SUMMARY

1. For isolation of rat-liver nuclei a medium is recommended having this composition: 0.3M sucrose, 0.065M potassium glycerophosphate, 0.001M magnesium chloride, 40% glycerol; pH 7.0.

2. Phase-contrast photographs are shown of nuclei in this medium, and in modifications of it causing various abnormal appearances.

3. Nuclei in the recommended medium retain their normal appearance for at least 6 hr. at room temperature, after which they are still able to form haloes of soluble viscous nucleic acid when transferred to molar sodium chloride.

4. If the glycerophosphate concentration is increased to 0.115M, with or without magnesium chloride, nuclei retain their normal appearance for 100 hr. at room temperature, apart from slight clumping; but the higher ionic strength of this medium may sometimes be a disadvantage.

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Cerebral Diphosphoinositide Breakdown: Activation, Complexity and Distribution in Animal (mainly Nervous) Tissues

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Suspensions of guinea-pig brain rapidly attack the 'inositol phosphatide' fraction of ox-brain kephalin, liberating into the medium acid-soluble inositol and phosphate (Sloane-Stanley, 1953). Presumably cerebral tissue contains enzymes catalysing the breakdown of the diphosphoinositide (DPI), shown by Folch (1942) to be an important constituent of brain kephalin. The interesting feature of this reaction is its rate, which is outstandingly higher than the rates previously reported for the *in vitro* hydrolysis of other brain lipids (Sloane-Stanley, 1952). On a molar basis it is comparable to the rate of oxygen uptake by cerebral preparations.

The present work is mainly concerned with the distribution of the enzyme system and its sensitivity to certain cations and drugs. For most experiments a saline suspension of the 'inositol phosphatide' fraction of kephalin has been used as substrate, but preliminary observations on the breakdown of the water-soluble potassium salt of cerebral DPI (Folch, 1949a) are also included.

EXPERIMENTAL

Enzyme activity was studied in two ways. (a) By following acid formation manometrically in bicarbonate-buffered saline solutions (Sloane-Stanley, 1953). The rate of CO₂

evolution during the first 15 min. was taken as a measure of the enzyme content of the tissue. Total P rendered acid-soluble was determined at the end of the experiment and in some cases inorganic P was also determined. (b) By determining phosphates (and in one experiment inositol) at various times during the course of the reaction.

Substrates

'*Inositol phosphatide*.' This was prepared from ox brain (from the slaughter house) or post-mortem human brain by the procedure of Folch (1942). Some samples (Table 1) were further fractionated with chloroform-methanol (Folch, 1949b). After dialysis the aqueous lipid emulsion was brought to pH 7 with ice-cold N-KOH before freeze-drying. The dried lipid was stored in a vacuum desiccator over silica gel at 0°.

It was found difficult to reduce the inorganic P content of the samples below about 0.13% by dialysis in bulk. This was done by washing small quantities of the substrate, as required, in the following manner. Lipid (100 mg.) was suspended in 3 ml. of ice-cold glass-distilled water (in an all-glass homogenizer), and centrifuged at 25 000 *g* for 20 min. at 0°. The supernatant, which was usually clear, was discarded and the washing process repeated. The deposit was then dried with acetone (in which case it was used within 2 days), or freeze-dried and stored as described above.

Inositol in the samples was determined and an estimate of their DPI content made by taking its inositol content to be 21% (Folch, 1949a) (Table 1). The structure or mol.wt. of DPI is not known, but analysis (Folch, 1949a) indicates that each molecule of inositol diphosphate is esterified to a mono- or di-glyceride; the mol.wt. of this would lie between 750 and 950, depending on its fatty acid composition. To assess the proportion of DPI degraded by the enzyme, a figure of 0.8 mg. has been taken as equivalent to 1 μ mole.

From previous work (Folch, 1942) the remaining lipids in the samples were assumed to consist of phosphatidyl serine and phosphatidyl ethanolamine, neither of which is attacked by brain suspensions (Sloane-Stanley, 1953).

Potassium diphosphoinositide (Folch, 1949a, b). This was prepared and generously given by Professor Folch-Pi. It dissolved in water (or 0.9%, w/v, NaCl) to form a clear pale-yellow solution, stable at 4° for at least 5 days. After dialysis for 48 hr. against distilled water it was freeze-dried,

stored at 4° in a vacuum desiccator, and immediately before an experiment was redissolved in the appropriate medium.

