A Comparison of the Native and Monomer Forms of Macroglobulin from Pathological Human Serum with Particular Reference to Electrophoretic Mobility Differences

By E. I. McDOUGALL* AND H. F. DEUTSCH

Department of Physiological Chemistry, University of Wisconsin, Madison, Wis., U.S.A.

(Received 19 June 1963)

Deutsch & Morton (1957) described the dissociation of human serum macroglobulins (native protein) by thiols and their subsequent reaction with alkylating agents to form a stable sub-unit (monomer protein). Deutsch & Morton (1958) further reported some macroglobulins which contained only one electrophoretic component but which formed two electrophoretic components on production of the monomer. Leder (1962), on the other hand, described five macroglobulin preparations that yielded monomers, which showed only one zone on paper electrophoresis. With the exception of one of the macroglobulins described by Deutsch & Morton (1958), all those so far reported produced monomers with a higher anionic mobility than that of the native protein. Deutsch & Greenwood (1960) observed that the monomer was not stable under acid conditions, but broke down reversibly to small units.

In the present work the native and monomer forms of five macroglobulin preparations of the type γ_1 M- or β_2 M-globulin from pathological human sera were compared by paper electrophoresis. One of them was available in quantity and the native and monomer forms of this protein were compared more extensively: their mobilities were studied in relation to pH and ionic strength, I ; their H^+ ion-dissociation curves were obtained by potentiometric titration and, for the tyrosyl residue, by spectrophotometric titration; their amino acid compositions were investigated and the state of the proteins was ascertained by ultracentrifugal-sedimentation experiments. These results are discussed in relation to the dissociation of the macroglobulin and to the difference in mobilities between the native and monomer proteins.

METHODS

Preparation of proteins. The native macroglobulin was prepared from suitable pathological sera by diluting 1:10 (v/v) with water, dissolving the precipitate in 0.15M-NaCl and reprecipitating at least twice more, as described by Deutsch & Morton (1958). Sub-units of these macro-

globulins were produced by reduction with 0.1M-mercaptoethanol and stabilized by alkylation with 0.02M-iodoacetamide to form the monomer, as described by Deutsch & Morton (1957).

Protein concentration. This was determined, when required for amino acid analyses or H^+ ion-dissociation studies, by evaporating the protein solution and the salt solution in which the protein had been dissolved, or against which it had been dialysed, at a low temperature and drying to constant weight *in vacuo* over phosphorus pentoxide.

Paper electrophoresis. This was carried out horizontally on Whatman 3MM paper in 0.05M-sodium veronal buffer, pH 8.6.

Urea-formate starch-gel electrophoresis. This was carried out in gels prepared according to the method of Edelman & Poulik (1961).

Moving-boundary electrophoresis. This was carried out in the Spinco model L apparatus at 1° and a protein concentration of 0.4–0.6 g./dl., either in sodium veronal buffer, pH 8.6 and I 0.1, or in a series of buffers of different pH values containing 0.02M uni-univalent buffer salt (potassium acetate, cacodylate, veronal or glycinate) and potassium chloride to make I 0.15, 0.1, 0.05 or 0.02. Conductance was measured at 0° at 1000 cyc./sec.

Hydrogen ion-dissociation curves. These were obtained by: (a) potentiometric titration, carried out by continuous manual microtitration of 20–95 mg. of protein in 5 ml. of 0.15M-KCl at 20° under nitrogen with 0.1N-HCl or 0.1N-KOH, by using a Radiometer PHM 4 pH-meter and calculating the results according to the method of Tanford (1955); (b) spectrophotometric titration for the tyrosyl residue, carried out at 295 m μ and room temperature on dilutions of the protein in a range of alkaline buffers at I 0.15; the buffers were made from different proportions of two solutions, one containing 0.1M-glycine in 0.15M-KCl and the other 0.1N-KOH in 0.05M-KCl; correction was made for non-specific absorption by the method of Beaven & Holiday (1952); the refractive increment of the protein in the solution used was measured in a differential refractometer; the results are expressed as the extinction due to a concentration of the diluted protein equivalent to a refractive increment (Δn) of 0.0002.

