Incorporation of Radioactive Phosphate into the Nucleic Acids of Brain Slices

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The unequivocal incorporation of radioactive phosphate (^{32}P) into the nucleic acids of brain slices has not as yet been demonstrated. Because of the great interest in the nucleic acids of the nervous system aroused by the work of Caspersson (1947) and Hydén (1943, 1947), it was decided to investigate the possible incorporation of ^{32}P into the pentosenucleic acid (PNA) and deoxypentosenucleic acid (DNA) of slices of brain tissue respiring in a suitable oxygenated medium.

In experiments with tissue slices only a small amount of material is available. For this reason most previous workers carrying out *in vitro* experiments have resorted to the method of Schmidt & Thannhauser (1945) for the separation of PNA and DNA. It has been shown by Jeener (1949), Davidson, Frazer & Hutchinson (1951), Szafarz & Paternotte (1951), and others, in experiments in which animals were killed after the administration of ^{32}P, that the specific activity of the Schmidt-Thannhauser PNA fraction of liver is much greater than that of more purified PNA preparations from the same tissue, even after the very active orthophosphate fraction, presumably derived from 'phosphoprotein', has been removed. Strickland (1952) reported similar findings for brain tissue. With tissue from the nervous system the situation is even less favourable than with liver, for Logan, Mannell & Rossiter (1952) have shown that more than one-half of the protein-bound phosphorus of brain tissue appears in the Schmidt-Thannhauser hydrolysate in the form of organic phosphate that has not the characteristic ultraviolet absorption of nucleotides. In white matter of brain this non-nucleotide phosphorus, which has a specific activity much greater than that of PNA, may account for as much as 80% of the phosphorus in the Schmidt-Thannhauser PNA fraction. Much of this phosphorus may be derived from the inositol-containing complex isolated from brain by Folch & Le Baron (1951), but other active phosphorus compounds may be present also. Davidson & Smellie (1952*b*) have recently shown that the Schmidt-Thannhauser PNA fraction of rat-liver tissue contains at least six non-nucleotide phosphate derivatives, which together make up 25% of the phosphorus of the fraction. Two hours after an injection of ^{32}P the specific activity of each of these fractions was greater than that of the isolated PNA nucleotides.

In view of these difficulties encountered when ^{32}P was administered *in vivo*, it is not surprising that similar difficulties arose when ^{32}P was added to brain slices respiring *in vitro*. In experiments with tissue slices it is convenient to add sufficient ^{32}P to make the specific activity of the inorganic phosphate of the incubating medium greater than the specific activity usually observed for the inorganic phosphate fraction of tissues from animals that have received ^{32}P *in vivo*. This presumably leads to a greater specific activity of the highly active contaminants present in the Schmidt-Thannhauser PNA fraction.

In the experiments described in this paper the nucleic acids were removed from the slices by the method of Hammarsten (1947), which separates them from most of the other active constituents (Strickland, 1952). The nucleotides of the PNA were then removed from the DNA by the alkaline hydrolysis procedure of Schmidt & Thannhauser (1945). The nucleotides so obtained were separated by paper chromatography, using the techniques of Markham & Smith (1949, 1951) and Magasanik, Vischer, Doniger, Elson & Chargaff (1950). For specific activity measurements the individual nucleotides were eluted from the chromatograms. Similar chromatograms were photographed with ultraviolet light, after which they were exposed to an X-ray film for radioautography.

A preliminary account of this work has appeared (Strickland, DeLuca & Rossiter, 1953).

METHODS

Preparation and incubation of brain slices. Slices were cut with a safety-razor blade from the cerebral hemispheres of a cat killed by decapitation. Each slice was rapidly weighed on a torsion balance and transferred to a standard Warburg reaction vessel containing 5 ml. Krebs-Ringer bicarbonate incubating medium, to which glucose had been added to make a final concentration of 0·2% (w/v). Sufficient slices were cut to give a weight of 500 mg. in each vessel. To each sample was added 24 μc. ^{32}P supplied in the form of $H_3{}^{32}PO_4$ by the Eldorado Mining and Refining (1944) Ltd. The vessels were filled with a 95% O_2 and 5% CO_2 mixture, after which they were incubated in a bath at 37·2° for 4 hr. During the

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incubation period the vessels were shaken in the usual way to ensure proper oxygenation of the incubating medium.

