REFERENCES

- Aboud, L. G. & Gerard, R. W. (1952). *Amer. J. Physiol.* **168**, 739.
- Baer, E. & Fischer, H. O. L. (1949). *J. biol. Chem.* **180**, 145.
- Brody, T. M. & Bain, J. A. (1952). *J. biol. Chem.* **195**, 685.
- Coxon, R. V. (1952). *Symp. biochem. Soc.* **8**, 3.
- Coxon, R. V. & Peters, R. A. (1950). *Biochem. J.* **46**, 300.
- Cross, R. J., Taggart, J. V., Covo, G. A. & Green, D. E. (1949). *J. biol. Chem.* **177**, 655.
- Dickens, F. & Greville, G. D. (1935). *Biochem. J.* **29**, 1468.
- Elliott, K. A. C., Greig, M. E. & Benoy, M. P. (1937). *Biochem. J.* **31**, 1003.
- Furchgott, R. F. & Shorr, E. (1948). *J. biol. Chem.* **175**, 201.
- Gore, M. B. R. & McIlwain, H. (1952). *J. Physiol.* **117**, 471.
- Himwich, H. E. (1951). *Brain Metabolism and Cerebral Disorders*. Baltimore: Williams and Wilkins.
- Jowett, M. & Quastel, J. H. (1937). *Biochem. J.* **31**, 565.
- Kratzing, C. C. (1951). *Biochem. J.* **50**, 253.
- Kratzing, C. C. & Narayanaswami, A. (1953). *Biochem. J.* **54**, 317.
- Krebs, H. A. & Eggleston, L. V. (1938). *Biochem. J.* **32**, 913.
- Krebs, H. A. & Johnson, W. A. (1937). *Biochem. J.* **31**, 645.
- Lipsett, M. N. & Crescitelli, F. (1950). *Arch. Biochem.* **28**, 329.
- Long, C. (1938). *Biochem. J.* **32**, 1711.
- McIlwain, H. (1951a). *J. ment. Sci.* **97**, 674.
- McIlwain, H. (1951b). *Biochem. J.* **49**, 382.
- McIlwain, H. (1952a). *Biochem. J.* **52**, 289.
- McIlwain, H. (1952b). *Symp. biochem. Soc.* **8**, 27.
- McIlwain, H., Buchel, L. & Cheshire, J. D. (1951). *Biochem. J.* **48**, 12.
- McIlwain, H. & Gore, M. B. R. (1951). *Biochem. J.* **50**, 24.
- Quastel, J. H. (1939). *Physiol. Rev.* **19**, 135.
- Quastel, J. H. & Wheatley, A. H. M. (1931). *Biochem. J.* **25**, 117.
- Quastel, J. H. & Wheatley, A. H. M. (1932). *Biochem. J.* **26**, 725.
- Slater, E. C. (1953). *Biochem. J.* **53**, 157.
- Webb, J. L., Saunders, P. R. & Thienes, C. H. (1949). *Arch. Biochem.* **22**, 451.

The Enzymic Determination of Energy-rich Phosphates in Brain

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Existing methods of measuring various phosphate esters in animal tissues are open to improvement in two ways. They are not sufficiently specific for certain constituents and they require relatively large amounts of tissue to carry out a complete analysis. In order to follow phosphate metabolism in preparations of cerebral tissues such as the slices used metabolically, it has been necessary to adapt methods of Kerr (1935) and Stone (1943) to deal with minimal quantities of material (see McIlwain, Buchel & Cheshire, 1951). Moreover, when such methods are used, even with the refinements which have been introduced to adapt them for minimal quantities of material, one can obtain values only for inorganic phosphate, creatinephosphate and total easily hydrolysable phosphate. Thus it is not possible to follow changes in adenosinetriphosphate or adenosinediphosphate specifically. Recent findings of Kornberg (1950) indicate that changes in inorganic pyrophosphate during metabolism are more extensive than previously realized. Changes in easily hydrolysable phosphate, therefore, may not necessarily be a true measure even of total nucleotide phosphate.

