SOME FACTORS AFFECTING RAT BRAIN PHOSPHATASE ACTIVITY IN FRESH TISSUE SUSPENSIONS AND IN HISTOCHEMICAL METHODS

by

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The histological sites of action of acid and alkaline phosphatase, 5'-nucleotidase, adenosine triphosphatase, or thiamine pyrophosphatase, have been studied in sections prepared from freeze-dried paraffin, infiltrated brain tissue[1,2]. The enzyme has been caused to act in a medium containing a lead or a calcium salt in order to precipitate the inorganic phosphate formed, and to make possible staining of the sites of enzyme action. Previous estimations[3] have shown that the activity of any of the five enzymes studied is not significantly lower in the histochemical localisation method than the activity in a fresh tissue suspension in corresponding conditions. These results suggest that, in so far as the activity of a fresh tissue suspension provides a standard, the activities of these enzymes are not seriously reduced by the preparation of the histochemical material which includes freeze-drying, infiltration with paraffin wax, section cutting, and removal of the wax.

However, the presence of lead or calcium in the histochemical medium may lead to the limitation, through solubility considerations, of the substrate concentration, or of the choice of buffer or pH. The activity found in these special conditions, even in fresh tissue suspensions, is sometimes much less than that reported by other workers using more favourable conditions, and some of the enzyme properties appear to differ. Thus, the activity shown in the histochemical staining depends both on the treatment to which the tissue is subjected and on the special conditions in which the enzyme acts.

The present work describes some of the effects of lead, of the tissue preparation procedures, and of calcium or magnesium on phosphatase activity in fresh tissue suspensions and in the histochemical methods.

EXPERIMENTAL

Tissue preparation. Normal WISTAR rats were provided with water and a commercial rat diet (No. 41; Associated Flour Millers, London) without restriction. Brains were removed under ether anaesthesia at 80–100 days of age. For histological work, 2 mm coronal slices comprising the middle third of the cerebrum were frozen rapidly in *iso*pentane, cooled to between $-40°$ and $-60°$. The tissue was placed in a previously cooled pyrex glass tube 15 × 3 cm containing a 2 cm deep layer of paraffin wax and attached by a B 34 ground joint to a wide bore glass adapter leading via a rubber diaphragm-type isolation valve to the P_2O_5 trap of an Edwards 2S50 mechanical vacuum pump with a non-return valve. The tube containing the tissue was immersed in a solid CO_2: ethyl

References p. 389.

phenyl ether mixture, initially containing excess solid CO_2. The temperature was allowed to rise to $-30°$ (the m.p. of the ethyl phenyl ether) and maintained at this temperature by occasional additions of solid CO_2.

A measure of the rate of water evolution from the tissue could be obtained by closing the isolation valve and recording the rate of pressure change by a Pirani type gauge attached to the glass adapter. The final vacuum reached was usually less than 10^{-2} mm Hg and the rate of pressure rise less than 10^{-2} mm Hg/min at $-30°$. When the tissue was judged to be sufficiently dried by this test, the vacuum flask containing refrigerant was removed. After 30 min at room temperature followed by a further test for adequacy of drying, the isolation valve was closed and the pump stopped. The layer of paraffin wax on which the tissue was resting was melted cautiously by placing the tube in a water bath to a depth of 1 to 2 cm, after the admission of a little dry air to prevent boiling of the wax. When the wax was molten, the pressure was alternately lowered and raised a few times so that good infiltration of the tissue with wax could be secured in about 20 min at 52–56°. Sections cut 15 μ thick were mounted dry on lightly albuminised glass slides. Paraffin blocks containing tissue could be stored at 0° over anhydrous silica gel without deterioration for at least 4 weeks and probably longer. A period of 30–60 min in an oven at 38° assisted in flattening the sections without affecting enzyme activity appreciably. The sections were dewaxed by treatment with light petroleum (b.p. 100–120°; 1 min; b.p. 60–80°; 1 min), and *iso*pentane (1 min). The last solvent was allowed to evaporate from the sections before their immersion in the incubation medium. Thus, the use of acetone or of aqueous ethanol was avoided.