The contents of each flask were dispersed at 0° in 10 ml. 10% (w/v) trichloroacetic acid (TCA) with a Potter-Elvehjem type homogenizer. The suspension was centrifuged and the supernatant was discarded. To remove last traces of contaminating acid-soluble phosphorus, the residue was extracted seven times with carrier phosphate (5 ml. 0·04 M-KH_2PO_4 in 10% TCA) at 0–4° and finally twice with 5 ml. 10% TCA. The final extracts contained negligible activity. The lipid phosphorus was removed by two cold extractions with 5 ml. 95% (v/v) ethanol, four extractions with 5 ml. ethanol:ether, (3:1, v/v) heated to boiling, and the residue was finally washed with 5 ml. ether. All experiments were done in duplicate. For experiments at 0 hr. the ^{32}P was added to the flask containing the brain slices. The flask was shaken to ensure thorough mixing and the entire contents, including the ^{32}P, were then dispersed at 0° in 10 ml. 10% TCA as described for the incubated slices.

Extraction of nucleic acids by method of Hammarsten (1947)

The nucleic acids were extracted from the protein residue as follows: 1·0 ml. of 10 M-urea was pipetted on to the residue and allowed to stand for 5 min. To this was added 3·0 ml. of S solution (a saturated solution of NaCl saturated with $(NH_4)_2SO_4$ at a boiling temperature and allowed to cool). The nucleic acids were then extracted by heating the solution to a boiling temperature for 1 min. Three more extractions were carried out with 2·0 ml. of S-urea solution (150 g. urea/l. S solution) at boiling temperature for 1 min. The nucleic acids were precipitated from the combined extracts by the addition of 1·0 ml. saturated $CuSO_4$. After standing overnight the tube was centrifuged and the supernatant discarded. The precipitate was washed twice with 1·8% $CuCl_2$ and then the copper nucleates were decomposed and the nucleic acids were extracted by successive additions (0·8 ml. and three 0·4 ml. portions) of a freshly prepared 1:1 mixture of acetate buffer, pH 6·4 (100 g. potassium acetate/100 ml. water titrated to pH 6·4 with glacial acetic acid) and 10 M-urea. This was followed by three extractions using 0·2 ml. 5 M-urea. The nucleic acids were then precipitated at −15° by the addition of 10 ml. 95% (v/v) ethanol and 1 drop saturated NaCl to the combined extracts. The precipitate was redissolved in 1·0 ml. water and reprecipitated by the addition of 0·11 ml. N-HCl at 0° followed by 10 ml. acid-alcohol (95% ethanol: N-HCl, 9:1, v/v) at −15° for at least 2 hr. This precipitate was dissolved in 0·01 N-NaOH and the nucleic acids were precipitated as before. This precipitate was then separated into DNA and PNA by the method of Schmidt & Thannhauser (1945). The precipitated DNA was washed with 5% TCA and the nucleotides of the PNA subjected to the magnesia treatment described by Friedkin & Lehninger (1949). Both fractions were then ashed and the specific activities determined as described below. The recovery of the total amount of nucleic acid in the tissue was of the order of 70–80%.

Chromatography

For the chromatographic experiments the precipitates of mixed DNA and PNA from five reaction vessels were pooled and hydrolysed overnight in 0·15 ml. N-NaOH at room temperature to liberate the PNA nucleotides. The hydrolysate was brought to pH 8 with HCl. The DNA was not precipitated, since it did not interfere with the separation of the nucleotides. Portions of the hydrolysate (10 μl.) were applied to filter-paper strips (19 × 45 cm. no. 597, Schleicher and Schüll). The assembly was as described by Magasanik *et al.* (1950). Either two or three chromatograms were run in each of the following solvents:

(1) *iso*Butyric acid, 10 vol., and 0·5 N-ammonia solution, 6 vol. Two 30 ml. portions were placed in flat dishes at the bottom of the tank. The solvent system was that described by Magasanik *et al.* (1950). It separated adenylic acid and cytidylic acid from the other nucleotides. The running time was 16–18 hr.