The introduction of spectrophotometric methods of phosphate analysis, based on the enzymic formation of dihydroxyacetonephosphate and its reduction of reduced diphosphopyridine nucleotide (Racker, 1947; Slater, 1951), offered a means of following the metabolism of phosphates in small pieces of tissue with the advantage of a high degree of specificity. Since we were mainly interested in the changes in individual energy-rich phosphates (adenosinetriphosphate, adenosinediphosphate and creatinephosphate) under various metabolic conditions, we have adapted Slater's (1953) methods of phosphate determination to our particular needs for cerebral tissues and have compared analyses by this method with that of Stone (1943). Previous publications from these laboratories have reported on the ability of different substrates and metabolic conditions to support the resynthesis of creatinephosphate and other labile phosphates in respiring slices of mammalian cerebral cortex (McIlwain *et al.* 1951; McIlwain & Gore, 1951; McIlwain, 1951, 1952a). It was of interest to know whether similar changes took place in the adenosinepolyphosphates of the slices, and whether there was an interrelation between changes in creatinephosphate and changes in the adenosinepolyphosphates. As the phospho-creatine and adenosinepolyphosphate content of

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such slices maintained under various metabolic conditions had been determined by the calcium-ethanol separation method before the enzymic method was adapted, results with this method also are included here.

METHODS

Abbreviations. ATP, adenosinetriphosphate; ADP, adenosinediphosphate; CP, creatinephosphate; HMP, hexosemonophosphate; ~P, energy-rich phosphate; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide.

Principles of analysis. Slater's (1953) procedure *B(b)* measures the total ~P content of an extract. The energy-rich phosphate compounds which react are ATP, ADP, CP and phosphopyruvate as well as phosphoglycerate (potentially an energy-rich compound). Since the phosphopyruvate and phosphoglycerate content of cerebral tissues is usually low (LePage, 1949; Stone, 1943), it can be assumed that $\sim P = 2ATP + ADP + CP$. Slater's procedure *C* measured ATP, so that from the two measurements it is possible to determine ADP + CP. We wished to measure ADP and CP separately. This was done by modifying Slater's procedure *C* by the addition of myokinase, so that the total HMP formed equalled $2ATP + ADP$, according to the following reactions: (1) $2ATP + 2 \text{ glucose} \rightarrow 2ADP + 2HMP$; (2) $2ADP \rightarrow ATP + \text{adenylic acid}$.

The possible incompleteness of reaction (2), due to inhibition by adenylic acid (Colowick & Kalckar, 1943) was overcome by the addition of excess enzyme.

In order to correct for phosphorylated sugars and other possible interfering substances (e.g. pyruvate and oxaloacetate) in extracts, these substances were determined in a separate sample of extract ('blank') and the correction applied to the ATP and ATP + ADP determinations. In experiments with a pure solution of phosphoglycerate, it was found that phosphoglycerate was quantitatively estimated by the 'blank' procedure. In the case of ~P determinations it was realized that hexosediphosphate, triosephosphate, pyruvate, and oxaloacetate would be measured as well as the energy-rich phosphate compounds, but all these compounds would be determined in the 'blank', together with hexosemonophosphates. As the extracts contained little phosphorylated sugar, we have assumed all reactive material to be monophosphorylated sugar and have applied no correction to the ~P determinations. The earlier work of LePage (1946) suggested that the total monophosphorylated sugar content of brain was much greater than the sum of the hexosediphosphate and triosephosphate.

Salines and conditions of metabolism. Glycylglycine saline was used for all the metabolic experiments. The composition was the same as that described by McIlwain *et al.* (1951). Glucose was used at 0.012M and glutamate at 0.02M. Usually 80–120 mg. slices and 3 ml. of medium were contained in each vessel. The gas phase was O_2 in all the manometric experiments where aerobic conditions were used. For anaerobic experiments the gas phase was 95% N_2 and 5% CO_2 . When 2:4-dinitrophenol was used in the experiments, the appropriate amount was added to the medium before slices were exposed to it. For measurement of phosphates after cutting, the slices were kept in the medium at room temperature until they were dropped into the deproteinizing solution. This was done within 30 min. of the death of the animal.

