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Factors Affecting the Incorporation of Radioactive Phosphate into the Pentosenucleic Acids of Brain Slices

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In the preceding paper (DeLuca, Rossiter & Strickland, 1953) it was shown that radioactive phosphate (^{32}P) was incorporated into the pentosenucleic acid (PNA) of cat-brain slices respiring in a glucose-containing Krebs-Ringer bicarbonate solution. Factors affecting this incorporation are now described in more detail. Evidence is presented that the incorporation is dependent upon an active phosphorylating mechanism within the tissue slice.

METHODS

The methods were essentially those described in the preceding paper (DeLuca *et al.* 1953). Where necessary, glucose was omitted from the incubating medium, or replaced by other substrates. All substrates and inhibitors were made up in the Krebs-Ringer bicarbonate and, if necessary, the solution was titrated to the pH of the buffer with HCl or NaOH. For the anaerobic experiments N_2 replaced O_2 in the gas mixture and the gas mixture was passed over heated Cu filings to remove the last traces of O_2.

The nucleic acids were extracted from the tissue and purified by the method of Hammarsten (1947). The specific activity of the PNA nucleotides was determined after two magnesia treatments. The mixed nucleotides so obtained have a specific activity within the range of that given by the individual nucleotides separated chromatographically (De Luca *et al.* 1953). All experiments were done in duplicate. The results reported have been corrected for decay and calculated on the basis of a standard count of $8{\cdot}1 \times 10^5$/min./ml. incubating medium.

RESULTS

Glucose as substrate

Table 1 shows the incorporation of ^{32}P into the PNA of the brain slices. The figures reported for 0 hr. represent slices that were placed in the medium containing the ^{32}P. The flask was shaken to ensure thorough mixing and the entire contents, including the ^{32}P, were then dispersed at 0° in trichloroacetic acid as described for the incubated slices (DeLuca, *et al.* 1953). It can be seen from Table 1 that when glucose, which is well known to increase the oxygen use of brain slices, was omitted from the medium there was a decreased incorporation of ^{32}P into the PNA. The stimulatory effect of glucose became greater as the concentration was increased from 0 to 100 mg./100 ml. Glucose concentrations as great as 800 mg./100 ml. were inhibitory. These experiments suggest a relation between the incorporation of ^{32}P into the PNA and the carbohydrate metabolism of the slice.

Table 1. *Specific activity of PNA of slices of cat brain*

(Slices incubated at 37·2° in Krebs-Ringer bicarbonate containing ^{32}P. Glucose, 0·011 M. Counts/min/μg. P.)

	Expt. 1	Expt. 2	Expt. 3
0 hr.	0·11	0·09	0·08
4 hr.	18·2	26·8	25·8
4 hr. glucose omitted	13·7	18·5	21·3

Fig. 1 shows the time course of the incorporation. After a slight initial lag the incorporation was linear with time for the first 4 hr. Other experiments with longer incubation times confirmed this result and showed that during the period 4 to 6 hr. the incorporation was faster. Since no special precautions were taken to prevent bacterial contamination, these experiments are not reported in detail.

The incorporation of ^{32}P into PNA was greatly suppressed when the conditions were anaerobic

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(Table 2). The incorporation was also much less when the cellular structure of the slice was destroyed by dispersing the tissue in a Potter-Elvehjem type homogenizer (Table 3). The medium used for the preparation of the suspension was the same as that used for the slice experiments, with the exception that nicotinamide was added to inhibit the destruction of cozymase. The suspension was reinforced with adenosine triphosphate (ATP) and cozymase. Fluoride was added to prevent the destruction of the ATP. Each of these additions slightly increased the incorporation of ^{32}P into the PNA, but despite this, the incorporation with the suspension was very poor compared with that obtained with slices (cf. Table 1). The observation that there was at least some incorporation with suspensions would suggest that further experiments with actively respiring suspensions might yield a system capable of incorporating ^{32}P into PNA.