Ultracentrifugal sedimentation measurements. These were made in the Spinco model E ultracentrifuge in phosphate buffer, pH 7.4 and I 0.2.

Amino acid analyses. These were made by: (a) the ion-exchange-chromatographic method of Spackman, Stein & Moore (1958) by using the Spinco Automatic Amino Acid Analyzer; for this 4–14 mg. of protein was hydrolysed in 2 ml. of 6N-HCl at 110° for 22 hr. in evacuated sealed tubes; (b) the ultraviolet-spectrophotometric method of Benzke & Schmid (1957) for tyrosine and tryptophan.

* Present address: Rowett Research Institute, Aberdeen, Scotland.

RESULTS

The paper electrophoretogram of the five macroglobulin preparations and the monomers produced from them is shown in Fig. 1. In each case the monomer migrated as a single zone of greater anionic mobility than the native protein. The protein referred to as GE had lower mobilities for both native and monomer protein than the other preparations. It is this one that was available in quantity and studied in some detail.

Some diagrams from the moving-boundary-electrophoresis experiments are shown in Fig. 2. Fig. 2 (a) was obtained with mixed solutions of the

Fig. 1. Zone electrophoresis on paper of five macroglobulin preparations and their monomers in sodium veronal buffer, pH 8.6 and I 0.05. S, Starting position; N, native protein; M, monomer. The arrow indicates the direction of migration. The code letters identify the different preparations.

Fig. 2. Moving-boundary-electrophoresis diagrams of the macroglobulin GE from experiments at 1° . N, Native protein; M, monomer. The arrow indicates the direction of migration. ϵ and δ , Stationary boundaries in descending and ascending limbs respectively. (a) Mixture of native and monomer protein in sodium veronal buffer, pH 8.6 and I 0.1, E 5.7 v/cm., protein concn. 0.6 g./dl., after 347 min. (b) Monomer in KCl-acetate buffer, pH 4.3 and I 0.15. E 3.4 v/cm., protein concn. 0.4 g./dl., after 334 min. (c) Monomer, after treatment with acid buffer used in experiment (b), in sodium veronal buffer, pH 8.6 and I 0.1, E 5.7 v/cm., protein concn. 0.4 g./dl., after 616 min.

native and monomer proteins in sodium veronal buffer, pH 8.6 and I 0.1: the mobilities of the two proteins are different, being

$$-0.71 \times 10^{-5} \text{ cm.}^2 \text{ sec.}^{-1} \text{ v}^{-1}$$

and

$$-1.65 \times 10^{-5} \text{ cm.}^2 \text{ sec.}^{-1} \text{ v}^{-1}$$

respectively, calculated from the descending boundary. This compares with

$$-0.70 \times 10^{-5} \text{ cm.}^2 \text{ sec.}^{-1} \text{ v}^{-1}$$

and

$$-1.72 \times 10^{-5} \text{ cm.}^2 \text{ sec.}^{-1} \text{ v}^{-1}$$

when the measurements are made separately. Fig. 2 (b) shows the behaviour of the monomer under acid conditions at pH 4.3, when a second component appears; Fig. 2 (c) shows that there are still two components present when the acid-treated monomer is re-examined in the sodium veronal buffer, pH 8.6 and I 0.1; their mobilities then are

$$-0.79 \times 10^{-5} \text{ cm.}^2 \text{ sec.}^{-1} \text{ v}^{-1}$$

and

$$-1.63 \times 10^{-5} \text{ cm.}^2 \text{ sec.}^{-1} \text{ v}^{-1},$$

calculated from the descending boundary.

The mobility measurements in relation to pH at I 0.15 are given in Fig. 3. The difference in mobility is maintained over the pH range 4.5–8.6, and the native and monomer proteins have markedly different isoelectric points of pH 8.1 and 5.6 respectively.

