05
Reagent (c)	0.1	0.1
Reagent (d)	0.1	0.1
Reagent (e)	0.1	0
Demineralized water	0	0.1

These mixtures were placed in a water bath at 25°C for 5 min, following which 50 μl of diluted lipoyl dehydrogenase (Reagent (i)) was added to all tubes, and the samples were again allowed to stand in a water bath at 25°C for 10 min. Then the reaction was interrupted by the addition of 2.5 ml of 90% ethanol. The samples are then carefully shaken and centrifuged at 3,000 RPM for about 10 min. One ml of Pd-PAPHY complex (Reagent (h)) was added to 2.0 ml of the clear supernatant, and the samples were allowed to stand at room temperature for 10 min before the absorption was read in 1-cm cuvettes at 540 nm against 90% ethanol.

It is of the greatest importance that the temperature of the water bath is kept at 25 ± 1°C, as an appreciable fall in the enzymatic activity occurs at a higher temperature.

Determination of lipoyl dehydrogenase activity in intact erythrocytes. The analytic method was as described above with the exception that the incubation time at 25°C was extended to 60 min in contrast with the method of Seet and Lee (1). This long incubation period was determined experimentally on the basis of studies on its influence on the results of the analysis (Fig. 3).

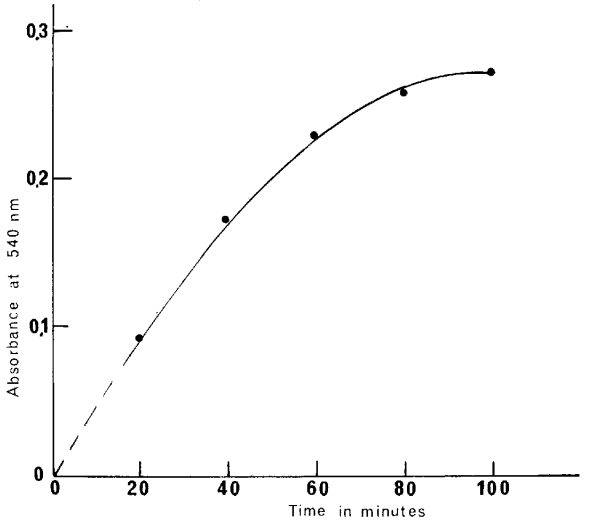

Fig. 3. The influence of the incubation time on the lipoyl dehydrogenase activity of the erythrocytes.

Another modification of the method was that the addition of the lipoyl dehydrogenase reagent has been replaced by the addition of 0.15 ml of packed, unwashed erythrocytes.

Studies on the amount of erythrocytes to be employed showed that 0.15 ml of packed erythrocytes gave a suitable result. Smaller amounts gave too low values, and the use of larger amounts showed that there was no longer proportionality between the amount of erythrocytes used and the absorption measured

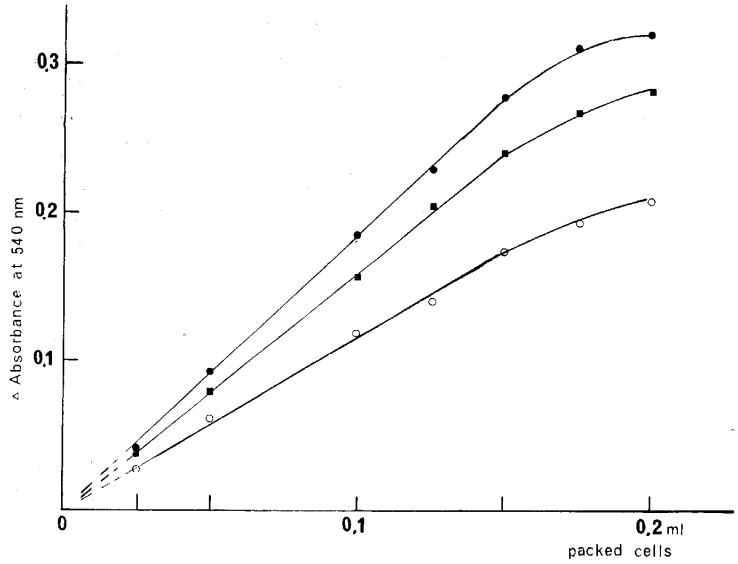

Fig. 4. The influence of the amount of erythrocytes used on the lipoyl dehydrogenase activity.