Tissue suspensions

Tissue was obtained as soon as possible after death and suspended in about four times its wet wt. of ice-cold 0.9% (w/v) NaCl in a glass homogenizer. The dry wt. of tissue was determined by heating 1.0 ml. samples of suspension at 110° for 3 hr., allowance being made for added salt. To convert the dry wt. into wet wt., a factor of 5 was used for whole-brain preparations from small laboratory animals; in other cases the dry wt.:fresh wt. ratio was determined by drying a sample at 110°.

Saline suspensions were stable for 2-3 weeks at 4° with respect to the properties of the system being studied, provided that toluene was added. For example, a preparation of whole brain lost less than 4% of its activity in 14 days.

For experiments in which a low initial P was desirable, suspensions were dialysed in cellophan bags for 48 hr. against 0.9% (w/v) NaCl, at 4°; such treatment did not alter enzyme activity.

Laboratory animals were killed by decapitation and bled before removal of the brain. Sheep brain was obtained from the slaughter house about 1 hr. after death and brought to the laboratory packed in ice. Infant-human brain from a child 9 days old, who had died from anoxia, was obtained some 24 hr. after death. Adult-human brain was obtained from neurosurgical operations and brought to the laboratory in ice immediately after removal (for further details see McIlwain, Ayres & Forda, 1952).

The stability of the system in intact brain after death was investigated by control experiments. Guinea-pig brain was removed in the usual manner, one half was immediately suspended in saline and the other left (a) at room temp., and (b) at 4° for 6 hr. before suspending. No loss of activity could be detected.

Reaction with 'inositol phosphatide'

Acid formation. Small Warburg vessels of conventional pattern but of 4-5 ml. capacity (Barker, 1949) were used to conserve substrate. Tissue preparations were diluted to the desired concentration with a bicarbonate saline at pH 7.4 and freshly equilibrated with N₂+CO₂ (95:5, v/v). The final concentration of constituents in the reaction mixture was: 120 mm-NaCl, 4.7 mm-KCl, 2.5 mm-CaCl₂, 1.2 mm-MgSO₄, 25 mm-NaHCO₃. Substrate was freshly weighed and suspended in the medium with a glass homogenizer. Added compounds were incorporated in the saline used for diluting the tissue suspensions.

In a typical experiment 0.4 ml. of dilute tissue suspension containing about 12-15 mg. of fresh wt. of tissue was pipetted into the main compartment, and 0.1 ml. (5 mg.) of lipid suspension into the side bulb. After gassing with N₂+CO₂ (95:5, v/v) for 10 min., and equilibrating in the thermostat for 15 min., the reaction was started by tipping the flasks and readings were taken every 3 or 5 min. for 30-40 min. Control flasks containing tissue or substrate only were run in parallel and any gas changes allowed for in calculating the rate of CO₂ evolution. Blank values were of the same magnitude as those described by Sloane-Stanley (1953), i.e. sometimes up to 20% of total, but usually between 10 and 15%. Preliminary experiments with varying amounts of substrate established the level required to produce the

Table 1. *Inositol and approximate diphosphoinositide contents of samples of crude substrate*

Average of results obtained by yeast-growth method (Sloane-Stanley, 1953) and HIO₄-oxidation method (Platt & Glock, 1943). Approx. DPI content was calculated from the data of Folch (1949a); inositol content of purified DPI = 21%.

Source	Inositol (%)	Approx. DPI (%)
Ox brain A*	9.9	47
B	4.8	23
C*	6.1	29
D	6.5	31
Human brain*	11.5	55

* Precipitated with chloroform-methanol (after Folch, 1949b).

maximum initial rate with a constant weight of tissue. The quantity of tissue added was sufficient to produce a smooth evolution of CO_2 , and small variations of a few mg. did not significantly alter the initial rate per unit wt. of tissue.

The reaction was stopped by adding 1.0 ml. of ice-cold 0.45N- HClO_4 . After centrifuging the supernatant was filtered through 4.5 cm. Whatman no. 542 papers (a few early experiments), or more often through small pads of acid-washed asbestos, supported by cotton wool in the drawn-out stem of a 3 cm. filter funnel. A sample of the filtrate was diluted with sufficient NaOH solution nearly to neutralize the acid present, and the solution was stored at -20° for P estimation. No increase in the inorganic fraction of the total P occurred under these conditions.

Phosphate liberation. In most cases the reaction was run in air in 0.067M glycylglycine buffer (pH 7.2) diluted from 0.5M stock solution with a salt solution of the desired composition. Samples were withdrawn at intervals for P estimation. In a few experiments P liberation was followed in bicarbonate saline in manometric apparatus by including several flasks of the same composition and removing them from the thermostat at intervals. No difference in the kinetics of the reaction was observed between experiments run in bicarbonate buffer in $\text{N}_2 + \text{CO}_2$ mixture and in glycylglycine buffer in air.