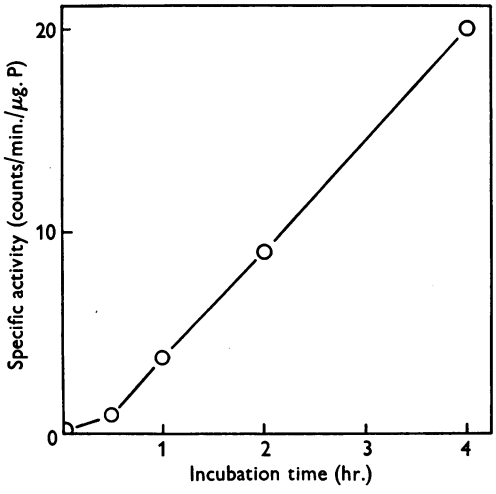

Fig. 1. Specific activity of the PNA of slices of cat brain incubated at 37·2° in Krebs-Ringer bicarbonate. Glucose, 0·011 M.

Table 2. *Effect of anaerobic conditions on the specific activity of the PNA of slices of cat brain*

(Slices incubated at 37·2° in Krebs-Ringer bicarbonate containing ^{32}P, glucose, 0·011 M. Incubation time, 4 hr. Counts/min./μg. P.)

Gas phase	Expt. 1	Expt. 2
95% O_2–5% CO_2	21·1	28·9
95% N_2–5% CO_2	2·1	2·7

Table 3. *Specific activity of PNA of suspensions of cat brain*

(Suspension incubated at 37·2° in Krebs-Ringer bicarbonate containing ^{32}P. Glucose, 0·011 M; cozymase, 0·004 M; ATP, 0·004 M; sodium fluoride, 0·025 M. The brain was dispersed in Ringer containing 0·04 M-nicotinamide. Incubation time, 4 hr. Counts/min./μg. P.)

	Expt. 1	Expt. 2	Expt. 3
0 hr.	1·32	—	0·25
4 hr.	1·95	0·82	0·87

Other substrates

In Table 4 the effect of a number of monosaccharides is shown. Mannose gave an incorporation comparable with that obtained with glucose, but fructose and galactose were generally more variable and less effective. These results were somewhat surprising for, although mannose is known to increase the oxygen consumption of brain tissue respiring *in vitro*, fructose is reported to be equally active (Loebel, 1925; Quastel & Wheatley, 1932). Galactose also increases brain respiration, but the effect is much smaller (Loebel, 1925; Quastel & Wheatley, 1932).

Table 4. *Effect of monosaccharides on the specific activity of the PNA of slices of cat brain*

(Slices incubated at 37·2° in Krebs-Ringer bicarbonate containing ^{32}P. Incubation time, 4 hr. Counts/min./μg. P.)

	Without substrate	With substrate	Percentage increase
Glucose, 0·011 M	18·0	22·0	22
	13·7	18·2	33
	18·5	26·8	45
	21·3	25·8	21
	17·0	26·6	56
	17·2	26·5	54
Mannose, 0·01 M	21·3	30·0	41
	26·7	28·0	5
	17·2	22·3	30
Fructose, 0·01 M	21·3	24·2	14
	26·7	19·3	−28
	17·2	20·1	17
Galactose, 0·01 M	21·3	28·3	33
	26·7	25·0	−6
	62·5	64·5	3

Table 5. *Effect of pyruvate and lactate on the specific activity of the PNA of cat brain*

(Slices incubated at 37·2° in Krebs-Ringer bicarbonate containing ^{32}P. Incubation time, 4 hr. Counts/min./μg. P.)

	Without substrate	With substrate	Percentage increase
Pyruvate, 0·01 M	13·7	24·9	82
	18·5	22·6	22
Lactate, 0·01 M	17·0	19·7	16
	26·7	31·1	16
	17·2	19·9	16

Table 5 shows that both pyruvate and lactate, substrates that are known to increase the oxygen consumption of brain tissue *in vitro* (Loebel, 1925; Quastel & Wheatley, 1932), increased the incorporation of ^{32}P into the PNA. However, both

succinate and L(+)-glutamate were without effect (Table 6), although each of these substrates is oxidized by brain *in vitro* (Quastel & Wheatley, 1932; Krebs, 1935). Weil-Malherbe (1936) showed that L(+)-glutamate increased the oxygen uptake of brain slices, but that D(−)-glutamate was inhibitory. Table 6 shows that D(−)-glutamate also inhibited the incorporation of ^{32}P into PNA. The tricarboxylic acid cycle intermediates, α-ketoglutarate, citrate and malate, did not support the incorporation.