Enzyme activity. The methods used for measurement of enzyme activities in fresh tissue suspensions or in section staining procedures have been described previously[3]. Sections were incubated with gentle agitation at 37.5° in the appropriate medium for 10, 20 or 40 min. The compositions of the media were as given in Table I, except for the variations indicated. The inorganic phosphate, deposited in the tissue after incubation as the Pb or Ca salt, would normally be converted to Pb or Co sulphides for visual study. Instead, it was redissolved in dilute H_2SO_4 and estimated colorimetrically after extraction as phosphomolybdic acid by an organic solvent. This analytical procedure was used with minimum modification for the measurement of inorganic phosphate liberation by fresh tissue suspensions. The media corresponded to those used in the histological methods except that the Pb^{+2} was omitted. The effect of Pb^{+2} (or of Ca^{+2} or Mg^{+2}) was investigated by mixing the other constituents, including the tissue suspension, at 0° and transferring samples (2 ml) at 30 sec intervals to tubes which were previously placed in the thermostat bath and contained the addition where appropriate. The standard incubation time was 30 min with rapid agitation. All determinations were duplicated, and corrected where necessary for enzyme and substrate blanks.

TABLE I

NORMAL COMPOSITION OF HISTOCHEMICAL MEDIA

Substrate	*Buffer*	*pH*	*Substrate* M	*Buffer* M	*Lead acetate* M	$CaCl_2$ M	$MgCl_2$ M	*Apparent solubility of inorganic phosphate in medium* 10^6 M
Glycerophosphate	Veronal	9.1	0.0200	0.025	—	0.10	—	3.0
Glycerophosphate	Acetate	5.3	0.0100	0.050	0.002	—	0.0033	3.6
Adenosine 5′-phosphate	Succinate	6.5	0.0005	0.050	0.001	—	—	1.2
ATP	Succinate	6.5	0.0005	0.050	0.001	—	—	24
Thiamine pyrophosphate	Maleate	6.9	0.0005	0.050	0.001	0.05	—	1.4

The apparent solubility of inorganic phosphate in the histochemical media was determined as follows. A small excess of 0.1 *M* Na_2HPO_4 or NaH_2PO_4 was added to the medium (30 ml) which was shaken for 30 min at 37.5°. After centrifugation, the supernatant was filtered at the same temperature and 2 ml samples of the filtrate were taken for estimation of inorganic phosphate.

Materials

Thiamine pyrophosphate (Roche) was reprecipitated twice by solution in a little *N* HCl and addition of 5 vol of acetone. Na glycerophosphate (Hopkin and Williams) was reprecipitated twice by solution in a little water and addition of acetone. Traces of heavy metal ions were removed from adenosine 5′-phosphate (Lights) as follows, all operations being carried out at 0°. A sample (0.347 g) was dissolved in 4 *N* NH_4OH (2 ml), H_2S was passed and 0.1 *M* Na_2SO_4 (0.1 ml) added. Sufficient acetone (about ¾ vol) was added to produce a slight further precipitate. After centrifugation and filtration, 4 *N* HCl was added to bring the supernatant to pH 3.4 (bromophenol blue).

References p. 389.

After crystallization, the product was redissolved in the minimum of water and reprecipitated with acetone. The stock solution (0.01 *M*) was neutralised with *N* NaOH.

