strong denaturing agents. The rotary diffusion constants corresponding to this denatured state are, however, relatively high, $\eta\theta/T$ ranging from about 5 to perhaps 20. These relatively high rotary diffusion constants suggest rather strongly that no drastic unfolding of the protein molecule has occurred under such conditions, but at most only an expansion. To actually draw any conclusions as to the nature of the change would require additional physical data such as intrinsic viscosity, as has been emphasized recently by Scheraga and Mandelkern.[10] It would be of considerable interest to obtain such data in the systems used, but measurement of intrinsic viscosity in a mixed solvent such as the glycerol–water systems employed poses a considerable problem.[11]

One final argument, favoring the idea that no substantial unfolding takes place in the case of serum albumin, appears worth mentioning. The low values of the reduced birefringence in the case of guanidine denaturation have been pointed out above. One can generalize further and say that in only a few cases are these figures comparable to those obtained with ovalbumin denatured under various conditions.[3] Interpretation of such data is uncertain since they depend on not only the intrinsic anisotropy of the molecule being oriented but also on the form birefringence. On the other hand, the intrinsic anisotropy of a swollen molecule such as envisaged here would almost certainly be less than that of an elongated, more highly ordered molecule. The form birefringence, too, would be less, other factors being the same. The low reduced birefringence is thus in accord, at least, with the swelling concept.

Acknowledgment.—We are indebted to Dr. Lawrence L. Lachat of Armour and Company for generous donations of bovine serum albumin.

AMES, IOWA

(10) H. Scheraga and L. Mandelkern, THIS JOURNAL, **75**, 179 (1953).

(11) A few such experiments have been carried out in our laboratory on denatured ovalbumin and have yielded results in close agreement with those obtained in absence of glycerol.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOPHYSICAL CHEMISTRY, HARVARD UNIVERSITY]

Preparation and Properties of Serum and Plasma Proteins. Plasma Cholinesterase[1]

BY DOUGLAS M. SURGENOR AND DRUMMOND ELLIS[2]

RECEIVED APRIL 2, 1954

Under controlled conditions of *p*H, ionic strength, ethanol concentration and temperature, plasma cholinestarase has been concentrated 3400 times over plasma on a protein basis. Although not yet pure, it has been estimated from ultracentrifugal analyses that the pure enzyme has an activity at least 11,000 times that of plasma, and comprises not more than 0.01% of the plasma proteins.

Among the enzymes found in human plasma is an esterase which catalyzes the hydrolysis of choline esters. In this respect, as well as in other physical properties, the plasma enzyme is distinguishable from the acetylcholinesterases which are found in nerve tissue and in intimate association with the envelope of the red cell,[3] both of which split acetylcholine faster than any other choline ester. The physiological function of the nerve enzyme is undoubtedly concerned with the hydrolysis of acetylcholine. The role of the plasma enzyme is, however, unknown.

In the fractionation of plasma, cholinesterase was separated into fraction IV-4,[4] together with the β_1-metal-combining protein. In the subfractionation of fraction IV-4, the enzyme was concentrated into fraction IV-6, which was rich in α_2-globulins.[5] Since our earlier studies had indicated that some denaturation of the rather labile carbohydrate-containing proteins in this fraction had occurred, we have confined our present efforts to concentration of the enzyme whose activity appeared to have been maintained in these separations. Preparations of the enzyme have been obtained which were over three thousand-fold as active, on a dry weight basis, as plasma; it has not yet been obtained in the pure state, however. The newer methods, recently described,[6] for the fractionation of proteins, which open up several new avenues of approach to difficult separations, while at the same time maintaining optimal conditions for protein stability, thus averting the danger of partial denaturation of at least certain of the components in the concentrates here described, make possible considerable improvement in the yields and purifications of this as well as other enzymes.

(1) This work has been supported by funds of Harvard University and the Eugene Higgins Trust, by grants from the Rockefeller Foundation and the National Institutes of Health, and by contributions from industry. It was presented at the 119th Meeting of the American Chemical Society, April 2, 1951, Boston, Massachusetts.

(2) The award to one of us (D. E.) of a Travelling Fellowship by the Kemsley Foundation, Glasgow, Scotland, and of a Fellowship Grant by the Cross Trust, Edinburgh, Scotland, in 1947–1948, is gratefully acknowledged. Present address: Blood Transfusion Service, Royal Infirmary, Edinburgh 9, Scotland.

(3) K. Augustinson, *Acta Physiol. Scand.*, **15**, Suppl. 52 (1948).

