Changes in Enzymes of the Uterus of the Ovariectomized Rat after Treatment with Oestradiol

By DWIGHT B. M. SCOTT AND A. G. LISI

Section on Cytology and Genetics of the Department of Physiology of the School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, U.S.A.

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The enzymes involved in the oxidation of glucose 6-phosphate are the dehydrogenases for glucose 6-phosphate and phosphogluconate, both of which require triphosphopyridine nucleotide as a co-enzyme. In *Escherichia coli*, the extractable activity of these enzymes was found to be remarkably constant under certain conditions and could be induced to decrease or increase under other conditions. When the cells were grown in synthetic medium in which ammonium ions supplied nitrogen and only the carbon source was an organic compound, there was no significant difference in enzyme activities, between cells which were resting or growing, under aerobic or anaerobic conditions, or with several different carbon sources, such as glucose, gluconate or lactate (Scott, 1956*a*). The ratio of glucose 6-phosphate dehydrogenase to phosphogluconate dehydrogenase was 1.9 ± 0.4 . However, when *E. coli* were grown in enriched medium, supplying preformed cell constituents, such as nutrient broth or casein hydrolysate, but not yeast ribonucleic acid, the glucose 6-phosphate-dehydrogenase activity was decreased whereas the phosphogluconate dehydrogenase was essentially unchanged and the ratio fell as low as 0.70 (Scott & Chu, 1959).

On the other hand, treatment with certain drugs, such as 2:4-dinitrophenol (Scott, 1956*b*) and nitrated benzimidazoles (Scott, Rogers & Rose, 1958), increased the glucose 6-phosphate dehydrogenase. The increase of activity was proportional to the concentration of the drug up to concentrations which inhibited growth. The increase was proportional also to the time of exposure to the drug. Again the activity of phosphogluconate dehydrogenase was not changed or was increased slightly so that ratios could more than double within 30 min. of growth in the presence of drug and could attain values as high as 12, i.e. six times the ratio of enzyme activities in synthetic medium without drug.

In animal tissues, Glock & McLean (1954) found evidence that these enzymes increased in the mammary gland during lactation. Glock & McLean (1955*b*) found a decrease in the phosphogluconate-dehydrogenase activity in the livers of

alloxan-diabetic rats and increases of both dehydrogenases after insulin. An increase of these enzymes in livers of rats treated with thyroxine was reported by Glock, McLean & Whitehead (1956) and by Spiro & Ball (1958). Tepperman & Tepperman (1958) reported increases up to 15-fold in glucose 6-phosphate-dehydrogenase activity in the livers of rats which were fed with a high carbohydrate diet for 48 hr., after starvation for 48 hr. The liver glucose 6-phosphate-dehydrogenase activity was also increased after changing the rats' diet from normal mixed to high carbohydrate, without a period of starvation. Weber & Cantero (1957) have found that glucose 6-phosphate dehydrogenase is about five times as high in Novikoff hepatoma as in normal rat liver. Thus this constitutive enzyme would seem to be adaptively responsive in the mammal as well as in bacteria to changes in nutrition, to changes in hormone balance, and to whatever constitutes the change from normal to tumour tissue.

In order to investigate the induction of changes in enzyme activity when a normal tissue changes from a maintenance state to an actively growing state, we chose a mammalian organ which grows under the influence of a steroid hormone and regresses when the hormonal effect wears off: the rat uterus under the influence of oestrogen.

MATERIALS AND METHODS

Chemicals. Adenosine triphosphate (disodium salt; ATP), triphosphopyridine nucleotide (monosodium salt; TPN), and reduced triphosphopyridine nucleotide (tetrasodium salt, tetrahydrate; TPNH, 90% pure) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Diphosphopyridine nucleotide (sodium salt; DPN) and reduced diphosphopyridine nucleotide (disodium salt; DPNH) were obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A. Glutathione and oxidized glutathione were obtained from Schwarz Laboratories Inc., Mt. Vernon, N.Y., U.S.A.