Preparation of extracts

Extracts from slices of guinea-pig cerebral cortex. Slices from the outer grey matter of guinea pig cerebral hemispheres were cut and weighed as described by McIlwain *et al.* (1951). The following procedure was adopted for preparation of extracts whether or not the slices had been used for manometric studies. Two slices of cerebral cortex (total 0.2–0.3 g.) with any adhering saline were dropped into 1 ml. ice-cold 19% (w/v) $HClO_4$ in a tared Potter-Elvehjem homogenizer, promptly disintegrated and reweighed. After standing for a few minutes in ice, the contents of the homogenizer tube were centrifuged at high speed in a refrigerated centrifuge and the largest possible quantity of supernatant removed, measured and neutralized to approx. pH 7.6 with a measured quantity of 5N-KOH, using phenol red as an external indicator. The addition of a small volume of glycylglycine buffer, pH 7.6, appreciably helped the final adjustment of pH. The neutralized extracts were stored at -20° until analysed. All analyses were based on the initial fresh weight of the tissue. For analyses by the Ca-ethanol method, two slices of cortex were similarly added to tared homogenizer tubes containing 3.0 ml. ice-cold 10% (w/v) trichloroacetic acid, reweighed and the contents disintegrated and centrifuged in the cold after a few minutes standing in ice. The maximum practicable volume of the supernatant was removed, measured and subjected to the Ca-ethanol procedure.

Extracts from rapidly frozen brain. Young guinea pigs, weighing 80–100 g., were dropped head first into about 500 ml. liquid air in a wide-mouthed thermos flask. When the vigorous boiling which this produced had ceased, the animal was judged to have reached the temperature of liquid air. It was removed from the flask, the head scraped to remove the fur and the skull opened by a medial split with a $\frac{3}{8}$ in. chisel cooled in liquid air. The skull was prised open to expose the cortex from which pieces were chipped. During this operation the tissue was immersed in liquid air every few seconds. The chips of brain (mainly cortex) were either added to ice-cold 19% (w/v) $HClO_4$ and disintegrated as already described or ground to a fine powder under liquid N_2 in a Wedgewood mortar and approx. 200–300 mg. powder added to each homogenizer tube. In one experiment, the animals were guillotined; the heads were immersed in liquid air within 1 sec. of decapitation. The frozen brain was then removed as before.

Enzyme preparations

Myokinase was prepared by the method of Colowick & Kalckar (1943). The material precipitated by 80% saturation with $(NH_4)_2SO_4$ was redissolved and stored in the frozen state. It was diluted 1:10 before use. Other enzyme preparations and DPNH were as described by Slater (1953). The activity of the enzymes was of the order obtained by Slater. We are grateful to Dr B. A. Askonas for the gift of creatinephosphokinase used in this work.

Method of preliminary incubation for determination of ATP and ADP

The method of incubation for ATP was similar to that described by Slater (1953) except that the 0.15 ml. 0.25M-NaF was replaced by 0.15 ml. 0.043M-ammonium molybdate. In experiments undertaken to compare fluoride and

molybdate as inhibitors of phosphatases in this incubation, it was found that molybdate was as efficient as fluoride for this purpose. With the hexokinase preparation used, 2 min. at room temperature was found to be an adequate incubation time after which 0.32 ml. 38% (w/v) HClO_4 was added to deproteinize the solution. After centrifuging, the supernatant was neutralized with 5N-KOH. HClO_4 was considered preferable to trichloroacetic acid because, after deproteinization, the former could be nearly completely removed as the K salt. It was important to keep these neutralized extracts ice-cold while samples were being taken for spectrophotometric measurements.

ADP was converted to HMP under the same conditions as those used for ATP. In addition to hexokinase, the incubation mixture contained 0.05 ml. of dilute myokinase, prepared from the stock solution by diluting 0.1 ml. to 1.0 ml. Incubation for 4 min. was found necessary for complete reaction.

Spectrophotometric measurements

Changes in the optical density, caused by reaction of DPNH with the dihydroxyacetonephosphate eventually formed, were measured in a Beckman model DU quartz spectrophotometer as described by Slater (1953). For the determination of $\sim\text{P}$ in the extract, 0.1 ml. creatinephosphokinase (0.2 mg./ml.) was added to the spectrophotometer cells in addition to the reagents described in Slater's procedure *B(b)*.