Table 6. *Effect of other substrates on the specific activity of the PNA of slices of cat brain*

(Slices incubated at 37·2° in Krebs-Ringer bicarbonate containing ^{32}P. Incubation time, 4 hr. Counts/min./μg. P.)

	Without substrate	With substrate	Percentage increase
Succinate, 0·05 M	13·7	14·5	6
	18·5	12·8	−31
L(+)-Glutamate, 0·01 M	13·7	14·6	7
	18·5	18·5	0
	17·0	15·5	−9
	17·0	14·0	−18
D(−)-Glutamate, 0·01 M	17·0	11·9	−30
α-Ketoglutarate, 0·01 M	34·3	30·1	−12
Citrate, 0·01 M	34·3	29·4	−14
Malate, 0·01 M	34·3	30·9	−10

Table 7. *Effect of inhibitors on the specific activity of the PNA of slices of cat brain*

(Slices incubated at 37·2° in Krebs-Ringer bicarbonate containing ^{32}P. Glucose, 0·011 M. Incubation time, 4 hr. Counts/min./μg. P.)

Inhibitor	Without inhibitor	With inhibitor	Percentage inhibition
Cyanide, 3×10^{-2} M	21·1	1·2	94
	25·6	0·7	97
	33·9	1·7	95
Azide, 10^{-2} M	35·5	0·6	98
	28·9	0·4	99
Malononitrile, 10^{-2} M	27·5	0·8	97
	40·0	2·3	94
Chloretone, 4×10^{-3} M	35·5	5·6	84
	28·9	6·1	79
Iodoacetate, 10^{-3} M	42·0	14·0	67
Nembutal (pentobarbitone sodium), 10^{-3} M	35·5	21·3	40
	26·8	13·9	48
2:4-Dinitrophenol			
10^{-3} M	42·0	2·45	94
10^{-3} M	27·5	0·5	98
10^{-4} M	27·5	4·7	83
10^{-5} M	27·5	22·0	20

Inhibitors

It has been shown that the incorporation of ^{32}P into the PNA of brain slices is dependent upon the aerobic metabolism of the slice. When the metabolism of the slice was inhibited by any of a number of metabolic inhibitors, the incorporation was greatly decreased (Table 7). With the exception of the lower concentrations of 2:4-dinitrophenol (DNP), all the inhibitors were used in concentrations that are known to decrease the oxygen consumption of the slice. These experiments provide good evidence that the incorporation is a metabolic phenomenon and not solely a simple exchange reaction.

DISCUSSION

The finding that the incorporation of ^{32}P into the PNA of brain slices was increased in the presence of substrates such as glucose, mannose, lactate, or pyruvate, and inhibited by a wide range of metabolic inhibitors indicates that the incorporation is dependent upon the metabolism of the slice. The absence of oxygen or the fragmentation of the tissue, as in the preparation of a suspension, greatly decreased the incorporation. In this regard, the incorporation of ^{32}P into the PNA of brain slices is similar to other synthetic reactions brought about by brain slices, such as the aerobic formation of acetylcholine (Quastel, Tennenbaum & Wheatley, 1936) and the incorporation of ^{32}P into phospholipids (Fries, Schachner & Chaikoff, 1942; Schachner, Fries & Chaikoff, 1942). In general, the incorporation of labelled precursors into the cholesterol, phospholipids, nucleic acids, or proteins of tissue slices is inhibited by anaerobic conditions or by the destruction of the tissue slice.

