PRECURSORS IN THE METABOLISM OF PHOSPHOPROTEINS IN CEREBRAL TISSUE

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SUMMARY

Dispersions of cerebral tissues carrying out oxidative phosphorylation at 37.5° have been used to study precursors in the phosphorylation of intrinsic phosphoproteins of cerebral tissue. It has been shown that ATP is a more immediate precursor than is inorganic phosphate and that GTP is a more immediate precursor than is ATP.

INTRODUCTION

When slices of cerebral tissues are stimulated by electrical pulses, applied for a few seconds only, rapid changes take place in the metabolism of various phosphate derivatives. Levels of phosphocreatine fall, those of inorganic phosphate rise while levels of adenosine triphosphate remain constant (see HEALD[1] for relevant literature). If the slices have been incubated in saline containing radioactive phosphorus, the changes are accompanied by equally marked alterations in the specific radioactivities of these and certain other phosphates. Thus, the specific radioactivities of phosphocreatine, adenosine triphosphate and inorganic phosphate were found to fall while those of the phosphoprotein fraction and guanosine triphosphate increased[2-4]. These changes have been interpreted as indicating that the phosphorylation and dephosphorylation of the above substances are part of a chain of reactions linking the metabolic response of nervous tissue to the changes caused by electrical stimuli and schemes illustrating possible relationships have been described[1,2,5]. Although the connections between the metabolism of phosphocreatine and adenosine triphosphate have been reasonably well delineated in brain[6-8], connections between the metabolism of the various nucleotides are still partly obscure and the finding that guanosine triphosphate was involved in the metabolic response of the tissue to electrical stimulus raised the question as to the position occupied by this nucleotide in the proposed sequence of changes. Thus, it was not clear whether guanosine triphosphate acted as an intermediate transferring phosphate from phosphoprotein to an unknown compound. The possibility of guanosine triphosphate being an intermediate in the phosphorylation of adenosine diphosphate to the triphosphate had been discounted, on other grounds, as being a major process[4].

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McMURRAY *et al.*[9] described a technique enabling some choice to be made as to which of two possible precursors A or B is the closer to the product C. Radioactive A is added to a system containing inactive B and radioactive B to a system containing inactive A, inactive C being present in each system. At the end of the incubation the specific activities of A, B and C are determined and the specific activity of C relative to A is calculated. If B is converted to A before forming C then the relative specific activity of C in both experiments would be equal. If B is a more likely immediate precursor than A, then the relative specific activity of C should be higher than in the case where A is the precursor. The advantage of this technique appears to be that experiments can be performed with dispersions of whole tissue and do not require the use of purified systems. Since purified preparations of cerebral phosphoprotein were not available, it was considered appropriate to examine by the above technique the problem of the position of guanosine triphosphate in the sequence of phosphorylations involving the phosphoprotein. Although cerebral tissues contain at least two different phosphoproteins[10] previous experiments leading to the implication of guanosine triphosphate in their metabolism had been carried out before such information was available. In the following experiments therefore no attempt was made to distinguish between them.

METHODS

Tissue preparations

Guinea pigs were killed by a blow on the neck, exsanguinated, and the brain rapidly removed. The cortical tissue was dispersed by grinding with a plastic pestle in 5 volumes ice cold aqueous sucrose (0.25 *M*) containing EDTA ($5 \cdot 10^{-4}$ *M*) at pH 7.4. Slices were prepared as described by McILWAIN[11].

Medium

Dispersions were incubated in a medium similar to that described by McMURRAY *et al.*[12] and contained adenosine triphosphate, 2.5 m*M*; $MgCl_2$, 8 m*M*; pyruvate, 13 m*M*; fumarate, 1.6 m*M*; KCl, 50 m*M*; diphosphopyridine nucleotide, 0.5 m*M*; cytochrome *c*, $8.3 \cdot 10^{-6}$ *M*; phosphate buffer (pH 7.4) 0.01 *M*; and Tris, 50 m*M*. When required, sodium fluoride, 12 m*M*; glucose, 2.8 m*M*; and yeast hexokinase (200 units) were included. Dispersions were incubated in a gas phase of air. Slices were incubated under an atmosphere of oxygen in a medium described previously[13]. Anaerobic experiments were conducted under an atmosphere of nitrogen. A stick of yellow phosphorus was placed in the centre well of the Warburg vessels to remove traces of oxygen.

