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Phosphate Esters in the Uterus

By J. M. CAWKWELL

Department of Biochemistry, University of Cambridge

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Hollmann (1949) observed that more than half the acid-soluble phosphate esters extracted from cow uterus resisted acid hydrolysis. After 10 hr. in *N*-HCl at 100°, 72.5 % of the organic phosphate was still unhydrolysed, and he suggested that the acid-resistant ester was phosphoglyceric acid.

In a preliminary experiment on an extract from rat uteri, 35 % of the acid-soluble phosphate resisted *N*-HCl at 100° for 3 hr., but very little phosphoglyceric acid was detectable by the enzymic method of Slater (1953). Since Awapara, Landua & Fuerst (1950) have demonstrated the presence of phosphorylethanolamine in extracts of rat uterus, it was decided to make a further study of the phosphate esters in the uterus. Extracts were prepared from non-pregnant uteri of a number of mammalian species, and from pregnant and post-partum rabbit uteri. Phosphate analyses were carried out on all the extracts. The extracts from pregnant and post-partum rabbit uteri were also investigated by paper chromatography.

EXPERIMENTAL

Extracts were prepared in three ways.

(1) A method described by Menkes & Csapo (1952) was used. Rats in oestrus were anaesthetized with Nembutal and the uteri dissected out. Four uteri were extracted together. After 20 min. in ice-cold oxygenated saline, they were blotted dry, frozen with liquid nitrogen and powdered in a mortar. The powder was extracted with 10 vol. of 5 % (w/v) trichloroacetic acid, and, after being left for 30 min. at 0°, the extract was filtered.

(2) The uterus was removed from a non-pregnant anaesthetized rabbit and, after the internal surface had been gently scraped, the tissue was frozen in liquid nitrogen. It was ground to a powder and extracted with 5 % trichloroacetic acid as in method (1). This method was also used for non-pregnant pig and cow uteri which were brought from the slaughter house packed in ice, immediately after the

death of the animal. Human uterus, which was obtained immediately after removal at operation, was extracted in this way.

(3) Extracts were prepared from the uteri of rabbits in the fourth week of pregnancy and just post partum, by the method of Dawson (1955*a*). In this method the danger of the liberation of glycerylphosphorylcholine and glycerylphosphorylethanolamine from the tissue phospholipids is diminished by the substitution of boiling water for trichloroacetic acid.

The animals were stunned and the uteri removed and packed in ice. Further steps were carried out in the cold. Remnants of the placenta were gently removed with a blunt scalpel, care being taken to remove as little as possible of the endometrium. After freezing in liquid nitrogen, the uteri were powdered. The powder was transferred to a beaker, extracted with 4 vol. of boiling water and kept in a boiling-water bath for 2 min. Absolute ethanol (6 vol.) was added and the solution was centrifuged. The supernatant was shaken with chloroform to extract the ethanol, and the aqueous layer was re-centrifuged and passed through a column of Amberlite IRC 50 H⁺ to remove metal ions. It was then shaken with 2 vol. of butan-2-ol to remove any turbidity and centrifuged. The supernatant was neutralized and dried *in vacuo*, the temperature being kept below 50°. The residue was dissolved in a known volume of water. Each rabbit uterus was extracted separately, and two or three extracts were combined before the material was dried *in vacuo*.

One uterus from a pregnant rabbit was extracted in two different ways: with water and with trichloroacetic acid. The frozen powder was divided into two parts, one part extracted with trichloroacetic acid as in method (1) and the other part with boiling water as in method (3).

Phosphoglyceric acid estimation. The enzymic method of Slater (1953) was used to estimate the phosphoglyceric acid in the trichloroacetic acid extracts from non-pregnant pig, rat, cow and human uteri, and in the water extract from pregnant rabbit uterus. Since this method does not distinguish phosphoglyceric acid from adenosine triphosphate and adenosine diphosphate, the trichloroacetic acid extracts were left overnight at room temperature to allow the hydrolysis of the acid-labile phosphates to occur.

