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Comparative Studies on the Molecular Properties of Purified Acetylcholinesterase from Human Erythrocytes and from the Electric Organ of *Electrophorus electricus*

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Acetylcholinesterases from human erythrocytes and from the electric organ of *Electrophorus electricus* were purified by affinity chromatography to high degrees of purity. The molecular parameters of both enzymes were compared following density gradient centrifugation, gel filtration, isoelectric focusing and polyacrylamide gel electrophoresis.

Sucrose density gradient centrifugation revealed that the human enzyme exists in a more complex state of aggregation than the eel enzyme. Upon isoelectric focusing both enzymes could be resolved into multiple molecular forms; the eel enzyme however focused at a lower pH than the red cell enzyme.

The Stokes radii of the human enzyme forms range between 6.8 and 12.9 nm; the ones for the eel enzyme forms are between 6.4 and 15.0 nm. SDS-polyacrylamide gel electrophoresis gave one subunit only for both enzymes with apparent molecular weights of 80 000 and 93 000 for the human and the eel enzyme respectively.

It is well established that acetylcholinesterase from a number of different sources exists in multiple molecular forms. The eel as well as the *Torpedo* enzyme can be extracted from fresh or frozen tissue as elongated clusters of 4—12 subunits apparently attached to a tail like structure[1-5] or from partially autolyzed tissue as a more globular tetrameric entity devoid of the tail like extension[6-8]. Much less information on the nature of acetylcholinesterase from red cell membranes is available.. Berman[9] compared some structural properties of the bovine red cell enzyme to the ones of the eel enzyme and found evidence by SDS-polyacrylamide gel electrophoresis for the existence of two dislike subunits in the bovine enzyme. Shafai and Cortner[10] postulated that human red cell acetylcholinesterase has a dimeric structure with two unlike subunits α and β. A more complex state of aggregation has been described by Wright and Plummer[11,12] and by Whittaker and coworkers[13,14]. From these data it should be possible to compare the properties of the red cell enzyme with eel acetylcholinesterase. However the human enzyme preparations described in the literature[15-18], although enriched more than 100 times over the initial concentration in membrane preparations, must be regarded as highly impure. The eel enzyme on the other hand was shown to be devoid of contaminating proteins[19,20]. In order to compare the molecular properties of the two enzymes we have purified both enzymes to apparent homogeneity upon polyacrylamide gel electrophoresis[21,22]. In this paper

their molecular parameters are compared to each other following density gradient centrifugation, gel filtration and isoelectric focusing experiments.

METHODS

Acetylcholinesterase was extracted from frozen electric tissue of *Electrophorus electricus* by a modified procedure of Hopff *et al.*[23] as described elsewhere[24] and purified by affinity chromatography according to Berman and Young[25]. The human red cell enzyme was solubilized by Triton X-100 and purified by the same technique[22,26]. Density gradient centrifugation was performed according to Martin and Ames[27], isoelectric focusing according to Vesterberg and Svenson[28] and SDS--polyacrylamide gel electrophoresis according to Weber and Osborn[29].

RESULTS

When a freshly Triton X-100 depleted preparation of human erythrocyte acetylcholinesterase is subjected to sucrose density gradient centrifugation