Comparison of the mobilities of the two components formed from the monomer under acid conditions (Figs. 2b and 2c) with the pH-mobility curves of Fig. 3 shows that, at both pH 4.3 and pH 8.6, one lies near the pH-mobility curve of the native protein and the other near the pH-mobility curve of the monomer.

The isoelectric points of the two proteins as determined by mobility measurements, at different

Fig. 3. pH-mobility curves for the macroglobulin GE from moving-boundary electrophoresis. $u = 10^5 \times$ mobility ($\text{cm.}^2 \text{ sec.}^{-1} \text{ v}^{-1}$) at 0° , I 0.15. ●, Native protein; ○, monomer. Values of mobility calculated from both ascending and descending limbs are shown when sufficiently different.

ionic strengths, show that the isoelectric point of the monomer is very dependent on the ionic strength and its value extrapolates from pH 5.6 at I 0.15 to pH 7.7 at zero ionic strength. The results that could be obtained for the native protein were limited by its solubility at I less than 0.1 and the difficulty of making accurate measurements at I greater than 0.15 where the conductivity is high and the slope of the pH-mobility curve very slight. In addition to a value of pH 8.1 at I 0.15, a value of pH 7.9 at I 0.1 was obtained. This makes extrapolation of the isoelectric point uncertain but it would appear to be less affected by ionic strength than the monomer and to give an isoionic point fairly close to that of the monomer.

Fig. 4. Zone electrophoresis of macroglobulin GE as monomer protein in urea-formate starch gel. S, Starting position. The arrow indicates the direction of migration.

The results of urea-formate starch-gel electrophoresis of the monomer are shown in Fig. 4: this shows two main components and small traces of other components.

The H^+ ion-dissociation curves obtained by potentiometric titration of the native and monomer proteins are shown in Fig. 5. For comparative purposes the results have been adjusted to zero H^+ ions bound or lost at pH 6.0 and are expressed as m-equiv./g. of protein. The curves for the two proteins are approximately the same over the pH range 4.5–11. However, a definite drift in the pH measurements was detectable at pH less than 3.2 for the native protein and at pH less than 4.5 for the monomer, indicating that reactions other than ionic ones were taking place.

The H^+ ion-dissociation curves for the tyrosyl residues in the two proteins are shown in Fig. 6. The curve for the native protein has a smaller slope than that of the monomer and shows an abrupt transition at pH 12.

Ultracentrifuge-sedimentation diagrams of the native and monomer proteins are shown in Fig. 7(a). These show that the native protein contains, in addition to the major 18s component and small amounts of a 29s component, a significant amount of a component sedimenting at about 22s appearing as a shoulder on the 18s component. In this respect it resembles the crystalline macroglobulin described by Kratochvil & Deutsch (1956). The monomer shows only one sedimentation boundary at 6.5s. Fig. 7(b) shows the diagrams of the two proteins after titrating through to pH 2 and then dialysing against the phosphate buffer for the ultracentrifuge examinations. This shows that, although some aggregated material is formed, the

Fig. 5. Hydrogen ion-dissociation curves of macroglobulin GE at I 0.15 and 20°. —, N, Native protein; ----, M, monomer. h , H^+ ions bound or lost (m-equiv./g. of protein). The arrows indicate the onset of drifting pH values. The results shown are for four experiments on the native protein and two on the monomer.

Fig. 6. Spectrophotometric titration curves of the tyrosyl residues in the macroglobulin GE at I 0.15. ●, Native protein; ○, monomer. $E_{295}^{1\text{cm.}}$, Extinction at 295 $m\mu$ for a protein concentration giving $\Delta n = 0.0002$.

Fig. 7. Ultracentrifuge diagrams of macroglobulin GE, examined in phosphate buffer, pH 7.4 and I 0.2. The sedimentation direction is from right to left.