For experiments of 60 min. duration or less blank determinations were made by mixing tissue and substrate, and immediately precipitating by adding the 0.45N- HClO_4 . With freshly washed substrate and dialysed brain these were never more than 15% of the total P evolved enzymically. For experiments extending over a longer period this method was unsatisfactory, since it was found that although no non-enzymic production of P took place in the substrate when shaken in buffer in the thermostat for several hours, the acid-soluble P content of brain suspensions under these conditions tended to increase (sometimes by 100%), and this invalidated the zero blank. In these cases a separate tissue blank was run and analysed at the end of the experiment; from the difference between this and a zero brain blank, corrections for intermediate times were calculated.

Experiments with potassium diphosphoinositide

Only a limited quantity of this substance was available and consequently the scale of the experiments was reduced to one-quarter of that employed with the cruder substrate. Thus in a typical experiment 0.025 ml. of a solution of the lipid containing 16 mg./ml. was added to 0.1 ml. of brain suspension in a 75 mm. $\times$ 9 mm. test tube; after incubation 0.125 ml. of ice-cold N- HClO_4 in 0.1M- CaCl_2 was added, followed by 0.125 ml. of 0.95N-KOH. After centrifuging phosphates were determined in the supernatant fluid; filtration was unnecessary. The addition of Ca ions to the perchloric acid improved the completeness of precipitation of unchanged lipid.

Analytical methods

Phosphates. These were determined by a micro modification of the method of Martin & Doty (1949), omitting the silico-tungstate reagent. Colours were read at 700 m μ . in a Beckman DU Quartz or Hilger Uvispek spectrophotometer. For the determination of total P, organically-bound P was hydrolysed by boiling with 10–11M- HClO_4 , and neutral instead of acid ammonium molybdate was

used. The recovery of added inorganic P with this procedure was satisfactory.

In experiments with 'inositol phosphatide' the method covered the range 0–6 μg . of P in a final volume of 4 ml. With potassium diphosphoinositide the scale was reduced to estimate 0–2 μg . of P in a volume of 1 ml.

Inositol. Inositol was determined in lipid samples by the yeast-growth method as described by Sloane-Stanley (1953), and by the chemical method (oxidation by HIO_4) of Platt & Glock (1943) as adapted by Sloane-Stanley (1953).

In reaction products the chemical method only was used. A similar procedure to that described by Sloane-Stanley (1953) was used except that the succinate buffer was omitted and filtration effected through asbestos. Good recovery of added inositol was obtained; blank determinations of the amount of inositol originally present and of that produced non-enzymically from both substrate and brain suspensions during the experiment were made (combined value less than 10% of the total liberated).

RESULTS

Activation of the reaction with 'inositol phosphatide' as substrate

Preliminary experiments. Fig. 1A illustrates the release of H ions (judged by CO_2 evolution) and phosphates from 'inositol phosphatide' by a whole-

Fig. 1. Release of H ions (CO_2 evolution) and phosphates from 'inositol phosphatide' by a suspension of whole guinea-pig brain in bicarbonate-buffered salines. Conical Warburg vessels, capacity 18–20 ml.; gas phase $\text{N}_2 + \text{CO}_2$ (95:5, v/v). The curve 'combined acid-soluble P' represents the difference between the curves for total acid-soluble P and inorganic P. A, medium contained: 120 mM-NaCl, 4.7 mM-KCl, 2.5 mM- CaCl_2 , 1.2 mM- MgSO_4 and 25 mM- NaHCO_3 . B, only 129 mM-NaCl and 25 mM- NaHCO_3 were present. (For reasons of clarity only one line has been drawn in B for both CO_2 and combined P.)

brain preparation (guinea pig) in bicarbonate medium containing K, Mg and Ca. Fig. 1B illustrates the release of the same ions when the reaction was carried out in a bicarbonate medium of the same tonicity and pH, but with the omission of K, Mg and Ca ions. Clearly the presence in the first medium of one of these ions had activated the release of combined P (total acid-soluble P less inorganic P), and to a less extent had inhibited the release of inorganic P. Other experiments with different samples of substrate and various brain preparations gave essentially the same result.

Nature of activation. This was studied in glycylglycine-buffered media by withdrawing samples of the reaction mixture for P estimation at various times (Table 2). Calcium greatly increased the release of combined P, but Mg, K and Ba were without action in this respect; Mg, Ca and Ba all slightly decreased the release of inorganic P. The possibility that this smaller amount of inorganic P found in the presence of bivalent cations was due to losses incurred in working up the reaction mixtures was excluded by adding inorganic P to media containing Ca in the same and greater concentrations than occurred in the experiments, when satisfactory recovery was obtained.

