geometrical optics, cf. Longsworth, 1947; Gosting & Morris, 1949) show a pronounced tendency to decrease as the fringe number j increases, indicating a departure from the ideal form, defined by the probability integral. Calculations were made from the fringe $j=1$, this would approximate to D_A (diffusion constant determined by height-area method).

The partial specific volume of the substance was 0·691.

The molecular weight was calculated from the usual equation (Svedberg & Pedersen, 1940), using the sedimentation and diffusion constant values obtained in a solution containing 0·5 g./100 ml. mucoid and the value for the partial specific volume, already determined, giving a value of 87 000. The frictional ratio f/f_0 calculated from the same data was 1·51. This corresponds to an axial ratio of 10 or may indicate some degree of hydration.

DISCUSSION

The results appear to indicate that electrophoretically the mucoid is essentially homogeneous, and, the nitrogen of the mucoid is therefore an integral part of the molecule, and not attributable to protein contaminants.

The appearance of the ultracentrifugal boundary indicates a fairly homogeneous material, taking size and shape into consideration. Some deviation from an ideal relationship is shown in the diffusion measurements, which is not large, but suggests the presence of more than one molecular species. The molecular size and degree of asymmetry are considerably smaller than those reported by Tamm & Horsfall (1952) for a urinary mucoid of similar biological properties.

The conclusion is that the mucoid is slightly polydisperse, but the precise range and distribution of molecular size have not been calculated, since insufficient data were available.

SUMMARY

1. The mucoid has been shown to be essentially homogeneous electrophoretically.

2. The molecular weight of the mucoid calculated from sedimentation and diffusion data is 87 000 with a frictional ratio of 1·51.

3. The mucoid is probably slightly polydisperse and not greatly asymmetrical in molecular shape.

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The Proportion of Phosphatase Activity Demonstrable in Brain by Histological Techniques

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Characteristic differences have been found in brain tissue in the histological sites of hydrolysis of adenosine triphosphate (ATP), adenosine 5-monophosphate (A5MP), aneurin pyrophosphate (APP), and of glycerophosphate (GP) by the acid and alkaline phosphatases (Naidoo & Pratt, 1951, 1952). Evidence that separate enzymes were represented was provided by differences in the conditions needed and by the preferential suppression of each reaction by suitable inhibitors. The validity of these results would be more readily assessable if the proportion of the potential enzyme activity of the tissue they represented was known. This is the purpose of the present work.

Histological study of the sites of phosphatase activity is made possible by the precipitation of inorganic phosphate at the site of its formation. The hydrolysis of a suitable substrate in the presence of

either lead or calcium ions, according to the pH of the medium, produces a precipitate which is converted to a coloured compound in order to make possible a microscopical examination. Earlier workers have applied these methods most frequently to tissue fixed by treatment with acetone or ethanol. Danielli (1946, 1950) has drawn attention to the danger of incorrect localizations in such procedures, due to diffusion of enzymes or of reaction products and to the possibility of enzyme loss which may not be uniform; other workers have confirmed that such losses may be considerable (Doyle, 1948; Rabinovitch, Junquiera & Fajer, 1949). The acid and alkaline phosphatase activities remaining in tissues at various stages of acetone or ethanol fixation and paraffin-wax embedding were determined by Stafford & Atkinson (1948) who found that the alkaline phosphatase activity of prepared sections had fallen to 20–30 % and that of acid phosphatase to about 5 % of the original value. Comparable values were obtained by Berenbom, Yokoyama & Stowell (1952) in liver after procedures involving acetone treatment.

A large proportion of the activity of five brain phosphatases was represented in histological procedures using freeze-drying instead of chemical fixation (Pratt, 1952). Assessment of activity by direct examination of the stained sections was rejected because of the irregular distribution of the deposit. Instead, the inorganic phosphate precipitated in the sections was estimated directly, making use of the Martin & Doty (1949) phosphomolybdic acid extraction by an organic solvent; the inorganic phosphate precipitated in a single microtome section after incubation in a histological medium could be estimated thus. Standards for comparison were provided by estimation of the activity of saline suspensions of fresh tissue by ordinary biochemical methods. Doyle (1950) has described a method for the estimation of lead sulphide deposits in sections after alkaline phosphatase staining. This method should be applicable after other phosphatase procedures, but direct estimation of the phosphate was preferred and was convenient also for the estimation of fresh tissue activity.