Inorganic phosphate and traces of heavy metals were removed from adenosine triphosphate (ATP) (Boots, dibarium salt) as follows, at 0°. A sample (1 g) was dissolved in 2 *N* HCl (15 ml) and water (20 ml) added. After the addition of *M* barium acetate (2 ml) sufficient 5 *N* NaOH was added to bring the pH to 4.7–4.9. The precipitate was separated by centrifugation, washed three times with a little water and redissolved in 2 *N* HCl (10 ml) and water 15 ml. After passing H_2S, a slight excess of 0.2 *M* Na_2SO_4 was added. After centrifugation and filtration, air was blown through the supernatant to remove excess H_2S, the solution neutralised with *N* NaOH and water added to give a 0.01 *M* solution on the basis of acid labile phosphate.

All reagents, even though of analytical grade, were redistilled, recrystallised, or reprecipitated as appropriate, and stock solutions of substrates stored at —78°. Previously, inconsistent or irreproducible results were often traced to some impurity in the reagents or substrates which inhibited activity. Consideration of earlier results[3] shows that minor impurities in the substrate may interfere with study of enzyme specificity. Thus, a single coronal section of rat cerebrum 15 μ thick might contain as little as $2 \cdot 10^{-8}$ mole of lead phosphate after an incubation period sufficient to make possible adequate staining of the sites of enzyme action. Yet, the medium in which the section was incubated might contain 1.5 to $30 \cdot 10^{-5}$ mole of substrate, so that the staining seen represented considerably less than 0.1 % hydrolysis of the available substrate.

RESULTS

Effect of Pb^{+2}. The enzyme activity appearing in the histological method depended on the Pb^{+2} concentration, the rate of inorganic phosphate deposition in the sections reaching a maximum at a Pb^{+2} concentration of about 10^{-3} *M* and falling again at higher concentrations (Fig. 1). In fresh tissue suspensions, each of the enzymes, except perhaps ATPase, was inhibited to some extent by Pb^{+2} at the usual concentrations in the histological medium (Table II). The inhibition was greater at higher Pb^{+2} concentrations. For instance, adenosine 5′-phosphatase was inhibited 55% by $2 \cdot 10^{-3}$ *M* Pb^{+2}. Further recrystallisation of the lead acetate did not reduce the inhibition. By contrast, alkaline phosphatase was activated by Ca^{+2} at the concentration present in the medium at pH 9.1 to precipitate the inorganic phosphate. At the lowest Pb^{+2} concentrations studied, the sections were stained less intensely and in an uneven manner, the deposit appearing black instead of brown. Irregular unstained patches were often apparent in regions which stained most intensely at the higher Pb^{+2} concentrations. This effect tended to be more pronounced in the absence of agitation and was not prevented by saturation of the medium with inorganic phosphate before adding the sections. Even when the sections were largely unstained, lead phosphate deposit was often apparent on the glass slide or in the medium.

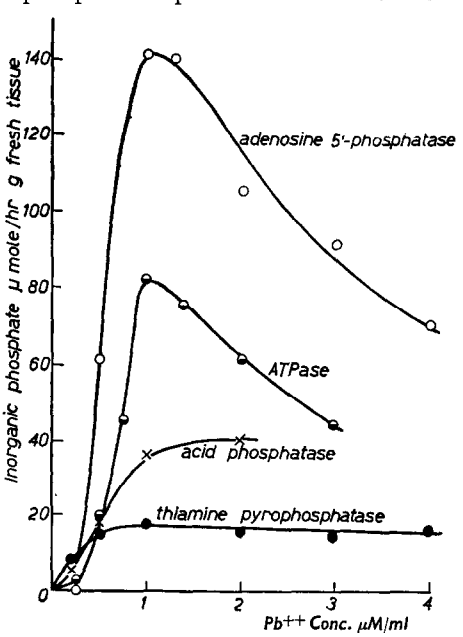

Fig. 1. Effect of variation of Pb^{+2} concentration on apparent enzyme activity in the histochemical method.

The apparent solubility of inorganic phosphate in each medium is shown in the final column of Table I. No signs of supersaturation were seen; precipitate formed immediately upon the addition of a small excess of inorganic phosphate.

References p. 389.