Phosphate estimations. Total phosphate was estimated by the method of Allen (1940), or by a modification of the method of Weil-Malherbe & Green (1951) (Needham & Cawwell, 1956). Inorganic phosphate was estimated by the method of Fiske & Subbarow (1925) or by that of Weil-Malherbe & Green (1951). All the extracts prepared were analysed for total phosphate, inorganic phosphate and for inorganic phosphate produced after heating for 10 min. and for 3 hr. in $N-HCl$ at 100° . The increase in inorganic phosphate after 10 min. gave a value for the easily hydrolysable phosphate. The further increase in inorganic phosphate after heating for 3 hr. was assumed to be due to the partial breakdown of ribose phosphate from the nucleotides. According to LePage (1945), ribose phosphate is 58.7% hydrolysed after heating in $N-HCl$ for 3 hr. at 100° and this figure was used in calculating the total ribose phosphate present in the extracts. The acid-resistant phosphate fraction was the phosphate remaining unhydrolysed after 3 hr. in $N-HCl$ at 100° , when corrected for the breakdown of ribose phosphate.

Ribose estimations. Ribose was estimated by the method of Mejsbaum (1939).

Ethanolamine estimations. Ethanolamine was estimated by measuring the ammonia production after periodate oxidation (Artom, 1945). To remove free ammonia from the samples before oxidation, borate buffer, pH 9.6, was added to the neutralized sample in a Markham (1942) distillation apparatus, and the free ammonia distilled off before the periodate reagent was added.

The ethanolamine estimations were carried out on extracts from pregnant and post-partum rabbit uteri before and after acid treatment, after heating in $N-HCl$ for 1 hr. at 100° and after heating in a sealed tube with $6N-HCl$ at 123° for 24 hr. The short acid treatment liberates ethanolamine from glycerylphosphorylethanolamine, and the long acid treatment liberates ethanolamine from phosphorylethanolamine.

Choline estimations. Choline was estimated by the reinkate-precipitation method of Engel, Salmon & Ackermann (1954) in material eluted from paper chromatograms. The choline periodide-precipitation method of Appleton, La Du, Levy, Steele & Brodie (1953) was used to estimate the choline in extracts from pregnant and post-partum rabbit uteri before acid treatment, after heating for 30 min. in $N-HCl$ at 100° and after heating for 24 hr. in $2N-HCl$ at 123° in a sealed tube. Glycerylphosphorylcholine is hydrolysed by the short acid treatment to give free choline and glycerol phosphate, whereas phosphorylcholine is hydrolysed by the prolonged acid treatment to give free choline and inorganic phosphate (Dawson, 1955b).

Chromatography

Removal of nucleotides. Before chromatography the extracts were freed from nucleotides by adsorption on charcoal (Norit SX Special, washed with $N-HCl$ to remove inorganic phosphate). The charcoal was removed by filtering through a no. 3 sintered-glass filter. Ribose estimations before and after the charcoal treatment showed that 90% of the nucleotides had been removed.

Two-dimensional chromatograms were run on Whatman no. 1 paper as described by Dawson (1955b). The first solvent was phenol saturated with 0.1% (w/v) NH_3 soln.; the second solvent was propanol-aq. NH_3 soln.-water (60 ml. of propanol, 30 ml. of aq. NH_3 soln., sp.gr. 0.880,

10 ml. of water). After running, the chromatograms were sprayed first with 0.1% ninhydrin in water-saturated butanol and then with the acid-molybdate solution of Hanes & Isherwood (1949). The phosphate-containing spots were developed by exposure to ultraviolet light.

Preparative chromatograms were also used. A quantity of charcoal-treated extract corresponding to a total P content of 0.2 mg. was applied as a streak to the paper and run for 18 hr. in the phenol solvent. Marker strips were cut from the middle of the paper and sprayed with ninhydrin and acid molybdate. Bands of the unstained paper with R_f values of 0.0-3, 0.3-0.5, 0.5-0.8 and 0.8-1.0 were eluted with water. The eluates were concentrated *in vacuo* and diluted to a known volume.