Glucose 6-phosphate (G 6-P) and phosphogluconate were the barium salts, kindly prepared by Dr M. L. Lanning by the method of Seegmiller & Horecker (1951). Solutions of the sodium salts were prepared by precipitation of the barium with sodium sulphate.

Oestradiol-17 β was kindly supplied by the Schering Corp., Bloomfield, N.J., U.S.A.

Operation. The rats were Wistar-strain adult females obtained from the Wistar Institute and weighed between 140 and 240 g. They were maintained on Purina Dog Chow. Ovariectomy was accomplished under ether anaesthesia. A midline dorsal incision was made, the skin was retracted to the side and the ovary was extracted through a small, lateral incision on each side as described by Griffith & Farris (1942). A ligature was placed near the end of each horn of the uterus, which was then sectioned distally. Thus complete removal of the ovary was assured by inclusion of the Fallopian tube and a small section of the horn.

Injection. Oestradiol (1 mg./ml.) was dissolved in 95% ethanol. For injection, 0.1 ml. was drawn into a 1 ml. syringe, which was then filled to 1 ml. with sterile 0.9% NaCl solution. This 100 μ g. was injected into the peritoneum of the rats under light ether anaesthesia, 10 days after the operation.

Preparation of extracts. At selected times after the injection, the animals were killed by cervical concussion. After the uterus was exposed and its gross appearance noted, it was quickly excised at the fundus. The two horns and the small section of fundus were first freed of fat and connective tissue and then washed with NaCl solution, blotted and weighed. The piece was placed on a dry Petri dish and the horns were slit lengthwise with scissors. The endometrial surface was flushed with 0.5 ml. of ice-cold 0.25 M-sucrose solution and scraped with a scalpel to remove as much of the endometrium as possible. The scrapings were washed and collected in additional cold sucrose solution to a total volume of 1.0–1.5 ml. The suspension of broken cells (10–30 mg. dry wt.) was placed in a centrifuge tube in an ice bath without homogenizing.

The myometrium was blotted and weighed. The tissue was cut finely with scissors and placed in a steel chamber which had been chilled in the freezer at -20° . A similarly chilled steel plunger was fitted on to a collar around the tissue and hit repeatedly with a large hammer. The fragmented frozen tissue was then homogenized in a small Elvehjem tube with a Teflon plunger with 10 vol. of ice-cold sucrose solution for 5 min. The tube was immersed in an ice bath throughout the homogenization.

When the adrenals were also analysed they were removed before the uterus and placed in the freezer while the uterus was being processed. After being cleaned, the adrenals were weighed and homogenized with approx. 2 ml. of cold sucrose solution in a small glass hand homogenizer for 2 min.

The homogenates and the suspension of endometrial scrapings were centrifuged for 1 hr. in an International refrigerated centrifuge at 2200 g. The supernatant extract contained not only the soluble protein but also the small particles, probably most of the microsomes. The dehydrogenases studied have not been found to act differently from those of extracts centrifuged at 10 000 g for 1 hr. The particulate residues were dried at 105° to constant weight.

These enzymes are unstable and may lose activity during preparation, especially during the longer procedure for the myometrium. This was minimized by maintaining a low but not freezing temperature and made uniform by standardizing the procedures as much as possible. Frozen extracts lost from 40 to 70% of the glucose 6-phosphate-dehydrogenase activity in 4 days. The loss in activity for

glucose 6-phosphate dehydrogenase at 4° was about 15–30% in 2–4 hr. Backward extrapolation shows that it is possible that 10–15% of activity was lost during preparation of the extracts. The dehydrogenase activities of extracts which had been frozen or had been standing at 4° for several hours could be partially reactivated by the addition of bovine-serum albumin to the mixture. The addition of albumin had little effect on the activity of fresh extracts. The addition of cysteine either during homogenizing or to the reaction mixtures had a variable effect, usually inhibitory.