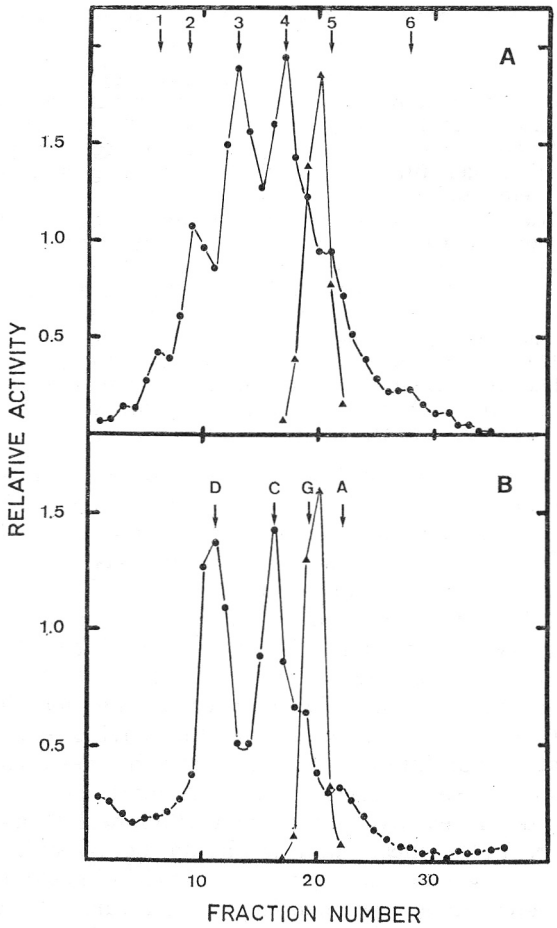

Fig. 1 — Sucrose density gradient centrifugation of human erythrocyte acetylcholinesterase (A) and of the eel enzyme (B). Centrifugation was performed in 20 mM Tris-HCl buffer pH = 7.4 containing 1% α-methyl-D-mannoside in case of the human enzyme and 1.0 M NaCl in case of the eel acetylcholinesterase. Enzyme; 100 µl containing 15—20 IU were applied together with 0.2 mg catalase. The collected fractions were assayed for acetylcholinesterase (● — ●) and catalase activity (▲ — ▲). Numbers in A refer to isolated forms obtained after recentrifugation of pooled fractions. Letters in B designate the multiple molecular forms according to the nomenclature of Massoulié (1).

in presence of 1% α-methyl-D-mannoside a number of multiple molecular forms are separated (Fig. 1A). The sedimentation pattern is more complex than the one obtained after ultracentrifugation of the eel enzyme extracted from frozen electric tissue (Fig. 1B). The sedimentation coefficients procured after recentrifugation of pooled peak fractions as well as the Stokes radii estimated by gel filtration are listed in Table I. When a freshly Triton X-100 depleted human enzyme was subjected to ultracentrifugation in absence of α-methyl-D-mannoside a somewhat different sedimentation pattern was obtained (Fig. 2A). Upon addition of 0.02% Triton X-100, the critical micelle

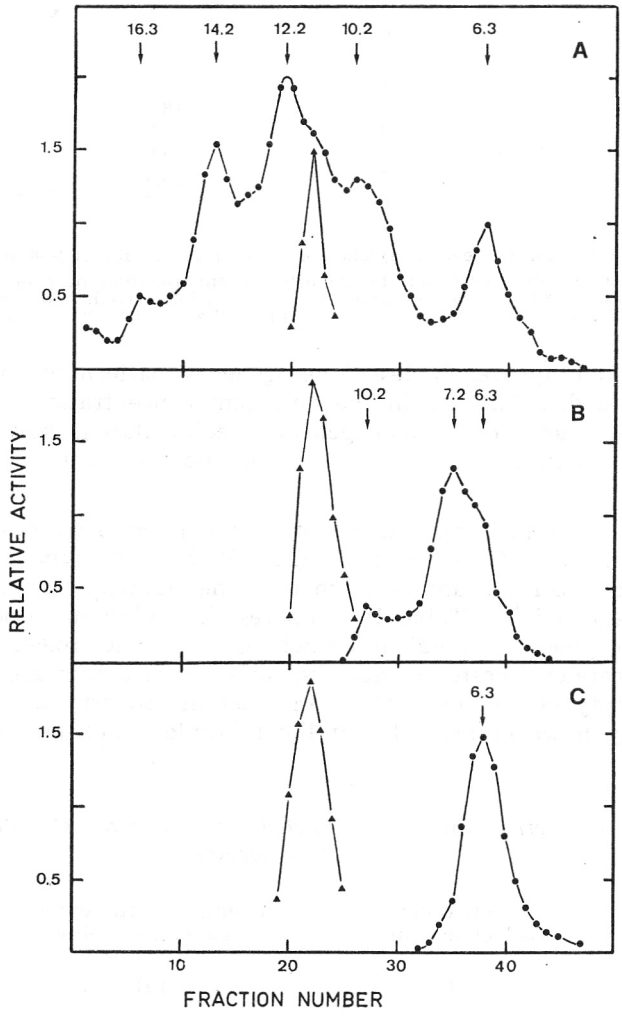

Fig. 2 — A: Sucrose density gradient centrifugation of freshly Triton X-100 depleted human erythrocyte acetylcholinesterase in absence of α-methyl-D-mannoside. Otherwise centrifugation was carried out as described in Fig. 1A. B: Sedimentation pattern obtained after readdition of 0.02% Triton X-100 to the detergent depleted enzyme preparation. C: Sedimentation pattern in 0.1% Triton X-100. Acetylcholinesterase (● — ●) and catalase activity (▲ — ▲). Numbers above arrows give the sedimentation coefficients estimated using catalase as marker protein.