(Fig. 4).

Calculation. A unit of enzymatic activity (U) is defined as 1 μmole Lip $(SH)_2$ formed per min per ml of packed erythrocytes.

The molar absorption of Pd-PAPHY at 540 nm is 13.6×10^3, which gives:

$$2A \times \frac{3}{13.6} \times \frac{1}{60} \times \frac{1000}{0.15} = A \times 49 \text{ mU}$$

Blood samples. Fresh venous blood stabilized with EDTA was used for the analysis.

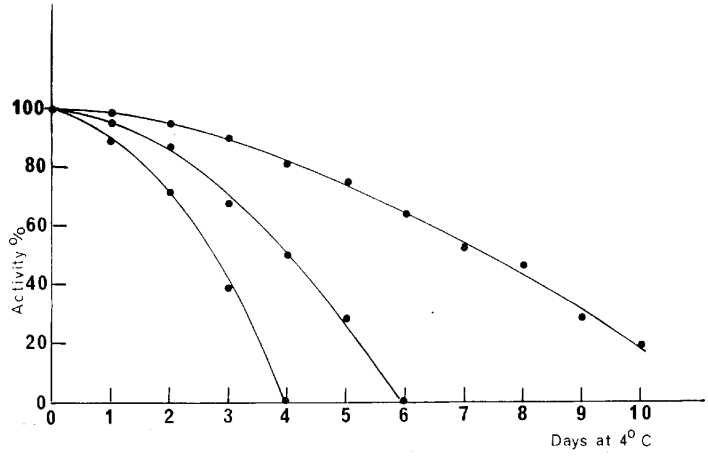

Fig. 5. The fall in the lipoyl dehydrogenase activity during standing at 4°C, expressed as percentages of the initial level.

Immediately on withdrawal, the blood samples were centrifuged at 1,500 g for 15 min. The plasma and leucocyte layer were removed by suction, and the packed erythrocytes were used as enzyme sources.

The blood samples were used at once, or at least on the next day, in which case they were stored at 4°C until used. A very rapid fall in the activity occurred on standing at 4°C; as early as the third day it was reduced by 10-40% of the initial activity (Fig. 5).

Clinical and control subjects. The series studied comprised a group of 12 patients with Spielmeyer-Vogt-Batten's disease and 223 men and women-either normal subjects or patients with epilepsy. As it soon appeared that there was no significant difference in the levels of activity observed in analyses of erythrocytes from normal subjects and patients with epilepsy, these individuals were grouped together and used as controls.

In the individuals studied, age and sex were disregarded, because the investigations of Seet and Lee (1) have shown that there is no interdependence between age, sex and enzymatic activity.

RESULTS

Fig. 6 shows the distribution curves for the values obtained in the control group and the group of patients with Spielmeyer-Vogt-Batten's disease. The values in the control group extended over a fairly wide range (7.5–13.6 mU), whereas, in the small, specifically studied group of 12 patients with Spielmeyer-Vogt-Batten's disease, the activity was around the lower limit of normal (7.0–8.2 mU) (Table 1).

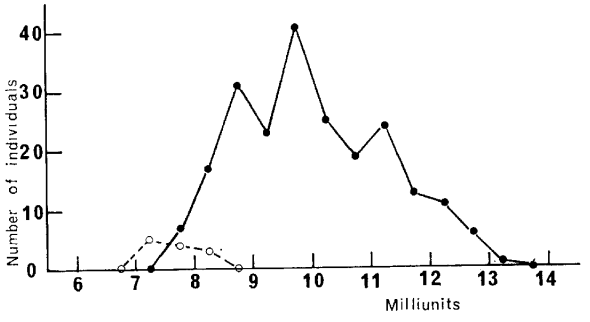

Fig. 6. Distribution curves for the enzymatic activity in the control group (●——●) and in 12 patients with Spielmeyer-Vogt-Batten's disease (○——○).