	(i) Native	(ii) Monomer
Concn. of protein (g./dl):		
(a) Untreated	0.8	1.1
(b) After titrating to pH 2	0.5	1.1
Time (min.):		
(a) Untreated	12	64
(b) After titrating to pH 2	12	16

major part of the native protein is still in the form of an 18s component, which, however, shows a broader peak and has therefore become less homogeneous. The monomer has also formed some aggregated material but otherwise has broken down to a component sedimenting at about 3s.

The results of the amino acid analyses are given in Table 1. They are expressed as the numbers of residues per molecule of sub-unit or monomer of assumed molecular weight 160 000. They show good

Table 1. Amino acid analyses of the macroglobulin GE

Details are given in the text. Results are expressed as the numbers of residues/molecule of native sub-unit or of monomer of mol.wt. 160 000.

Amino acid	Native sub-unit	Monomer
(a) Analyses by ion-exchange chromatography		
Lysine	58	56
Histidine	19	18
Arginine	51	50
Aspartic acid	90	87
Threonine	110	106
Serine	127	125
Glutamic acid	125	122
Proline	88	82
Glycine	74	72
Alanine	72	70
Cystine (half)	27	19
Valine	90	87
Methionine	10	8
Isoleucine	42	42
Leucine	88	87
Tyrosine	42	40
Phenylalanine	42	43
S-Carboxymethylcysteine	—	8
(b) Analyses by ultraviolet spectrophotometry		
Tyrosine	41	40
Tryptophan	35	34

agreement in the composition of the two proteins, except for the sulphur-containing amino acids. The sub-unit of the native protein contains 27 half-cystine residues/molecule: the monomer contains eight residues/molecule less but also contains eight residues of *S*-carboxymethylcysteine/molecule: this indicates that about one-third of the disulphide bonds of the macroglobulin have been broken in forming the monomer. The monomer also contains slightly less methionine than does the native protein and shows traces of three additional ninhydrin-positive components. From the work of Gundlach, Stein & Moore (1959) and their position on the chromatogram, they are identified as *S*-carboxymethylhomocysteine, homoserine and homoserine lactone, formed by the breakdown of the methionine sulphonium salt formed in the production of the monomer, as a result of the acid hydrolysis.

DISCUSSION

The amino acid analyses and physico-chemical measurements made in the present work give some information on the changes taking place on the production of the monomer in terms of structural sub-units in the macroglobulin. The five macroglobulins examined here and those studied by Leder (1962) all yield a monomer containing only one electrophoretic component and this has a greater anionic mobility than the native protein. In contrast with this, two of the macroglobulins

described by Deutsch & Morton (1958) yielded monomers containing two electrophoretic components; it is not clear whether this is due to individual differences among macroglobulins or to some unknown variation in their preparation.

The experiments with moving-boundary electrophoresis and the urea-formate starch-gel electrophoresis on the monomer obtained from the macroglobulin GE show that under acid conditions it breaks down irreversibly to two main electrophoretic components. The ultracentrifugal examination under these conditions shows that it breaks down to smaller components. In these respects the macroglobulin substructure is in agreement with that described by Edelman & Poulik (1961) and Cohen (1963).

The amino acid analyses show that eight out of 27 disulphide bonds in each molecule of 6.5S sub-unit of the macroglobulin have been reduced on production of the monomer: these disulphide bonds may be both between and within the sub-units. For comparison it may be noted that the conditions used for the production of the monomer are as mild as those employed by Fleischman, Pain & Porter (1962) and that these workers found 5 out of 20 disulphide bonds/molecule to be broken in the reductive cleavage of γ -globulin; if a similar number of disulphide bonds/molecule form part of the internal structure of the sub-units of the present macroglobulin and are reduced on production of the monomer, then three disulphide bonds/molecule would be available for holding the sub-units together in the native protein: this would allow them to form a three-dimensional covalent structure in the native protein.