TABLE I

Comparison of molecular parameters of acetylcholinesterase from electric eel and from human erythrocytes

	Form[a]	Sedimentation coefficient[b] S	Stokes radius nm
Electric eel	E	21.6	17.0
	D	18.4	15.0
	C	14.2	14.4
	A	8.8	12.4
	G_p	11.4	8.2
Human erythrocytes	1	18.1	12.9
	2	16.8	11.1
	3	14.8	10.7
	4	12.9	10.4
	5	10.9	10.1
	6	7.3	6.3

[a] Numbers 1—6 of human red cell acetylcholinesterase refer to forms 1—6 shown in Fig. 1A.

[b] Sedimentation coefficients were determined after recentrifugation of pooled fractions using catalase as marker protein. The resulting peaks were pooled again and used for the determination of the Stokes radii by gel filtration of Sepharose 4-B (22).

concentration, an apparently less heterogeneous pattern could be observed (Fig. 2B). A further increase in the detergent concentration to 0.1% Triton X-100 gave the apparently homogeneous sedimentation pattern shown in figure 2C. This pattern is identical to the one obtained before removal of the detergent.

Upon isoelectric focusing both eel and human acetylcholinesterase showed considerable amount of heterogeneity (Fig. 3). Most notably the eel enzyme focused between pH 2.7 and 3.8, whereas the human enzyme is stabilized between pH 4.5 and 5.2. Table II compares the individual isoelectric points of the two enzymes. As detailed elsewhere[22,30] the heterogeneity of human erythrocyte acetylcholinesterase does not arise from a mixture of genetically different phenotypes but seems to be an inherent property of each individual. The possibility however existed that the multiple molecular forms observed

TABLE II

Comparison of isoelectric points of acetylcholinesterase from electric eel and from human erythrocytes

Electric eel Isoelectric point	Human erythrocytes Isoelectric point
pH	pH
2.77	4.55
3.24	4.68
3.53	4.81
3.71	4.98
	5.18

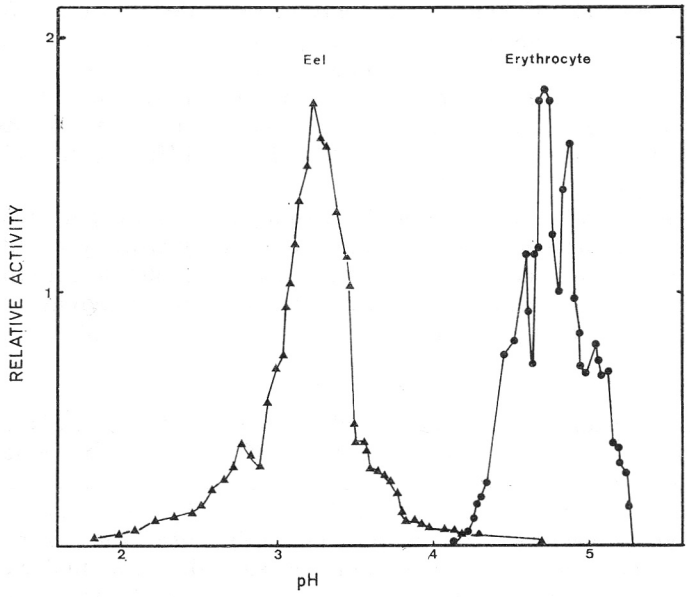

Fig. 3 — Isoelectric focusing of acetylcholinesterases in a Uniphor Column Electrophoresis system. As support a density gradient was prepared from 0—45% sucrose containing 1% α-methyl-D-mannoside to stabilize the enzyme. The eel enzyme was focused in a gradient of pH = 2.5—4.0, the red cell enzyme in a gradient of pH = 4—6.