Four separate levels of enzyme activity must be considered: (1) the activity of a fresh tissue suspension under optimal conditions; (2) the activity of prepared tissue sections in the present histological procedure; (3) the activity of a fresh tissue suspension under the conditions of the histological method; (4) the activity of tissue subjected to chemical fixatives during its histological preparation, including paraffin removal. Work to which reference was made above (Doyle, 1948; Stafford & Atkinson, 1948; Rabinovitch *et al.* 1949; Berenbom *et al.* 1952) has shown that level (4) was considerably below the activity of fresh tissue. In the present work comparisons have been made between levels (2) and (3) in order to detect enzyme loss in the histological preparation of the tissue or in the incubation procedure which might reduce the proportion of the enzyme demonstrated. This was believed to be more important than comparison with the level (1) which involved in addition such factors as the effects of the cations which were necessarily present to precipitate inorganic phosphate.

EXPERIMENTAL

Wistar rats, 40–100 days old, were used. The histological material was prepared as described by Naidoo & Pratt (1951). The principal steps were the removal of the brain under ether anaesthesia, rapid freezing of 2 mm. coronal slices at $-40°$, and drying of the tissue under vacuum below $-30°$. The dried material was embedded in melted paraffin wax at 52–56° for 20 min., the pressure being allowed to rise sufficiently to avoid boiling of the wax. Serial sections were cut 15 μ. thick, mounted dry on albuminized glass slides and de-waxed by treatment with three changes of light petroleum of which the last, *iso*pentane, was allowed to evaporate before immersing sections in the incubation media.

Sections were weighed on a quartz-fibre balance before the removal of the wax in order to check their thickness and uniformity. The area of the tissue was measured on the cut surface of the block. Tracings were made on a Vickers projection microscope and the areas measured by a planimeter. Sections were cut by a Reichert rotary microtome. In order to obtain uniformity, it was essential to cut serial sections, turning the handle at a slow steady rate without a break, rejecting the first section, and any which appeared to be abnormal. The mean thickness in a series of thirty consecutive sections was 14·8 μ. (S.D. of one section, $\pm 0{\cdot}40$ μ.). The weight of fresh tissue was usually 0·5–1·5 mg./section. It was necessary to use three medulla sections to provide sufficient tissue for one determination.

Glass-distilled water was used throughout. The solutions of substrates were prepared as in earlier work, except that a more concentrated (0·005 M) ATP stock solution proved more convenient and acetone precipitation of the APP was advantageous in order to reduce the inorganic PO_4 content of the solution. The silicotungstic acid was freed from inorganic PO_4 by ether extraction (cf. North, 1939). Suitable blanks were included for each procedure.

Enzyme activity in sections. Slides (three to five), each bearing a pair of adjacent sections, were incubated at 37·5° with gentle agitation in 30 ml. of medium contained in a Coplin staining jar. One slide was removed after a minute (the others at various intervals) and rinsed in water (10 sec.). Repetition of this rinse or the use of dilute lead acetate solution in place of water yielded similar inorganic PO_4 values. The sections were allowed to drain but not to dry. One section from each pair was reserved for visual examination. The other was scraped from the slide by means of a safety razor blade held at an angle of about 60° to the glass. The section, rolled upon the cutting edge of the blade, was transferred by means of a finely drawn glass rod to a glass-stoppered test-tube containing extraction fluid, and also the

Table 1. *Composition of media for the incubation of sections*

Substrate	Concn. (M)	pH	Buffer	Buffer concn. (M)	Lead acetate (M)	$CaCl_2$ (M)	$MgCl_2$ (M)
GP	0·0100	5·3	Acetate	0·050	0·002	—	0·0033
GP	0·0200	9·1	Veronal	0·025	—	0·10	—
A5MP	0·0005	6·5	Succinate	0·050	0·001	—	—
ATP	0·0005	6·5	Succinate	0·050	0·001	—	—
APP	0·0005	6·9	Maleate	0·050	0·001	0·05	—
PP	0·0005	6·9	Glycylglycine	0·10	0·001	—	0·02

protein precipitate used in the Martin & Doty method of inorganic PO_4 estimation. Each tube contained N-H_2SO_4 (2·0 ml.) and 0·02 M-silicotungstic acid (0·5 ml.). The tube was shaken gently to ensure that the section unfolded. The benzene:*iso*butanol (1:1, v/v) mixture (3·0 ml.) and ammonium molybdate (5% w/v; 0·5 ml.) were added. The tube was stoppered and shaken vigorously for 15 sec. Separation of the layers was completed by centrifugation. A portion of the organic solvent layer (2·0 ml.) was added to 2% H_2SO_4 (v/v) in absolute ethanol (2·0 ml.), followed by $SnCl_2$ in N-H_2SO_4 (0·1 ml.). The latter solution was prepared freshly by the 100-fold dilution of the 40% solution of $SnCl_2.2H_2O$ (40 g. in 100 ml. 11 N-HCl). Absorptions at 700 mμ. were measured in a Hilger Uvispek spectrophotometer, using as a blank the extract from the section removed at the start. The results were expressed as μmoles inorganic PO_4 liberated/g. fresh tissue/hr. The proportion of the substrate hydrolysed was always less than 10%.