Chemical determination of tissue phosphates

Inorganic phosphate, CP, and adenosinepolyphosphate were determined in both slices and frozen tissue by Ca-ethanol separation followed by colorimetric determination of the inorganic phosphate according to the method of Stone (1943). In the case of extracts from slices, hydrolysis of the Ca-insoluble fraction in N-HCl was carried out on separate samples for 10 min. and 30 min. The former gave the value for adenosinepolyphosphate and the latter an indication of other hydrolysable substances present in the fraction. All volumes recommended by Stone were reduced to one-eighth so as to adapt the method to the smaller quantity of tissue available. For comparison of the present method with that of Stone, a pooled HClO_4 extract was prepared from powdered frozen brain and neutralized with KOH as already

described. Before separation was begun, trichloroacetic acid was added to the neutralized extract in the concentration used by Stone.

RESULTS

Analysis of various phosphate compounds

To test the efficiency of the myokinase modification for separate measurement of the adenosinepolyphosphates, it was applied to a laboratory preparation of ATP of high purity and also to a preparation expected to consist largely of ADP (Table 1).

When both hexokinase and myokinase were used for the preliminary incubation, twice as much HMP was formed as on incubation with hexokinase alone. The value for $2\text{ATP} + \text{ADP}$ agreed closely with the $\sim\text{P}$ value obtained by Slater's procedure *B(b)*. It has been reported by Slater (1953) that most laboratory preparations of ATP contain insignificant amounts of ADP, and the results of this analysis therefore indicate that the second phosphate group of ATP is being estimated quantitatively by this method.

The ADP preparation had been obtained from ATP by Bielschowsky's (1950) method. Again the total adenosinepolyphosphate value obtained by Slater's procedure *B(b)* and by the myokinase procedure were in close agreement and both led to ADP values also in close agreement. The ATP content was higher than expected, an observation also reported by Slater (1953) for ADP prepared by this method.

Recovery experiments were carried out using a synthetic sample of sodium creatinephosphate. The sample was free from inorganic phosphate and contained 8.0% total phosphorus (calc. for the hexahydrate, 8.5%). Determination of CP after calcium ethanol separation gave 7.95% CP phosphorus. The CP was determined as the increase in the $\sim\text{P}$ content of a solution of either ATP or ADP on addition of CP and creatinephosphokinase. The

Table 1. *Comparison of methods of analysis*

(Analytical methods are described in the text. The ATP was a laboratory preparation received from Dr E. C. Slater. The ADP was prepared from commercial ATP by Bielschowsky's (1950) method. The CP, a laboratory preparation, was dissolved to give two solutions (a) and (b).)

Preparation	Values obtained by chemical determination of acid-labile P ($\mu\text{moles/ml.}$)	Values by enzymic method ($\mu\text{moles/ml.}$)					
		$\sim\text{P}$	CP	ATP	2 ATP + ADP	ADP	
						2 ATP + ADP - 2 ATP	$\sim\text{P} - 2\text{ATP}$
ATP	—	46.0	—	22.6	47.1	1.9	0.8
ATP	—	44.8	—	22.4	45.2	0.4	0.0
ADP	7.33	6.16	—	1.97	6.20	2.26	2.22
ADP	—	6.26	—	1.97	6.26	2.32	2.32
CP (a)	4.25	—	4.06	—	—	—	—
CP (a)	4.25	—	4.10	—	—	—	—
CP (b)	17.37	—	15.50	—	—	—	—
CP (b)	17.37	—	16.10	—	—	—	—

Table 2. *Comparison of methods of analysis for determining the phosphate content of frozen brain*

(Methods of determination are described in the text. Pooled extracts from the same young guinea pigs (80–100 g.) were used for comparison of the two methods.)

Method of killing	Enzymic method (μ moles/g. fresh tissue)					Ca-ethanol separation (μ moles/g. fresh tissue)				
	$\sim$ P	CP	ATP	ADP	'Blank'	Sum of CP + adenosine-polyphosphate	CP	Adenosine-polyphosphate	Inorganic P	Total P
Whole animal dropped into liquid air (A)	8.41, 8.56, 8.66, 8.58	2.14	3.28, 3.18	0.0, 0.0	0.26	10.06	2.66, 2.74	7.35, 7.19	3.58, 3.67	26.7
Guillotined; head dropped into liquid air (B)	8.30, 8.16, 7.82, 8.09	1.96	3.10, 3.04	0.0, 0.0	0.33	10.36	1.75, 1.85	8.56, —	5.04, 4.91	29.4

recoveries obtained under these conditions agreed well with the amounts expected from the phosphorus content of the material. With the enzymes at our disposal Slater's procedure *C*, with the inclusion of creatinephosphokinase in the incubation mixture, proved unsatisfactory; only 50–60 % of the CP could be accounted for as increased HMP even after prolonged incubation. The direct determination of CP would have been preferred to that of calculation by difference, but in view of the poor recoveries obtained, the latter method was used in all further experiments.