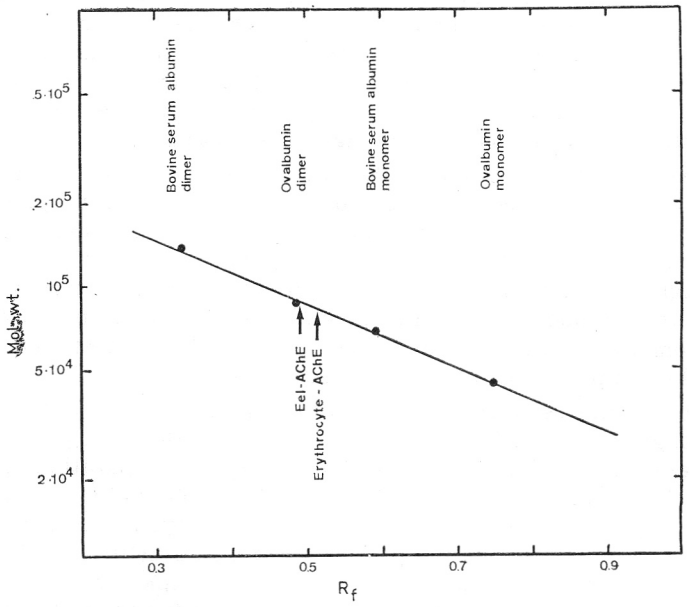

Fig. 4 — Determination of the subunit molecular weights by SDS-polyacrylamide gel electrophoresis of acetylcholinesterases from human red cells and from the electric eel. Ovalbumin and bovine serum albumin served as marker proteins. Electrophoresis was carried out on slab gels using the Ortec equipment.

upon sucrose density gradient centrifugation coincided with the forms separated by isoelectric focusing. Consequently an isolated form of the human enzyme (form 4, Fig. 1A) was subjected to isoelectric focusing. However the activity pattern obtained was identical to the one shown in Fig. 3 suggesting that the heterogeneity observed in this experiment is not caused by the presence of a number of multiple molecular forms differing in their sedimentation coefficients.

Despite of these heterogeneities, SDS-polyacrylamide gel electrophoresis in presence of mercaptoethanol gave one subunit only with apparent molecular weights of 93 000 for eel acetylcholinesterase and 80 000 for the red cell enzyme (Fig. 4). In absence of mercaptoethanol slower moving bands were obtained with molecular weights corresponding to multiples of the above numbers.

DISCUSSION

Acetylcholinesterase from eel as well as from erythrocytes is a membrane bound enzyme. From the mode of solubilization it can be concluded that the human enzyme is more firmly attached to the membrane than the eel enzyme. The use of detergents such as Triton X-100 is necessary to solubilize red cell acetylcholinesterase, but it most probably causes the enzyme to appear as artefactual homogeneous entity and conceals the true molecular heterogeneity of this enzyme. Only after removal of the detergent become the oligomeric forms apparent.

The sedimentation pattern observed with red cell acetylcholinesterase is more complex than the one of the eel enzyme. This suggests that the red cell enzyme exists in a more complex state of aggregation than the eel enzyme. From gel filtration studies[5] and electron micrographs[3], Massoulié and coworkers showed that forms A, C and D of the eel enzyme are non-spherical proteins. Although it is not known to present whether the red cell enzyme too is made up of clusters of subunits attached to a tail like extension, the behaviour of this enzyme upon gel filtration and rate sedimentation experiments implies that the human enzyme too is of elongated structure.

By comparing the S values listed in Table I it is seen that the three forms A, C and D of the eel enzyme roughly span the same range of Svedberg units as the six forms of red cell acetylcholinesterase. As detailed by Powell *et al.*[20] forms A, C and D are made of 4, 8 and 12 subunits attached to the tail. Although the subunit molecular weight of the human enzyme is known at present, the number of subunits in each form remains obscure. The data obtained from rate sedimentation experiments might suggest that the human enzyme is built up in multiples of two subunits.

Contrary to the eel enzyme the sedimentation pattern of the human enzyme is strongly influenced by the presence of Triton X-100. Upon readdition of this detergent to a Triton X-100 depleted enzyme preparation an apparent decrease in the sedimentation coefficient to 6.3 is observed. As detailed elsewhere[22] the binding of Triton X-100 to red cell acetylcholinesterase increases the partial specific volume of the protein yielding an artificially low sedimentation coefficient (6.3 S). This enzyme detergent complex has a Stokes radius of 8.7 nm which together with the low *S* value strongly supports the view that the apparent homogeneity observed in Triton X-100 is an artefact of the solubilization procedure.