(2) *tert.*-Butanol, 70%, water, 30% (v/v) and enough HCl to make the solvent 0·8 M. Two 30 ml. portions of the solvent were placed in flat dishes at the bottom of the tank. This solvent, described by Smith & Markham (1950), was used to separate uridylic acid from the other nucleotides. The running time was about 24 hr.

(3) Saturated $(NH_4)_2SO_4$, 79%, 0·2 M-acetate buffer, pH 5·9, 19%, and *iso*propanol, 2% (v/v/v). Two 30 ml. portions of the solvent with excess *iso*propanol were placed in flat dishes at the bottom of the tank. This solvent system, described by Markham & Smith (1951), was used to separate the isomeric adenylic acids *a* and *b* and guanylic acids *a* and *b* from the other nucleotides. The running time was 6 to 8 hr.

Ultraviolet photography and radioautography

The nucleotides were localized on the chromatograms with a 'Mineralight' short-wave ultraviolet lamp. Photographs of the chromatograms were made with an assembly similar to that described by Markham & Smith (1949, 1951). An 8 w germicidal lamp (General Electric) emitting a high percentage of ultraviolet light at 254 mμ. was used as a light source. The light beam was filtered through a Corning no. 9863 filter and a cobalt-nickel liquid filter ($CoSO_4$, $7H_2O$, 7 g. and $NiSO_4$. $7H_2O$, 23 g.) contained in a round-bottomed quartz flask. The chromatogram was placed in contact with Kodagraph Contact paper and exposed for 3 min. at 6 ft.

The chromatograms were then placed in contact with a strip of X-ray 'No Screen Film' for times varying from 17 to 28 days according to the activity of the nucleotides.

Elution of nucleotides

The nucleotide spots located with the 'Mineralight' lamp were marked out in pencil. The spots, with comparable spots from blank lanes, were cut out and eluted with 5 ml. 0·1 N-HCl for 18 hr. The extinction of the eluted nucleotides was measured at $\lambda_{max.}$ in the Beckman DU spectrophotometer. The following are the molecular extinction coefficients used and the $\lambda_{max.}$ found:

	$\lambda_{max.}$	ϵ
Adenylic acid	258	13 900
Guanylic acid	257	11 800
Cytidylic acid	278	12 700
Uridylic acid	261	9 900

The values for the extinction coefficients are those reported by Marrian, Spicer, Balis & Brown (1951) for the purine nucleotides and those reported by Ploeser & Loring (1949) for the pyrimidine nucleotides. They do not differ greatly from those reported by Holiday (1930) or from those used by Markham & Smith (1951). The values found for

$\lambda_{max.}$ of each of the nucleotides are in agreement with those reported in the literature.

From the concentrations of the nucleotide in the eluate the molar ratios of the nucleotides were calculated. The figures have a relative significance only, for the separation procedure of Hammarsten (1947) is not quantitative. This lack of quantitative recovery is offset by the finding that the method yields a PNA preparation sufficiently free from contaminating substances for satisfactory specific activity determinations.

Specific activity measurements

A portion of the solution of each of the eluted nucleotides, or of the solution of PNA or DNA separated by the method of Hammarsten (1947), was ashed with 60% perchloric acid. The inorganic phosphate and the radioactivity of the resulting inorganic phosphate was determined by the method of Ernster, Zetterström & Lindberg (1950). The counting was done with a liquid counter (20th Century Electronics, M-6).

RESULTS

Comparison of methods of Schmidt & Thannhauser (1945) *and Hammarsten* (1947)

In the experiments reported in Table 1 the PNA from similar slices of brain from the same animal was separated either by the standard procedure of Schmidt & Thannhauser (1945) or by the method of Hammarsten (1947). The Schmidt-Thannhauser hydrolysate was then subjected to the magnesia treatment described by Friedkin & Lehninger (1949) to remove inorganic phosphate. It can be seen that in each instance the activity of the fraction from the incubated slices was much greater than that of the corresponding fraction from the 0 hr. control. The magnesia treatment reduced the activity of both the unincubated and the incubated slices, but not to levels approaching the values given by the Hammarsten method. The reason for this is the high concentration of active non-nucleotide ester phosphorus in the Schmidt & Thannhauser hydrolysate (Logan *et al.* 1952; Strickland, 1952).