TABLE II

INHIBITION OF PHOSPHATASE ACTIVITY IN FRESH BRAIN SUSPENSIONS BY LEAD ACETATE (10^{-3} *M*)

Media as Table I. Mean ± S.D. followed by no. of experiments in parentheses

Substrate	*pH*	*% Inhibition by Pb*
Glycerophosphate	5.3	29.8 ± 3.0 (4)
Adenosine 5′-phosphate	6.5	35.1 ± 4.5 (7)
ATP	6.5	−12.5 + 25.8 (6)
Thiamine pyrophosphate	6.9	29.8 ± 13.1 (5)

Commercial samples of the substrates used, except adenosine 5′-phosphate, always contained sufficient inorganic phosphate to saturate the medium, but after reprecipitation they were often practically free of phosphate. However, the tissue itself still contained the inorganic phosphate which was present in the tissue at the time of its removal from the animal. In this respect the freeze-dried sections differed from chemically fixed tissue or freeze-dried tissue subsequently exposed to aqueous ethanol or similar solvents. This inorganic phosphate, amounting to 14–16 μ mole/g, accounted for part of the deposit found in the sections after incubation in the enzyme localisation medium.

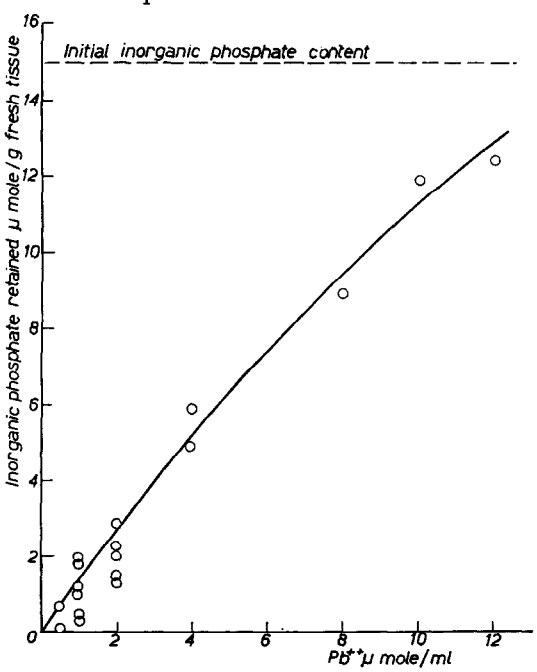

Fig. 2. Proportion of initial tissue content of inorganic phosphate retained when sections are added to the histochemical medium.

The size of the non-enzymic part of the deposit in the sections could be estimated by extrapolation of each series of estimations to zero time, or from controls without substrate (Fig. 2). The non-enzymic inorganic phosphate deposit in sections from the medium at pH 9.1 (which contained Ca^{+2} 10^{-1} *M*) approximated to that found in unincubated sections. On the other hand in the acid pH range media, the proportion of inorganic phosphate retained in the tissue at the start of incubation depended on the Pb^{+2} concentration. At the Pb^{+2} concentrations commonly used (about 10^{-3} *M*), most of the initial inorganic phosphate content of the tissue escaped into the bulk of the medium, probably owing to local exhaustion of the Pb^{+2}. Corresponding visual differences were seen in sections; the non-enzymic inorganic phosphate, when retained in the tissue, gave rise to a weak diffuse background staining, which was absent if the sections were rinsed in saline before incubation.

Effect of tissue preparation. A fresh saline suspension of rat brain was freeze-dried and treated successively with molten wax and light petroleum, so as to simulate as closely as possible the treatment to which tissue was subjected in the course of section preparation. After separation of the tissue residue by centrifugation, and regrinding in

References p. 389.

water, the acid phosphatase activity appeared to be about 25% lower. Therefore, the alternative possibility was investigated that the fresh tissue method might yield only part of the enzyme activity. Table III shows that losses of this enzyme and of thiamine pyrophosphatase and of ATP-ase did occur on keeping the suspension at 0°. High values were obtained by grinding the tissue directly in the assay medium instead of in saline. Therefore, the method of grinding directly in medium containing the substrate was used when possible and assays were made without delay. Tissue ground in sucrose medium as described by BERTHET AND DE DUVE[4] did not show much lower activity. Repeated freezing and thawing of the suspension[4] caused only a small increase in the acid phosphatase activity.