Alternate sections were stained for visual examination in order to ensure that comparable anatomical structures were present in the series used in any one determination. If the deposit was $Pb_3(PO_4)_2$, the section was washed in four changes of water, treated with dilute ammonium sulphide solution, dehydrated and mounted. If the deposit consisted of calcium phosphate, the section was treated directly with $Co(NO_3)_2$ solution, and then washed, treated with ammonium sulphide solution and mounted.

The conditions used for the incubation of sections are detailed in Table 1. They were those considered to be preferable in previous work on the basis of visual comparisons of substrate specific staining of sections. There appeared to be no activation by the addition of Mg or Ca to the ATP medium. The use of low Pb concentrations reduced the intensity of the diffuse background staining both in experimental and in control sections incubated in the absence of substrate, probably owing to a reduction of the proportion of the initial inorganic PO_4 content (14–16 μmoles/g.) retained in the tissue at the start of incubation. All the media were found to be saturated with respect to inorganic PO_4.

Enzyme activity in fresh tissue. The whole brain (removed as for histological work) was weighed and ground rapidly in 0·89% NaCl at 0°, using a Pyrex glass tube and pestle. The motor was run at 2000 rev./min. for 30 sec. with a pestle diameter of 1·4 cm. The degree of grinding was checked histologically in order to ensure that it was sufficient to disrupt cell bodies. The preparations were diluted with further saline to contain 50 mg./ml. of fresh tissue and stored when necessary at −78°. Incubations were carried out at 37·5° with agitation. The test solution volume was usually 9 ml. so that four samples of 2 ml. could be withdrawn. Proportions of the constituents corresponding to those used in the histological methods were mixed, the solution centrifuged to remove any inorganic PO_4 precipitate and made up to the final volume less that of the tissue suspension. The latter was added as soon as temperature equilibrium was reached. The first sample was withdrawn immediately and 0·02 M-silicotungstic acid (0·5 ml.) added, followed by ammonium molybdate (5% in 4-N H_2SO_4; 0·5 ml.). The subsequent procedure was the same as that used for the estimation of inorganic PO_4 in microtome sections. Since a precipitate of lead or calcium phosphates formed as the reaction proceeded, it was preferable to pipette the other samples into separate tubes soon after the start. The proportion of the substrate hydrolysed was always less than 5%.

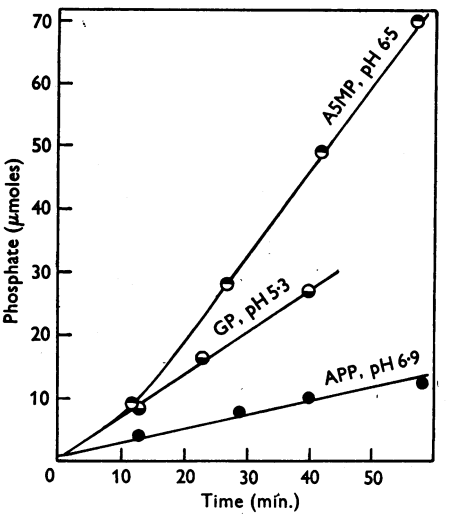

Fig. 1. Rate of accumulation of inorganic phosphate in microtome sections during incubation with adenosine-5-monophosphate, glycerophosphate and aneurin pyrophosphate. Coronal sections at the level of the posterior cerebrum and midbrain. Incubation conditions as in Table 1 at 37·5°.

RESULTS

Enzyme activity in sections. Fig. 1 shows typical determinations of inorganic phosphate in adjacent sections incubated for various times in the same medium. The inorganic phosphate content of the sections increased linearly with time over practically all the range studied. This increase in inorganic phosphate content was accompanied always by a corresponding increase in the intensity of specific staining of the adjacent section incubated on the same slide.

Table 2. *Phosphatase activity in fresh tissue suspensions and histological sections*

(Results are expressed in μmoles P/g. fresh tissue/hr. The levels at which coronal sections were made are indicated as follows: (I) Anterior cerebrum; (II) middle cerebrum; (III) posterior cerebrum and midbrain; (IV) cerebellum and pons; (V) medulla.)