Table 1. *Specific activity of PNA 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	
	0 hr.	4 hr.	0 hr.	4 hr.
Schmidt-Thannhauser (1945) hydrolysate	2·7	281	5·7	354
Schmidt-Thannhauser (1945) hydrolysate after magnesia treatment	0·4	141	0·1	162
Hammarsten (1947)	0·3	17·9	0	22·5

Table 2 shows similar data for DNA. Again it can be seen that the magnesia treatment reduced the activity of the Schmidt-Thannhauser DNA, but it was not reduced to figures comparable to those found for the DNA separated by the Hammarsten method.

Table 2. *Specific activity of DNA 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	
	0 hr.	4 hr.	0 hr.	4 hr.
Schmidt-Thannhauser (1945)	0·3	5·3	0	12·7
Schmidt-Thannhauser (1945) after magnesia treatment	—	3·4	0·6	6·6
Hammarsten (1947)	0·1	2·4	0	2·1

Incorporation of radioactive phosphate into the nucleotides of PNA

Brain slices were incubated in the bicarbonate buffer containing both glucose and ^{32}P either with or without the addition of cyanide (0·03 M). The nucleotides of the PNA obtained by the method of Hammarsten (1947) were separated chromatographically. Fig. 1 shows the chromatogram and the radioautograph obtained with solvent 1. This

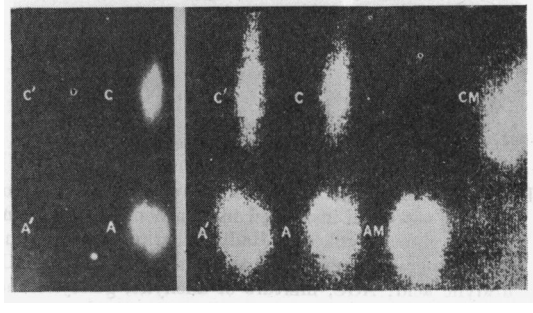

Fig. 1. Chromatogram (solvent 1) of nucleotides of PNA of slices of cat brain incubated in bicarbonate buffer containing glucose and ^{32}P. Radioautograph of the same chromatogram is shown on the left. The spots are: C, cytidylic acid; A, adenylic acid; C′ and A′, cytidylic acid and adenylic acid from PNA of slices incubated in the presence of cyanide (0·03 M); CM, cytidylic acid marker, AM, adenylic acid marker.

solvent separated cytidylic acid (C, C′) and adenylic acid (A, A′) from the other nucleotides. To the right can be seen the separation of the nucleotides. C represents the cytidylic acid and A the adenylic acid of the PNA of the slices incubated without the addition of cyanide. C′ and A′ represent the cytidylic acid and adenylic acid of the slices incubated in a medium to which cyanide had been added. To the left is the radioautograph of the same

chromatogram. The cytidylic acid (C) and adenylic acid (A) from the slices incubated without cyanide darkened the X-ray film, whereas the same nucleotides (C′, A′) from the slices incubated in the presence of cyanide produced no darkening. In the presence of the metabolic inhibitor no ^{32}P was incorporated into the PNA of the slice.

Fig. 2 shows the results obtained with solvent 2. This solvent separated uridylic acid (U, U′) from the other nucleotides. Again it can be seen from the radioautograph, shown on the left, that for slices incubated in the absence of cyanide the ^{32}P was incorporated into the uridylic acid (U) and also into the mixture of adenylic, guanylic and cytidylic acids represented by the spot AGC. When the slices were incubated in the presence of cyanide the incorporation was abolished (U′, AGC′).