TABLE III

EFFECT OF SUSPENSION MEDIUM AND OF STORAGE ON ENZYME ACTIVITY

Media: as Table I, except ATP medium contains $MgCl_2$ ($7.7 \cdot 10^{-3}$ *M*) but no lead acetate.
μmole inorganic phosphate/g h

Tissue ground in	*Treatment*	*Glycerophosphate pH 5.3*	*Adenosine 5'-phosphate pH 6.5*	*ATP pH 6.5*	*Thiamine pyrophosphate pH 6.9*
assay medium	used immediately	33	98	545	14.5
0.9% NaCl	stored < 10 min	32	96	476	16.2
0.9% NaCl	stored 5 h	27	106	421	12.9
0.9% NaCl	stored 19 h	25	107	395	11.1
0.9% NaCl	stored 30 h	23	99	309	11.8
0.25 *M* sucrose	stored < 10 min	27*	—	—	—
0.25 *M* sucrose	frozen and thawed 6 times	34*	—	—	—

* sucrose (0.2 *M*) added to assay medium.

Effect of Ca^{+2} or Mg^{+2}. Examples of the effects of these cations on enzyme activity are given in Table IV. The hydrolysis of adenosine 5'-phosphate did not appear to be

TABLE IV

COMPARISON OF ACTIVATION BY $CaCl_2$ OR $MgCl_2$ IN HISTOCHEMICAL SECTIONS AND IN FRESH BRAIN SUSPENSIONS

Media as Table I

Substrate	*pH*	*Addition*	*M*	*Activity (μmole/g hr)*	
				Fresh suspensions	*Histochemical*
Glycerophosphate	9.1	—		7.6	—
		$CaCl_2$	0.1	22.0	—
Glycerophosphate	5.3	—		30	35
		$MgCl_2$	0.03	38	41
		$CaCl_2$	0.05	28	39
Adenosine 5'-phosphate	6.5	—		161	129
		$MgCl_2$	0.03	152	108
		$CaCl_2$	0.03	137	116
Thiamine pyrophosphate	6.9	—		2	2
		$MgCl_2$	0.03	18	14
		$CaCl_2$	0.03	31	19

References p. 389.

activated by these cations either in the histochemical method or in fresh tissue suspensions. On the other hand, either Ca^{+2} or Mg^{+2} raised the activity of thiamine pyrophosphatase in both methods. Acid phosphatase was activated slightly by Ca^{+2} or Mg^{+2} in the histochemical method and by Mg^{+2} in fresh saline suspensions. The effect of Mg^{+2} on ATPase activity depended on whether Pb^{+2} was also present. Mg^{+2} but not Ca^{+2}, activated ATPase in fresh tissue suspensions. In the histochemical method, in which Pb^{+2} was present, this enzyme was probably activated to a certain extent by Mg^{+2} although results were not consistent. Pb^{+2} had little effect on fresh tissue ATPase activity in the absence of Mg^{+2} but it reduced activity considerably when Mg^{+2} was present (Table V).