Values from individual experiments

Substrate	pH	Exp. group	Fresh whole brain	Coronal sections
A5MP	6·5	A	106	99 (III)
			94	94 (III)
		B	140	65 (I), 112 (II), 94 (III)
			154	—
		C	82	60 (II), 81 (III), 106 (IV), 105 (V)
			110	—
GP	5·3	A	21	50 (I), 46 (II), 60 (III)
			26	—
			16	—
		B	24	37 (II), 36 (III), 40 (IV), 58 (V)
			31	39 (III), 43 (V)
			28	—
			24	—
GP	9·1	A	26	24 (I), 15 (II), 25 (III)
			22	—
			21	—
		B	26	20 (II), 20 (III), 28 (IV), 18 (V)
			34	46 (III)
APP	6·9	A	11·5	14 (II), 12 (III)
			14·5	18 (I), 11 (II), 13 (III)
		B	8·6	25 (II), 16 (III)
			15·6	23 (II)
			13·0	—
		C	15·1	11 (II), 22 (IV), 15 (V)
			13·4	—
			10·2	—
ATP	6·5	A	41	32 (I), 82 (III)
			71	—
		B	65	91 (I), 37 (II), 47 (III), 40 (IV), 53 (V)
			68	—
			45	—
PP	6·9	—	122	9 (II), 1 (III), 3 (IV)
			130	—

Summary and comparison with the results of other workers

Substrate	pH	Fresh tissue suspension (mean ± S.D.)	Histological sections (mean ± S.D.)	Values from literature*
A5MP	6·5	114·3 ± 25·2	90·7 ± 17·3	58† (Reis, 1937)
GP	5·3	24·3 ± 4·5	45·4 ± 8·5	7·1 (Gordon, 1950)
GP	9·1	25·8 ± 4·7	24·5 ± 9·0	18 (Gordon, 1950)
APP	6·9	12·7 ± 2·3	16·4 ± 4·6	0·7–1·4 (Westenbrink, Steyn Parvé & Goudsmit, 1943)
ATP	6·5	58·0 ± 12·5	54·6 ± 21·2	900 (DuBois & Potter, 1943) 1040‡ (Gore, 1951)
PP	6·9	126	4·3	250 (Gordon, 1950) 1150‡ (Gore, 1951)

* Rat brain except for values marked ‡ which are from guinea pig.
† Inosine-5-phosphoric acid.

Comparison of sections and fresh tissue. The phosphatase activities of frozen, dried, paraffin sections and fresh tissue suspensions are compared in Table 2. Some comparisons were made by dividing the brain into left and right halves, freeze-drying one half and preparing a fresh saline suspension from the other. Otherwise, litter mates or rats of similar ages were included in any one experimental group. Sections were cut coronally at five different anatomical levels. A series of adjacent sections was used in making each determination. The absence of large differences in activities for different anatomical levels was consistent with the presence of stained structures in all sections although the staining was localized. The enzyme activity of the prepared sections was slightly below or of a similar order to that found in the procedure using fresh tissue in those comparisons in which the substrate was A5MP (pH, 6·5), ATP (pH, 6·5), or GP (pH, 9·1). Table 2 shows a considerable overlap in the ranges of the two series of determinations for these substrates. The differences between the means were not significant in relation to the spread of the results. On the other hand, the activity of sections appeared to be rather higher than that obtained with fresh tissue when the substrate was APP (pH, 6·9) or GP (pH, 5·3). The ranges partly overlapped when APP was used. There was no overlap and a significant difference ($P < 0·01$) was present when the substrate was GP at pH 5·3.

The hydrolysis of inorganic pyrophosphate (PP) showed a different kind of behaviour. Repeated attempts to detect this activity by a histological method were unsuccessful and the rate of inorganic phosphate deposition in incubated sections was little greater than in the absence of substrate. Nevertheless, fresh tissue suspensions showed high PPase activity under similar conditions.