The phosphate content of frozen cerebral tissue

In order to check in biological material the results obtained by these methods of analysing phosphates, extracts of rapidly frozen brain (see Methods) were prepared and analysed both enzymically and by the calcium-ethanol separation of Stone (1943). Results are given in Table 2.

Values for the CP content obtained by the two methods are of the same order for both extracts. The value for CP as determined by the chemical method on extract *A* are comparable with those obtained by McIlwain *et al.* (1951) for guinea-pig brain cortex. However, only 80 % of this value was measured enzymically. In extract *B*, the enzymic CP determination gave a slightly higher value than the chemical method. There was no detectable amount of ADP in either extract. The sum of the CP and adenosinepolyphosphate obtained by the chemical method is 1.5–2.2 μ moles/g. higher than the $\sim$ P value determined enzymically. This difference may possibly be due to the presence of substances like inorganic pyrophosphate which are separated and determined in the adenosinepolyphosphate fraction but are not measured by the more specific enzymic method.

The 'blank' value reported in Table 2 is a measure of the total hexosemono- and di-phosphates, triose-phosphates and phosphoglyceric acid as well as of

pyruvate and oxaloacetate. By independent analysis using lactic dehydrogenase, only barely detectable traces of pyruvate were found. The constituents of this fraction were not further differentiated. These values are lower than those previously reported by Stone (1943), LePage (1946) and Dawson & Richter (1950).

The values for brain constituents obtained by Kerr (1935) and Dawson & Richter (1950) indicate that freezing the whole animal causes less stimulatory activity in the animal than other methods of killing, such as decapitation. The present findings (Table 2) are in agreement with these observations.

Phosphates of cerebral cortex slices

The results of several experiments in which the phosphates of cerebral cortex were measured are given in Tables 3 and 4. The resynthesis of creatinephosphate reported to occur after respiration in glucose-containing saline (McIlwain *et al.* 1951) has been confirmed by both methods of analysis. While the level of CP increased two- to three-fold and that of inorganic phosphate decreased significantly, there was a far smaller increase in the ATP content and a fall in the 'blank' value. No significant amounts of ADP could be found in slices either before or after respiration in glucose saline. It was of interest, however, to find that in the presence of 0.05 mm-2,4-dinitrophenol, simultaneously with a failure to resynthesize CP and a fall in ATP content, significant amounts of ADP were formed. The same observation was made with slices respiring in glutamate saline, but phosphate values obtained on extracts from them using the enzymic method tended to vary widely and are not reported here. Due to the presence of glutamate or some metabolic product formed from glutamate in these extracts, the enzymic method of determining phosphates was not entirely satisfactory. The oxidation of DPNH normally continues to take place at a slow rate throughout the period of spectrophotometric

Table 3. *Phosphate content of cerebral cortex slices by enzymic method*

(Slices were cut in glycylglycine saline with 0.012M-glucose and incubation, when carried out, was in the same medium for 60 min. Mean values are given $\pm$ standard error; number of determinations are given in parentheses.)

Respiration (μ moles/g./hr.) (Slices after cutting, not incubated)	2:4-Dinitrophenol in medium (mm)	Phosphates (μ moles/g.)				
		$\sim$ P	CP	ATP	ADP	'Blank'
	0	2.15 $\pm$ 0.07 (3)	0.68 $\pm$ 0.13 (3)	0.73 $\pm$ 0.17 (3)	0.00 $\pm$ 0.02	0.35 $\pm$ 0.02 (3)
55.5 $\pm$ 1.5	0	3.12 $\pm$ 0.14 (12)	1.12 $\pm$ 0.04 (6)	1.01 $\pm$ 0.09 (5)	0.01 $\pm$ 0.04 (4)	0.23 $\pm$ 0.02 (5)
107.5 $\pm$ 6.5	0.05	1.52 (2)	0.18 (2)	0.57 (2)	0.19 (2)	0.16 (2)

Table 4. *Phosphate content of cerebral cortex slices by Ca-ethanol separation*

(Respiration was for 60 min. in glycylglycine medium with 0.012M-glucose or 0.02M-glutamate and anaerobic glycolysis for 60 min. in bicarbonate saline with 0.012M-glucose. Methods of determination and experimental details are given in text. Inorganic phosphate values (as μ moles/g. wet weight of tissue) and the standard error are given.)