Action of calcium on release of combined acid-soluble inositol. Sloane-Stanley (1953) reported the release of acid-soluble inositol (85–90% of which was combined) from 'inositol phosphatide' when incubated with brain suspensions. The influence of Ca on this aspect of the reaction is shown in Table 3. Calcium activated combined-inositol release to the same extent as it did that of combined P; approximately equimolar amounts of each were found in both media at all stages of the reaction studied.

Optimum concentration of calcium for activation. The initial rate of combined P release was greatest in a medium containing mM-CaCl₂, but the reaction was maintained longer and the amount of combined P eventually formed was greater in 2 mM-CaCl₂ (Fig. 2). Increasing concentrations of Ca also progressively inhibited the release of inorganic P.

The 'inositol phosphatide' used in these experiments contained DPI in the form of its Ca-Mg salt. Analysis of one sample gave: Ca 0.13%, Mg 1.12%, figures of the same order as reported by Folch (1942). The question whether this intrinsic Ca was able to activate the enzyme, and might therefore account for the small quantity of combined P released even in a Ca-free medium (Table 2),

Table 2. *Action of calcium, magnesium and barium salts on release of phosphates from 'inositol phosphatide' by brain suspensions*

Whole brain (guinea pig) was suspended in 0.9% (w/v) NaCl, about 120 mg. of fresh tissue/ml., and dialysed for 48 hr. at 4°. Before an experiment the suspension was diluted to 30 mg./ml. with an isotonic NaCl-glycylglycine medium (pH 7.2), containing CaCl₂, MgSO₄ or BaCl₂ where stated, so that the final concn. of buffer was 0.067M. A portion (2 ml.) was incubated at 38° with 0.5 ml. of 'inositol phosphatide' (50 mg./ml.). Samples (0.5 ml.) were withdrawn at 0, 30 and 60 min. DPI available was approx. 125 μ moles/g. of fresh tissue (see text). Combined P = total acid-soluble P less inorganic P.

Addition to medium	Total acid-soluble P (μ moles/g. of fresh tissue)		Inorganic P (μ moles/g. of fresh tissue)		Combined P by difference (μ moles/g. of fresh tissue)		Ratio combined P: inorganic P	
	30 min.	60 min.	30 min.	60 min.	30 min.	60 min.	30 min.	60 min.
None	31	36	24	30	7	6	0.3	0.2
3.3 mM-CaCl ₂	33	51	10	17	23	34	2.3	2.0
2.0 mM-MgSO ₄	13	23	9	16	4	7	0.5	0.4
3.3 mM-BaCl ₂	14	18	11	15	3	3	0.3	0.2

Table 3. *Action of calcium on release of combined acid-soluble inositol from 'inositol phosphatide' by brain suspensions*

Whole brain (guinea pig) prepared as in Table 2 and diluted to 43 mg./ml. with NaCl-glycylglycine medium (pH 7.2) containing CaCl₂ where stated. A portion (14 ml.) was incubated at 38° with 1 ml. of 'inositol phosphatide' suspension (80 mg./ml.). Samples (3 ml.) were withdrawn at 0, 1, 2 and 4 hr. Total DPI available was approx. 40 μ moles/g. of fresh tissue (see text). Combined P = total acid-soluble P less inorganic P.

Addition to medium	Total acid- soluble P (μ moles/g. of fresh tissue)			Inorganic P (μ moles/g. of fresh tissue)			Combined P by difference (μ moles/g. of fresh tissue)			Combined acid- soluble inositol (μ moles/g. of fresh tissue)		
	1 hr.	2 hr.	4 hr.	1 hr.	2 hr.	4 hr.	1 hr.	2 hr.	4 hr.	1 hr.	2 hr.	4 hr.
None	29	34	39	19	21	21	10	13	18	11	15	18
2 mM-CaCl ₂	35	43	48	10	14	18	25	29	30	22	25	31

Table 4. *Release of phosphates from potassium diphosphoinositide by brain suspensions*

Whole brain (guinea pig) prepared as in Table 2 and diluted to 30 mg./ml. with NaCl-glycylglycine medium (pH 7.2) containing CaCl_2 or MgSO_4 where stated. Samples (0.1 ml.) were incubated at 38° with 0.025 ml. of a 'solution' of potassium diphosphoinositide (16 mg./ml.). Total DPI available was approximately $166 \mu\text{moles/g.}$ of fresh tissue (see text). Combined P = total acid-soluble P less inorganic P. The results quoted for Expts. 2-6 are mean values followed by their S.E.

