Glucose Metabolism in the Superovulated Rat Ovary *in vitro*

EFFECTS OF LUTEINIZING HORMONE AND THE ROLE OF GLUCOSE METABOLISM IN STEROIDOGENESIS

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1. Superovulated rat ovary slices from rats treated with 20 μ g. of luteinizing hormone/100g. body wt. 2 hr. before death and from control animals have been incubated *in vitro*. Output of Δ^4 -3-oxo steroids (0.2 μ mole/g. wet wt./hr. in control tissue) was linear for 4 hr., and was increased by approx. 70% in slices from luteinizing hormone-treated rats. Rate of oxygen consumption ($90.0 \pm 4.6 \mu$ moles/g. wet wt./hr.) was linear for 3 hr. and unaltered by luteinizing hormone treatment or addition of glucose (1 mg./ml.) to the medium. 2. In slices from control animals, steady-state rate of glucose uptake was $78.0 \pm 2.9 \mu$ g. atoms of carbon/g. wet wt./hr.; steady-state rates of lactate output, pyruvate output and incorporation of [U- ^{14} C]-glucose carbon atoms into carbon dioxide and total lipid extract were 60.7 ± 0.9 , 2.4 ± 0.1 , 18.0 ± 1.1 and $0.7 \pm 0.1 \mu$ g. atom of carbon/g. wet wt./hr. and accounted for $104.5 \pm 1.9\%$ of the glucose uptake. In slices from luteinizing hormone-treated rats, glucose uptake and outputs of lactate, pyruvate and [^{14} C]carbon dioxide were increased by approx. 25%, and $108.4 \pm 3.2\%$ of the glucose uptake could be accounted for. 3. The total lipid extract was separated by thin-layer chromatography and saponification. Of the ^{14} C incorporated into this fraction during incubation with [U- ^{14} C]glucose 97% was found in the fractions containing glyceride glycerol and less than 3% in the fractions containing sterols, steroids or fatty acids. Appreciable quantities of ^{14} C were incorporated into these lipid fractions from [1- ^{14} C]acetate. 4. From a consideration of the tissue glycogen content, the specific activities of [^{14} C]lactate and glucose 6-phosphate (C-1) derived from [1- ^{14} C]-, [6- ^{14} C]- and [U- ^{14} C]-glucose, and the ratio of [^{14} C]carbon dioxide yields from [1- ^{14} C]glucose and [6- ^{14} C]glucose, it was concluded that there was no appreciable glycogenolysis or flow through the pentose phosphate cycle. 5. In ovary slices from both control and luteinizing hormone-treated animals, glucose *in vitro* raised the incorporation rate of ^{14} C from [1- ^{14} C]acetate into sterols and steroids. Luteinizing hormone *in vivo* stimulated the incorporation rate *in vitro* but only in the presence of glucose. 6. In slices incubated in medium containing [^{3} H]water, [^{14} C]sorbitol and glucose (1 mg./ml.), the total water space ($865 \pm 7.1 \mu$ l./g.) and the extracellular water space ($581 \pm 22 \mu$ l./g.) were unchanged by luteinizing hormone treatment *in vivo* but the glucose space was raised from $540 \pm 23.6 \mu$ l./g. to $639 \pm 31.3 \mu$ l./g. 7. Luteinizing hormone treatment was found to lower the tissue concentration of the hexose monophosphates and to increase the total activity of hexokinase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase and possibly of phosphofructokinase. 8. The kinetic properties of a partially purified preparation of phosphofructokinase were found to be qualitatively similar to those from other mammalian tissues. 9. The results are discussed with reference to both the role of glucose metabolism in steroidogenesis and the mechanism by which luteinizing hormone increases the rate of glucose uptake.

The administration of LH* to the superovulated rat *in vivo* increases rates of steroidogenesis

* Abbreviations: LH, luteinizing hormone; PFK, phosphofructokinase (ATP-D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11).

(Armstrong, O'Brien & Greep, 1964) and of glucose metabolism (Armstrong & Greep, 1962; Armstrong, 1963; Channing & Vilee, 1966) measured subsequently *in vitro*. It is not known whether these two effects of the hormone are related. The effect of

LH administered *in vivo* on glucose uptake *in vitro* could be exerted at either a membrane-transport step for glucose or on subsequent steps involved in intracellular metabolism of the sugar. The evidence available is conflicting: Channing & Vilee (1966), using tissue minces and no extracellular or total water markers, have reported small increases in tissue glucose and galactose accumulation after prior treatment with LH *in vivo*. This led these authors to conclude that LH acts on the cell membrane. Sowerby (1968), using tissue slices, found that the rate of intracellular accumulation of the non-metabolizable sugar D-xylose (which in other tissues shares a common transport system with glucose) was not changed by treatment with LH *in vivo*. In many tissues PFK has been shown to control the rate of glycolysis. Two ways in which LH may bring about an increase in the activity of PFK are: as a result of an increased concentration of 3',5'-(cyclic)-AMP (which has been shown in bovine corpus luteum in response to LH added *in vitro*; Marsh, Butcher, Savard & Sutherland, 1966) or from an elevated total activity of the enzyme.

An increased rate of glucose metabolism could act as an additional source of carbon, reducing equivalents (as NADPH₂) or energy (as ATP) for steroidogenesis. The rate of conversion of glucose carbon atoms into steroids in this tissue has not been reported, but only up to 2% of the total radioactive glucose carbon atoms utilized has been found to be incorporated into lipid-soluble components during incubation for 2 hr. *in vitro* (Armstrong, 1963). From the relative rates of incorporation of C-1 and C-6 of [1-¹⁴C]- and [6-¹⁴C]-glucose to ¹⁴CO₂ and ¹⁴C-labelled lipids, Armstrong & Greep (1962) have suggested that there is no pentose-cycle activity in this tissue *in vitro*. However, Kidwell, Balogh & Wiest (1966) have since reported the presence of glucose 6-phosphate dehydrogenase and find that the total activity of this enzyme is increased by treatment with LH *in vivo*.

The studies described here were designed to clarify the relationship between glucose metabolism and steroidogenesis, and the site of action of LH on glucose uptake and glycolysis.

MATERIALS

Animals. Superovulated ovaries were obtained from immature female Wistar rats treated with pregnant-mare serum gonadotrophin and human chorionic gonadotrophin as described by Stansfield & Flint (1967). The rats were fed with stock laboratory diet 41B (Short & Parkes, 1949) and given water *ad libitum*.

LH was administered intraperitoneally in doses of 20 µg./100 g. body weight in 0.5 ml. of 0.9% NaCl 2 hr. before death. Control animals received the saline.

Chemicals. Radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks. Dowex 1

(formate form) (AG1-X8) was from Bio-Rad Laboratories, Richmond, Calif., U.S.A. DEAE-cellulose (Whatman DE-11; W. and R. Balston Ltd., Maidstone, Kent) was prepared for use by the procedure described by Pogson & Randle (1966). Silica gel G was from E. Merck A.-G. through Anderman and Co. Ltd., London S.E.1. Bovine plasma albumin (fraction V) was obtained from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex. 2,5-Diphenyloxazole and 1,4-bis-(5-phenoxazol-2-yl)benzene were obtained from Thorn Electrical, Tolworth, Surrey; Hyamine hydroxide (1 M in methanol) was from the Packard Instrument Co. Inc., La Grange, Ill., U.S.A.