Isoelectric focusing resolved both enzymes into a number of multiple molecular forms that can not at present be correlated with the different forms appearing in rate sedimentation experiments. It is however well established that acetylcholinesterase from both eel tissue and red cell membranes contains carbohydrate residues[6,20,22,24]. The microheterogeneity observed upon isoelectric focusing thus may reflect differences in the sialic acid content of the glycoprotein. It is of interest to note that the eel enzyme, prepared under exclusion of autolysis, is a rather acidic protein. On the other hand when the eel enzyme is solubilized after limited proteolysis (under conditions where a heavy and a light subunit become apparent) the average isoelectric point of the enzyme increases by approximately 1.5 pH units.

Although both enzymes show considerable amount of heterogeneity, it is noteworthy that SDS-polyacrylamide gel electrophoresis gives one subunit only in both cases. It can not be excluded at present that the apparently homogeneous protein observed after SDS-gel electrophoresis is made up of two subunits equal in size but of different charge, a hypothesis that might offer an alternative explanation for the existence of multiple molecular forms differing in net charge but of apparently equal size.

Despite some differences in the above mentioned parameters, the molecular properties known so far of human red cell acetylcholinesterase are quite comparable to the ones of the eel enzyme. In addition the purified erythrocyte enzyme has similar regulatory properties as eel acetylcholinesterase (unpublished observations). The specific activities of Triton X-100 solubilized and of the purified red cell enzyme preparation are 1—2 and 4000 IU per mg of protein. They are considerably lower than the corresponding values for eel acetylcholinesterase suggesting that the human enzyme is less concentrated in the membrane and has a lower turnover number than eel acetylcholinesterase. To present little is known about the function of the human enzyme embedded in the erythrocyte membrane. The recent discovery by Huestis and McConnell[32] of a functional acetylcholine receptor in the human erythrocyte adds new incentive to the old question: What is the physiological role of a cholinergic system located in red cell membranes? The answer however remains to be given.

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DISCUSSION

M. R. Pavlič:

Have you found any differences in the catalytic center activities in enzymes by different solubilization procedures, *e. g.* by Triton X-100?

U. Brodbeck:

We have not yet determined the catalytic center acticity of the human red cell acetylcholinesterase. We know, however, that the activity of the Triton X-100 depleted enzyme is enhanced by a factor of 2 when the assay is carried out in presence of 0.1% Triton X-100.

R. D. O'Brien:

Can one solubilize some erythrocyte enzyme without detergent, *e. g.* by proteinase or high salt?

U. Brodbeck:

It has been about 6 years since we studied in some detail the release of the human enzyme from erythrocyte ghosts. If I recall correctly we found that without detergents neither high ionic strength, chaotropic ions and chelating agents nor attack of the membrane by proteolytic and lipolytic enzymes gave satisfactory results. For instance extraction of red cell stroma by 1—2 M NaCl released up to 70% of the initial enzyme activity into a 1.6×10^6 $g \cdot$ min supernatant thus confirming the observation of Mitchell and Hanahan (*Biochemistry* **5** (1966) 51—57). This »soluble« activity, however, sedimented quantitatively after 6.2×10^6 $g \cdot$ min centrifugation in agreement to the observation of Burger *et al.* (*Biochemistry* **7** (1968) 3682—3700). However, we could solubilize the enzyme (6.2×10^6 $g \cdot$ min

supernatant fraction) in yields up to 35% of the initial activity in the hemolysate by treating lyophilized red cell ghosts with 1-butanol followed, after solvent evaporation, by extraction with 10 mM Na-phosphate buffer containing 10 mM NaCl.

P. W. Taylor:

Since α-methyl-D-mannoside will assist in the displacement of Triton X-100 and will minimize the AChE-sepharose interaction, is it your opinion that Triton X-100 is eliminating the carbohydrate-carbohydrate interaction (or protein-carbohydrate interaction) rather than eliminating hydrophobic interactions as a detergent is normally thought to do? Is there any lectin activity in your preparation?