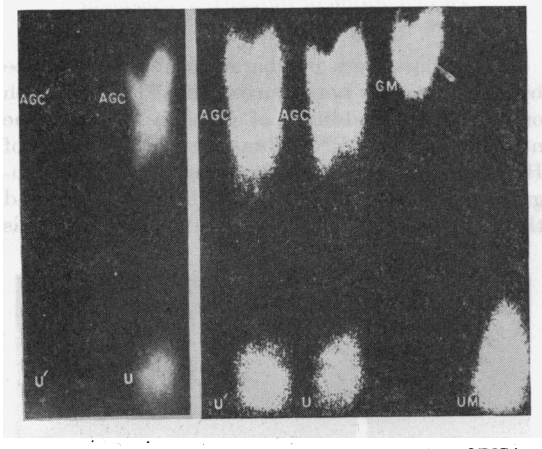

Fig. 2. Chromatogram (solvent 2) of nucleotides of PNA of slices of cat brain incubated in bicarbonate buffer containing glucose and ^{32}P. Radioautograph of the same chromatogram is shown on the left. The spots are: U, uridylic acid; AGC, mixture of adenylic, guanylic and cytidylic acids; U′ is the uridylic acid and AGC′ the mixture of nucleotides from the PNA of slices incubated in the presence of cyanide (0·03 M); GM, guanylic acid marker; UM, uridylic acid marker.

Fig. 3 shows similar data for solvent 3. This solvent system, as described by Markham & Smith (1951), separated adenylic acid *a* (AA), adenylic acid *b* (AB), guanylic acid *a* (GA) and guanylic acid *b* (GB) from the other nucleotides. Again it can be seen from the radioautograph that for the slices incubated in the absence of cyanide there was an incorporation of ^{32}P into all four nucleotides. Cyanide inhibited this incorporation. Fig. 3 also shows the starting point of the chromatogram (D, D′). The intense ultraviolet absorption of these spots is due to the fact that they contain all the DNA, which was not moved in this solvent system. The darkening of the X-ray film by spot D, seen in the radioautograph to the left, does not necessarily mean that ^{32}P was incorporated into the DNA. The darkening was probably caused by traces of much more active contaminants, possibly unhydrolysed PNA, that were not moved on the chromatogram. In obtaining the figures for DNA reported in Table 2, the DNA was precipitated from the solution and thoroughly washed. Nevertheless, contaminating substances

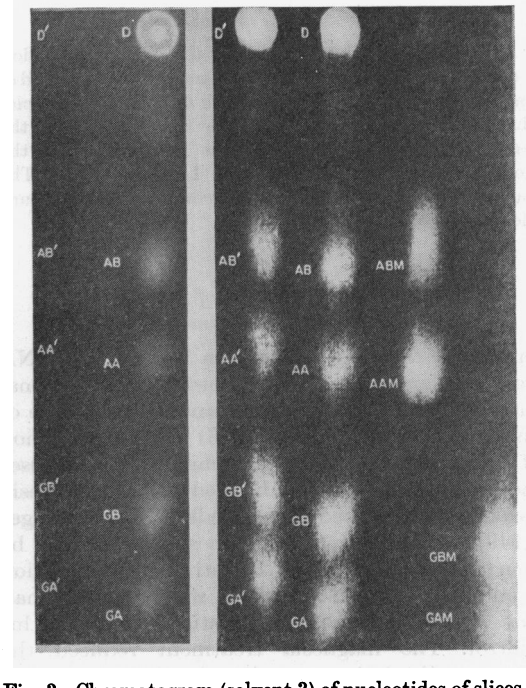

Fig. 3. Chromatogram (solvent 3) of nucleotides of slices of cat brain incubated in bicarbonate buffer containing glucose and ^{32}P. Radioautograph of the same chromatogram is shown on the left. The spots are: D, deoxypentosenucleic acid; AB, adenylic acid *b*, AA, adenylic acid *a*; GB, guanylic acid *b*, GA, guanylic acid *a*; AB′, AA′, GB′, GA′, the same nucleotides from the PNA of slices incubated in the presence of cyanide (0·03 M); ABM, adenylic acid *b* from marker; AAM, adenylic acid *a* from marker; GBM, guanylic acid *b* from marker; GAM, guanylic acid *a* from marker.

may be responsible for the radioactivity of the DNA fraction obtained by the Hammarsten (1947) procedure. It is possible that no ^{32}P was incorporated into the DNA. Fig. 3 also shows that for slices incubated in the presence of cyanide there was no incorporation of ^{32}P into spot D′.