DISCUSSION

It might be concluded from the work to which reference was made in the introduction that histological methods for the study of phosphatase distribution can demonstrate only a small part of the enzyme activity originally possessed by the tissue. However, it has been found in the present work that the activities in histological sections are, with one exception, close to those shown by fresh tissue suspensions under similar conditions. Probably, a major cause of enzyme loss during tissue preparation has been removed by avoiding chemical fixation, whereas infiltration of the tissue with melted paraffin wax and its removal by light petroleum have only a slight effect on these enzymes. This conclusion might seem to contradict the results of earlier workers (Danielli, 1946; Stafford & Atkinson, 1948; Rabinovitch *et al.* 1949; Berenbom *et al.* 1952) who found considerable phosphatase losses during paraffin infiltration and subsequent treatment. These losses are probably explained by the use of acetone or ethanol to displace the xylene or benzene used to dissolve the paraffin, and are avoided in the procedure of Naidoo & Pratt (1951) since rehydration of the tissue takes place in the incubation medium. Doyle (1948) used carbon disulphide for paraffin removal with only moderate losses (alkaline phosphatase 55 % and acid phosphatase 60 % of the values before embedding), although he used more severe conditions of paraffin infiltration (1 hr. at 60°) than in the present work.

Sensitivity of PPase to the paraffin treatment may explain the absence of appreciable activity in the histological procedure, but attention is drawn to the ease with which PPase can be separated from the tissue in soluble form. The supernatant of a saline suspension of brain after centrifugation contains 80–90 % of the activity, whereas the major part of brain ATPase remains in the tissue residue under similar conditions (Gore, 1951).

The use of the activity of a fresh tissue suspension as a standard with which to compare the values obtained in the histological methods was an arbitrary choice so that undue emphasis should not be placed on the higher values found in the latter method when APP is used as the substrate. The basis of comparison is extended by consideration of some previously reported values for the activities of these enzymes in brain, which are given in the final columns of Table 2. Much higher figures have been reported by previous workers for brain ATPase but not for the other enzymes. The present work suggests that brain ATPase activity is not greatly reduced by freezing, drying and paraffin treatment in the histological procedure. However, there are other special factors which apply equally to histological sections and fresh tissue suspensions. Lead was present in the histological incubation medium and it was used also in fresh tissue suspensions. Solubility considerations restrict the choice of buffer and pH and limit the substrate concentration. Lower activity can be expected under these conditions (ATP, 0·0005 M; no Mg; pH 6·5) since minimum values for maximal activity are ATP, 0·0025 M, Mg 0·0077 M and pH 7·4 (Gore, 1951). It is believed that further investigation of the effects of lead and magnesium will explain the lower ATPase values found in the present work. However, the possibility exists also that the ATPase activity of brain is a complex system and not all the enzymes are represented in the histological results.

The histological methods for 5-nucleotidase, APPase and the phosphatases hydrolysing GP all yield values which are higher than those previously reported by workers using purely biochemical methods. Although inorganic phosphate in solution

in the assay medium may reduce enzyme activity, it is not considered likely that the precipitation of this inorganic phosphate by lead or calcium in the present technique can produce a sufficient effect to account for the increased rates. The largest difference, that for APPase, may be explained by the low substrate concentration, probably much below the optimum, which Westenbrink, Steyn Parvé & Goudsmit (1943) used on account of their analytical method. With GP, previous workers have used acetone in the preparation of enzyme extracts from brain. Such treatment is likely to reduce activity and has been avoided in the present method. Brain acid phosphatase was not found by Giri & Datta (1936) to be activated by magnesium, whereas magnesium addition to the incubation medium has been found advantageous in the present work. For 5-nucleotidase, magnesium addition has not been found desirable, although it was used by Reis (1937). The existence of liver acid phosphatase in a form inactive to GP (Berthet & de Duve, 1951; Berthet, Berthet, Appelmans & de Duve, 1951) does not appear to be important to the present work since all the procedures under consideration seem likely to activate the enzyme. Probably, the relatively higher activities found in some of the histological procedures are due to a failure to obtain the full enzyme activities in the fresh tissue estimations. The enzyme activity represented in five of the histological procedures is of a similar order to or above that estimated by purely biochemical methods so that suitably chosen histological methods can justifiably take a place alongside those more commonly used for enzyme study.

SUMMARY

1. The phosphatase activity in microtome sections has been assessed by the estimation of phosphate deposited in these sections in the presence of lead or calcium and compared with that in fresh tissue suspensions under the conditions of the histological method.

2. The activities of acid and alkaline phosphatases, 5-nucleotidase, adenosine triphosphatase and aneurin pyrophosphatase are of a similar order in frozen, dried, paraffin-infiltrated brain tissue and in fresh tissue. These values are often much greater than previously reported values, probably largely because the tissue was prepared without using acetone or ethanol.

3. It is concluded that the staining obtainable in these methods represents a high proportion of the potential activity of the tissue under the conditions used.

4. Pyrophosphatase activity was negligible in the histological preparations, possibly owing to a high solubility of this enzyme in saline media.

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