TABLE 1. LIPOYL DEHYDROGENASE ACTIVITY OF PACKED ERYTHROCYTES IN TERMS OF MU PER ML OF ERYTHROCYTES

	No.	Mean	S.D.	Minimum	Maximum
Control group	223	10.07	1.33	7.6	13.6
Spielmeyer-Vogt-Batten's disease	12	7.51	0.38	7.0	8.2

Serum/plasma analyses. Repeated analyses of serum and plasma did not reveal any enzymatic activity. This observation is in agreement with the results reported by Seet and Lee (1).

Activity of the erythrocytes. All the values plotted in Fig. 6 and those listed in the Tables are results of determinations made on intact erythrocytes.

In erythrocytes washed once with 0.15 M NaCl, the enzymatic activity was reduced by 45% (range 30–55%). Repeated washings gave a further reduction in the activity.

Individual variation in enzymatic activity. The individuals studied were randomly selected. In the patients with epilepsy, the blood samples used were obtained when samples were taken for other purposes. For this reason, a few patients appear in the series several times as the studies extended over several months. As already mentioned, the total control group consisted of 223 individuals. Of these, 33 were studied twice, 17 three times, and 10 four times. The results are shown in Table 2, from which it appears that only small variations in the same individual occurred from time to time.

TABLE 2. VARIATIONS IN THE ENZYMATIC ACTIVITY IN A NUMBER OF CONTROLS OBSERVED OVER SEVERAL MONTHS

No.	Mean	S.D.	No. of analyses
223	10.07	1.33	1
33*	10.08	1.02	2
	10.05	1.01	
17*	10.09	1.05	3
	10.25	1.02	
	10.16	1.01	
10*	10.06	0.54	4
	10.20	0.72	
	10.06	0.55	
	10.14	0.54	

* The mean values for each of these individuals were used in the calculation of that for n=223.

DISCUSSION

Lipoyl dehydrogenase catalyzes the reversible dehydrogenation of oxidized lipoic acid ($LipS_2$) (DL-6.3 thioctic acid) with NADH. Lipoyl dehydrogenase is similar to glutathione reductase, both in structure and function. Lipoyl dehydrogenase has been demonstrated in the mitochondria of hepatic and cardiac cells in at least three forms: 1. bound to the 2-oxoglutarate dehydrogenase complex, 2. bound to the pyruvate dehydrogenase complex, and, finally, 3. as a free enzyme, which constitutes the greater part (Lusty and Singer (4), Hayakawa *et al.* (5)).

The physiological role of free lipoyl dehydrogenase is still unclear.

As in the investigations reported by Seet and Lee (1), the presence of lypoyl dehydrogenase was demonstrated in intact erythrocytes in the present study. It was possible to demonstrate this activity only in intact cells, whereas a haemolysate did not show any activity. This observation suggests that the enzyme is localized on, or bound to, the erythrocyte membrane, and that it functions there as an "SH-protector enzyme". It is thus possible that the two disulphide reductases, glutathione reductase and lipoyl dehydrogenase together, play a part in maintaining the content of sulph-hydryl groups in the erythrocyte membrane.

The present study also showed that the lipoyl dehydrogenase activity of the erythrocytes was reduced if the cells were washed with physiological saline. This may be taken as evidence in favour of the assumption that the enzyme is either very weakly bound to the membrane, or that washing of the erythrocytes removes activators of the enzyme.

Seet and Lee (1) showed that the enzymatic activity is markedly reduced in 23% of neonates with hyperbilirubinaemia, whereas the activity returned to normal when normal conditions are restored in the infants.

In our series consisting of 223 controls and 12 patients with Spielmeyer-Vogt-Batten's disease specifically studied, the latter 12 revealed an enzymatic activity around the lower limit of normal. A sign test showed that the difference between the two groups is statistically significant.

The reduction in the activity was not so pronounced as that observed by Seet and Lee (1) in newborn infants with icterus neonatorum, but the reduced activity detected in patients with Spielmeyer-Vogt-Batten's disease may nevertheless affect the function and metabolism of the erythrocytes.

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