Human chorionic gonadotrophin and pregnant-mare serum gonadotrophin (Pregnyl and Gestyl) were obtained from Organon Laboratories Ltd., Morden, Surrey; LH (NIH-LH-S11, ovine) was the gift of the National Institutes of Health, Bethesda, Md., U.S.A.

Nucleotides, sugar phosphates and enzymes were obtained from the Boehringer Corp. (London) Ltd., London W.5 [with the exception of 3',5'-(cyclic)-AMP, from Sigma Chemical Co., St. Louis, Mo., U.S.A., who also supplied tris]. Thiosemicarbazide was recrystallized from methanol and dried overnight *in vacuo* (Sommerville, Pickett, Collins & Denyer, 1963). Chloroform, methanol, benzene, *n*-hexane and ethyl acetate were redistilled shortly before use. Diethyl ether was passed down a column of alumina (grade I; Woelm, Eschwege, Germany) before redistillation over FeSO₄ and storage over copper gauze. All other chemicals were of the purest grade commercially available.

Media. Ovary slices were incubated in a bicarbonate-buffered medium (Krebs & Henseleit, 1932) gassed with O₂ + CO₂ (95:5) and containing additions as given in the text and tables.

PROCEDURES AND METHODS

Radioactivity assay

Radioactivity was assayed with a Nuclear-Chicago liquid-scintillation system (model mark 1). Quench corrections were made by the channel-ratios technique with an external standard of ¹³³Ba. Counting efficiency was always at least 60%, and at least 2000 counts were recorded.

Non-aqueous solutions were counted in a toluene-based scintillator and aqueous solutions in a dioxan-based scintillator as described by Denton & Randle (1967).

Simultaneous assay of ³H and ¹⁴C in samples added to dioxan-based scintillator was carried out by the balance-point procedure as described by the makers of the scintillation system.

Incubation of ovary slices

Preparation and incubation of slices. Slices were cut by hand, from ovaries (trimmed free of adipose tissue) held between ice-cold ground-glass plates, and were immersed in ice-cold medium for up to 10 min. before use. After blotting and weighing, the slices were transferred to stoppered flasks gassed with O₂ + CO₂ (95:5) and incubated with shaking at 37°. The volume of the incubation medium was either 1.5 ml. (30–60 mg. of tissue) or 5.0 ml. (100–200 mg. of tissue).

When ¹⁴CO₂ evolved during incubation was collected, incubations were carried out in 5.0 ml. of medium, as above, in Marie flasks. At the end of the incubation, 0.5 ml. of

Hyamine hydroxide (1M) was injected into the inner annulus followed by 0.5 ml. of HClO_4 (25%, v/v) into the outer annulus through self-sealing rubber caps. To ensure collection of all the $^{14}\text{CO}_2$ the flasks were incubated with shaking for a further 2 hr. The Hyamine hydroxide solution was then transferred to scintillation bottles with 15 ml. of toluene-based scintillation fluid.

Measurement of oxygen uptake. Oxygen uptake was followed continuously with a Clark type of O_2 electrode (Chappell, 1964) calibrated at 37° with incubation medium gassed with $\text{O}_2 + \text{CO}_2$ (95:5) and with medium treated with excess of sodium dithionite. Incubations were made in 5 ml. of gassed medium in a closed vessel surrounded by a water jacket at 37° and stirred magnetically. Air was excluded from the vessel as the electrode was lowered into it. Oxygen uptakes were linear for about 15 min., at which time approximately 30% of the O_2 dissolved in the medium had been utilized. The apparatus was designed so that it was possible to gas the medium again with water-saturated $\text{O}_2 + \text{CO}_2$ (95:5) through a small inlet tube passing through the side of the water-jacketed chamber without removing the slices. In this way a series of rate measurements could be made between periods of gassing. Such series of uptake rates measured over 5–10 min. periods and followed by 5–10 min. periods of gassing were constant for up to 3 hr.

Extraction of medium and slices after incubation. For the assay of medium glucose, lactate and pyruvate concentrations after incubation, media were deproteinized with 5% (v/v) HClO_4 and neutralized with saturated KHCO_3 .

For the assay of tissue concentrations of glucose, ^3H -water, $[\text{U-}^{14}\text{C}]$ sorbitol, glucose 6-phosphate and fructose 6-phosphate and for the determination of glucose 6-phosphate specific activity, the slices were lightly blotted and immediately plunged into liquid N_2 . The frozen slices (approx. 500 mg.) were weighed and extracted with 2 ml. of ice-cold 5% (v/v) HClO_4 in an all-glass grinder (Kontes Glass Co., Vineland, N.J., U.S.A.). After centrifugation the extracts were neutralized with saturated KHCO_3 .

For the estimation of the incorporation of ^{14}C into lipid fractions and the rate of Δ^4 -3-oxo steroid production a total lipid extract was prepared as follows. Media and tissue slices were extracted three times in an all-glass Potter-Elvehjem homogenizer with 20 ml. of diethyl ether. The pooled ether extracts were washed with water (5.0 ml.), which in turn was extracted with a further 20 ml. of diethyl ether. The combined ether extracts were evaporated to dryness in a rotary evaporator under vacuum at 45° and the residue was dissolved in 10 ml. of chloroform-methanol (2:1, v/v). Recovery of $[\gamma\text{-}^3\text{H}]$ progesterone added before homogenization was greater than 98%.

For the assay of glycogen, the tissue was extracted by the procedure of Walaas & Walaas (1950) and glycogen measured as glucose after acid hydrolysis as described for heart by Randle, Newsholme & Garland (1964).

Analysis of total lipid extract

Assay of Δ^4 -3-oxo steroids. Steroids containing the Δ^4 -3-oxo grouping were assayed without prior separation in a sample of the total lipid extract by a modification of the method described for serum progesterone by Sommerville *et al.* (1963).

The sample (containing 5–60 μg . of Δ^4 -3-oxo steroid) was evaporated to dryness at 45° under a stream of O_2 -free N_2

and the residue treated with 2 ml. of a solution containing thiosemicarbazide (180 mg.), 0.1 M-HCl (20 ml.) and 95% (v/v) ethanol (100 ml.) (Sommerville *et al.* 1963) and allowed to stand for 1.5 hr. at room temperature in the dark. The extinctions at 292, 302 and 312 $\text{m}\mu$ were then determined in a 1 cm.-light-path cuvette and the Allen (1950) correction was applied. The corrected readings were compared with those given by standard progesterone solutions (previously calibrated by measurement of the extinction in ethanol at 240 $\text{m}\mu$ by using the extinction coefficient determined by Dorfman, 1953).