In Table 3 are reported the specific activities of the eluted nucleotides for three experiments. For the slices incubated in the presence of cyanide the specific activities were all very low as was to be anticipated from their failure to darken the X-ray film. When cyanide was absent from the incubating

Table 3. *Specific activities of the nucleotides of the PNA of slices of cat brain incubated at* 37·2° *in Krebs-Ringer bicarbonate containing* ^{32}P. *Glucose*, 0·011 M. *Incubation time*, 4 *hr.*

(Counts/min./μg. P.)

	Expt. 1		Expt. 2		Expt. 3	
	Cyanide (0·03 M)	No cyanide	Cyanide (0·03 M)	No cyanide	Cyanide (0·03 M)	No cyanide
Adenylic acid	1·5	41·7	0·8	35·8	0	50·0
Adenylic acid *b*	1·5	42·9	0	24·4	4·2	48·1
Adenylic acid *a*	2·5	37·1	—	26·4	0	55·8
Guanylic acid *b*	0·9	26·3	0·8	17·3	0·5	35·0
Guanylic acid *a*	1·4	26·5	1·3	14·0	0·4	38·6
Cytidylic acid	1·1	27·4	1·7	25·0	0	30·4
Uridylic acid	2·7	42·6	0	36·8	1·8	35·5
PNA	1·0	29·6	0·6	25·1	1·7	33·9

medium there was an appreciable incorporation of ^{32}P into all of the nucleotides. The specific activities of the individual nucleotides appeared to range around that found for the phosphorus of the hydrolysate reported as PNA in Table 3.

Concerning the relative incorporation of ^{32}P into the various nucleotides, little can be said at present. The figures varied somewhat from experiment to experiment. For purine nucleotides, adenylic acid was always found to be more active than guanylic acid, and for the pyrimidine nucleotides, uridylic acid was more active than cytidylic acid. These findings are similar to those reported by Volkin & Carter (1951) and Davidson & Smellie (1952*b*) for the specific activities of the nucleotides of the PNA of liver tissue after the administration of ^{32}P *in vivo*.

The small differences between the *a* and *b* isomers of adenylic and guanylic acids are not regarded as significant.

Nucleotides of brain PNA

Table 4 shows the molar ratios of the four nucleotides of brain PNA. The figures represent the means from twelve different brain samples. The standard errors give an indication of the significance that may be attached to these figures. Recoveries of total nucleotide (as phosphorus) were of the order of 90–95 %. It will be noted that, on a molar basis, brain PNA contains more guanylic acid than adenylic acid and more cytidylic acid than uridylic acid. It may be more than coincidence that the molar ratios of the four nucleotides are almost the inverse of the ratio of the specific activities, guanylic acid occurring in higher concentration than adenylic acid and having a lower specific activity. Cytidylic acid occurs in higher concentration than uridylic acid and also has a lower specific activity.

As far as we are aware, there are no previous reports in the literature on the distribution of nucleotides in brain PNA. The data presented in Table 2 are similar to those reported by Chargaff *et al.* (1950) for PNA of mammalian liver, with the exception that the proportion of uridylic acid is greater. The figures more closely resemble those reported by Marshak (1951) for the PNA of calf liver and Davidson & Smellie (1952*a*) for the PNA of rat liver.

Table 4. *Molar ratios of the nucleotides in the hydrolysate of PNA of cat brain*

(Mean ± S.E.M. of 12 samples. Adenylic acid = 1.)

Adenylic acid	1·00
Adenylic acid *b*	0·59 ± 0·01
Adenylic acid *a*	0·41 ± 0·01
Guanylic acid *b*	0·82 ± 0·03
Guanylic acid *a*	0·64 ± 0·01
Guanylic acid (by addition)	1·47 ± 0·03
Cytidylic acid	1·20 ± 0·05
Uridylic acid	0·95 ± 0·04

For adenylic acid and guanylic acid, the *b* isomer accounted for 59 and 56% of the total respectively. This value is in agreement with the figures of 50–65 % reported by Carter (1950), Cohn (1951) and Marrian *et al.* (1951) for the nucleotides of yeast PNA, and with the similar figures reported by Marrian *et al.* (1951) for the nucleotides of PNA from the mixed organs of the rat.