Experimental conditions			Phosphates (μ moles/g.)				
Substrate	2:4-Dini- trophenol (mm)	Respiration or glycolysis (μ moles/g./hr.)	Inorganic P	CP	Adenosine- polyphosphate (10 min. hydrolysis)	Phosphate liberated on 30 min. hydrolysis	Adenosine- poly- phosphate + CP
Glucose	0	(After cutting, not incubated)	7.15 $\pm$ 0.54 (4)	0.45 $\pm$ 0.12 (3)	1.79 $\pm$ 0.09 (4)	2.59 $\pm$ 0.19 (3)	2.24
	0	Respiration, 62.8 $\pm$ 0.1 (12)	4.92 $\pm$ 0.39 (6)	1.19 $\pm$ 0.09 (4)	2.18 $\pm$ 0.09 (6)	2.47 $\pm$ 0.05 (6)	3.37
	0.05	Respiration, 118 $\pm$ 5.4 (8)	4.07 $\pm$ 0.13 (4)	0.33 (2)	1.85 $\pm$ 0.19 (4)	—	2.18
	0.20	Respiration, 56 $\pm$ 5.7 (8)	5.34 $\pm$ 0.14 (4)	0.35 $\pm$ 0.09 (4)	0.73 $\pm$ 0.06 (4)	—	1.08
	0	Anaerobic glycolysis, 40 $\pm$ 1.3 (8)	6.38 $\pm$ 0.40 (4)	0.24 (2)	1.05 $\pm$ 0.33 (4)	1.53 $\pm$ 0.29 (4)	1.29
Glutamate	0	(After cutting, not incubated)	10.12 $\pm$ 0.64 (3)	0.35	1.27 $\pm$ 0.14 (3)	2.10 (2)	1.62
	0	Respiration, 65.9 $\pm$ 1.5 (10)	7.63 $\pm$ 0.20 (5)	0.30 $\pm$ 0.09 (4)	1.06 $\pm$ 0.07 (5)	1.42 $\pm$ 0.15 (5)	1.36

measurement even after the dihydroxyacetone-phosphate formed has completely reacted. With extracts from slices maintained in glutamate saline it was found that the rate of this extraneous oxidation of DPNH was considerably faster, so that the extrapolation to zero time would give rise to greater error than in the case of other extracts.

The CP values determined by both methods of analysis were consistent and were in close agreement with those reported previously (McIlwain *et al.* 1951; McIlwain & Gore, 1951). Again the sum of the ATP and ADP values could not wholly account for the adenosinepolyphosphate value obtained by the chemical method. Consequently, the sum of the CP and adenosinepolyphosphate values obtained by the chemical method was greater than the corresponding $\sim$ P.

It would seem from Table 4 that the adenosinepolyphosphate concentration is maintained at a steadier level than that of CP under adverse condi-

tions. In the presence of 0.05 mm-2:4-dinitrophenol, the CP of the slice is not maintained, but there is only a small decrease in the adenosinepolyphosphate. At a higher concentration of the dinitrophenol (0.2 mm), when the stimulatory effect on respiration disappears, the adenosinepolyphosphate level falls by more than 50 %. Significant decreases in adenosinepolyphosphate are observed also in slices maintained under anaerobic conditions in glucose saline and in slices respiring in the presence of glutamate as substrate. Slices tend to lose adenosinepolyphosphate even in the first few minutes after cutting if they are kept in a medium containing glutamate instead of glucose. The values for phosphate liberated on hydrolysis (30 min. in N-HCl) of the calcium-precipitable fraction deserve comment. These 30 min. values tend to be considerably higher than the adenosinepolyphosphate values. The difference between the 10 and 30 min. values may be considered to be a