The Δ^4 -3-oxo steroid content of total lipid extracts assayed as above was $101 \pm 4\%$ (mean $\pm$ S.E.M. for four determinations) of the values obtained by using the method of Armstrong *et al.* (1964) involving thin-layer chromatography and extinction at 240 $\text{m}\mu$ for the assay of progesterone and 20α -hydroxyprogesterone in tissue extracts.

The formation of a thiosemicarbazone with an extinction maximum at 302 $\text{m}\mu$ is reported to be characteristic of Δ^4 -3-oxo steroids; other oxo steroids give maxima at different wavelengths (Bush, 1953). We have found the assay to be unaffected by the presence of the following in amounts greatly in excess of the Δ^4 -3-oxo steroids: pregnenolone, 17α -hydroxypregnenolone, dehydroepiandrosterone, cholesterol, various cholesterol esters, squalene, tripalmitate, phospholipids, oestrone and oestradiol. Thus this method appears to give a valid and rapid assay of Δ^4 -3-oxo steroids in this tissue.

Partial separation of the lipids in total lipid extract by thin-layer chromatography. Samples of the total lipid extract (containing the lipid from 50–200 mg. of tissue) were evaporated to dryness under a stream of O_2 -free N_2 at 45° and plated on glass thin-layer-chromatography plates (20 cm. $\times$ 20 cm.) coated (0.5 mm.) with silica gel G and activated before use at 110° for 30 min. The plates were developed in benzene-ethyl acetate (19:1, v/v) in unsaturated tanks until the solvent front was 16 cm. from the origin. Where necessary spots were identified either by I_2 vapour or by spraying with 10% (w/v) phosphomolybdate (Smith & Foell, 1962). The R_f values of the lipid classes in this system were: squalene, 0.95; cholesterol esters, 0.85; triglycerides, 0.7; diglycerides, 0.25; cholesterol, 0.2; Δ^4 -3-oxo steroids, 0.15; pregnenolone, 0.1; monoglycerides, 0.05; phospholipids, 0.00.

To elute the lipids, silica gel was scraped from the plates in three bands between the R_f values 0.5–1.0 (fraction A), 0.05–0.5 (fraction B) and 0.00–0.05 (fraction C) and extracted three times with 3 ml. of chloroform-methanol (2:1, v/v). The pooled extracts were evaporated to dryness under a stream of O_2 -free N_2 at 45° . Overall recovery (after chromatography) of radioactivity in total lipid extracts of tissues incubated in $[\text{I-}^{14}\text{C}]$ acetate was $91 \pm 2.3\%$ (mean $\pm$ S.E.M. for ten determinations).

Saponification of lipid fractions separated by thin-layer chromatography. Samples of fraction A (containing sterol esters, squalene and triglycerides) and fraction B (containing free sterols, steroids and diglycerides) were evaporated to dryness after elution under a stream of O_2 -free N_2 at 45° and the residue was saponified with 2% (w/v) KOH in 95% (v/v) ethanol (5 ml.) at 60° for 2 hr. This prolonged saponification was necessary to ensure complete hydrolysis of the sterol esters. After saponification samples were cooled and extracted three times with 10 ml. of *n*-hexane. The remaining aqueous phase was acidified with 50% (v/v) HClO_4

(1 ml.) and re-extracted three times with 10 ml. of *n*-hexane to remove fatty acids. The pooled (30 ml.) *n*-hexane fractions were evaporated to dryness with O_2 -free N_2 at 45° and the residues assayed for radioactivity with toluene-based scintillator. The aqueous fraction was assayed for radioactivity, after neutralization, with dioxan-based scintillator. Recoveries of radioactivity from [4- ^{14}C]-cholesterol, glycerol tri[1- ^{14}C]palmitate and [U- ^{14}C]-glycerol tripalmitate in the *n*-hexane extract before acidification, in the *n*-hexane extract after acidification and in the aqueous phase respectively were greater than 98%.

Analysis of neutralized perchloric acid extracts of tissue and incubation media

Glucose and glucose specific activity. Glucose was assayed in the initial and final incubation media and in tissue extracts with hexokinase and glucose 6-phosphate dehydrogenase (Slein, 1963). Medium glucose specific activity was calculated from the concentration of glucose in the initial medium and the radioactivity assayed in dioxan-based scintillator.

[^3H]Water and [^{14}C]sorbitol. These were assayed simultaneously as given under 'Radioactivity assay'.

Glucose 6-phosphate and fructose 6-phosphate. These were assayed in tissue extracts both spectrophotometrically and fluorimetrically by the method of Hohorst (1963a).

Glucose 6-phosphate (C-1) specific activity. The specific activity of C-1 of glucose 6-phosphate in samples of tissue extracts was determined from the reduction of NADP and the $^{14}CO_2$ formed on addition of 6-phosphogluconate dehydrogenase (EC 1.1.1.43) after the glucose 6-phosphate had been converted into 6-phosphogluconate with glucose 6-phosphate dehydrogenase (Randle, Denton & England, 1968).

Medium lactate and pyruvate concentrations and lactate specific activity. Lactate was assayed in samples of neutralized extracts of medium with lactate dehydrogenase and NAD in the presence of hydrazine as a trapping agent (Hohorst, 1963b); pyruvate was assayed with lactate dehydrogenase and $NADH_2$ (Bücher, Czok, Lamprecht & Latzko, 1963).

The specific activity of lactate in a sample of medium extract (1 ml.) was determined after separation of [^{14}C]-lactate from other metabolites of [^{14}C]glucose on Dowex 1 (formate) with an exponential formic acid-water gradient by the method of Denton & Randle (1967). A known amount of carrier [^{12}C]lactate was added before chromatography; pyruvate was not converted into lactate before chromatography since it is well separated from lactate in this system.

Measurement of tissue enzyme activities

Enzyme activities were measured at 37° by following the disappearance of $NADH_2$ or the production of $NADPH_2$ at $340 m\mu$ with a Hilger spectrophotometer and Gilford kinetic recording attachment.

Pairs of ovaries were extracted in a hand-operated glass Potter-Elvehjem homogenizer with 50 mM-tris acetate buffer, pH 7.5 (5.0 ml.), at 4° . Activities were assayed where possible with this crude homogenate. In some instances, particularly when the rate of $NADH_2$ oxidation or adenosine triphosphatase activity or both in the absence of substrate(s) was greater than 25% of the rate in the presence

of substrate(s), activities were assayed in supernatants obtained after centrifugation at 4° either at 2000g for 5 min. (LSS) or at 25000g for 20 min. (HSS).

Activities were measured under conditions of maximum velocity, as follows. All ancillary enzymes were present at 1 unit/ml. or greater.

Hexokinase (EC 2.7.1.1). Assays were performed in 100 mM-triethanolamine-HCl buffer, pH 7.4, containing $MgCl_2$ (10 mM), glucose (1.8 mM), NADP (0.1 mM), ATP (2 mM) and glucose 6-phosphate dehydrogenase. Since the addition of 6-phosphogluconate dehydrogenase (1 unit) caused the observed rate of NADP reduction to be doubled, it is assumed that endogenous 6-phosphogluconate dehydrogenase did not contribute significantly to NADP reduction.