Expt.	Addition to medium	Total acid-soluble P ($\mu\text{moles/g.}$ of fresh tissue)		Inorganic P ($\mu\text{moles/g.}$ of fresh tissue)		Combined P by difference ($\mu\text{moles/g.}$ of fresh tissue)		Ratio combined P: inorganic P	
		10 min.	30 min.	10 min.	30 min.	10 min.	30 min.	10 min.	30 min.
1 (brain D)	None	85	118	29	50	56	68	1.9	1.4
	mm- CaCl_2	89	134	17	38	72	96	4.2	2.5
	2 mm- CaCl_2	85	128	15	29	80	98	5.3	3.4
	mm- MgSO_4	94	122	25	49	69	73	2.8	1.5
	2 mm- MgSO_4	95	134	25	50	65	84	2.6	1.7
2-6 (brains B, C and D)	None	70	106	28 ± 0.9	46 ± 2.7	42 ± 4.2	60 ± 5.1	1.5	1.3
	2 mm- CaCl_2	78	109	19 ± 1.7	31 ± 1.8	59 ± 6.5	76 ± 7.1	3.1	2.5

Fig. 2. Action of various concentrations of CaCl_2 on release of phosphates from 'inositol phosphatide' by a suspension of whole guinea-pig brain. The reaction mixture was buffered at pH 7.2 with glycylglycine (0.067M) and contained CaCl_2 in the concentrations shown. The curve 'combined acid-soluble P' represents the difference between the curves for total acid-soluble P (not shown here) and inorganic P. A, phosphates found after 1 hr.; B, phosphates found after 4 hr.

was considered. However, when the reaction was studied in a Ca-free medium containing 10 mM-NaF there was no further reduction in the amount of combined P released, which suggests that this intrinsic Ca was not available.

To investigate the effect of ions on the physical state of the suspended substrate, dilute suspensions of 'inositol phosphatide' were prepared and their turbidity was measured in a Spekker absorptiometer. The addition of either 2 mM- CaCl_2 or MgSO_4 to suspensions in glycylglycine-buffered isotonic NaCl medium increased their turbidity by about 15%, but microscopical examination of the centrifuged deposit revealed no marked differences in the size of the clumps of lipid particles. The addition of 10 mM- CaCl_2 resulted in a much greater turbidity, and flocculation and rapid sedimentation of the lipid occurred on standing.

Experiments with potassium diphosphoinositide

Table 4 summarizes the results of six experiments with whole guinea-pig brain suspensions in which a solution of potassium diphosphoinositide was used as substrate at a final concentration of 3.2 mg./ml., equivalent to approx. $166 \mu\text{moles/g.}$ of fresh wt. of tissue. Compared to the reaction with 'inositol phosphatide' the following points are worthy of comment: (a) In media containing Ca the proportion of combined P to inorganic P released after incubation for 30 min. was roughly the same with both substrates. (b) The breakdown of potassium diphosphoinositide was very rapid, when assessed by the amount of combined P released after 10 min. in media containing Ca. That the rates quoted in Table 4 were still considerably less than the maximum was shown in an experiment in which substrate concentrations of 2, 4 and

6 mg./ml. were employed; the 10 min. total acid-soluble P figures were then respectively 43, 92 and 130 μ moles. This extremely rapid breakdown was confirmed in bicarbonate-buffered saline containing Ca, with a rabbit-brain preparation; at a substrate concentration of 4 mg./ml. the initial rate of CO_2 evolution was 300 μ moles/g. fresh wt. of tissue/hr., compared to figures of 24 and 46 μ moles/hr. obtained with crude substrate and the same species (Table 5). (c) An activation by Ca of combined P release from potassium diphosphoinositide could not be unequivocally demonstrated, since the difference between the means quoted in Table 4 for media with and without Ca (at either 10 min. or 30 min.) was only significant to the 0.1 level of probability. On the other hand, as was found with the cruder substrate, Ca significantly inhibited the release of inorganic P ($P < 0.01$). A solution of potassium diphosphoinositide in the Ca-free medium was clear and stable; on addition of CaCl_2 to 2 mM concentration, a slight turbidity appeared, but no precipitation of the lipid occurred on standing at room temperature.

Distribution of enzyme activity

In experiments described in this section the substrate was 'inositol phosphatide'.