Of the substances that failed to increase the incorporation of ^{32}P into PNA, glutamate and succinate are of some interest. Both increase the oxygen uptake of brain slices. As pointed out by Weil-Malherbe (1950, 1952), it is unlikely that L(+)-glutamate is a major energy-yielding substrate for brain tissue. The same is probably true for succinate, for it failed to support the aerobic formation of acetylcholine by brain slices (Quastel *et al.* 1936) and it also failed to support the active transport of glutamate into brain tissue against a concentration gradient (Stern, Eggleston, Hems & Krebs, 1949).

In the concentrations used, most of the inhibitors probably affected the incorporation of ^{32}P into PNA as a result of their inhibition of respiration. Because it is representative of a group of metabolic inhibitors whose mode of action may possibly be different, 2:4-dinitrophenol was studied at lower concentrations. Loomis & Lipmann (1948) reported that low concentrations of DNP inhibited the uptake of inorganic phosphate, but not of oxygen, by washed suspensions from rabbit kidney. Although there is still considerable doubt as to the mechanism whereby DNP exerts its effect (Teply, 1949; Judah, 1951), subsequent work in a number of laboratories with a wide variety of tissue preparations, including brain suspensions (Case & McIlwain, 1951), has

shown that DNP is one of a series of substances that may prevent the formation of energy-rich phosphate compounds without decreasing the oxygen consumption. The incorporation of ^{32}P into the PNA of cat-brain slices was greatly inhibited by DNP in a concentration of 10^{-4} M and was slightly inhibited by a concentration as low as 10^{-5} M. This range of DNP concentrations stimulates the oxygen consumption of brain slices (Peiss & Field, 1948; Tyler, 1950; McIlwain & Gore, 1951).

The evidence indicates that in circumstances where the formation of energy-rich phosphate compounds is inhibited, the incorporation of ^{32}P into PNA is also inhibited. It is possible that it may be necessary for the ^{32}P to be incorporated into some such labile phosphate compound before it can be incorporated into the PNA. Under the appropriate conditions ^{32}P is readily incorporated *in vitro* into the labile phosphate compounds of other tissues (Friedkin & Lehninger, 1949*a*, *b*; Green, Atchley, Nordmann & Teply, 1949; Hummel & Lindberg, 1949).

It is also possible that the incorporation of ^{32}P into the PNA of brain slices may be dependent upon the concentration of energy-rich phosphate donors. McIlwain, Buchel & Cheshire (1951) have shown that slices of guinea-pig brain respiring in glucose are able to form phosphocreatine and that the concentration of this substance is maintained at levels approaching those attained *in vivo*. Adenine polyphosphates are probably similarly maintained (McIlwain, 1952). There is a remarkable similarity between the conditions that adversely affect the incorporation of ^{32}P into the PNA of slices of cat brain and the conditions, studied by McIlwain and his collaborators, that are responsible for the failure of guinea-pig brain slices to maintain their level of phosphocreatine. The absence of oxygen or glucose (McIlwain *et al.* 1951; McIlwain, 1952), replacing the glucose by L(+)-glutamate (McIlwain, 1951, 1952), or succinate (Kratzing, 1952), or the presence of a wide range of metabolic inhibitors (Buchel & McIlwain, 1950), is responsible for the failure of both guinea-pig brain slices to maintain the concentration of phosphocreatine and cat-brain slices to incorporate ^{32}P into PNA. Dinitrophenol, which may affect the pattern of brain metabolism differently (McIlwain & Gore, 1951; McIlwain, 1952), also inhibits creatine phosphate formation and the incorporation of ^{32}P into PNA.

SUMMARY

1. The incorporation of radioactive phosphate into the pentosenucleic acid of cat-brain slices respiring in a Krebs-Ringer bicarbonate medium was increased by the addition of glucose or mannose, but not by the addition of fructose or galactose, to the medium.

2. The incorporation of ^{32}P into the pentosenucleic acid was decreased greatly by rendering the conditions anaerobic, or by dispersing the tissue.

3. Pyruvate or lactate supported the incorporation of ^{32}P into the pentosenucleic acid, but not succinate, L(+)-glutamate, D(−)-glutamate, α-ketoglutarate, citrate or L-malate.