DISCUSSION

It should be pointed out that in these experiments only a small percentage of the ^{32}P of the medium was incorporated into the PNA of the slice. Much more was incorporated into the phospholipid, 'phosphoprotein', and the residual ester phosphorus, i.e. the organic phosphorus remaining after the complete extraction of the nucleic acids. After an incubation period of 4 hr. the specific activity of the PNA was of the order of one-thousandth of that of the inorganic phosphate originally in the medium. For tissues other than brain, and for incubation times longer than 4 hr., the incorporation of ^{32}P into PNA was much greater. During the period of incubation there was no demonstrable increase in the amount of PNA present in the slice.

For studies involving the *in vitro* incorporation of ^{32}P into the nucleic acids of tissue slices, a complete separation of the nucleotides is desirable. It has been shown that the isolation procedure of Hammarsten (1947) is satisfactory for PNA, since the specific activity of the PNA so isolated fell within the range observed for the individual nucleotides. The experiments of Davidson & Smellie (1952*b*) would suggest that their shortened partial isolation method (their procedure 2) would be equally satisfactory. However, both methods suffer from the disadvantage that they are not quantitative. The experiments reported here do not indicate whether the Hammarsten procedure is satisfactory for DNA, or whether there is indeed any incorporation of ^{32}P into DNA *in vitro*. The activity of the DNA obtained by the Hammarsten method may be due to contaminating traces of other active substances, possibly traces of PNA not hydrolysed by the alkali. Unequivocal incorporation of ^{32}P into DNA could best be demonstrated by the isolation of the constituent deoxynucleotides, a procedure that would require the enzymic hydrolysis of the DNA. It is clear that for both DNA and PNA the method of Schmidt & Thannhauser (1945) is unsatisfactory, especially for tissue from the nervous system where so much of the protein-bound phosphorus is non-nucleic acid phosphorus (Logan *et al.* 1952).

Previous *in vitro* experiments in which the Schmidt & Thannhauser (1945) method was used, such as those of Mann & Gruschow (1949) with tissue slices, those of Rafelson, Winzler & Pearson (1949) and Rafelson, Pearson & Winzler (1951) with brain mince, and those of Friedkin & Lehninger (1949) with particles from rat liver should be interpreted with caution, although they may show a general trend. The preliminary note of Hokin (1952), who studied slices of pancreas, and the findings of Findlay (1953) in this laboratory would indicate that ^{32}P is readily incorporated into the PNA of slices of a wide variety of tissues.

Objections similar to those made to *in vitro* experiments also apply to many studies in which ^{32}P was administered to animals *in vivo*. The results reported here, taken in conjunction with the findings of Logan *et al.* (1952) and Strickland (1952), suggest that the specific activities of the 'nucleoprotein P' of Dawson & Richter (1950) and the 'residual P' of Bodian & Dziewiatkowski (1950) were greater than the true specific activity of the PNA, and that they were considerably greater than the specific activity of the total nucleic acid, for the DNA is relatively inactive. Similar high specific activities were obtained for the 'nucleic acid' fraction in studies such as those of Erickson, Larson & Gordon (1949), Samuels, Boyarsky, Gerard, Libet & Brust (1951) and Stern & Marshall (1951) in which the TCA extraction procedure of Schneider (1945) was applied to tissue of the nervous system. Experiments have shown that the TCA removes much non-nucleic acid phosphorus (Logan *et al.* 1952) and that this phosphorus has a specific activity higher than that of the PNA and much higher than that of the DNA.

The nucleotides of PNA may be separated by paper chromatography with the use of appropriate solvents. Unfortunately, no one solvent system was found that gave a complete separation of the nucleotides on the same chromatogram. In this regard the methods of ionophoresis developed by Davidson & Smellie (1952*a*) and Markham & Smith (1952) should prove of great value. Magasanik *et al.* (1950), Carter (1950), Marshak & Vogel (1950) and Markham & Smith (1951) achieved a degree of separation, but in each instance the spots for two or more of the nucleotides overlapped to such an extent that specific activity measurements were impossible. Boulanger & Montreuil (1951) reported good separation with systems containing phenol, but under such conditions localization of the spots is difficult.