Nervous tissues. A preliminary survey of the distribution of the enzyme system in anatomically defined regions of the central nervous system was made by measuring acid formation, supplemented by total acid-soluble P determinations at the end of the experiments (Table 5). The results suggest that the enzyme activity is fairly uniformly distributed in nervous tissue, although peripheral nerve showed less activity than brain or cord preparations; the higher proportion of connective tissue in

nerve may, however, account for this difference. In the human material, adult-brain preparations from biopsy specimens were less active than those from a 9-day old infant brain (*post-mortem*) in which myelination was incomplete.

Further determinations of the rate of phosphate release in glycyglycine media, both with and without Ca, failed to reveal any great variation in activity (Table 6), although the ratio of combined P to inorganic P released tended to be higher with the peripheral nerve and spinal cord preparations. Activation by Ca was demonstrated in all preparations (except spinal cord, which was not tested).

Non-nervous tissues. Suspensions of rat liver, kidney and spleen released H^+ ions and acid-soluble P from the substrate at about the same rate as did cerebral tissues; no activity was shown by either serum or lysed erythrocytes (guinea pig).

When the release of both inorganic and total P was followed some differences in the course of the reaction were found (Table 6). With kidney (either in the presence or absence of Ca) the main end-product of the reaction was inorganic P. A skeletal-muscle preparation released inorganic P at a rate approx. 20% of that found with brain, but no combined P. When Ca was omitted from the medium less combined P appeared with all tissues.

Attempts to influence the reaction with drugs and other compounds

The compounds examined were chosen for one of the following reasons: (a) for their known action as inhibitors of other enzyme systems; (b) for their known action on the central nervous system in the intact animal; (c) for the structural similarity of their molecule (or groups) to inositol.

Table 5. *Diphosphoinositide-splitting enzyme activity in nervous tissues from several species*

Tissues were suspended in 0.9% (w/v) NaCl at about 120 mg./ml. and diluted to 30 mg./ml. with a bicarbonate-buffered medium containing NaCl, KCl, CaCl_2 and MgSO_4 (see text). A portion (0.4 ml.) was incubated at 38° with 0.1 ml. of 'inositol phosphatide' suspension (50 mg./ml.) under manometric conditions in small Warburg vessels; CO_2 evolution was followed for 30–45 min. after adding the lipid suspension from the side bulb.

Species	Source of tissue	Initial rate of hydrolysis (μ moles of CO_2 /g. of fresh tissue/hr.)	Total acid-soluble P after 45 min. (μ moles/g. of fresh tissue/hr.)
Guinea pig	Whole brain	50, 52, 62	49, 56, 76
Guinea pig, rat, rabbit, sheep and monkey	Cerebrum,* cerebellum, thalamus, medulla, pons and spinal cord	24–60	37–74
Cat and monkey	Sciatic nerve	14, 19, 20	12, 16
Human (infant 9 days)	Cerebrum,* cerebellum, olivary nucleus, medulla and pons	42–70	60–82
Human adult (3 cases)	Cerebrum*	24, 25	38, 46
	Grey matter (cortex)	22, 36	36, 50
	White matter (cortex)	20, 20	30, 36

* Cerebral cortex together with some underlying white matter.

Table 6. *Release of phosphates from 'inositol phosphatide' by suspensions of nervous and non-nervous tissues: action of calcium salts*

Tissues were prepared and their enzyme activity was determined as described in Table 2. The DPI available in the substrate was approx. 200 μ moles/g. of fresh tissue (see text). Combined P = total acid-soluble P less inorganic P.

Species	Source of tissue	Addition to medium (2 mm)	Inorganic P (μ moles/g. of fresh tissue)		Combined P by difference (μ moles/g. of fresh tissue)	
			25 min.	60 min.	25 min.	60 min.
Sheep	Cortex	None	26	42	15	15
	Cortex	CaCl ₂	16	26	37	38
	Subcortical white matter	None	21	31	11	10
	Subcortical white matter	CaCl ₂	14	26	36	38
Rabbit	Cortex	None	20	31	7	7
	Cortex	CaCl ₂	10	16	30	45
	Brain stem	None	27	39	3	11
	Brain stem	CaCl ₂	15	25	36	45
	Sciatic nerve	None	2	6	2	4
	Sciatic nerve	CaCl ₂	2	5	18	20
Monkey	Spinal cord	CaCl ₂	5	10	46	57
			20 min.	60 min.	20 min.	60 min.
Guinea pig	Liver	None	6	16	4	14
		CaCl ₂	5	10	31	44
	Kidney	None	17	28	0	0
		CaCl ₂	27	36	6	9
	Spleen	None	10	26	31	38
		CaCl ₂	8	16	45	50
	Skeletal muscle	None	4	7	1	0
		CaCl ₂				

Table 7. *Action of added compounds on breakdown of diphosphoinositide by brain preparations*

Whole brain (guinea pig) was prepared and its enzyme activity determined in the presence and absence of the compounds as described in Table 5; compounds were incorporated in the saline medium used for diluting the tissue suspension. Values quoted are (a) the initial rate of hydrolysis (μ moles of CO₂/g. of fresh tissue/hr.) in the presence of compound, expressed as a percentage of the rate in its absence, and (b) the total and inorganic P released in 30 min. similarly expressed.