(4) E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin and H. L. Taylor, THIS JOURNAL, **68**, 459 (1946).

(5) D. M. Surgenor, L. E. Strong, H. L. Taylor, R. S. Gordon, Jr., and D. M. Gibson, *ibid.*, **71**, 1223 (1949).

Materials and Methods

Fraction IV-6 was the starting materials in these studies. It was prepared by the method and from the sources described previously.[5]

Plasma esterase determinations were carried out by Miss M. Dougherty, working under the direction of Dr. Avram Goldstein in the Department of Pharmacology, Harvard Medical School, to whom we are indebted for continued interest. They measure release of CO_2 from a bicarbonate–Ringer solution (0.025 *M* bicarbonate), buffered to *p*H 7.8

(6) E. J. Cohn, F. R. N. Gurd, D. M. Surgenor, B. A. Barnes, R. K. Brown, G. Derouaux, J. M. Gillespie, F. W. Kahnt, W. F. Lever, C. H. Liu, D. Mittelman, R. F. Mouton, K. Schmid and E. Uroma, *ibid.*, **72**, 465 (1950).

with a 95% N_2–5% CO_2 gas phase, at 37°, in the Warburg constant volume manometer. Freshly prepared recrystallized acetylcholine was used, at a concentration of 0.0805 *M* in the reacting solution. Enzyme solutions, appropriately diluted with the bicarbonate–Ringer solution, so as to hydrolyze acetylcholine at a rate of approximately 10 millimoles per liter per hour, were equilibrated with the gas phase before adding the substrate. Readings were made during the first 20 minutes. Under these conditions, at least 97% of maximum enzyme activity was obtained, and determinations were reproducible to ±3%.[7]

The unit of enzyme activity has been defined as that amount of enzyme which would hydrolyze 1 millimole of acetylcholine per hour under the above conditions.

Carbohydrate determinations were carried out by a modification of the method of Sørensen and Haugaard,[8] as previously described.[5]

Other methods and the equipment used in these studies have also previously been described.[4,5]

Experimental

Fraction IV-6 was adjusted to the conditions: *p*H 4.9, ionic strength 0.01, 9% ethanol, 2% protein at −3°. Each kilogram of paste (or an equivalent amount of dry powder) was suspended at 0° in 9 liters of ice and water. To each liter of suspension was added a total of 12 mmoles of sodium, dissolved in 100 ml., consisting of the sodium bicarbonate required to adjust the *p*H to 4.92 and *p*H 4.92 sodium acetate buffer to increase the ionic strength. Then 203 ml. of 53.3% ethanol (for each liter of suspension) was added through capillary jets, with cooling and stirring, so that the final temperature was −3°. The resulting suspension was stirred for 3 hours and the precipitate, largely denatured mucoprotein, was removed by centrifugation at −3°.[9]

The solution was next adjusted to *p*H 4.40–4.45, ionic strength 0.02 and 18% ethanol by adding to each liter, (a) 17 mmoles of sodium acetate and the required amount of acetic acid for the *p*H adjustment, in 50 ml. of water, and. (b) 280 ml. of 53.3% ethanol. The usual precautions were taken during addition of the reagent and the temperature was lowered to −5°.

Fraction IV-6-1 was removed by centrifugation at −5°. The small amount of mucoprotein which still remained was then removed by returning to the conditions of the first step; *p*H 4.90–4.94, ionic strength 0.01, 2% protein, 10% ethanol at −3°. Removal of this small amount of inert protein was essential to the carrying out of later separations.

Solution IV-6-2 was obtained after centrifugation at −3°. Plasma cholinesterase was preferentially precipitated from this solution at *p*H 3.92–3.93 and 18% ethanol at −5°. The *p*H at this point was critical: a small aliquot of the solution was adjusted and its enzyme distribution determined before proceeding. To decrease the time necessary for equilibration, and to take advantage of the greater stability of proteins in the solid state, the final conditions were approached from the solid phase rather than from solution. This was done by adding sufficient 53.3% ethanol to the solution to raise the concentration to 18%, while the temperature was lowered to −5°; the enzyme and most of the protein precipitated. The resulting suspension was then adjusted to the acid reaction by the cautious addition of precooled *p*H 3.5, ionic strength 0.01; acetate buffer in 18% ethanol. This brought about the resolution of most of the protein and left the highly concentrated enzyme in the solid state. The final ionic strength was approximately 0.013. After stirring for 30 minutes the small precipitate was removed.

