THE RESPIRATION OF THE RABBIT EPIDIDYMIS AND ITS SYNTHESIS OF GLYCERYLPHOSPHORYLCHOLINE

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Summary

The synthesis of glycerylphosphorylcholine (GPC) has been studied in the caput epididymis of the rabbit *in vitro* using [^{32}P]orthophosphate and [*Me*-^{14}C]choline. The incorporation of ^{32}P or ^{14}C into GPC was much greater than into lecithin and the ratio of ^{32}P to ^{14}C was much less in the GPC than in lecithin. Coenzyme A and stearate caused a sixty-fold stimulation of the incorporation of ^{32}P into lecithin but only a sevenfold stimulation of incorporation into GPC. The results of these experiments are not consistent with lecithin being the major immediate precursor of GPC, *in vitro*.

The Q_{O_2} values for caput epididymis tissue varied from a maximum of 2·2 for tissue slices to 0·5 for some homogenates. Oxygen uptake of tissue slices in Ringer–glucose was similar to that in a complex medium containing most cofactors. The addition of 10 μg/ml testosterone to the incubation mixture did not affect the oxygen uptake of either tissue slices or homogenates. However, tissue slices of mature epididymides respired at a greater rate than those obtained from immature animals.

I. INTRODUCTION

Glycerylphosphorylcholine (GPC) occurs in the seminal plasma of many species (Dawson, Mann, and White 1957) and is formed chiefly in the epididymis Scott, Wales, *et al.* 1963).

Dawson and Rowlands (1959) found that when [^{32}P]orthophosphate was injected into rats it was incorporated into the GPC of the epididymis within several hours and we have confirmed and extended this observation using the rabbit (Scott, Wales, *et al.* 1963). The synthesis occurs when the epididymis is separated from the testes and will take place even when slices of rabbit epididymis are incubated with ^{32}P *in vitro*. Both the head and the tail regions of the epididymis can synthesize GPC, but the head is more active (Dawson and Rowlands 1959; Scott, Wales, *et al.* 1963; Scott, Dawson, and Rowlands 1963).

Phospholipid has been demonstrated in vacuoles of the epithelial cells of the mouse epididymis (Christie 1955) and this has been suggested as a possible precursor of GPC (Dawson, Mann, and White 1957; Dawson and Rowlands 1959). Further experiments to elucidate the mode of synthesis of GPC *in vitro* by the caput epididymis of the rabbit are described in this paper. Since little is known concerning the respiratory activity of this organ, some studies of the oxygen uptake of epididymal tissue *in vitro* under various conditions are also described.

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II. Materials and Methods

(a) *General Procedure*

For the study of GPC synthesis, adult albino rabbits with normal genitalia and producing good quality semen were used in all experiments. In experiments 1 and 2, fat-free caput epididymis tissue was chopped finely with scissors and homogenized for 1–2 min at 0–2°C in 4 volumes of 0·3M sucrose. Since the sucrose complicated the chromatography in the first two experiments, the tissue in the third experiment was homogenized in a Tris-buffered (pH 7·4, 20 mM) medium containing glucose, sodium acetate, sodium citrate, potassium malate, and potassium 2-oxoglutarate at the concentrations stated below.

Unless otherwise stated, aliquots (2 ml) equivalent to approximately 300 mg of homogenized tissues were mixed with equal volumes of Tris-buffered (pH 7·4, 20 mM) sucrose (100 mM) containing [^{32}P]orthophosphate (75 μc), glucose (100 mM), sodium acetate (10mM), sodium citrate (30 mM), potassium malate (3 mM), potassium 2-oxoglutarate (3 mM), magnesium chloride (6·6 mM), choline chloride (0·4 mM), cytidine triphosphate (0·6 mM), adenosine triphosphate (3·2 mM), cytochrome *c* (0·6 mM), cysteine (6 mM), coenzyme A (0·6 mM), and stearic acid (4 mM, bound to albumin). In experiment 2, the choline chloride used was [*Me*-^{14}C]choline chloride (from the Radiochemical Centre, Amersham, England) of specific activity 0·18 μc/μmole.

The homogenates were incubated for different times in Warburg flasks at 37°C and their oxygen utilization measured by the direct method. The incubation period was taken as the time from adding the medium containing the isotope to the homogenate until the tissue was inactivated by heating.

In some of the later respiratory studies the tissue slices were incubated in calcium-free Krebs–Ringer phosphate (Umbreit, Burris, and Stauffer 1961) containing 11 mM glucose. In these experiments, the tissue of the caput epididymis was finely sliced (0·5 mm thick) immediately after removal from the animal and incubated 3 hr at 37°C. The oxygen uptakes have been expressed as Q_{O_2} (μl O_2 per mg dry weight of tissue per hour).