Compound added to medium	Rate of hydrolysis (%)	Total acid-soluble P (%)	Inorganic P (%)
NaCN, 10 mM; 2:4-dinitrophenol, 10 mM; Na L-glutamate, 10 mM	100-123	100-120	83-104
Na iodoacetate, 10 mM	72	83	90
NaF, 10 mM	77	85	125
Glycerol <i>o</i> -tolyl ether (Myanesin), 6.6 mM, 13 mM; mescaline, 30 mM; chloral hydrate, 10 mM; diethylbarbitone, 10 mM; diallylbarbitone, 10 mM	89-108	77-125	78-101*
β -Diethylaminoethyl <i>p</i> -aminobenzoate, HCl (Procaine), 10 mM	130	138	160
Streptomycin, 2.5 mM, 10 mM; 4- <i>n</i> -hexylresorcinol, 2 mM; inositol, 3.3 mM	91-93	96-115	98-100
γ -Hexachlorocyclohexane (Gammexane), about 1 mM	83	63	82

* Values for Myanesin, mescaline and dial not available.

None of the compounds showed any marked action (Table 7); such changes as were observed were either within experimental error or too small to warrant further investigation considering their high concentrations in the medium.

DISCUSSION

Complexity of the reaction between 'inositol phosphatide' and cerebral tissues. This is illustrated

by its time course in media with and without Ca. In the presence of appropriate concentrations of Ca ions, the concentration of acid-soluble organically-combined P, inositol and H⁺ ions is increased by 80 %, whereas that of inorganic P is reduced by about 40 % (Fig. 1 and Table 3).

These observations tend to confirm the suggestion of Sloane-Stanley (1953) that there are at least two enzymes in brain concerned in the breakdown of

the diphosphoinositide in 'inositol phosphatide'; furthermore, they suggest that one of these enzymes is a phosphodiesterase releasing inositol monophosphate, and another a phosphomonoesterase releasing inorganic P. The two reactions appear to occur concurrently, since the present evidence renders it unlikely that the first product of the reaction is inositol metadiphosphate (which is a product of the acid hydrolysis of DPI: Folch, 1949*a*), from which another molecule of P is then split by a second enzyme. If this were the case, the reduction in the amount of inositol monophosphate released in the Ca-free medium should be accompanied by a corresponding reduction in inorganic P; in fact, in the absence of added Ca the inorganic P released is always greater in amount (in some experiments by as much as three molar equivalents) than the acid-soluble inositol.

Nature of calcium activation. The diphosphoinositide present in 'inositol phosphatide' exists as its practically insoluble Ca-Mg salt (Folch, 1942, 1949*a, b*), but it is not clear whether the particles of lipid suspended in the medium unite directly with the enzyme, or whether solution occurs first. Certainly the physical state of the substrate is likely to be an important factor influencing the reaction, and it is one which might well be altered by the ionic composition of the medium. Physiological concentrations of Ca are known to modify the interfacial tension in oil-water systems (Netter, 1953, for example).

The possibility cannot be excluded, therefore, that Ca activates the reaction by changing the physical properties of the suspended-lipid particles. If this were the case, however, other bivalent cations might be expected to have a similar action; in fact, although both Ca and Mg increase the turbidity of substrate suspensions only Ca has been found to activate the reaction.

The activation of certain enzymes by Ca is well known. Examples in cerebral tissues are thiamine pyrophosphatase and acid phosphomonoesterase (Pratt, 1954), although both these are also activated by Mg. Calcium has also been implicated in enzymes hydrolysing other lipids: its activation of the hydrolysis of neutral fats by lipases has long been known and has recently been re-examined (Schönheyder & Volquartz, 1945), as has its similar action on snake-venom lecithinase (Holden, 1934; Braganca & Quastel, 1953). Of possibly greater relevance to this work is the lecithinase 'C' of *Clostridium welchii* described by MacFarlane & Knight (1941). This is a phosphodiesterase, releasing phosphocholine, but not free P, from lecithin, and is maximally activated by Ca in concentrations similar to the optimum found for the reaction that splits DPI. Activation by Mg is said to be slight in comparison with the Ca effect.