Fraction IV-6-3 was used immediately; if not, it was dried from the frozen state after neutralization to *p*H 5. The final purification was similar to the above. Fraction IV-6-3 was redissolved in ice water at 0° to a concentration of 1.5% protein and then the ethanol concentration was raised to 18% while the temperature was lowered to −5°. The suspension was then titrated, as before, with *p*H 3.3 sodium acetate buffer in 18% ethanol, until the *p*H was 3.85–3.88. The precipitate was centrifuged at −5° after 15 minutes.

Fraction IV-6-4 was immediately redissolved in 2 volumes of partly frozen 0.05 *M* sodium bicarbonate and was dried from the frozen state.

Fraction IV-6-4 had an esterase activity of over 9 units per milligram. A third acid precipitation did not increase the activity. A less acid reaction, *p*H 4.8–5.0, resulted in slight but insignificant further purification. Since the isoelectric zone of the esterase appeared to be acid to *p*H 4.4, an attempt was made to form an insoluble complex between the enzyme and an added protein of more alkaline isoelectric point. β_1-Metal-combining globulin and γ-globulin were used. Although a precipitate formed under conditions where either component alone would have been soluble, the enzyme was not significantly concentrated.

Fraction IV-6-G.—The proteins remaining in solution after separation of fractions IV-6-3 and IV-6-4, largely glycoproteins, were reprecipitated by adjusting the *p*H to 4.4 without change in the ethanol concentration or temperature. The cholinesterase activity of this fraction was 0.5 units per milligram.

Discussion

The analytical data for the fractions obtained are presented in Table I. In the assay of the enzyme, extremely low protein concentrations were used particularly with the most active fractions—and some inactivation of the enzyme occurred. Addition of albumin to the system diminished this effect. This stabilizing action of albumin has been studied in some detail by Goldstein,[10] who found that addition of solutions of crystallized human serum albumin, which had no enzyme activity, resulted in approximately 10% higher enzyme activities in the purest fractions. The carbohydrate content of the protein increased as the enzymatic activity increased. These results suggest but do not prove that plasma cholinesterase is a glycoprotein. Fraction IV-4 and IV-6-1 consisted primarily of α_2 globulins. The electrophoretic mobility of the enzyme is not proved, however, because of the very small amounts of enzyme in the fractions.

TABLE I

Fraction	Plasma protein, %	Hexose, %	Plasma cholinesterase Yield %	Activity units/mg.	Concn. × plasma
Plasma	100	..	100	0.003	1
IV-4	7	2.9	90	...	..
IV-6	1.4	3.8	61	.09	35
IV-6-1	0.05	6.9	28	.3	111
IV-6-2	.025	8.1	21	.5	185
IV-6-3	.005	9.8	14	2.0	740
IV-6-4	.0008	11.1	7	9.2	3400

Ultracentrifugal analyses, which required less than 20 mg. of protein, revealed a new component, not previously observed, in fraction IV-6-3. Amounts of this component, proportional to enzyme activity, were found in all subfractions. The sedimentation constant of the new component varied with protein concentrations, extrapolating to approximately $S_{20,w} = 12$, at zero concentration (Table II). An ultracentrifuge diagram of fraction IV-6-4 with an enzyme activity of 9.2 units per milligram is reproduced in Fig. 1.

(7) O. H. Straus and A. Goldstein, *J. Gen. Physiol.*, **26**, 559 (1943).

(8) M. Sørensen and G. Haugaard, *Compt. rend. trav. lab. Carlsberg*, **19**, No. 12, 1 (1933).

(9) The persistent separation of a part of the enzyme into this precipitate, and its resistance to extraction therefrom suggested the possibility that the precipitated enzyme might not be identical with that which remained in solution. Studies kindly carried out by Dr. Goldstein using two different substrates and a series of inhibitors failed, however, to reveal any significant differences between the two fractions. These results are in agreement with the studies recently reported by D. H. Adams and V. P. Whittaker, *Biochem. J.*, **44**, 62 (1949), which indicate the existence of but a single chlorinesterase in plasma.

(10) A. Goldstein and M. E. Dougherty, *Arch. Biochem. and Biophysics*, **33**, 22 (1951).

TABLE II

ULTRACENTRIFUGAL ANALYSES FOR S = 9 TO 11 COMPONENT OF FRACTIONS RICH IN HUMAN PLASMA CHOLINESTERASE

Total protein[a] mg./ml. in soln.	S = 9 to 11 protein mg./ml. in soln.	Plasma cholinesterase Units/ml. in soln.	Plasma cholinesterase Units/mg. of protein
8.0	0	6	0.75
9.3	1.0	18	1.87
18.5	1.2	35	1.87
4.0	0.9	22	5.80
8.0	1.5	44	5.80
16.0	2.3	87	5.80
9.1	2.5	84	9.20
18.3	4.3	168	9.20

[a] In 0.15 M NaCl at pH 6.5. The air-driven ultracentrifuge was used.