Preparation of radioactive intermediates

Radioactive adenosine and guanosine triphosphates were prepared by incubating adenosine triphosphate (2.5 m*M*) and adenosine triphosphate + guanosine triphosphate (both at 2.5 m*M*) with dispersions made as above in the medium containing added fluoride and radioactive, carrier free, orthophosphate. Usually each Warburg vessel contained 100 μC ^{32}P. After 30 min incubation the reaction was stopped by the addition of trichloroacetic acid to a concentration of 10% (w/v) and, after cooling, the protein was removed by centrifuging. The supernatants were made alkaline to phenolphthalein with KOH and the nucleotides were precipitated as the barium salts[14]. Generally with ten vessels containing a total of 35 ml of solution, 7 ml of

barium acetate (25% w/v) were required. After standing 2–3 h at +2° the barium salts were collected, washed with ice cold water, and decomposed by shaking in water with Amberlite-IR 120 H^+. The clear solution was made 1 *M* with respect to ammonia and the nucleotides were separated on a column of Dowex-1 by a procedure essentially similar to that of AYENGAR, GIBSON, PENG AND SANADI[15]. The column (17 × 1 cm) contained 7.5 g of resin (8% cross-linked, 200–400 mesh). A preliminary wash of 900 ml 0.04 *M* NaCl in 0.01 *N* HCl, was given to remove inorganic phosphate and mono- and dinucleotides, followed by 0.08 *M* NaCl in 0.01 *N* HCl to remove adenosine triphosphate. When this had been eluted, guanosine triphosphate, if required, was eluted with 0.25 *M* NaCl in 0.01 *N* HCl. If guanosine triphosphate was not added to the original incubation medium adenosine triphosphate was usually eluted with the higher concentration of acidified saline. The positions of the nucleotides in the eluates were checked by absorption at 260 mμ and each fraction of the eluent was made alkaline to phenolphthalein with dilute KOH. The nucleotides were precipitated as the barium salts, decomposed with Amberlite-IR·120 H^+ and, after making the solution alkaline with dilute ammonia, were obtained as the solid ammonium salts by freeze drying. When radioactive adenosine triphosphate was prepared by this method the yield was some 70% of the amount originally added. When both guanosine and adenosine triphosphates were prepared simultaneously the yields were reduced to 30–60% of the amounts added to the medium. In these experiments the specific radioactivity of guanosine triphosphate was approx. 50% of that of adenosine triphosphate. Attempts to prepare labelled guanosine triphosphate from guanosine monophosphate, either in the system described above or with a rat-liver cytoplasmic system[16] were unsuccessful. The identity of the nucleotides was checked by electrophoresis at pH 3.5, chromatography on paper in propanol–ammonia[4] and by hydrolysis in 1 *N* HCl to release the purine bases followed by chromatography in propanol–HCl[17].

Analytical methods

Inorganic phosphate: This was estimated by the method of BERENBLUM AND CHAIN[18] as modified for use in a single tube by LONG[19]. The final blue solutions were also used for the determination of radioactivity by means of an M-6-H liquid counter (20th Century Electronics Ltd.).

Acid labile phosphorus: This was determined as inorganic phosphate after hydrolysis of the nucleotide with 1 *N* HCl at 100° for 10 min.

Phosphoprotein: Radioactivity incorporated into phosphoprotein was determined after hydrolysis of the phosphoprotein fraction and separation of O-phosphorylserine by methods previously described[20].

Chemicals

Adenosine triphosphate and guanosine triphosphate were obtained as the sodium salts from the Sigma Chemical Co. Ltd.

DL-O-phosphorylserine was obtained from the California Foundation for Biochemical Research.

Radioactive phosphate was obtained as carrier free orthophosphoric acid from the Radiochemical Centre (Amersham, Bucks, England). It was treated as previously described[2] before use.