As reported by Markham & Smith (1951), solvent 3 separated the *a* and *b* isomers of adenylic acid and guanylic acid. These isomers, obtained by the alkaline hydrolysis of PNA, were first reported in hydrolysates of yeast PNA by Carter & Cohn (1949), Carter (1950) and Cohn (1950). Adenylic acids *a* and *b* have been separated one from the other chemically (Riechard, Takenaka & Loring, 1952) and have been synthesized (Brown & Todd, 1952*a*). Adenylic acids *a* and *b* are adenosine-2′-phosphate and adenosine-3′-phosphate, but not necessarily in that order. The finding that the specific activities of the *a* and *b* isomers of either adenylic acid or guanylic acid did not differ significantly from each other, whereas adenylic acid, for example, differed significantly from guanylic acid, is in accord with present concepts of PNA structure and hydrolysis. Brown & Todd (1952*b*) postulated that on alkaline hydrolysis the polynucleotide gives rise to a mixture of the *a* and *b* isomers of each of the mononucleotides by way of an intermediate cyclic 2′:3′-phosphate. Added support is given to this suggestion by the finding of Markham & Smith (1952) that all four cyclic nucleotides (2′:3′-phosphates) were present in both alkaline hydrolysates and ribonuclease digests of yeast PNA and turnip yellow mosaic virus PNA. The natural cyclic nucleotides were found to be indistinguishable chromatographically from the corresponding synthetic nucleotides prepared by Brown, Magrath & Todd (1952). If the theory of Brown & Todd (1952*b*) concerning the origin of the *a* and *b* nucleotides is correct, the similar specific activities of the *a* and *b* isomers of adenylic and guanylic acids would be expected. In experiments in which tracer substances were administered *in*

vivo, the *a* and *b* isomers of adenylic and guanylic acid were shown to be metabolically indistinguishable (Marrian *et al.* 1951, for ^{14}C- and ^{15}N-labelled adenine; Volkin & Carter, 1951, for ^{32}P).

The experiments reported above indicate that ^{32}P is readily incorporated into the PNA of brain slices and that the incorporation is dependent upon the metabolism of the slice. Findlay, Rossiter & Strickland (1953), demonstrate in the following paper that the incorporation is probably related to an active phosphorylating mechanism. The finding that the addition of cyanide to the incubating medium inhibited the incorporation indicates that the separation procedure was satisfactory, for in these experiments all the manipulations were carried out in the presence of concentrations of ^{32}P similar to those used when the cyanide was omitted. As an additional check a small quantity of ^{32}P was added to a mixture of the inert nucleotides and the nucleotides were separated by the usual chromatographic methods. No radioactivity was detected in the spots corresponding to each of the nucleotides.

The active metabolism of brain PNA *in vitro* is in sharp contast to the results of early experiments in which ^{32}P was administered *in vivo* (summarized by Hevesy, 1947, 1948). These showed that little ^{32}P was incorporated into the nucleic acids of brain, suggesting that both the PNA and the DNA of brain were rather inert metabolically. However, as Bakay & Lindberg (1949) and Lindberg & Ernster (1950) have demonstrated, when ^{32}P is injected into the cerebro-spinal fluid it is rapidly taken up by the brain. This ^{32}P is readily incorporated into the PNA and phospholipid fractions (Strickland, 1952). Thus experiments carried out both *in vivo* and *in vitro* have shown that ^{32}P can be rapidly incorporated into the PNA of brain. It follows that the active metabolism of PNA in the nervous system postulated by Caspersson (1947) and Hydén (1943, 1947) may be possible.

SUMMARY

1. Radioactive phosphate is incorporated into the pentosenucleic acid of cat-brain slices respiring in a Krebs-Ringer bicarbonate medium containing glucose and ^{32}P. This was demonstrated by measuring the specific activity of the constituent nucleotides isolated by paper chromatography.

2. The incorporation is abolished by the addition of cyanide to the medium.

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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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