TABLE V

EFFECT OF Mg^{+2} AND Pb^{+2} ON ATPase

Fresh brain suspension from 7 rats ($MgCl_2$ $7.7 \cdot 10^{-3}$ *M* or lead acetate 10^{-3} *M*)

Inorganic phosphate μmole/g hr				*Rates as % of "Mg only" rate*		
Mg only	*Mg and Pb*	*No additon*	*Pb only*	*Mg and Pb*	*No addition*	*Pb only*
395	272	150	—	69.0	38.0	—
324	126	86	87	38.9	26.5	26.9
594	346	151	169	58.3	25.4	28.5
394	152	56	90	38.6	14.2	22.9
324	173	—	84	53.4	—	25.9
231	—	76	—	—	32.9	—
567	—	144	—	—	25.4	—
	Mean %			56.1	27.1	26.1

DISCUSSION

When comparisons were made made previously[3] of enzyme activity in a histochemical method and in fresh brain suspensions, the histochemical acid phosphatase activity was found to be nearly twice that in a fresh brain suspension, and the histochemical thiamine pyrophosphatase activity was slightly higher. Either activity was lost in the fresh tissue assay, or there was an increase during the preparation of the histochemical sections. The former possibility now seems more likely since fresh brain suspension acid phosphatase activity is not raised by treatment similar to that necessary to prepare freeze-dried paraffin wax sections. One possible cause of fresh tissue loss is suggested by the slow fall in suspensions stored at 0° (Table III). The results of the present work are in agreement with the observation of GORDON[5] that 25% of the ATPase activity in a rat brain suspension is lost after 18 hr at 0°, and with the findings of RICHTER AND HULLIN[6] who report a slow fall in acid phosphatase activity in brain suspensions at 0°. Acid phosphatase has been reported to exist in liver suspensions in an inactive form[4] and an osmotic barrier impermeable to glycerophosphate has been postulated[7]. Any barrier of this type in brain tissue is likely to be lost in the histochemical procedure by treating the tissue successively with molten paraffin wax and with light petroleum. However, no evidence was found of the existence of any large part of brain acid phosphatase in an inactive form since activity did not differ greatly in a sucrose suspension,

References p. 389.

and repeated freezing and thawing, which is reported to activate the enzyme in liver[4], caused only a small activity increase.

Some inhibition of enzyme activity in fresh tissue suspensions is found when the medium contains lead, except possibly the ATPase medium, which will be considered separately as it shows special features. The inhibition of acid phosphatase by lead is in agreement with the results of BERTRAN AND BERTRAN[8] for dog liver suspensions. The fall in apparent activity in the histochemical method when the lead concentration exceeds about 10^{-3} *M* suggests that a similar inhibition occurs in this method also. Such inhibition is likely to be less than the losses which may occur in the preparation of tissue sections other than by freeze-drying. Thus, thiamine pyrophosphatase activity is reduced by 30% in the presence of 10^{-3} *M* lead acetate, whereas brief treatment of sections with acetone or with 80% aqueous ethanol (both commonly used in tissue fixation) causes a loss of 80–90% of the activity[9].