When the eluates were treated with HCl and then re-chromatographed the acid was removed by repeated distillation *in vacuo*.

Chromatographic markers. Ethanolamine phosphate was prepared by Mr B. R. Slater according to the method of Outhouse (1937). Glycerylphosphorylcholine was a gift from Dr R. M. C. Dawson.

RESULTS

The results of the ribose and phosphate analyses are given in Table 1. The percentage of acid-resistant phosphate in the total phosphate does not vary greatly with the animal and type of extraction used. The two different methods of estimating ribose phosphate, by ribose and by phosphate estimations, gave results in agreement in two cases and within 20% in the other two cases.

Very little phosphoglyceric acid was found in the extracts in which this compound was estimated. The extracts from rat uteri contained $65 \mu\text{moles}/100 \text{ g. wet wt.}$ The extracts from pig, cow, human and pregnant rabbit uteri contained no detectable phosphoglyceric acid.

Chromatography of the charcoal-treated extracts from the rabbit uteri showed the presence of the esters phosphorylethanolamine, phosphorylcholine and glycerylphosphorylcholine. Glycerylphosphorylethanolamine could be estimated in the extracts from pregnant rabbit uteri, and the chromatograms showed a faint spot in the position of this compound, but enough material was not obtained to identify the products of acid hydrolysis. Phosphoglyceric acid was not detected on the chromatograms.

Identification of phosphorylethanolamine

Two-dimensional chromatograms of the charcoal-treated extracts showed a spot in the position of phosphorylethanolamine which was ninhydrin-positive and stained blue with the acid-molybdate spray. Phosphorylethanolamine, which has R_f 0.39 in the phenol solvent (Dawson, 1954), was eluted in the fraction with R_f 0.3-0.5 from the chromatogram of a streak of the charcoal-treated extract. A sample of this fraction was cochromato-

graphed in two dimensions with a synthetic sample of phosphorylethanolamine. Only one ninhydrin-positive, phosphate-containing spot could be seen. A further sample was given the prolonged acid treatment as described and then chromatographed in phenol. A ninhydrin-positive spot near the solvent front with the same R_f as the marker ethanolamine and an inorganic phosphate spot were seen, but there was no spot in the position of phosphorylethanolamine.

Identification of phosphorylcholine and glycerylphosphorylcholine

Two-dimensional chromatograms of the charcoal-treated extracts showed two spots which stained blue with acid molybdate but which were not ninhydrin-positive. Phosphorylcholine has R_f 0.93, glycerylphosphorylcholine R_f 0.88 in the phenol

solvent (Dawson, 1955b), therefore both compounds would be present in the fraction with R_f 0.8–1.0 eluted from the chromatogram of a streak of the charcoal-treated extracts. When a sample of the eluted fraction was chromatographed in the propanol-aq. NH_3 soln. solvent, two spots which stained blue with the acid-molybdate spray were seen, with R_f values 0.23 and 0.41, corresponding to phosphorylcholine and glycerylphosphorylcholine respectively.

The presence of combined choline in the eluted fraction was shown by reineckate precipitation and by chromatography. After the prolonged acid treatment of a sample, choline could be precipitated as the reineckate. No reineckate precipitate could be obtained from the untreated fraction. A further sample was given the prolonged acid treatment and then chromatographed in phenol. Free

Table 1. *Soluble phosphates extracted from the uterus*

Trichloroacetic acid is indicated by TCA. All values are expressed as $\mu\text{moles}/100 \text{ g. wet wt.}$

Animals	Extraction	Total P	Inorganic P	Easily hydrolysable P	Ribose P	Ribose	Acid-resistant P
Rats (4)	TCA	1450	356	356	219	266	522
Rats (4)	TCA	1420	355	533	—	—	—
Rabbit	TCA	1680	371	500	290	280	515
Rabbit	TCA	1630	355	290	194	232	790
Pig	TCA	1580	680	74	290	280	538
Cow	TCA	1490	623	216	—	—	—
Human	TCA	1480	555	187	238	—	500
Pregnant rabbit	TCA	1470	455	261	232	—	520
	Water	1140	500	100	164	—	410
Pregnant rabbits (3)	Water	1370	635	77	148	—	510
Rabbit (1), 6 hr. post-partum	Water	1400	570	110	155	—	560
Rabbits (2) 24 hr. post-partum	Water	1190	465	91	155	—	485
Rabbits (2) 24 hr. post-partum	Water	1140	452	91	181	—	420

Table 2. *Ethanolamine in extracts of the uterus before and after acid treatment*

All values are expressed as μmoles of ethanolamine/100 g. wet wt.