Phosphofructokinase (EC 2.7.1.11). Assays were performed in 20 mM-imidazole buffer, pH 7.4, containing potassium acetate (150 mM), $(NH_4)_2SO_4$ (20 mM), $MgCl_2$ (5 mM), bovine plasma albumin (0.01%), $NADH_2$ (0.1 mM), glucose 6-phosphate (1.0 mM), ATP (2 mM), phosphoglucose isomerase (EC 5.3.1.9), aldolase (EC 4.1.2.13), triose phosphate isomerase (EC 5.3.1.1) and L-glycerol 3-phosphate dehydrogenase (EC 1.1.1.8).

Pyruvate kinase (EC 2.7.1.40). Assays were performed in 100 mM-triethanolamine-HCl buffer, pH 7.4, containing $MgCl_2$ (10 mM), phosphoenolpyruvate (1.5 mM), ATP (1.5 mM), $NADH_2$ (0.1 mM) and lactate dehydrogenase (EC 1.1.1.27).

Lactate dehydrogenase (EC 1.1.1.27). Assays were performed in 100 mM-triethanolamine-HCl buffer, pH 7.4, containing $MgCl_2$ (10 mM), pyruvate (1.0 mM) and $NADH_2$ (0.1 mM).

Glucose 6-phosphate dehydrogenase (EC 1.1.1.49). Assays were performed in 100 mM-triethanolamine-HCl buffer, pH 7.4, containing $MgCl_2$ (10 mM), glucose 6-phosphate (1.5 mM) and NADP (0.1 mM).

6-Phosphogluconate dehydrogenase (EC 1.1.1.43). Assays were performed in 100 mM-triethanolamine-HCl buffer, pH 7.4, containing $MgCl_2$ (10 mM), 6-phosphogluconate (2 mM) and NADP (1.0 mM).

Protein. Assays of protein in homogenates and supernatants were carried out by the method of Lowry, Rosebrough, Farr & Randall (1951).

Partial purification of superovulated ovary PFK

Extraction of PFK. Ovaries (1–2 g.) from 10–15 rats were trimmed, washed in 50 mM-tris acetate buffer, pH 7.5, blotted and extracted in 3 vol. of the same buffer at 4° in a hand-operated Potter-Elvehjem homogenizer. The homogenate was centrifuged at 25000g for 30 min. at 4° .

DEAE-cellulose fractionation. The supernatant (from the above) was layered on to a fast-flowing (8–10 ml./min.) column of DEAE-cellulose (2.5 cm $\times$ 4.1 cm.) equilibrated with 50 mM-tris acetate buffer, pH 7.5. After being washed with 40 ml. of the same buffer, the column was eluted with a continuous linear gradient of tris acetate buffer, pH 7.5, generated by the addition of 500 mM-tris acetate (250 ml.) to 50 mM-tris acetate (250 ml.) in an open vessel. PFK was eluted as a single peak after 100 ml. of this gradient.

Assay of PFK activity. Activity was measured as described by Denton & Randle (1966); sulphate activation of PFK by $(NH_4)_2SO_4$ present in suspensions of the ancillary enzymes in the assay was avoided by dialysis against 10 l. of the assay medium. All activities are expressed in terms of

the maximum activity of each preparation of PFK measured (with sulphate activation) in medium containing ATP (2mM), fructose 6-phosphate (0.3mM) and $(\text{NH}_4)_2\text{SO}_4$ (20mM).

RESULTS

Effects of time of incubation on the uptake of oxygen, output of Δ^4 -3-oxo steroids, and the uptake and metabolism of glucose by superovulated rat ovary slices. The rate of uptake of oxygen by ovary slices was linear over the first 3 hr. of incubation (see Procedures and Methods section for further details). The rate was not altered by administration of LH *in vivo* or by the presence of glucose (1mg./ml.) in the incubation medium, in agreement with the observations of Channing & Vilee (1966). The mean rate of oxygen uptake was $90.0 \pm 4.6 \mu\text{moles/g. wet wt./hr.}$ (mean $\pm$ S.E.M. of 40 observations). This value is considerably lower than that obtained manometrically by Channing & Vilee (1966).

The rate of Δ^4 -3-oxo steroid production by slices from animals not treated with LH *in vivo* was

Fig. 1. Effect of administration of LH *in vivo* on the rate of formation of Δ^4 -3-one steroids by slices of superovulated rat ovaries incubated *in vitro*. ●, Control; ▲, LH-treated. Slices were incubated in 1.5ml. of Krebs bicarbonate medium containing glucose (1mg./ml.) for various periods of time up to 4 hr. Δ^4 -3-oxo steroids were then assayed in slices+media as described in the Procedures and Methods section.

$0.20 \mu\text{mole/g. wet wt./hr.}$ and was constant for up to 4 hr. of incubation (Fig. 1). After administration of LH 2 hr. before death, the rate of production was increased by some 70% and was again constant for the first 4 hr. of incubation.

The uptake of glucose and output of lactate and pyruvate were found to be linear during the first 3 hr. of incubation (Fig. 2a).

The rate of incorporation of $[\text{U-}^{14}\text{C}]$ glucose into $^{14}\text{CO}_2$ and ^{14}C lactate (Fig. 2b) was found to be linear only after about 30 min. of incubation. The specific activity (as d.p.m./ $\mu\text{g. atom}$ of carbon) of ^{14}C lactate released from slices, expressed as a

Fig. 2. Effect of incubation time on glucose metabolism by slices of superovulated rat ovaries incubated *in vitro*. The slices were incubated in 5ml. of Krebs bicarbonate medium containing $[\text{U-}^{14}\text{C}]$ glucose (1mg./ml., $0.1 \mu\text{C/ml.}$) for various lengths of time. (a) Rate of glucose uptake (○), lactate+pyruvate output (▲) and $^{14}\text{CO}_2$ output (●). (b) Rate of incorporation of ^{14}C into lactate (▲) and carbon dioxide (●). Each point is the mean of three determinations in each case.

Table 1. *Effect of LH administered in vivo on the rates of glucose uptake, lactate and pyruvate outputs and the incorporation of [U-¹⁴C]glucose into carbon dioxide and total lipid extract by superovulated rat ovary slices in vitro*

Slices were incubated in 5 ml. of medium containing [U-¹⁴C]glucose (1mg./ml. and 0.1 μ C/ml.). The glucose uptake, lactate and pyruvate outputs and the incorporation of [U-¹⁴C]glucose into the total lipid extract are calculated from the rates during a 2 hr. incubation. The incorporation of [U-¹⁴C]glucose into carbon dioxide was calculated from the incorporation during the second and third hours of incubation observed in parallel experiments. Results are given as means $\pm$ s.e.m. for the numbers of observations given in parentheses. * $P < 0.01$; otherwise $P > 0.2$ versus control.