It is not possible to avoid inhibition of the enzymes by reducing the lead concentration much below 10^{-3} *M* since less phosphate is deposited and staining becomes uneven. GOMORI[10] has reported on an increase in intensity and uniformity of acid phosphatase staining as the lead concentration is increased, within limits, which he explains by a reduction in the solubility of inorganic phosphate. However, the persistence of the artefact in the present work even when the media are previously saturated with inorganic phosphate suggests that too high a lead phosphate solubility is not the only cause. It has been suggested by JOHANSEN AND LINDERSTRØM-LANG[11,12] that supersaturation will lead to artefacts, especially in the intracellular localisation of alkaline phosphatase. However, it does not seem likely that such artefacts can be of any great importance in acid media since the distribution of the deposit changes with different substrates[1]. GOMORI AND BENDITT[13] find that a histochemical medium containing 0.01 *M* $CaCl_2$ at pH 9.3 appears to be saturated by about $4 \cdot 10^{-6}$ *M* inorganic phosphate and that supersaturation is not likely to interfere with enzyme localisation under these conditions. They recommend that a suitable quantity of inorganic phosphate should be added to the medium if not present in the reagents. The persistence of inorganic phosphate in freeze-dried sections may help to make staining in such tissues more reliable than in chemically-fixed tissue. The tissue content of about 15 μ mole/g of inorganic phosphate is more than sufficient, at the usual ratio of medium to tissue of about 1.5 l/g, to saturate the medium (except when ATP is present). According to JOWETT AND PRICE[14], the stable form of lead phosphate, except under extreme conditions, is pyromorphite $Pb_5(PO_4)_3Cl$ provided that the chloride ion concentration is not less than 10^{-6} *M*. However, it seems that the solubility products reported for this salt or the tertiary lead phosphate do not have any meaning for the histochemical media as their inorganic phosphate contents are much higher than would be expected and perhaps are better expressed as "phosphate tolerances", as suggested by GOMORI AND BENDITT[13]. These tolerances are of the same order as that found for the alkaline medium but the lead concentration in the acid media is much lower than the corresponding calcium concentration in the alkaline medium. The possible importance of this difference is suggested by the finding that the inorganic phosphate present in the sections is retained in the tissue when plunged into the medium containing 0.1 *M* $CaCl_2$ or a high lead concentration, but largely escapes when media have lead concentrations as specified in Table I. Probably, phosphate ions diffuse out of the tissue more rapidly than lead ions diffuse into it, and the loss occurs after the phosphate tolerance of the medium has been exceeded. Such conditions are undesirable in

References p. 389.

enzyme localisation. Local depletion of the lead at low concentrations could account for the apparent "reversal" of staining, *i.e.* the failure to stain of regions normally the most intensely stained, although transient supersaturation may also play a part.

The highest activity in the histochemical method is shown by adenosine 5'-phosphatase of which the activity found at a lead concentration of 10^{-3} *M* will exhaust the lead in the medium after about 17 sec if replenishment from the bulk of the solution does not take place. The time is likely to be reduced more than proportionately by reduction of the lead concentration since the enzyme activity will rise also. The rate of diffusion of lead ions into the tissue is not known but it may depend on the nature of the tissue and on the pH of the medium. The optimal overall rate of staining in the rat brain in the histochemical method is found at a lead concentration of about 10^{-3} *M*, but the choice may be affected by the level of enzyme activity and a slightly higher concentration is probably preferable in view of the unevenness in distribution of the enzymes. Still higher concentrations may be desirable for the study of fine cytological detail in regions of high activity. Some form of gentle agitation during incubation may be helpful. Artefacts due to too low lead concentrations should not cause serious difficulty in assessing the results of enzyme localisation since they can be recognised readily when produced intentionally. A further safeguard can be provided by confirming findings at a different lead concentration in the medium. At pH 9.1 difficulty is avoided since a relatively high concentration of $CaCl_2$ can be used without seriously reducing alkaline phosphatase activity. However there is an upper limit to the lead concentration owing to inhibition or to limited solubility of the lead salt of the substrate. Both these factors are likely to depend on the pH of the medium so that the apparent pH optimum of the enzyme may appear to differ from that found by other methods. Some other cation may prove more suitable than lead. TANDLER[15] has suggested cobalt, but its phosphate is more soluble than that of lead and there is little evidence of other advantages.

Although ATPase is not inhibited appreciably by lead under the histochemical conditions, the magnesium activation of this enzyme appears to be reduced considerably in the presence of lead. This effect of lead seems likely to provide the main explanation of the lower values for ATPase activity found histochemically[3] compared with those of other workers using fresh tissue methods[5, 16, 17]. Somewhat analogous competition with magnesium as an activator by beryllium has been reported for alkaline phosphatase[18, 19]. Further study is needed to determine whether magnesium activates ATPase in the histological method since appearance of such activation may have been prevented in earlier work by too low a lead concentration in relation to the enzyme activity. The higher phosphate tolerance of the ATP medium is consistent with the ability of 0.2 *M* solutions of the sodium salt of ATP to prevent the precipitation of lead phosphate[20]. If ATP forms a complex with lead ions in the medium, a possible mechanism is suggested for the blocking of magnesium activation by lead.