(b) *Extraction of Tissues to Obtain Water-soluble Fractions*

(i) *GPC.*—The flask contents were extracted for GPC by the method based on that of Dawson (1955). The aqueous phase after washing with chloroform was applied to a mixed-bed column (2 parts deacidite FF : 1 part Zeocarb 226) to remove cations and to separate GPC from the bulk of the inorganic phosphate and other phosphate esters. Preliminary tests showed that this treatment removed 99% of the inorganic phosphate, phosphorylcholine, and α-glycerophosphate. Aliquots of the deionized samples were chromatographed on Whatman No. 1 paper using phenol–ethanol–acetic acid as solvent (Dawson 1960). Sections of the chromatograph with an R_F similar to that of authentic GPC were eluted with water – ethanol (1 : 1 v/v) and the identity of the eluates confirmed by chromatography using propanol–ammonia–water (6 : 3 : 1 v/v) and phenol (water-saturated)–ethanol (4 : 1

v/v) which will separate GPC (R_F 0·82 and 0·42) from phosphorylcholine (R_F 0·65 and 0·23). Aliquots of the isolated GPC were assayed for radioactivity and phosphorus content.

(ii) *Inorganic Phosphate.*—The inorganic phosphate and phosphate esters were eluted from the mixed-bed columns with 0·2M potassium bicarbonate (up to 14 bed volumes) and the eluate freed of potassium bicarbonate by making it slightly acid (about pH 4·5) with perchloric acid and then centrifuging in the cold to remove the insoluble potassium perchlorate. Inorganic phosphate was isolated from an aliquot of the bicarbonate-free eluate by repeated precipitation in the cold with calcium ions at pH 8·3 (Leloir and Cardini 1957). The calcium phosphate precipitate was dissolved finally in the minimum of dilute HCl and aliquots assayed for phosphorus and radioactivity. In some cases, to confirm the identity of the fraction, the phosphomolybdic acid was extracted into isobutanol (Berenblum and Chain 1938).

(*c*) *Extraction of Tissues to Obtain Lipid Fraction*

The lipids were extracted by twice dispersing the tissue residues which had been extracted with phenol–water in 20 volumes of chloroform–methanol (2 : 1 v/v) and pooling the solution with the chloroform phase from Section II(*b*)(i) above. The lipid extract was washed free of water-soluble contaminants by shaking with 2 volumes of ice-cold 0·1N HCl. Aliquots of the washed lipid extract were taken for the assay of phosphorus and radioactivity or for the isolation of lecithin by the hydrolytic method of Dawson (1960).

(*d*) *Assay of Radioactivity*

The ^{32}P-radioactivity was determined in most cases with an M6 liquid counter. Carbon (and in some cases ^{32}P-) radioactivity was determined on plated samples of infinite thinness with either a Geiger–Müller end-window tube or windowless gas-flow counter. The ^{32}P- and ^{14}C-radioactivity in the same sample were differentiated by counting with and without the addition of an aluminium cover. Samples were counted in duplicate for at least 1000 counts above background and all readings were corrected for background, decay, coincidence, and relative efficiency. Specific activities are expressed as counts per second per μmole or μatom of phosphorus. The chromatograms were scanned for the distribution of radioactivity by passing between two Geiger–Müller end-window tubes.

III. Results

(*a*) *GPC Synthesis*

(i) *Experiment 1.*—The first experiment was a study of the incorporation *in vitro* of [^{32}P]orthophosphate into GPC, lecithin, and other phosphate-containing compounds by homogenates of the caput epididymis during periods of 2, 20, 60, and 180 min. Aliquots of the tissue homogenate were incubated in a fortified medium and continued to respire actively throughout the experimental period. From the results presented in Table 1, it is evident that insufficient isotope has been incorporated into lecithin for this substance to be the sole precursor of GPC. The specific activity

values for GPC at 60 and 180 min when considered in relation to the relative pool sizes correspond to an incorporation of about 2–3% of the added radioactivity, while those for lecithin amount to only about 0·1%.

(ii) *Experiment 2.*—A study of the simultaneous incorporation of [^{32}P]orthophosphate and [*Me*-^{14}C]choline by epididymal tissue homogenates incubated for periods of 20 and 60 min was made. The ratios of the specific activities of ^{32}P to ^{14}C incorporated into GPC over these two periods were 2·7 and 6·6 respectively, whilst the corresponding ratios for incorporation into lecithin were 16·9 and 17·3. These results indicate that there is no close relationship between these ratios. In addition total incorporation of isotope, either ^{32}P or ^{14}C, into GPC was greater than that into lecithin.