Rates (μ g.atoms of C/g. wet wt. of tissue/hr.)	Control animals	LH-treated animals
Glucose uptake	78.0 $\pm$ 2.85 (6)	98.7 $\pm$ 4.65 (6)*
Lactate output	60.7 $\pm$ 0.93 (6)	82.2 $\pm$ 3.96 (6)*
Pyruvate output	2.4 $\pm$ 0.08 (6)	2.5 $\pm$ 0.43 (6)
Incorporation of [U- ¹⁴ C]glucose (as μ g.atoms of [U- ¹⁴ C]glucose C/g. wet wt. of tissue/hr.) into:		
Carbon dioxide	18.0 $\pm$ 1.10 (4)	21.7 $\pm$ 1.49 (4)
Total lipid extract	0.7 $\pm$ 0.1 (3)	0.6 $\pm$ 0.1 (3)

percentage of the medium [U-¹⁴C]glucose specific activity, was found to be 59.3 $\pm$ 3.7 at the end of $\frac{1}{2}$ hr. incubation, and 75.9 $\pm$ 2.8, 87.5 $\pm$ 4.1, 93.3 $\pm$ 1.5 at the end of 1, 2 and 3 hr. of incubation respectively (mean $\pm$ s.e.m. of two observations). The average specific activity of lactate released between the first and third hours of incubation was 102% of the [U-¹⁴C]glucose specific activity.

During the first hour of incubation, intermediates between glucose and the end products of glucose metabolism (such as lactate and carbon dioxide) are presumably becoming labelled until isotopic equilibrium is reached. This has to be taken into consideration when calculating the steady-state rates of incorporation of [¹⁴C]glucose into metabolites derived from it from isotopic data (see the next section).

Effects of administration of LH in vivo on the rates of glucose uptake and metabolism by superovulated rat ovary slices in vitro. Table 1 shows the effect of LH administered 2 hr. before death on the rates of glucose uptake and lactate and pyruvate outputs and the steady-state rate of incorporation of [U-¹⁴C]glucose into ¹⁴CO₂. In agreement with results of other workers (Armstrong & Greep, 1962) both the rate of glucose uptake and output of lactate are significantly increased.

Also shown in Table 1 is the rate of incorporation of [U-¹⁴C]glucose into the total lipid extract during the first 2 hr. of incubation. Since the steady-state rates of incorporation of [U-¹⁴C]glucose into other end products of its metabolism (lactate and carbon dioxide) are not reached until after about 30 min. of incubation, this rate of incorporation will be less than the steady-state rate. However, it is unlikely that more than 2% of the glucose taken up is

incorporated into the total lipid extract in slices of ovaries from either control or LH-treated animals.

In ovary slices from control animals, the output of lactate + pyruvate accounts for 80.9% of the glucose carbon atoms taken up. The steady-state rate of incorporation of [U-¹⁴C]glucose into carbon dioxide accounts for a further 23.1%. Allowing for the small amount of glucose carbon atoms incorporated into the total lipid extract, a total of 104.5 $\pm$ 1.89% (mean $\pm$ s.e.m.; data from Table 1) of the glucose uptake of slices from control animals can be accounted for. In ovary slices from LH-treated animals, lactate + pyruvate output accounts for 85.8% and the steady-state rate of incorporation into carbon dioxide for 22.0% of the glucose uptake; in all a total of 108.4 $\pm$ 3.17% (mean $\pm$ s.e.m.; data from Table 1) of the glucose uptake can be accounted for.

Thus the end products of glucose metabolism appear to be lactate, pyruvate, carbon dioxide and some components of the total lipid extract. Treatment with LH *in vivo* appears to have little or no effect on the proportion of glucose carbon atoms converted into these end products. In control tissues 22.6 $\pm$ 3.87% of the oxygen taken up by the slices will be utilized in the oxidation of glucose to carbon dioxide: 27.1 $\pm$ 6.00% with slices from LH-treated animals. The balance of the oxygen consumption must be utilized in the oxidation of endogenous substrate.

The possibility of glycogen or other polysaccharides supplying glucose carbon atoms for glycolysis and therefore invalidating the above calculations can be excluded. As stated in the previous section and in Table 2 the specific activity

of lactate released by slices after the first hour of incubation is very similar to that of the medium glucose. Furthermore, superovulated ovaries contain only small amounts of glycogen (Stansfield & Robinson, 1965). In these studies ovaries from control animals contained only 0.64 ± 0.03 $\mu\text{mole/g. wet wt.}$ and animals treated with LH 0.64 ± 0.04 $\mu\text{mole/g. wet wt.}$ (mean $\pm$ S.E.M. of five observations in each case) of glycogen glucose. Lastly no lactate or pyruvate output was detected in tissues incubated in the absence of glucose.

Estimation of the flux through the pentose phosphate pathway in superovulated rat ovary slices in vitro. Armstrong & Greep (1962) have reported that the rates of conversion of $[1-^{14}\text{C}]\text{glucose}$ and $[6-^{14}\text{C}]\text{glucose}$ into $^{14}\text{CO}_2$ measured over the first 2 hr. of incubation were the same in superovulated ovaries from both control and LH-treated rats. In our studies the ratios of these rates, measured between the first and third hours of incubation (when an isotopic steady state is maintained) were 0.93 ± 0.024 and 1.05 ± 0.199 (mean $\pm$ S.E.M. for four determinations in each case).

Since there is no simple relationship between the ratio of yields of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]\text{glucose}$ and from $[6-^{14}\text{C}]\text{glucose}$ and the rate of glucose metabolism by the pentose cycle (Katz & Wood, 1960), the specific activities of the medium $[^{14}\text{C}]\text{lactate}$ and tissue $[^{14}\text{C}]\text{glucose 6-phosphate (C-1)}$ have been measured during incubations in the presence of $[1-^{14}\text{C}]\text{glucose}$. These results, together with the specific activity of $[^{14}\text{C}]\text{lactate}$ released in the presence of $[\text{U-}^{14}\text{C}]\text{glucose}$ and $[6-^{14}\text{C}]\text{glucose}$, are shown in Table 2.

In the absence of any pentose cycle activity or glycogenolysis the specific activity of $[^{14}\text{C}]\text{lactate}$ produced from $[1-^{14}\text{C}]\text{glucose}$ should equal 50% of

that of the $[1-^{14}\text{C}]\text{glucose}$ (in molar terms). In slices from both control and LH-treated rats, the specific activity of $[^{14}\text{C}]\text{lactate}$ released between the first and third hours of incubation did not differ significantly from 50% of that of $[1-^{14}\text{C}]\text{glucose}$. Furthermore the specific activity of glucose 6-phosphate (C-1) in slices incubated in $[1-^{14}\text{C}]\text{glucose}$ was not significantly different from that of the $[1-^{14}\text{C}]\text{glucose}$ (C-1) in either tissue. Thus there appears to be no appreciable flow of carbon atoms through the decarboxylative step of the pentose phosphate cycle under these conditions.