The ATP concentration ($0.5 \cdot 10^{-3}$ *M*) is already below the optimum level of $2.5 \cdot 10^{-3}$ *M*[17], but further reduction may prove useful by reducing the rather high phosphate tolerance of the medium and yielding an enzyme activity in the presence of magnesium which does not require an impracticable lead concentration in the medium.

References p. 389.

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SUMMARY

1. A study has been made of the effects of lead, calcium, magnesium, or of the tissue preparation on the activity of acid and alkaline phosphatases, adenosine 5'-phosphatase, ATPase, and thiamine pyrophosphatase in brain suspensions and in histochemical enzyme localisation methods.

2. Alkaline phosphatase is activated by calcium. The other enzymes are inhibited more or less by the lead present in the histochemical medium. However, if the lead concentration in the histochemical medium is too low, the inorganic phosphate may not be completely retained in the tissue, leading to artefacts.

3. The effects of calcium or magnesium on activity in the histochemical method correspond with the effects of these cations on the enzymes in fresh tissue suspensions, except that the activation of ATPase by magnesium is greatly reduced by lead which is present in the histochemical medium.

4. The special medium containing lead, in which the enzyme acts, appears to be the main factor limiting the activity found in the histochemical methods.

RÉSUMÉ

1. On a étudié les effets du plomb, du calcium, du magnésium, et de la préparation des tissus sur l'activité des phosphatases acide et alcaline, de l'adénosine 5'-phosphatase, de l'ATPase et de la thiamine pyrophosphatase; on a comparé des suspensions de cerveau et les méthodes de localisation histochimique des enzymes.

2. La phosphatase alcaline est activée par le calcium. Les autres enzymes sont plus ou moins inhibés par le plomb, qui se trouve dans le milieu de la réaction histochimique; mais, quand la concentration du plomb dans le milieu de réaction histochimique est trop basse, le phosphate inorganique n'est plus retenu complètement dans le tissu et il se produit des artefacts.

3. Les effets du calcium et du magnésium sur la méthode histochimique sont parallèles à ceux que ces cations exercent sur les enzymes dans les suspensions de tissu frais; mais l'activation de l'ATPase par le magnésium est largement diminuée par le plomb contenu dans le milieu de la réaction histochimique.

4. Le milieu spécial à base de plomb, dans lequel agissent les enzymes, semble être le facteur limitant principal de l'activité observée dans les méthodes histochimiques.

ZUSAMMENFASSUNG

1. Der Einfluss, den Blei, Magnesium, Kalzium und das Präparieren der Gewebe auf die Aktivität der sauren und alkalischen Phosphatasen, Adenosin-5'-Phosphatase, ATPase, und Aneurinpyrophosphatase in Gehirnsuspensionen und bei der Anwendung histochemischen Enzymlokalisierungs-Methoden ausüben, wurde untersucht.

2. Die alkalische Phosphatase wird durch Kalzium aktiviert. Die Wirkung der übrigen Enzyme wird durch Blei mehr oder weniger gehemmt. Wenn die Bleikonzentration zu niedrig ist, bleibt das anorganische Phosphat nicht vollständig im Gewebe, wodurch Artefakte entstehen.

3. Der Einfluss des Kalziums und des Magnesiums auf die Aktivität in der histochemischen Untersuchung entspricht der Wirkung dieser Kationen auf die Enzyme in frischen Gewebesuspensionen, aber die Aktivierung der ATPase durch Magnesium wird durch das in dem histochemischen Medium enthaltene Blei stark vermindert.

4. Das Blei enthaltende Medium, in welchem die Enzyme wirken, scheint für die in der histochemischen Methode beobachtete Verminderung der Aktivität hauptsächlich verantwortlich zu sein.

References p. 389.

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