TABLE 1

INCORPORATION OF [^{32}P]ORTHOPHOSPHATE BY HOMOGENATES OF RABBIT EPIDIDYMIS *IN VITRO*

Compound	Pool Size [μmoles/100 mg tissue (wet wt.)]	Specific Radioactivity (counts/sec/μmole) for Incubation Periods (min) of:			
		2	20	60	180
Inorganic phosphate	2·9	8384	5983	5890	5394
GPC	0·03	837	1426	2604	2015
Lecithin	0·8	2	40	25	7

(iii) *Experiment 3.*—Aliquots (2 ml) of homogenate equivalent to about 300 mg of tissue were mixed with equal volumes of Tris-buffered (pH 7·4, 40 mM) sodium chloride (70 mM) containing [^{32}P]orthophosphate, magnesium chloride, choline chloride, cytidine triphosphate, adenosine triphosphate, cytochrome *c*, and cysteine at the concentrations specified for experiment 1. Coenzyme A and stearic acid were also included in the media of four samples but omitted from four other flasks. The incubation procedure was as in experiment 1.

From the results presented in Table 2 it is evident that, despite a 60-fold stimulation of the incorporation of isotope into lecithin by coenzymes A and stearate, the labelling of GPC increased only about sevenfold. Statistical analysis of the results of this experiment after logarithmic transformation showed a significant interaction between incorporation of ^{32}P into GPC or lecithin and the presence or absence of coenzyme A and stearic acid. Again the degree to which lecithin was labelled with ^{32}P was far less than could account for the ^{32}P activity in GPC.

The specific radioactivity of the GPC in this experiment was much lower than that in experiment 1. The pool size, however, was correspondingly larger and the total incorporation of isotope into GPC in this experiment was comparable to that in the earlier experiment. Thus the difference in specific radioactivity may only reflect a difference in the pool size of GPC in the epididymis at the time of removal from the animal.

An indication of the significance of the effect of time on the incorporation of ^{32}P into GPC and lecithin was gained by statistical analysis of the combined results at 20 and 60 min for the three incubations in the complete medium described in Section II. Due to the differences in specific radioactivity of GPC and lecithin, the logarithmic transformation was again used. A significant interaction was found between the time of incubation and the incorporation of ^{32}P into GPC or lecithin ($P < 0{\cdot}05$). The specific radioactivity of the GPC increased over the time, but there was no change in the specific radioactivity of lecithin.

(*b*) *Respiration Studies*

The oxygen uptake of the tissue homogenized in sucrose (expt. 1) was somewhat higher (see Fig. 1) than that of tissue homogenized in the medium without sucrose (expt. 3). This difference may only reflect sample differences, since a further sample

TABLE 2

EFFECT OF STEARIC ACID AND COENZYME A ON THE INCORPORATION OF [^{32}P]ORTHOPHOSPHATE BY HOMOGENATES OF RABBIT EPIDIDYMIS *IN VITRO*

Compound	Pool Size [μmoles/100 mg tissue (wet wt.)]	Stearic Acid + Coenzyme A	Specific Radioactivity (counts/sec/μmole) for Incubation Periods (min) of:			
			2	20	60	180
Inorganic phosphate	3·8	−	10,029	7,753	6,755	6,681
	3·6	+	11,951	7,533	6,910	6,185
GPC	0·7	−	39	20	56	143
	0·4	+	38	142	447	106
Lecithin	0·5	−	0·06	0·09	0·03	0·16
	0·6	+	2·70	1·17	8·30	6·30

of tissue homogenized in Tris-buffered saline had an oxygen uptake over 180 min of 1·5 μl/mg dry weight of tissue. In all samples, the rate of oxygen uptake declined after 60 min and the mean value over 180 min was less than double the value over the first 60 min.

In the next experiment, the oxygen uptake of tissue homogenized as described for experiment 2 was compared with aliquots of the same epididymides incubated as tissue slices in the same medium. The effect of added testosterone (10 μg/ml) was also examined. In this experiment, the mean oxygen uptake of the homogenates over 60 min was 60% of that of the tissue slices and there was no significant effect of added testosterone.

The oxygen uptake of slices of caput epididymis tissue incubated in Krebs–Ringer diluent was compared with that of slices of the same epididymides incubated in the complex medium used for the studies of GPC synthesis. In the first hour the oxygen uptake was 1·89 (Krebs–Ringer diluent) and 1·74 (complex medium) μl/mg dry weight of tissue, whilst the mean oxygen uptake over 3 hr was 1·63 and 1·60, respectively (means for three samples). There was no significant effect of change

in incubation medium and the mean Q_{O_2} during the first hour of incubation was 1·82. There was a slight decrease in oxygen consumption during incubation and the mean Q_{O_2} over 3 hr was significantly lower than that measured during the first hour.