Effects of LH on the incorporation of ^{14}C from $[\text{U-}^{14}\text{C}]\text{glucose}$ and $[1-^{14}\text{C}]\text{acetate}$ into fractions of the total lipid extract of superovulated rat ovaries in vitro. The total lipid extract was separated by thin-layer chromatography into three fractions: A (including sterol esters and triglycerides), B (including free sterols and steroids) and C (including monoglycerides and phospholipids). Fractions A and B were then saponified and the products separated into: (a) those lipids extractable with *n*-hexane before acidification (including sterols and steroids); (b) those extractable with *n*-hexane after acidification (fatty acids); (c) those remaining in the aqueous phase (including glyceride glycerol).

In slices from both control and LH-treated rats more than 97% of the ^{14}C incorporated into the total lipid extract from $[\text{U-}^{14}\text{C}]\text{glucose}$ was found in the products remaining in the aqueous phase after saponification of fractions A and B, presumably as glycerol derived from tri- and di-glycerides respectively (Table 3). In contrast, less than 3% of the ^{14}C incorporated into the total lipid extract from $[\text{U-}^{14}\text{C}]\text{glucose}$ was found in the *n*-hexane-soluble products after saponification of fractions A and B. Thus less than 0.04% of the glucose taken

Table 2. *Effect of LH administered in vivo on the specific activities of glucose 6-phosphate (C-1) and lactate derived from $[^{14}\text{C}]\text{glucose}$ by superovulated rat ovary slices in vitro*

Slices were incubated in 5 ml. of medium containing glucose (1 mg./ml.) and either $[\text{U-}^{14}\text{C}]\text{glucose}$ or $[6-^{14}\text{C}]\text{glucose}$ or $[1-^{14}\text{C}]\text{glucose}$ (all at $0.1 \mu\text{C/ml.}$) as indicated. The specific activities of glucose 6-phosphate (C-1) and lactate were measured in neutralized perchloric acid extracts of tissue and media respectively as given in the Procedures and Methods section, and are expressed as percentages of the medium glucose specific activity (means $\pm$ S.E.M. of the number of determinations given in parentheses). The specific activity of lactate is the average value for lactate released into the medium during the second and third hours of incubation. The specific activity of glucose 6-phosphate (C-1) was determined after incubation for 1 hr. Maximum values of specific activity had been reached by both lactate and glucose 6-phosphate (C-1) at these times of incubation.

	Control animals	LH-treated animals
Specific activity (d.p.m./ μmole) of lactate as % of that of glucose after incubation with		
$[\text{U-}^{14}\text{C}]\text{glucose}$	50.5 ± 2.82 (4)	—
$[1-^{14}\text{C}]\text{glucose}$	50.0 ± 1.46 (4)	53.0 ± 0.75 (4)
$[6-^{14}\text{C}]\text{glucose}$	54.5 ± 3.46 (4)	46.0 ± 0.29 (4)
Specific activity of glucose 6-phosphate (C-1) as % of that of glucose after incubation with		
$[1-^{14}\text{C}]\text{glucose}$	92 ± 16 (2)	93 ± 4 (2)

Table 3. *Effect of LH administered in vivo on the incorporation of [U-¹⁴C]glucose and [1-¹⁴C]acetate (in the presence of glucose) into lipid fractions of superovulated rat ovary slices incubated in vitro*

Slices were incubated for 2 hr. in 5 ml. of medium containing glucose (1 mg./ml.) and either [U-¹⁴C]glucose (0.2 μ C/ml.) or [1-¹⁴C]acetate (0.1 mM and 0.2 μ C/ml.). Values are means of two determinations. —, Mean value was less than 0.1. Values are expressed as $10^{-3} \times$ d.p.m./g. wet wt. incorporated in 2 hr.

	$10^{-3} \times$ Incorporation of ¹⁴ C (d.p.m./g. wet wt./2 hr.)			
	From [U- ¹⁴ C]glucose		From [1- ¹⁴ C]acetate	
	Control animals	LH-treated animals	Control animals	LH-treated animals
Total lipid extract	24.6	30.3	131.0	157.8
Fractions of total lipid extract separated by thin-layer chromatography				
A (inc. sterol esters and triglycerides)	17.0	20.4	74.0	52.5
B (inc. free sterols, steroids and diglycerides)	3.1	5.0	33.4	71.1
C (inc. phospholipids and monoglycerides)	1.9	1.8	13.7	19.0
Products from saponification of fraction A				
(a) Hexane extract pre-acidification	—	—	6.6	8.3
(b) Hexane extract post-acidification	—	—	54.6	36.5
(c) Water-soluble components	15.6	18.2	6.7	2.5
Products from saponification of fraction B				
(a) Hexane extract pre-acidification	—	—	15.5	46.7
(b) Hexane extract post-acidification	—	—	13.4	14.1
(c) Water-soluble components	2.8	4.8	—	0.3

up by the tissue under these conditions appears to be converted into either fatty acids or sterols and steroids.

On the other hand, from tracer amounts (0.1 mM) of [1-¹⁴C]acetate added to medium containing glucose, ¹⁴C was readily incorporated into the *n*-hexane-soluble products after saponification of fractions A and B. In slices from control animal ovaries, 16.9% of the ¹⁴C incorporated into the total lipid extract was present in fractions containing sterols and steroids (Aa and Ba) and 51.9% in fractions containing fatty acids (Ab and Bb). In slices from LH-treated animals the percentages were 34.8 and 32.1 respectively. The rate of incorporation of [1-¹⁴C]acetate into fractions containing sterols and steroids was increased 2.5-fold in ovary slices from LH-treated animals.

Since the metabolism of glucose by the superovulated rat ovary appeared to supply neither carbon atoms nor reducing power (as NADPH₂ from the pentose phosphate pathway), the effect of omitting glucose on the incorporation of [1-¹⁴C]acetate into the lipid fractions containing sterols and steroids was investigated. Table 4 shows the effects of glucose added *in vitro* and LH treatment *in vivo* on the incorporation of [1-¹⁴C]acetate into free sterols and steroids (taken as the incorporation into fraction Ba) and that into esterified sterols (taken as the incorporation into fraction Aa). In the presence

of glucose, the rate of incorporation into both free sterols and steroids and esterified sterols was greatly increased in ovary slices from both control and LH-treated rats. The administration of LH *in vivo* had no significant effect on the incorporation of [1-¹⁴C]acetate into either fraction in the absence of glucose; in the presence of glucose, administration of LH *in vivo* increased the rate of incorporation of [1-¹⁴C]acetate into free sterols and steroids by a factor of 2.6.

In a typical incubation carried out under these conditions $49.2 \pm 1.44\%$ (mean $\pm$ s.e.m. of 16 determinations) of the [1-¹⁴C]acetate added initially was present at the end of the incubation time. The rate of utilization of [1-¹⁴C]acetate was not altered by the presence of glucose (1 mg./ml.) in the medium.