An increased anionic electrophoretic mobility of the monomer compared with that of the native protein appears to be a feature of many macroglobulins. This mobility difference could be due to differences in size and shape or to differences in charge on the protein occurring when the monomer is produced. The difference in charge can be estimated from the mobility results by using electrophoretic theory and from the H^+ ion-dissociation curves.

To calculate the number of elementary charges on the molecule it is necessary to have some simple geometrical model for the size and shape of the native and monomer protein. In the present discussion the native protein is represented by its major 18S component; this is taken to have a molecular weight of 1×10^6 and on dissociation to form six of the 6.5S monomer units with a molecular weight of 160000. This sub-unit weight has already been used in presenting the amino acid composition. The monomer is assumed to resemble the human γ -globulin molecule as described by Oncley, Scatchard & Brown (1947). This is a

prolate ellipsoid $235\text{\AA} \times 44\text{\AA}$ containing 0.2 g. of water of hydration/g. of protein. The native macroglobulin is here considered: (a) as a sphere of six times the volume of the monomer; (b) as an elongated rod of six sub-units in line. The true model probably is asymmetric, as Deutsch & Morton (1958) found a marked effect of concentration on the sedimentation coefficients of their macroglobulins. It is also necessary to make the additional assumptions that the bulk values for dielectric constant and viscosity apply in the region of the ionic atmosphere around the charged molecule in solution and that there is laminar flow of the solution past the migrating particle, and to simplify the calculations by considering the protein first as a sphere and then as a randomly orientated cylinder of the same volume. Values for Henry's correction and the correction for asymmetry were interpolated from the evaluations of these terms given by Abramson, Moyer & Gorin (1942). Gorin's correction, given in this reference, was made by using a value of $2\text{\AA}$ for the radius of the hydrated buffer ions, r_1 . The magnitude of the relaxation effect was obtained by calculating an approximate value for the ζ -potential and referring to the curves for the combined effect of Henry's correction and the relaxation effect for uni-univalent electrolytes given by Overbeek (1950). Correction for the relaxation effect appeared to be negligible for the proteins and mobilities encountered in the present work and was omitted. The electrophoretic equation used, therefore, had the form for the net number of elementary changes:

$$Z = u \frac{6\pi\eta r(1 + Kr + Kr_1)}{ef(Kr)(1 + Kr)f(Ka/b)} C$$

where C is a dimensional constant, a/b the axial ratio and the other symbols have their usual significance.

The pH-charge curves obtained in this way are shown in Fig. 8 where the charge refers to the sub-unit or monomer to facilitate comparisons; the actual values found for pH 8.6 are given in Table 2.

The pH-charge curves show that when the native protein is considered as a sphere the curves for the native and monomer protein intersect at approximately pH 4.7, above which there is an increase in the net negative charge on forming the monomer which becomes greater as alkaline pH values are approached. When the native protein is considered to be an elongated rod, the increase is approximately constant at a value of eight elementary charges over the pH range studied.

For comparison with the pH-charge curves, the H^+ ion-dissociation curves have been adjusted to zero H^+ ions bound or lost/molecule at the isoelectric points provided by the mobility results and

calculated as the number of H^+ ions for a sub-unit or monomer molecule and included in Fig. 8, and the values obtaining at pH 8.6 given in Table 2. The number of H^+ ions bound or lost/molecule is greater than the charge/molecule calculated from the mobility results, but the difference is not so great when the native protein is considered to be an elongated rod near its isoelectric point.

The H^+ ion-dissociation curves show that the increase in the number of H^+ ions lost/molecule on forming the monomer is approximately constant over the pH range in which the mobility was studied: in this respect they resemble the pH-charge curves obtained when an elongated-rod

Fig. 8. Comparison of the charge on the macroglobulin GE calculated from the electrophoretic mobility at 0° for the native protein and the monomer, with the H^+ ion-dissociation curves at 20° , in each case at I 0.15. Z , Charge expressed as net number of elementary charges; h , no. of H^+ ions bound or lost. Both Z and h are given for a sub-unit or molecule of mol.wt. 160 000. Δ , Charge on native protein (sphere); $\bullet$, charge on native protein (elongated rod); $\circ$, charge on monomer; ----, H^+ ion-dissociation. Curves adjusted to $h = 0$ at the appropriate isoelectric point.

model is assumed for the native protein: the actual value of 15 H^+ ions/molecule is greater than the calculated difference of eight charges/molecule.