The oxygen uptake of slices of caput epididymal tissue obtained from immature, pubertal, and adult male rabbits was also measured. The mean Q_{O_2} values over the first hour for two samples in each group were 1·47, 1·67, and 1·87, respectively. The variance between samples was found to be 0·003 (d.f. = 3) and there were significant differences between the group means.

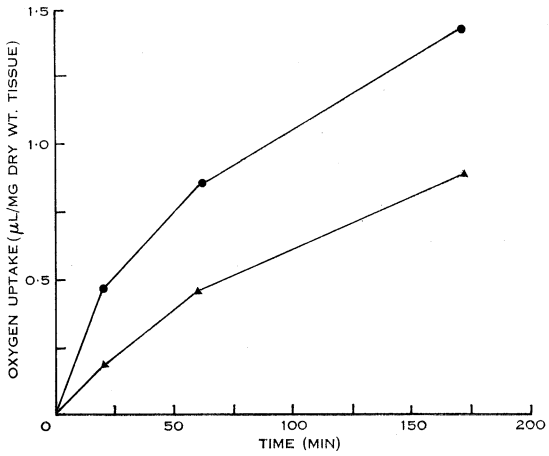

Fig. 1.—Oxygen uptake of homogenized epididymal tissue used in experiments 1 (●) and 3 (▲). The tissue was homogenized in sucrose (expt. 1) or a Tris-buffered medium (expt. 3).

IV. Discussion

The biosynthetic pathways leading to the secretion of seminal GPC are still unknown. Scott, Dawson, and Rowlands (1963) have reported that the incorporation of GPC into the rat epididymis *in vivo* is consistent with the formation of GPC from lecithin or choline plasmalogen. On the other hand, in similar *in vivo* studies on the rabbit the specific activity of epididymal phospholipids was less than that of GPC (Scott, Wales, *et al.* 1963). Since, however, GPC is secreted into the lumen of the epididymis, in the latter experiments it may have maintained a higher specific activity than the rapidly turning over intracellular phospholipids (Scott, Dawson, and Rowlands 1963) which would decline due to equilibration with phosphorus constituents of the blood and other tissues (Dawson 1958).

Without more knowledge of the state and movement of GPC and spermatozoal phospholipid in the epididymal tubules, Reiner's (1953) discussion of isotopic tracers in the determination of product–precursor relationships indicates that it might be

difficult to establish an unequivocal relationship between GPC and lecithin in the epididymis by studying ^{32}P-incorporation *in vivo* alone. It was felt, therefore, that an alternative approach *in vitro* might yield useful information.

The results suggest that GPC may be synthesized, *in vitro*, in the rabbit epididymis by an alternative pathway not involving lecithin. While the possibility cannot be excluded of isotopic reversal of the glycerylphosphorylcholine diesterase reaction even in the face of a net breakdown of GPC; this explanation seems unlikely since incorporation of ^{32}P into the diester continued over most of the incubation period. Had GPC been solely derived from lecithin by the action of phospholipases (see Kates 1960) the specific activity and total isotope content of lecithin would be expected to be equal to or greater than that of GPC. Also one would have expected the ratios of isotope labelling (^{32}P from orthophosphate/^{14}C from choline) of lecithin and GPC to have been comparable and a greater stimulation of lecithin turnover by coenzyme A and stearic acid. Such arguments against lecithin being the precursor of GPC might, however, be invalidated if a small but rapidly changing pool of lecithin was involved in the synthesis of GPC.

Support for a non-phospholipid precursor of GPC, *in vitro*, is seen in the results of Williams-Ashman and Banks (1956) who, working with homogenates of guinea pig seminal vesicles, found that under a number of experimental conditions for which cytidine diphosphocholine acted as a precursor in the synthesis of lecithin, no formation of GPC from this cytidine coenzyme could be detected.

In comparison with other mammalian tissues the respiratory activity of the caput epididymis would appear to be low. The highest Q_{O_2} value found in the present studies for tissue slices was 2·2, while the oxygen uptake of homogenates was often only 25% of this value. Even in those samples which respired actively, the oxygen uptake was much less than that found for several other rabbit tissues and for ejaculated rabbit spermatozoa (see Spector 1956). Since respiration was no greater in the complex medium than in Ringer–glucose, the low activity is apparently an inherent property of the tissue and is probably not due to lack of essential cofactors.

Although the addition of testosterone to the incubation mixture did not affect oxygen uptake, the hormone may have long-term effects in maintaining the metabolic activity of the organ. The fact that mature epididymal tissue respired at a greater rate than immature tissue would seem to support this. It must be remembered, however, that mature epididymal tissue contains a considerable proportion of spermatozoa and their activity may have contributed to the differences observed between mature and immature organs.

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