	Ethanolamine in original solution	Increase in ethanolamine after prolonged acid treatment	Increase in ethanolamine after short acid treatment
Pregnant rabbits (3)	510	296	55
Rabbits (2), 24 hr. post-partum	500	275	0
Rabbits (2), 24 hr. post-partum	394	252	0

Table 3. *Acid-resistant phosphate esters of the uterus*

All values are expressed as μmoles of P/100 g. wet wt. Esters are phosphorylethanolamine (PE), glycerylphosphoryl-ethanolamine (GPE), phosphorylcholine (PC) and glycerylphosphorylcholine (GPC).

	Acid-resistant phosphate	PE	GPE	PC	GPC
Pregnant rabbits (3)	510	296	55	36	135
Rabbit (1), 6 hr. post-partum	560	—	—	26	148
Rabbits (2), 24 hr. post-partum	485	275	0	42	84
Rabbits (2), 24 hr. post-partum	420	252	0	55	161

choline was detected as a spot on the chromatogram with the phosphomolybdate spray of Chargaff, Levine & Green (1948). The untreated material gave a very faint choline spot.

The presence of glycerylphosphorylcholine in the eluted fraction was confirmed by cochromatography with a marker and by the production of glycerol phosphate after a short acid treatment. When the treated sample was chromatographed in two dimensions, a spot in the position of phosphorylcholine was still present, but the glycerylphosphorylcholine spot had disappeared and a phosphate-containing spot in the position of glycerol phosphate had appeared.

Ethanolamine and choline were estimated in the water extracts from rabbit uteri before and after acid treatment, by the methods described. Serine as well as ethanolamine is estimated by the periodate-oxidation method of Artom (1945), and the large value obtained before acid treatment was due to the presence of free serine detected chromatographically in the extracts. No serine phosphate could be detected, so that all increase in ammonia production by periodate oxidation after acid treatment was assumed to be due to the ethanolamine produced from ethanolamine phosphate. It is, however, possible that serine peptides were present in the extracts and that these liberated free serine after acid treatment. Choline compounds other than phosphorylcholine and glycerylphosphorylcholine may also be estimated by the method of choline estimation used. The results for the ethanolamine estimation are given in Table 2. In Table 3 the results are expressed as μ moles of the phosphate compound which liberates ethanolamine or choline on acid treatment.

DISCUSSION

Phosphorylethanolamine was first isolated by Outhouse (1936) from extracts of bovine malignant tumour, and Awapara *et al.* (1950) identified and estimated this compound in extracts from a number of organs of the rat. Dawson (1955*b*) first identified phosphorylcholine in extracts from rat liver under conditions which ensured that the compound was not formed from phospholipids in the course of preparation of the extracts, and van Heyningen & Pirie (1958) have also found it in the lens. Glycerylphosphorylcholine was first isolated from tissue extracts by Schmidt, Hershman & Thannhauser (1945), who extracted it from incubated ox pancreas, and it was identified and estimated in water extracts from fresh liver by Dawson (1955*b*).