measure of some phosphate compound other than ATP or ADP which is present in the calcium-precipitable fraction and the variations in its level are interesting. The content of this phosphate compound seems to increase when the adenosine-polyposphate content decreases, as in slices after cutting, and resynthesis of adenosinepolyposphate is accompanied by a fall in the level of this compound. From the work of Stone (1943), the hexose-monophosphates may be expected to be present in this fraction, as well as hexosediphosphate. Glucose-6-phosphate, fructose-6-phosphate and hexosediphosphate are all known to release varying proportions of their phosphate on prolonged hydrolysis under the conditions used in this study (LePage, 1949), but since the maximum upper limit for their total concentration in brain slices is the 'blank' value obtained in the enzymic determinations, which is quite low, the much larger differences between the 10 and 30 min. values cannot be entirely accounted for by the presence of hexose-mono- and di-phosphates.

DISCUSSION

Because of the known specificity of the hexokinase and myokinase reactions, it may be expected that the enzymic method described in this paper will provide a truer measure of ATP and ADP than methods involving chemical separation followed by hydrolysis of the labile phosphate group. Slater (1953) has assessed the sensitivity of these enzymes in relation to the accuracy of the enzymic determinations based on them. The measurement of CP by the method described here is liable to a greater degree of error since it involves two separate measurements and because of the possible presence of interfering substances for which no correction could be applied. That the error caused by these factors is negligible in cerebral preparations is shown by the agreement between CP values obtained by this method and those obtained by chemical methods. The criticism of the chemical method on the basis of non-specificity for adenosine-polyposphate is less serious in the case of CP. Since CP appears to be the only substrate in mammalian tissue extracts with a soluble calcium salt and extreme instability in acid molybdate, the chemical method is adequate for its estimation.

Similar considerations of nonspecificity apply also to the measurement of the individual hexose-phosphates by methods of chemical separation and differential hydrolysis. While the present method does not distinguish between these compounds, it does give an upper limit for their total content in tissue and gives values considerably lower than those previously reported.

In various circumstances enzymic methods for the determination of ATP were previously reported

to give lower values than those obtained by acid hydrolysis (see Bailey, 1949; Kornberg, 1950; Rowles & Stocken, 1950). Consequently, calculations of the distribution of ATP and ADP based on acid-hydrolysable phosphate and total adenine or ribose are subject to considerable error. The errors due to the presence of non-nucleotide phosphates are magnified in the results of such calculations. This is borne out by our finding that in rapidly fixed brain and in cerebral slices kept under conditions capable of supporting phosphate resynthesis, there was no significant amount of ADP. Previous workers using chemical methods of separation have reported considerable quantities of ADP (up to $1.1 \mu\text{moles/g.}$) in rapidly fixed brain (LePage, 1946; Klein & Olsen, 1947; Bain & Klein, 1949). Klein & Olsen (1947) have found changes in the ADP content of brain in varying physiological conditions. Attempts to interpret changes in ADP by an equilibrium constant of creatinephosphokinase as do these authors, do not seem to be justified in view of the unreliability of methods of measuring the nucleotides. Our results with slices also do not support the idea that there is a direct relation between changes in CP, ADP and ATP. While the reserves of CP are rapidly depleted under unfavourable metabolic conditions, total adenosinepolyposphate does not decrease to the same relative extent under the same conditions.

The changes observed in the labile phosphates of cerebral cortex slices respiring under various conditions are consistent with the dependence of the brain *in vivo* on a steady supply of oxygen and glucose for normal functional activity. The sequence of changes in phosphocreatine and the adenosine-polyposphates follow a significant pattern. Both with 0.05 mm-2:4-dinitrophenol, and in slices kept at room temperature, it is the phosphocreatine that disappears first. Once the phosphocreatine is lost, ATP tends to fall and concomitantly with this ADP appears in detectable amounts in the tissue. This supports the opinion that the order of utilization of the phosphates on initiation of metabolic activity may be as follows: ATP is first utilized but, under normal conditions, the resulting ADP is rapidly rephosphorylated to ATP by the creatine-phosphokinase system utilizing the phosphocreatine reserves of the tissue. When the phosphocreatine concentration falls below a minimum level, this mechanism is no longer effective, and any further utilization of ATP results in a decrease in the total adenosine-polyposphate content, and the appearance of ADP in the tissue.