Effect of LH administered in vivo on the intracellular concentrations of glucose and hexose monophosphates in incubated superovulated rat ovary slices. Table 5 shows the effects of LH treatment *in vivo* on the [³H]water, [¹⁴C]sorbitol and glucose spaces in superovulated rat ovary slices incubated for 1 hr. LH treatment had no effect on the total water (TW) or extracellular water (EW) spaces as measured with [³H]water and [¹⁴C]sorbitol as markers respectively. The overall mean value for the volume of intracellular water (TW - EW) in tissue slices from both control and LH-treated rats was $301 \pm 22.9 \mu$ l./g. wet wt. (mean $\pm$ s.e.m. of 16

Table 4. *Effect of LH administered in vivo and glucose in vitro on the incorporation of [1-¹⁴C]acetate into sterol- and steroid-containing fractions by superovulated rat ovary slices*

Slices were incubated for 2 hr. in 5.0 ml. of medium containing [1-¹⁴C]acetate (0.1 mM and 0.2 μ C/ml.) with or without glucose (1 mg./ml.). Values are expressed as $10^{-3} \times$ d.p.m./g. wet wt. (mean $\pm$ S.E.M. of number of determinations given in parentheses) incorporated into the fractions designated Aa (including esterified sterols) and Ba (including free sterols and steroids); see Table 2 for further details. * $P < 0.005$ (effect of LH versus appropriate control); † $P < 0.05$; ‡ $P < 0.005$ (effect of glucose versus appropriate control). Other differences, $P > 0.1$.

	Without glucose		With glucose	
	Free sterols and steroids	Ester sterols	Free sterols and steroids	Ester sterols
Control animals	3.8 $\pm$ 0.86 (3)	1.2 $\pm$ 0.18 (3)	11.6 $\pm$ 2.40 (4)†	6.6 $\pm$ 0.80 (4)‡
LH-treated animals	6.6 $\pm$ 0.92 (4)	1.4 $\pm$ 0.12 (4)	30.4 $\pm$ 3.36 (4)*‡	9.0 $\pm$ 0.88 (4)‡

Table 5. *Effect of LH administered in vivo on [³H]-water, [¹⁴C]sorbitol and glucose spaces of superovulated rat ovary slices incubated in vitro*

Slices were incubated for 1 hr. in 5 ml. of medium containing glucose (1 mg./ml.), [³H]water (0.2 μ C/ml.) and [U-¹⁴C]-sorbitol (0.5 mg./ml. and 0.06 μ C/ml.). Spaces were calculated from assays of glucose, [³H]water and [U-¹⁴C]sorbitol in perchloric acid extracts of tissue and medium after incubation by the method of Randle & Smith (1958). Results are expressed as μ l./g. wet wt. (mean $\pm$ S.E.M. of eight determinations). * $P < 0.025$; ** $P < 0.01$ when compared with the appropriate control.

	Control animals	LH-treated animals
[³ H]Water space (TW)	865 $\pm$ 7.1	883 $\pm$ 29.1
[¹⁴ C]Sorbitol space (EW)	581 $\pm$ 22.2	566 $\pm$ 26.4
Glucose space (GS)	540 $\pm$ 23.6	639 $\pm$ 31.3*
TW—EW	285 $\pm$ 21.3	317 $\pm$ 26.0
GS—EW	—41 $\pm$ 22.2	74 $\pm$ 28.8**

determinations). The glucose space was increased from a value not significantly greater than the extracellular space in control slices to a value significantly greater than the extracellular space in slices from LH-treated rats. In the latter tissues the intracellular glucose concentration was 0.9 ± 0.30 mM (mean $\pm$ S.E.M. of eight determinations).

Tissue concentrations of glucose 6-phosphate measured after 1 hr. of incubation in medium containing glucose (1 mg./ml.) were significantly decreased by LH treatment from 22.2 ± 1.57 to 15.9 ± 0.97 μ moles/g. wet wt. (mean $\pm$ S.E.M. of eight determinations in each case; $P < 0.005$). These values are lower than those found in muscle or adipose tissue incubated under similar conditions (Denton, Yorke & Randle, 1966) but are comparable with those found in incubated kidney slices (Newsholme & Underwood, 1966). Tissue concentrations of fructose 6-phosphate were found to be approximately 20–25% of the concentration of

glucose 6-phosphate in slices from both control and LH-treated animals.

Effect of LH administered in vivo on the activities of some enzymes of glucose metabolism in superovulated rat ovaries. Table 6 shows the activities of the enzymes measured in either crude homogenates or supernatants obtained after low- or high-speed centrifugation, expressed per g. wet wt. of tissue and per g. of protein in the preparation assayed. Treatment with LH *in vivo* resulted in significant increases (1.14- to 1.47-fold) in the activities of hexokinase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase expressed in terms of both tissue weight and protein. There was also some indication of an increase in the activity of PFK. However, the assay of PFK in preparations of ovaries from LH-treated rats is subject to some error because of the increased instability of PFK (Fig. 3). After incubation of a low-speed supernatant for 1 hr. at 0°, activity of PFK from control tissues fell by about 5% and that from ovaries of LH-treated rats fell by about 30%. Similarly, after incubation of a low-speed supernatant at 25° for 1 hr., the activity from control tissues decreased about 25% whereas that from ovaries of LH-treated rats decreased by nearly 70%.

A brief study of the kinetic properties of a partially purified preparation of superovulated rat ovary PFK. Because of the relative instability of PFK from ovaries of LH-treated rats a partially purified preparation of PFK from ovaries of control animals was used in this study.

With respect to its activation by fructose 6-phosphate, ADP, AMP, 3',5'-(cyclic)-AMP, phosphate and sulphate, and to its inhibition by citrate and ATP, PFK from this tissue was qualitatively similar to that from other mammalian sources. 3',5'-(cyclic)-AMP, in the presence of 0.2 mM-ATP and 0.3 mM-fructose 6-phosphate, was an extremely potent activator with a K_m of activation (as determined by double reciprocal plot) of approximately 3 μ M. The enzyme that had been partially inhibited by citrate was also activated by 3',5'-(cyclic)-AMP.

Table 6. *Effect of administration of LH in vivo on the activities of some enzymes involved in the metabolism of glucose carbon atoms in superovulated rat ovaries*

Ovaries were extracted and enzyme activities assayed as described in the Procedures and Methods section in either crude homogenates (CH) or the supernatants after centrifugation at 2000g for 5 min. (LSS) or at 25000g for 20 min. (HSS). Results are expressed as units/g. wet wt. or units/g. of protein in the preparations and are the means $\pm$ s.e.m. of the numbers of observations given in parentheses. One unit converts 1 μ mole of substrate/min. (measured at 37°). * $P < 0.05$; ** $P < 0.02$; † $P < 0.005$; other differences, $P > 0.05$ versus control value.