RESULTS

Respiration and phosphorylation in the dispersions

Dispersions prepared as described respired linearly at rates between 100–120 μmoles O_2/g wet wt. tissue/h for periods up to 30 min after which respiration decreased markedly. These rates were not achieved if EDTA was omitted from the final medium, the oxygen uptake being 80 μmoles/g/h. The rates were greatly increased if fluoride was omitted, the oxygen uptake rising to 200 μmoles/g/h (*cf.* McMURRAY *et al.*[21]) presumably owing to the stimulatory effect of adenosine triphosphatase, the activity of which was suppressed by fluoride. Thus in this system the quantities of inorganic phosphate liberated in 30 min from adenosine triphosphate (7.5 μmoles/vessel) were; fluoride absent, 2.0 and 2.3 μmoles; fluoride present, 0.5 and 0.51 μmole, in each of two experiments.

Oxidative phosphorylation in the complete system was satisfactory. P/O ratios of 1.5 ± 0.18 (see ref. 9) being achieved over the 30-min period. These compare favourably with values previously found by other workers[12, 22, 23] for phosphorylation in dispersions in whole brain, or with preparations of brain mitochondria. As with oxygen uptake, EDTA and fluoride were found necessary for optimal phosphorylation. Under these conditions radioactive phosphorus was incorporated into the phosphoprotein fraction. These results are similar to those found with homogenates of liver or with particulate preparations from liver or rat-mammary gland[24–26]. This process

TABLE I

THE EFFECT OF ANAEROBIOSIS UPON THE INCORPORATION OF RADIOACTIVE PHOSPHATE INTO PHOSPHOPROTEINS

Slices were incubated in glucose–saline[2] with radioactive phosphate for 30 min. Dispersions were incubated in the system described in the METHODS section.

Expt.	*Tissue preparation*	*Radioactivity (counts/min) in phosphorylserine from phosphoprotein*	
		Aerobic	*Anaerobic*
1	Slices	1420	0.0
2	Dispersions	460	76.0
3	Dispersions	838	0.0

TABLE II

THE INCORPORATION OF RADIOACTIVE PHOSPHATE INTO THE PHOSPHOPROTEIN FRACTION OF CEREBRAL TISSUES IN THE PRESENCE AND ABSENCE OF FLUORIDE

Medium and conditions as in the METHODS section, omitting glucose and hexokinase. Where added, sodium fluoride was present at a concentration of 12 mM.

Expt.	*Addition*	*Oxygen uptake μmoles/g wet wt./h*	*Radioactivity of phosphoprotein*
1	NaF	104.0	35.1
	No NaF	220.0	31.2
2	NaF	106.0	20.2
	No NaF	240.0	14.8

was almost completely abolished by anaerobiosis, an effect also obtained with slices of cerebral tissue, showing that incorporation was not due to a simple chemical exchange (Table I).

In the presence of sodium fluoride incorporation into phosphoprotein was greater than in the absence of fluoride (Table II).

Experiments with labelled precursors

The above system was used to determine the relative effectiveness of possible precursors as a means of introducing the labelled phosphate into the phosphoprotein fraction. When inorganic phosphate and adenosine triphosphate were compared for their effectiveness (Table III, Expts. 1, 2 and 3) in each experiment the relative specific activity of the phosphoprotein fraction (measured as phosphorylserine) was higher when the nucleotide was the precursor than when inorganic phosphate was the precursor. This indicated that adenosine triphosphate is a closer precursor of phosphoprotein than is inorganic phosphate. When adenosine triphosphate and guanosine triphosphate were similarly compared, the relative specific activity was higher with the guanosine nucleotide as the precursor than with the adenosine nucleotide as the precursor. In these experiments (Expts. 4 and 5), to each vessel containing one of the labelled nucleotides was added the other nucleotide, unlabelled but equal in amount to the concentration used when it in turn was the labelled nucleotide. Thus in Expt. 4(a), 3.6 m*M* unlabelled GTP was added and in Expt. 4(b) 1.0 m*M* unlabelled adenosine triphosphate was added.