Thus both the mobility results and the titration experiments agree in showing that there is an increase in the net negative charge on the sub-unit on forming the monomer.

This increase in the net negative charge could be the result of a gain in anionic groups, either introduced on formation of the monomer or released from a hidden position where they could not ionize in the native protein: it could also be the result of a loss of cationic groups or be due to a difference in the binding of small ions by the native and monomer protein.

The main chemical change on forming the monomer is the reduction of cystine to give eight amido-*S*-carboxymethylcysteine residues/molecule. As these are not likely to be deamidated under the conditions of the present study of electrophoretic mobility, they should not be responsible for any differences in charge.

A consideration of the surface and volume relationship of the models that have been assumed for the native and monomer protein and of the H^+ ion-dissociation curves should indicate the possibility of hidden anionic groups being exposed on forming the monomer. When a spherical model is assumed for the native protein, its volume is six times that of the monomer but its surface area is only two-and-a-half times that of the monomer. However, when a more asymmetric model is assumed for the native protein, the relative gain in surface area by the monomer will be correspondingly less.

The H^+ ion-dissociation curves of the tyrosyl residues show that they behave differently in the two proteins in a way that indicates a greater change in the electrostatic free energy of ionization in the native protein at pH less than 12. The behaviour of the tyrosyl residues themselves is not likely to affect the charge in the pH range over which the mobility has been studied, but it does

Table 2. Charge on the macroglobulin GE, calculated from electrophoretic-mobility and titration-curve results for pH 8.6 and I 0.15

Details are given in the text. The results are expressed as the net numbers of elementary charges/molecule of mol.wt. 1×10^6 for the native protein and of mol.wt. 160 000 for the sub-unit or monomer. The native protein has been considered as (a) a sphere, (b) an elongated rod.

Protein	$10^5 \times \text{Mobility}$ ($\text{cm}^2 \text{ sec}^{-1} \text{ V}^{-1}$)	Charge/molecule		Difference in charge/ sub-unit on forming the monomer	
		From titration	From mobility	From titration	From mobility
Monomer	-1.7	-18	-12	—	—
Native	-0.6	-20	{ (a) -8	{ 15	{ 10
			{ (b) -24		{ 8

suggest the possibility of other ionizing groups behaving differently in the native protein compared with the monomer. It was therefore hoped that the H^+ ion-dissociation curves obtained by potentiometric titration would indicate the existence and nature of any hidden anionic groups. The potentiometric titrations, however, are limited in the information they provide by the instability of the proteins which has been noted at acid pH values, but they do show from the good agreement of the curves for the native and monomer proteins that groups ionizing in the pH range 4.5–11 are not involved. This is a different conclusion from that of Deutsch & Morton (1958), whose H^+ ion-dissociation studies on three macroglobulins suggested that histidine groups were liberated on forming the monomer. The present results do not exclude the possibility that some acidic groups such as sialic acid (pK approx. 2) which are known to occur in macroglobulins may be affected by the dissociation of the native protein: they could behave like the tyrosyl residues; in this case charge differences should be decreased as less acid pH values are approached; alternatively some of these groups may be hidden so that they cannot ionize at all in the pH range over which the proteins are stable, and in this case the pH-charge curves would simply be displaced relative to each other along the charge ordinate.

An alternative to a gain in negative ionizing groups would be the loss of positive ionizing groups on a small peptide fragment liberated on forming the monomer. The H^+ ion-dissociation curves indicate that, if this is the case, they must be very basic groups. However, the good agreement of the amino acid analyses of the two proteins shows that no significant basic fragment can have been lost.