Inhibition of the release of inorganic P appears to be a non-specific action of bivalent cations, being shown equally by Ca, Mg and Ba, and is presumably related to the increased turbidity of substrate suspensions in media containing these cations. The 5-nucleotidase of brain tissue is also inhibited by Ca and Mg to a similar extent (Pratt, 1954).

Breakdown of potassium diphosphoinositide. The faster reaction with the 'soluble' compound suggests that the rate-limiting factor with the crude substrate is the relative insolubility of the Ca-Mg salt of diphosphoinositide in the medium. The possible relevance of this observation to *in vivo* conditions cannot be judged at present; the Ca and Mg of the crude substrate appear native to it (Folch, 1942, 1949*a, b*).

Distribution of enzyme activity. The even distribution of the enzyme system in fairly grossly defined areas of the brain, particularly between white and grey matter, seems to exclude its special participation in highly localized events in the central nervous system. In non-nervous tissues the reaction has not been studied sufficiently to compare it in detail with that occurring in brain preparations. Information is also needed on the distribution and chemical nature of substrates for the enzyme system; inositol-containing lipids have been isolated from several tissues (Taylor & McKibbin, 1953), but their constitution has yet to be investigated.

SUMMARY

1. In a suitable medium the reaction between cerebral-tissue suspensions and the 'inositol phosphatide' fraction of brain kephalin results in the concurrent release of inositol monophosphate and smaller amounts of inorganic phosphate.
2. Inositol monophosphate release is activated by Ca salts, whilst inorganic phosphate release is slightly inhibited by Ca, Mg and Ba salts.
3. The purified potassium salt of diphosphoinositide is attacked by cerebral-tissue suspensions at a rate equivalent to 300 μ moles/g. fresh wt. of tissue/hr., which is at least six times as rapid as the reaction with 'inositol phosphatide'.
4. The enzyme system is present in suspensions prepared from the brains of the guinea pig, rat, rabbit, monkey, sheep and infant and adult humans. No great variations in activity have been found in gross anatomical regions of the central nervous system.
5. Suspensions of liver, spleen and kidney also attack 'inositol phosphatide'.
6. The reaction was not influenced significantly by several enzyme inhibitors, drugs and compounds structurally related to inositol.

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Techniques in Tissue Metabolism

4. APPARATUS FOR MAINTAINING AND RAPIDLY TRANSFERRING TISSUE SECTIONS*

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Enzymes of a given tissue can often cause the labile metabolites in that tissue to react completely in a minute or so. This speed of reaction is especially notable in excitable tissues, and in those of the central nervous system metabolites including lactic acid, phosphocreatine or acetylcholine can be doubled or halved in quantity in 2–10 sec.

Consequently, the study of such substances with preparations of isolated tissues has commonly been carried out under relatively artificial conditions in which the tissue is ground and diluted up to a 1000-fold, and quantities of substrate are added which are very great in comparison with those normally associated with a given quantity of tissue. While these conditions are often excellent for studying individual enzyme reactions, much additional information is usually required before such findings can be applied to the behaviour of the same substances in cell-containing tissues.

An alternative or complementary procedure, with which the present paper is concerned, is to examine events in cell-containing tissues over periods within the few seconds or minutes during which their native metabolites undergo reaction. The general

procedure has been to establish a steady state in the tissue, to apply a given agent, and to fix the tissue for analysis at brief intervals afterwards. The agents have been added chemicals or electrical pulses. Such arrangements have in the past been employed with intact organs as muscle or nerve, but rarely with tissue slices.

EXPERIMENTAL

Tissues and salines

From the guinea pig or larger animals large slices, 0.35 mm. thick, can readily be obtained from the liver, kidney cortex and brain (McIlwain, 1951). Such slices, up to 2 cm. × 1.5 cm. (100 mg.), were strong enough to be picked up on wires and drained of fluid for weighing and transferring. Slices rather larger than the size mentioned were floated from the blade or cutting plate into a shallow dish of the appropriate saline (Rodnight & McIlwain, 1954; Heald, 1954), and trimmed to size with a scalpel, picked out with the rider of a torsion balance and drained on a glass surface, weighed, and returned to the saline. A transfer-holder was opened, its lower jaw put in the saline, the slice was manoeuvred over it with a spatula and lifted from the saline with the holder. The jaw of the holder was then closed on the slice, and the holder and slice were placed into the beaker (Fig. 1) containing about 5 ml. of appropriate saline.

* Part 3: Rodnight & McIlwain (1954).