DISCUSSION

The experiments described above support the view that adenosine triphosphate is a more likely immediate precursor of phosphoprotein than is inorganic phosphate and that guanosine triphosphate is a more likely immediate precursor of phosphoprotein than is inorganic phosphate. Although it may be argued that the experiments in Table III do not exclude the possibility that the phosphoprotein acts as an intermediate between adenosine and guanosine triphosphate, for the differing relative specific activities might be due to different rates of obtaining equilibrium with the different precursors, this view is not supported by the data: the reactions considered are

$$\text{ATP} \longleftrightarrow \text{GTP} \longleftrightarrow \text{Phosphoprotein} \qquad (1)$$

$$\text{ATP} \longleftrightarrow \text{Phosphoprotein} \longleftrightarrow \text{GTP} \qquad (2)$$

In Expt. 5 (Table III) the specific radioactivity of the precursors was identical and identical quantities of the nucleotides were present in each system. At the end of the experiment, where adenosine triphosphate was the labelled precursor, the specific radioactivity of the adenosine triphosphate was higher than that of guanosine triphosphate. Where guanosine triphosphate was the labelled precursor, the specific radioactivity of both nucleotides, at the end of the experiment, was identical. In both experiments the final specific radioactivities of guanosine triphosphate were similar. Nevertheless the relative specific radioactivity of the phosphoprotein was lower where adenosine triphosphate was the precursor than where guanosine triphosphate was the precursor. If phosphoprotein was an intermediate between

TABLE III

LABELLING OF PHOSPHOPROTEIN OF CEREBRAL TISSUES WITH DIFFERENT RADIOACTIVE PRECURSORS

System as in the METHODS section. Period of incubation 30 min. Radioactive precursors were synthesized as described in METHODS. Radioactivity of phosphoprotein was determined as described in METHODS.

Expt.	*Precursor*	*Concentration (mM)*	*Specific radioactivity*				*Specific radioactivity of phosphorylserine*	*Relative specific activity* $\times 10^3$
			Before incubation	*After incubation*				
				Pi	*ATP*	*GTP*		
1	(a) ^{32}Pi	10.0	—	3760	—	—	49.0	13.0
	(b) [^{32}P]ATP	7.0	$3 \cdot 10^3$	1260	—	—	28.8	22.8
2	(a) ^{32}Pi	10.0	—	3150	—	—	25.8	8.4
	(b) [^{32}P]ATP	7.0	$2 \cdot 10^4$	1620	—	—	61.5	38.0
3	(a) ^{32}Pi	2.6	—	1070	—	—	6.54	6.1
	(b) [^{32}P]ATP	2.3	$2.6 \cdot 10^3$	994	—	—	52.9	53.4
4	(a) [^{32}P[ATP	1.0	$1.45 \cdot 10^3$	618	618	—	48.2	7.79
	(b) [^{32}P]ITP	3.6	$1.45 \cdot 10^3$	327	327	—	46.4	14.6
5	(a) [^{32}P]ATP	1.2	$1.68 \cdot 10^3$	—	531	352	5.7	1.08
	(b) [^{32}P]ITP	2.5	$1.68 \cdot 10^3$	—	391	371	10.0	2.55

* In Expts. 1–3. $\text{RSA} = \dfrac{\text{Specific activity (counts/min/}\mu\text{gP) of phosphorylserine}}{\text{Specific activity of inorganic phosphate}}$

In Expts. 4 and 5. $\text{RSA} = \dfrac{\text{Specific activity of phosphorylserine}}{\text{Specific activity of adenosine triphosphate}}$

adenosine triphosphate and guanosine triphosphate it seems reasonable to expect that its relative specific radioactivity, with adenosine triphosphate as the labelled precursor, would have been higher than or equal to that with guanosine triphosphate as the labelled precursor. This is clearly not the case. The results are also not interpretable on the basis of a preferential dilution of adenosine triphosphate via side reactions, since the relative specific activities should not have been affected. The data is thus more in conformity with Eqn. 1.

Previously a role for guanosine triphosphate in the phosphorylation of phosphoproteins had not been demonstrated in studies with particulate fractions from liver[24,25] from mammary gland[27] or in the purified yeast phosphokinase system capable of phosphorylating phosvitin[28]. Whether guanosine triphosphate is essential to this phosphorylation in brain is not yet known but its close association with phosphoprotein metabolism in intact cerebral tissues and the results presented above, strongly suggest this to be the case. However, this cannot be finally decided without further study, and investigation of this and other topics related to phosphoprotein metabolism in nervous tissue is proceeding.

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