These changes in tissue phosphates may be compared with concomitant changes in tissue respiration. Comparison previously made by McIlwain & Gore (1951) and McIlwain (1952*b*) suggested respiration (and glycolysis) to be conditioned in

part by the levels of inorganic phosphate and of phosphate acceptors in the tissue. The present results with 0.05 mM-2:4-dinitrophenol show increased respiration with little change in any component measured other than creatinephosphate. Creatine may therefore be important in such control. 0.2 mM-2:4-Dinitrophenol may inhibit respiration by its great reduction in energy-rich phosphates, possibly to a level too low for phosphorylation of glucose. Inorganic phosphate, as previously concluded (McIlwain, 1952b), cannot alone control respiratory level.

SUMMARY

1. The spectrophotometric method of phosphate analysis developed by Slater (1953) has been adapted for the determination of energy-rich phosphates in mammalian cerebral tissues. It is based on the enzymic formation of dihydroxyacetonephosphate and its enzymic reduction by reduced diphosphopyridine nucleotide.

2. Assays by this method gave values for creatinephosphate which were in close agreement with those obtained by a chemical method.

3. The adenosinediphosphate and adenosinetriphosphate content of cerebral preparations determined enzymically were less than those determined by the chemical method. Only traces of adenosinediphosphate could be found in cerebral cortex *in vivo* or in slices respiring in a glucose-containing saline.

4. Creatinephosphate, adenosinepolyphosphate and inorganic phosphate have been measured in cerebral cortex slices maintained under a variety of metabolic conditions.

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REFERENCES

- Bailey, K. (1949). *Biochem. J.* **45**, 479.
 Bain, J. A. & Klein, J. R. (1949). *Amer. J. Physiol.* **158**, 478.
 Bielschowsky, M. (1950). *Biochem. J.* **47**, 105.
 Colowick, S. P. & Kalokar, H. M. (1943). *J. biol. Chem.* **148**, 117.
 Dawson, R. M. C. & Richter, D. (1950). *Amer. J. Physiol.* **160**, 203.
 Kerr, S. E. (1935). *J. biol. Chem.* **110**, 625.
 Klein, J. R. & Olsen, N. S. (1947). *J. biol. Chem.* **167**, 747.
 Kornberg, A. (1950). *J. biol. Chem.* **182**, 779.
 LePage, G. A. (1946). *Amer. J. Physiol.* **146**, 267.
 LePage, G. A. (1949). In *Manometric Techniques and Tissue Metabolism*, ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F., 2nd ed. p. 185. Minneapolis: Burgess.
 McIlwain, H. (1951). *J. ment. Sci.* **97**, 674.
 McIlwain, H. (1952a). *Biochem. J.* **52**, 289.
 McIlwain, H. (1952b). *Symp. biochem. Soc.* **8**, 27.
 McIlwain, H., Buchel, L. & Cheshire, J. D. (1951). *Biochem. J.* **48**, 12.
 McIlwain, H. & Gore, M. B. R. (1951). *Biochem. J.* **50**, 24.
 Racker, E. (1947). *J. biol. Chem.* **167**, 843.
 Rowles, S. L. & Stocken, L. A. (1950). *Biochem. J.* **47**, 489.
 Slater, E. C. (1951). *Biochem. J.* **50**, vii.
 Slater, E. C. (1953). *Biochem. J.* **53**, 157.
 Stone, W. E. (1943). *J. biol. Chem.* **149**, 29.

On the Isolation and Properties of a Citrate Oxidation Factor from Yeast

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Recent experiments (Foulkes, 1951a, b) provided evidence for two distinct pathways of metabolism in cell-free preparations from baker's yeast. Pathway 1 is closely related to the tricarboxylic acid cycle normally found in mammalian tissues; all the citrate metabolized under suitable conditions by dialysed, unfortified preparations, can be accounted

for by the formation of α -ketoglutaric acid. The addition to such dialysed preparations of magnesium, adenosinetriphosphate (ATP) and a heat-stable organic fraction from yeast or various vertebrate tissues causes a large increase in the amount of citrate metabolized. The heat-stable factor involved in this reaction has been called the citrate oxidation factor (COF) (Foulkes, 1951b). The reaction or reactions catalysed by COF will be referred to as Pathway 2.

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