U. Brodbeck:

To answer your first question, I must emphasize that we do not have any direct proof that α-methyl-D-mannoside actually displaces Triton X-100 from the red cell enzyme, we only take the difference in sedimentation pattern obtained in the presence or absence of this sugar as suggestive evidence for this working hypothesis. On the other hand the 6.3 S form disappears by itself when a Triton X-100 depleted enzyme preparation is stored in solution at 4 °C for some weeks.

The binding of Triton X-100 possibly shields the carbohydrate moieties of the enzyme from interacting with similar moieties on the sepharose column and as a matter of fact also from binding to concanavalin-A sepharose, as I have pointed out. This is of course an additional effect observed with Triton X-100 besides its expected function in eliminating hydrophobic interactions.

The answer to your second question is that we do not know as we have not assayed our preparation for possible lectin activity.

D. T. Plummer:

Dr. Brodbeck is correct in pointing out the problems of working with Triton X-100 and one must always be aware of artifacts. For this reason we have used a wide range of solubilization procedures while investigating the pig brain enzyme and in this case the molecular weight-species obtained with detergent are similar to those obtained using methods involving sonication, EDTA extraction, enzyme treatment and extraction with organic solvents. Triton X-100 does not then appear to produce molecular weight-artifacts in the case of the brain enzyme. One other comment I would like to make is that for the human erythrocyte enzyme we have managed to separate the bulk of Triton from the enzyme on gel-filtration using Sephadex G-200. The reason for this may be the treatment prior to gel--filtration which differs from that of Dr. Brodbeck's group.

I. Silman:

(a) Does Triton stain both, Coomassie blue and Amido Schwarz? (b) Do you observe similar isoelectric focusing patterns with crude extracts as you observe with purified enzyme?

U. Brodbeck:

(a) I am not sure about Amido Schwarz. (b) Early isoelectric focusing experiments on hemolysates indicated that the crude red cell enzyme also shows heterogeneity with respect to overall charge. The results however were not very convincing as we had to load the column with large amounts of protein in order to see enough enzyme activity. At such a high protein concentration precipitation occurred which of course gave very unreliable results. On the other hand we have observed similar heterogeneity in the butanol solubilized partially purified red cell enzyme preparation. Three different forms of acetylcholinesterase were separated by isoelectric focusing. The activity pattern of this eöperiment shows less heterogeneity than the pattern of the red cell enzyme. It should be noted, however, that in the above experiment a gradient of pH = 3—10 was used whereas in latter the elution pattern originated from a pH=4—6 gradient. Besides the heterogeneity with respect to overall charge the butanol solubilized partially purified human enzyme showed considerable heterogeneity with respect to molecular dimensions as found by gel filtration and confirmed by preparative polyacrylamide gel electrophoresis. I cannot give you an answer on the crude eel enzyme as we have not done any isoelectric focusing with electric tissue extracts.

SAŽETAK

Komparativni studij molekularnih svojstava čišćene acetilkolinesteraze ljudskih eritrocita i električnog organa Electrophorus electricus

U. Brodbeck, P. Ott i Thérese Wiedmer

Acetilkolinesteraze ljudskih eritrocita i električnog organa *Electrophorus electricus*, čišćene su metodom afinitetne kromatografije do visokog stupnja čistoće. Molekularni parametri čišćenih enzima uspoređivani su nakon centrifugiranja uz različite gradijente gustoće, gel-filtracije, izoelektričnog fokusiranja i elektroforeze na SDS-poliakrilamidnom gelu.

Centrifugiranje u saharoznom mediju različitog gradijenta gustoće pokazalo je da se enzim ljudskih eritrocita nalazi u kompleksnijem obliku agregacije od enzima jegulje. Izoelektričnim fokusiranjem uspjelo je oba enzima razdvojiti u multiple molekularne oblike pri čemu se je enzim jegulje fokusirao pri nižem pH nego li enzim ljudskih eritrocita.

Stokesovi radiusi za enzim ljudskih eritrocita iznose 6,8 do 12,9 nm, a za enzim jegulje 6.4—15.0 nm. Elektroforeza na SDS-poliakrilamidnom gelu pokazala je za oba enzima samo jednu osnovnu jedinicu s prividnim molekularnim težinama 80 000 i 93 000 za enzim ljudskih eritrocita odnosno jegulje.

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