The binding of small ions can have an important effect on electrophoretic behaviour, particularly of proteins that are weakly buffered near their isoelectric point. A good example is the aldolase studied by Velick (1949); the isoelectric point of aldolase was changed by several pH units in phosphate buffer. If the native and monomer proteins behave differently with respect to the binding of small ions this could account for the difference in electrophoretic behaviour observed. The present results show that the isoelectric point of the monomer is more depressed by the concentration of electrolyte than is that of the native protein, and that both the titration and mobility results show an increased positive charge on the sub-unit on forming the monomer, and further that the isoionic point of the native protein is probably near to that of the monomer. This suggests there is greater anion-binding by the monomer than by the native protein and that most of the mobility difference observed is due to this anion-binding.

Comparison of the H^+ ion-dissociation curves with the pH-charge curves shows that the former are steeper than the latter. This, on the other hand, suggests cation-binding on the alkaline side of the isoelectric point. If the H^+ ion-dissociation results measure the net charge on the protein, then it cannot be adequately accounted for by present electrophoretic theory and anion-binding observed at the isoelectric point. A similar discrepancy to this is described by Velick (1949) in a study of anion-binding by aldolase by using dialysis-equilibrium methods.

SUMMARY

1. In five macroglobulin preparations from pathological human sera, the monomer produced by reduction with thiols followed by alkylation showed a single electrophoretic component of greater anodic mobility than that of the native protein.

2. One of these preparations was studied in detail through its electrophoretic behaviour, H^+ ion-dissociation curves and amino acid composition.

3. It was shown that: (a) eight disulphide bonds/molecule were broken for each sub-unit on forming the monomer; (b) the monomer breaks down irreversibly to two electrophoretic components under acid conditions; (c) the tyrosyl residues behave differently in the native protein and the monomer; (d) the difference in mobility is probably mostly due to anion-binding by the monomer, but the existence of hidden very acid groups in the native protein has not been excluded.

E. I. McD. thanks the Kellogg Foundation for an Agricultural Fellowship and the Agricultural Research Council of Great Britain for leave of absence from the Rowett Research Institute, Aberdeen, Scotland. This work was supported in part by a grant from the U.S. Public Health Service (C-1786).

REFERENCES

- Abramson, H. A., Moyer, L. S. & Gorin, M. H. (1942). *Electrophoresis of Proteins*, pp. 121, 132, 147. New York: Reinhold Publishing Corp.
- Beaven, G. H. & Holiday, E. R. (1952). *Advanc. Protein Chem.* **7**, 320.
- Bencze, W. L. & Schmid, K. (1957). *Analyt. Chem.* **29**, 1193.
- Cohen, S. (1963). *Nature, Lond.*, **197**, 253.
- Deutsch, H. F. & Greenwood, F. C. (1960). *Fed. Proc.* **19**, 344.
- Deutsch, H. F. & Morton, J. I. (1957). *Science*, **125**, 600.
- Deutsch, H. F. & Morton, J. I. (1958). *J. biol. Chem.* **231**, 1107.
- Edelman, G. M. & Poulik, M. D. (1961). *J. exp. Med.* **113**, 861.
- Fleischman, J. B., Pain, R. H. & Porter, R. R. (1962). *Arch. Biochem. Biophys.* Suppl. **1**, 174.

- Gundlach, H. G., Stein, W. H. & Moore, S. (1959). *J. biol. Chem.* **234**, 1754.
- Kratochvil, C. H. & Deutsch, H. F. (1956). *J. biol. Chem.* **222**, 31.
- Leder, P. (1962). *Nature, Lond.*, **193**, 1087.
- Oncley, J. L., Scatchard, G. & Brown, A. (1947). *J. phys. Chem.* **51**, 184.
- Overbeek, J. Th. G. (1950). *Advanc. Colloid Sci.* **3**, 97.
- Spackman, D. H., Stein, W. H. & Moore, S. (1958). *Analyt. Chem.* **30**, 1190.
- Tanford, C. (1955). In *Electrochemistry in Biology and Medicine*, p. 248. Ed. by Shedlovsky, T. New York: J. Wiley and Sons Inc.
- Velick, S. F. (1949). *J. phys. Chem.* **53**, 135.