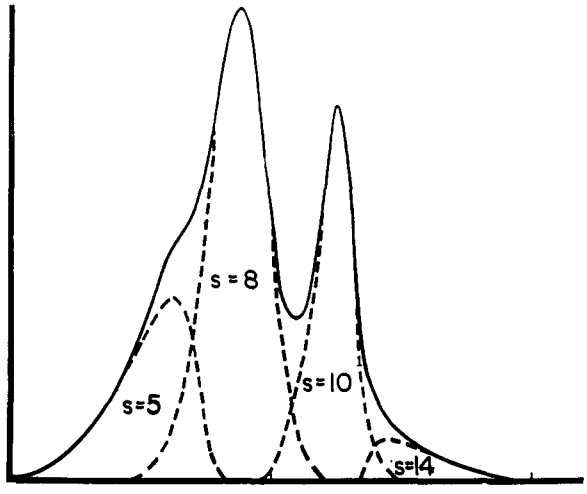

Fig. 1.—Ultracentrifuge diagram of plasma cholinesterase concentrate of activity 9.2 units/mg. protein.

If this new component were the enzyme, then pure plasma cholinesterase would have an activity of approximately 30 units per milligram (Fig. 2). Since normal plasma has an activity of the order of 200 units per liter, the concentration of the enzyme in normal plasma would be approximately 7 mg. per liter; it would comprise but 0.01% of the plasma proteins. Assuming a molecular weight of 300,000 for a molecule with this sedimentation constant, the molar concentration of cholinesterase in human plasma would be of the order of 2×10^{-8} M.[11]

(11) The assumption that the new component in the ultracentrifuge was indeed the enzyme would not be inconsistent with the following statistics: (a) For one active center per molecule, the turnover number of human plasma cholinesterase would be of the order of 1.5×10^{5} molecules of substrate (acetylcholine) hydrolyzed per molecule of enzyme per minute. This is a reasonable turnover number, although much higher turnover numbers have been observed. L. R. Easson and E. Stedman [*Proc. Roy. Soc.* (*London*), **B121**, 142 (1936)] have estimated a turnover number of 89,400 per minute for horse plasma cholinesterase, based on inhibition of acetylcholine hydrolysis: (b) Goldstein, on the basis of inhibition studies, has concluded that the *maximal* molar concentration of active enzyme centers in dog plasma is approximately 5×10^{-7} M. (A. Goldstein, *J. Gen. Physiol.*, **27**, 529 (1944)).

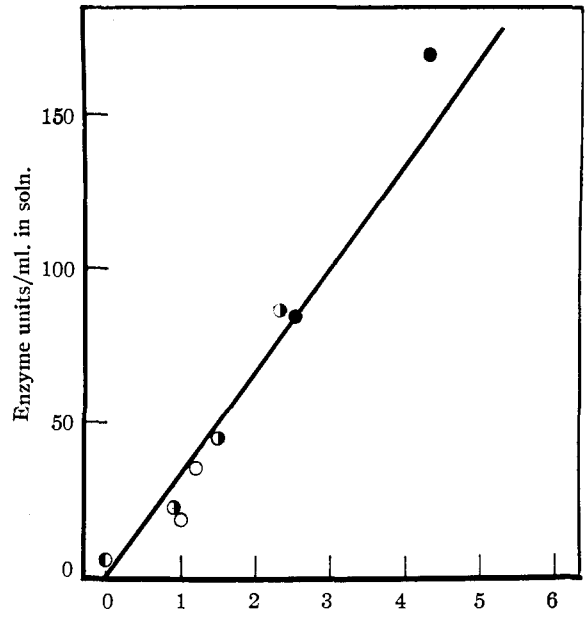

Fig. 2.—Ultracentrifugal analysis of some plasma cholinesterase concentrates: ◐, 0.75 unit/mg. protein; ○, 1.87 unit/mg. protein; ◑, 5.8 unit/mg. protein; ●, 9.2 unit/mg. protein.

These considerations do not prove, however, that the component of S = 12 was the enzyme. The enzyme could have been present in too low a concentration to be observable in the ultracentrifuge and have sedimented within the larger peak.

Acknowledgments.—We are most grateful to Dr. J. L. Oncley for his many suggestions for discussions and critical evaluation of the ultracentrifugal data.

BOSTON, MASS.