4. A wide range of metabolic inhibitors that depress the oxygen consumption of brain slices inhibited the incorporation of ^{32}P into the pentosenucleic acid.

5. 2:4-Dinitrophenol in a concentration that does not decrease the oxygen consumption of brain slices decreased the incorporation of ^{32}P into the pentosenucleic acid.

6. The similarity between the conditions that adversely affect the incorporation of ^{32}P into the pentosenucleic acid of brain slices and those that cause a failure of brain slices to maintain their concentration of phosphocreatine is commented upon.

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Effect of Starvation on the Composition of the Liver Cell

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It has long been known that starvation causes the liver as a whole to shrink, but it is only with the development of analytical and histological techniques during the last 100 years that this reduction in size has been attributed to a decrease in the size of the individual liver cells, caused by a rapid loss of glycogen and a slow but persistent loss of protein and water. Numerous descriptions of the changes produced by fasting in the livers of many species have appeared and many authorities have discussed and reviewed them (Benedict, 1915; Kosterlitz & Campbell, 1945; Peters & Van Slyke, 1946). Most workers were interested principally in the organic matter of the liver (Davidson, 1947; Davidson & Waymouth, 1944, 1946; Dible, 1932; Addis, Poo & Lew, 1936) or, more recently, in its enzymes (Miller, 1948; Lightbody & Kleinman, 1940), and few paid attention to the minerals. Moreover, no one has analysed the changes which take place in the liver cell itself, although Elman, Smith & Sachar (1943) and Kosterlitz (1947) correlated their chemical findings with the histological appearance of the liver.

A method by which the results of a fairly complete chemical analysis of samples of whole liver can be used to deduce the mass and composition of the liver cell was described in detail in a previous paper and is given here in outline (Harrison, 1953*a*). This method is based upon the assumption that the masses of the liver cells, like those of their nuclei, are dependent upon the number of chromosomes which they contain and are therefore related to their content of deoxynucleic acid. Moreover, the average mass of the tetraploid cell, whether it contains the tetraploid number of chromosomes in a single nucleus or is binucleate, is twice that of the diploid cell, containing the diploid number of chromosomes in its nucleus. Similarly, the octoploid cell is four times as large as the diploid cell. It follows that the average mass of the diploid cell, which is independent both of the degree of polyploidy and of the proportion of binucleate cells, may be obtained from the ratio of the amount of deoxynucleic acid (DNA) in a diploid nucleus to that in a unit weight of liver tissue. The amount of DNA in the diploid nucleus was determined in the kidney, a tissue known to contain only diploid nuclei. The average mass of the diploid cell determined in this way was considered a better unit for comparison than the mass of the 'mean liver cell', the average for all the cells in the liver. A correction was made for that portion of the mass of the liver which lies outside the cells, which was assumed to be equal to the mass of water, protein and minerals in the blood, lymph, bile and interstitial fluid, and was deduced from the chloride space of the liver and the composition of the serum. The water, protein and minerals in the extracellular fluids of the liver were subtracted from values for whole tissue to determine the composition of the cells; it was assumed that all of the neutral lipids, glycogen, phospholipids, ribonucleic acid (RNA), DNA, inorganic iron, copper and zinc was intracellular.

This method has now been used to follow the changes in the liver cells of adult male and female rats during a 6-day period of starvation and to define differences in the responses of the two sexes to fasting.

EXPERIMENTAL

Animals. Thirty-five male and thirty-five female black and white hooded rats of the Lister strain were used. The male and female rats were from the same litters and were 3–4 months old. Until the fast began they were fed on a stock diet consisting of bread, milk, whole wheat, yeast, fishmeal, oatflakes and bran. The rats were weighed twice each week for 3 weeks before the fast to ensure that they were growing at a normal rate. The males and females were divided into five comparable groups of seven rats, according to their mean weights on the 3 days before the fast, and to their increase in weight during the three pre-experimental weeks. One group of either sex was killed at the start between 11 a.m. and 12 noon and served as controls. Their food had been removed 2 hr. previously. The remaining rats were kept in cages which were raised to prevent coprophagy.