Other differences ...	Preparation	Enzyme activity			
		Units/g. wet wt.		Units/g. of protein	
		Control animals	LH-treated animals	Control animals	LH-treated animals
Hexokinase	CH	14.5 $\pm$ 0.51 (7)	16.6 $\pm$ 0.43 (8)**	111 $\pm$ 3.3 (7)	128 $\pm$ 3.4 (8)†
	HSS	1.4 $\pm$ 0.03 (7)	1.8 $\pm$ 0.10 (8)†	24.9 $\pm$ 1.00 (7)	30.5 $\pm$ 2.20 (8)*
Phosphofructokinase	LSS	8.0 $\pm$ 0.39 (4)	9.1 $\pm$ 0.11 (4)*	87.4 $\pm$ 4.80 (4)	97.4 $\pm$ 1.10 (4)
Pyruvate kinase	HSS	22.3 $\pm$ 0.92 (7)	22.6 $\pm$ 0.90 (8)	377 $\pm$ 13.2 (7)	376 $\pm$ 21.2 (8)
Glucose 6-phosphate dehydrogenase	CH	8.1 $\pm$ 0.43 (8)	9.8 $\pm$ 0.23 (8)†	66.7 $\pm$ 4.57 (8)	82.8 $\pm$ 2.17 (8)**
6-Phosphogluconate dehydrogenase	CH	6.4 $\pm$ 0.12 (5)	7.9 $\pm$ 0.12 (8)**	48.9 $\pm$ 4.10 (5)	61.4 $\pm$ 2.97 (8)*
	HSS	1.9 $\pm$ 0.07 (10)	2.8 $\pm$ 0.14 (10)†	32.0 $\pm$ 1.18 (10)	46.3 $\pm$ 2.31 (10)†
Lactate dehydrogenase	CH	115 $\pm$ 4.8 (8)	113 $\pm$ 2.5 (8)	936 $\pm$ 32.8 (8)	951 $\pm$ 25.4 (8)

Fig. 3. Stability of PFK extracted from superovulated rat ovaries from control animals (● and ○) and from animals treated with LH (▲ and △). The extract was centrifuged at 2000g for 5 min. and the supernatant (approx. 20mg. of protein/ml.) was incubated without additions at either 0° (●, ▲) or 25° (○, △). Each point is the mean of two determinations.

DISCUSSION

Effect of LH treatment on the rate of glucose metabolism. The increases in the rate of glucose uptake and in glycolysis in superovulated rat ovary slices

in vitro caused by LH treatment *in vivo* were associated with the following changes.

The glucose space was increased from equality with the extracellular space to a value significantly greater. The intracellular glucose concentration (assuming homogeneity within the cell) rose in tissues from LH-treated rats to about 20% of the extracellular glucose concentration. This suggests that the transport of glucose across the membrane limits the rate of glucose uptake and that LH treatment accelerates the transport process.

The tissue concentration of glucose 6-phosphate was decreased by LH treatment. Since the flow of carbon atoms through the hexose monophosphate pool is increased under these conditions, the removal of glucose 6-phosphate appears to be accelerated presumably (since the pentose cycle is not operative) via PFK. Superovulated rat ovary PFK has been shown to be extremely sensitive to activation by 3',5'-(cyclic)-AMP; since LH is known to raise the concentration of this activator in slices of bovine corpus luteum, LH treatment *in vivo* may well increase PFK activity in this way.

The activity of hexokinase, and perhaps also that of PFK, was raised in homogenates of superovulated ovary by LH treatment. The decreased stability of PFK observed *in vitro* in tissue from LH-treated animals renders the total activity assay less reliable.

The intracellular concentration of glucose does not reach the extracellular concentration in tissues from LH-treated rats, which suggests that the increased glucose uptake rate is primarily the result of the accelerated rate of membrane transport.

Relationship between glucose metabolism and steroidogenesis in incubated superovulated rat ovary slices. Steroidogenesis requires sources of carbon atoms, energy and reducing power. The extent to which each of these requirements may be met by the metabolism of glucose will be considered.

Since no detectable amounts of [$U-^{14}C$]glucose were incorporated into either free sterols and steroids or esterified sterols, glucose does not appear to be a source of carbon atoms for steroidogenesis under the conditions used in this investigation. In incubated slices of superovulated ovaries from both control and LH-treated rats, all the glucose taken up could be accounted for by its conversion into lactate, pyruvate, carbon dioxide and glyceride glycerol. Glycogenolysis does not appear to be a significant source of glucose carbon atoms for glycolysis.

From a knowledge of the oxygen consumption and the glycolytic flux measured under identical conditions, and assuming a P:O ratio 3, it can be calculated that less than 10% of the ATP produced will arise from substrate-linked phosphorylation in glycolysis. Since the oxygen consumption is unchanged by the addition of glucose to the incubation medium, and assuming no change in the P:O ratio, it can be seen that glucose has no effect on the rate of consumption or production of ATP. The oxidation of glucose to carbon dioxide accounts for less than 30% of the oxygen uptake: in the presence of glucose the balance of the oxygen utilized must be accounted for by the oxidation of some endogenous substrate. The same substrate may well supply carbon atoms for steroidogenesis.

The metabolism of glucose carbon atoms through the pentose cycle is usually regarded as an important source of cytoplasmic NADPH₂ for biosynthetic pathways. However, from measurements of the ratio of production of $^{14}CO_2$ from [$1-^{14}C$]glucose and [$6-^{14}C$]glucose and from the specific activities of [^{14}C]lactate during these incubations, there appears to be no appreciable flux through the decarboxylative step of the pentose cycle. This evidence is supported by measurements of the specific activity of the C-1 of glucose 6-phosphate in tissue slices incubated in [$1-^{14}C$]glucose. In view of the presence in the superovulated rat ovary of both glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, this result may be unexpected; however, a preliminary investigation has revealed the presence of other enzymes capable of reducing NADP (NADP-isocitrate dehydrogenase, EC 1.1.1.42; NADP-malate dehydrogenase, EC 1.1.1.40) in total activities 5–10 times in excess of that of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase.

The metabolism of glucose does not therefore appear to supply any of the above requirements of

steroidogenesis. Nevertheless the presence of glucose has been found to stimulate markedly the incorporation of ^{14}C from [$1-^{14}C$]acetate into sterols and steroids in tissue slices from both control and LH-treated rats. In addition the stimulatory effect of LH treatment was observed only in slices incubated in the presence of glucose. Two possible explanations of the stimulatory effect of glucose are either (i) that some intermediates between [$1-^{14}C$]acetate and the end products of steroidogenesis are lowered in concentration (and thus raised in specific activity) or (ii) that the rate of steroidogenesis is increased under these conditions. At the present time we are unable to distinguish between these two possible explanations; were the latter correct, the stimulatory effect of glucose on steroidogenesis may be due to the metabolism of glucose supplying pyruvate to prime a cycle involving the enzymes pyruvate carboxylase (EC 6.4.1.1), NAD-malate dehydrogenase (EC 1.1.1.37) and NADP-malate dehydrogenase (EC 1.1.1.40). Such a cycle could convert cytoplasmic or mitochondrial NADH₂ or both into the NADPH₂ required for steroidogenesis. Similar cycles involving these three enzymes have been suggested to operate in liver (Lowenstein, 1961; Shrago & Lardy, 1966; Walter, Paetkau & Lardy, 1966) and in adipose tissue (Kornacker & Ball, 1965; Ballard & Hanson, 1967).

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