Biochem. J. (1964) **90**, 170

The Purification and Properties of Adenosine Triphosphate-Lombricine Phosphotransferase

By T. J. GAFFNEY*, H. ROSENBERG AND A. H. ENNOR

Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T., Australia

(Received 20 May 1963)

The properties of the enzyme ATP-lombricine phosphotransferase (lombricine kinase), which catalyses the reaction:

have not yet been described in any detail. Pant (1959) studied the above reaction, in the forward direction, with crude homogenates and extracts of acetone-dried powders prepared from whole earthworms (*Lumbricus terrestris* L.). At pH 9.0 these preparations showed a low activity which was slightly increased by the addition of Mg^{2+} ions. In addition to lombricine (D-2-amino-2-carboxyethyl 2-guanidinoethyl hydrogen phosphate) two other guanidino compounds, taurocyamine (2-guanidinoethanesulphonic acid) and 2-guanidinoethyl phosphate, acted as phosphoryl group acceptors in the reaction. Lombricine-kinase activity was lost on prolonged dialysis, and the addition of concentrated diffusate to the enzyme system did not restore activity. Pant (1959) suggested that this loss of enzymic activity was due to the removal of a labile cofactor by dialysis.

In a preliminary note, Rosenberg, Rossiter, Gaffney & Ennor (1960) described the partial purification of lombricine kinase by ammonium sulphate fractionation of aqueous extracts of the muscle of the earthworm *Megascolides cameroni*. This enzyme preparation catalysed the phosphorylation of synthetic L-lombricine, as well as of D-lombricine and taurocyamine, and was activated by Mn^{2+} , Co^{2+} or Mg^{2+} ions. The isolation in high

yields of crystalline D-lombricine and N-phosphoryl-D-lombricine from earthworms (Ennor, Rosenberg, Rossiter, Beatty & Gaffney, 1960; Ennor & Rosenberg, 1962) has provided an opportunity for a more detailed study of the properties of lombricine kinase. The present paper reports a method for the preparation of highly purified lombricine kinase and some properties of the enzyme from earthworm muscle.

EXPERIMENTAL

Materials and Methods

All reagents were of analytical grade and glass-distilled water was used for the preparation of all solutions.

Magnesium chloride. Solutions of MgCl_2 were made from a stock solution (approx. 0.1M) of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and standardized by titration of H^+ ions displaced from Dowex 50 (H^+ form) by a known volume of the solution.

Ammonium sulphate solution. A solution of $(\text{NH}_4)_2\text{SO}_4$ saturated at 2° was prepared and adjusted to pH 7.4 (measured on a 1:20 dilution by the glass electrode) by the addition of N-NaOH.

Preparation of earthworm muscle. The body wall from large live earthworms (*Megascolides cameroni*), obtained in the Australian Capital Territory and southern New South Wales, was dissected free from viscera and rinsed in cold water. The muscle tissue was frozen in liquid air and stored at -18° until required.

Guanidino compounds. D-Lombricine was isolated from natural sources as described by Ennor *et al.* (1960), dried in air and stored at room temperature. The material was pure as judged by elementary analysis; after chromatography in two dimensions in ethanol-formic acid (98%)-water (7:1:2, by vol.) and in phenol-water (4:1, w/v), it gave a single ninhydrin-positive spot. Solutions of lombricine (0.05M) were adjusted to pH 8.6 by the addition of 0.1N-KOH and stored at -10°.

D-Phospholombricine was prepared enzymically and purified by a modification of the method described by

* Present address: Pathology Department, The North Brisbane Hospitals' Board, Brisbane, Queensland, Australia.