These compounds are known to be important in the metabolism of phospholipids, and this subject has been reviewed by Dawson (1957). Kornberg &

Pricer (1952) found that phosphorylcholine was incorporated intact into phosphatidylcholine in rat liver, and it was later found by Kennedy & Weiss (1955) that the nucleotide cytidine triphosphate was required for the incorporation, by the intermediate formation of cytidine diphosphate choline. Phosphorylethanolamine reacts in a similar way to form phosphatidylethanolamine. Phosphorylcholine and phosphorylethanolamine are therefore intermediates in the synthesis of phospholipids. On the other hand, isotopic evidence suggests that glycerylphosphorylcholine and glycerylphosphorylethanolamine are breakdown products of the phospholipids and are not concerned in their synthesis. Dawson (1955*a*) measured the rate of labelling of glycerylphosphorylcholine and glycerylphosphorylethanolamine with ^{32}P in intact rat liver, and compared it with the rate of labelling of the phospholipids. The experiments showed that phosphatidylethanolamine and phosphatidylcholine could be the precursors of the free glycerylphosphorylethanolamine and glycerylphosphorylcholine extracted from the liver. The acid-resistant compounds identified in the rabbit uterus may be similarly related to the metabolism of phospholipids.

Since glycerylphosphorylcholine and glycerylphosphorylethanolamine are breakdown products of phospholipids, it seemed likely that the post-partum uterus would contain more of these compounds than the pregnant uterus. Dawson (1955*c*) found that rat liver contained more glycerylphosphorylcholine and ethanolamine after starvation of the animal for 24 hr., which is known to decrease the total phospholipid of the liver. However, there do not appear to be large differences between the concentration of these compounds in pregnant and post-partum rabbit uteri.

The results of the phosphoglyceric acid estimations are not in agreement with Hollmann's (1949) suggestion that extracts from cow uterus contain a large quantity of phosphoglyceric acid. Purkayastha & Roy (1956), who fractionated the phosphate compounds from extracts of human uterus by the barium-precipitation method, found large quantities of phosphoglyceric acid, but this could not be detected by Slater's (1953) enzymic method.

SUMMARY

1. The phosphate esters in extracts from human, cow, pig and rabbit uteri have been investigated. About 35% of the total soluble phosphate is resistant to acid treatment for 3 hr. in N-HCl at 100° .

2. An extract from rat uteri contained a small quantity of phosphoglyceric acid, but extracts from human, cow, pig and rabbit uteri contained no phosphoglyceric acid.

3. Phosphorylcholine, phosphorylethanolamine and glycerylphosphorylcholine have been identified and estimated in the acid-resistant fraction of extracts from pregnant and post-partum rabbit uteri. Glycerylphosphorylethanolamine was found in the pregnant-rabbit uterus.

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The Reaction of Peptides and Insulin with Methylhydrazine

By J. H. BRADBURY

Wool Textile Research Laboratories, C.S.I.R.O., Geelong, Australia

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The hydrazine method for the determination of the C-terminal amino acids of proteins (Akabori, Ohno & Narita, 1952; Ohno, 1953) has been modified by Bradbury (1956) to give improved recoveries of most of the amino acids. Subsequent kinetic studies showed that the increased selectivity of fission of peptide bonds possible in hydrazinolysis, as against acid hydrolysis, is due to the greater importance of steric factors in hydrazinolysis (Bradbury, 1958a). In an attempt to increase still further this selectivity the larger molecule methylhydrazine has been reacted with glycylglycine and DL-leucylglycine. The possibilities of the use of methylhydrazine in the determination of the C-terminal amino acids of proteins and as a protein solvent have been examined.

EXPERIMENTAL AND RESULTS

Materials

Glycylglycine, DL-leucylglycine and trypsin (Light and Co., Colnbrook, Bucks), insulin (Boots Pure Drug Co., Ltd., Nottingham) and lysozyme (Armour and Co., Chicago) were used without further purification. Merino 64's wool was purified as described previously (Bradbury, 1958b). Anhydrous hydrazine was obtained from the hydrate by drying over CaO and distilling (Bradbury, 1958a).

Methylhydrazine sulphate (Fisons Chemicals, Wigmore Street, London, W. 1) and a 100% molar excess of solid A.R. NaOH were placed in a flask fitted with a reflux condenser. Sufficient water was added to dissolve the NaOH and the flask cooled to slow the vigorous reaction. The mixture was then distilled under reduced pressure, two fractions being collected, the first of which was rich in