Enzyme determinations

Dehydrogenases. Enzyme activities of the extracts were determined immediately. The dehydrogenase activities in terms of rate of reduction of TPN at 35° were measured by increase of absorption at 340 m μ in a Beckman spectrophotometer. Samples of the supernatant fluids chosen to give an increase of extinction of 0.010–0.050/min. were added to 1 ml. cuvettes containing 0.05 M-2-amino-2-hydroxymethylpropane-1,3-diol (tris) buffer, pH 7.4, and 0.5 μ mole of substrate, either glucose 6-phosphate or phosphogluconate, in double-distilled water to give a final volume of 1 ml., which had been brought to 35° . Addition of Mg^{2+} ions was not necessary. TPN solution (0.3 mg.) was added and readings were taken at 0.5 min. intervals for 5 or 6 min. In blanks with no added substrate the reduction of TPN by endogenous substrate was found to be almost finished in the first minute except by the most active extracts. Therefore, except in the last-named cases, no correction was made for endogenous reduction of TPN. The glucose 6-phosphate-dehydrogenase activities were not corrected for reduction of TPN by dehydrogenase acting on the resulting phosphogluconate, for the maximum concentration of phosphogluconate produced in the first 5 min. was less than 0.04 mM, a concentration at which the dehydrogenase has less than half the maximum activity. Therefore the rates of both enzymes were chosen as the mean increase in extinction/min. between the first and fourth minutes.

Results are reported as change in extinction at 340 m μ /min./mg. of protein, or have been converted into rates in μ moles of TPN reduced/min./mg. of protein by division by 6.22 (Horecker & Kornberg, 1948).

The difference between duplicate determinations of glucose 6-phosphate dehydrogenase was usually less than 10% unless the time between the duplicates was greater than 2 hr. The difference between duplicate phosphogluconate-dehydrogenase determinations was less than that of the other dehydrogenase. The second determination was the lower one except when the sample size of the first was below the set limits of activity. Because of the loss of activity with time the results recorded here are the first determinations within the set limit.

Determinations of dehydrogenase activities and protein content were performed on all the extracts. Hexokinase, transhydrogenase and/or TPNH-oxidase activities were determined whenever there was sufficient extract remaining.

Transhydrogenase. Transhydrogenase was determined by the methods of Kaplan, Colowick & Neufeld (1953) and Talalay & Williams-Ashman (1958). Fig. 1 illustrates the measurement of the transhydrogenase reaction of extracts of the adrenal and myometrium and the absence of it in the

endometrial extract of rat no. 51. The transhydrogenase activity was calculated as the increase in $E_{340\text{ m}\mu}/\text{min.}/\text{mg.}$ of protein due to production of DPNH after all the TPN was reduced. Insufficient material was available in this series for a study of the transhydrogenase activity of the unfractionated homogenate.

Hexokinase. Hexokinase of the extracts was determined by either the direct or the indirect method or both (Slein, Cori & Cori, 1950). By the first method samples of the extracts containing approx. 0.5 mg. of protein were added to cuvettes containing 1.1 μmole of glucose, 1 μmole of Mg^{2+} ions and 1 μmole of ATP in 1 ml. of 0.05 M-tris buffer, pH 7.4. After 10 min. 0.4 μmole of TPN was added and from the curve of TPNH formation the amount of glucose 6-phosphate which had been formed was calculated with corrections for endogenous substrate and rate of glucose 6-phosphate formation. By the direct method, more

commonly employed, the extract was added to cuvettes containing 1.1 μmole of glucose, 1 μmole of MgSO_4 , 0.4 μmole of TPN and 0.05 M-tris buffer in a total volume of 1 ml. After reduction, caused by endogenous ATP and substrate, had become minimal, 1 μmole of ATP was added and the rate of reduction of TPN determined. In these determinations the glucose 6-phosphate dehydrogenase of the extracts was considered to be not rate-limiting. No attempt was made to determine the hexokinase activity either of the whole homogenate or of the particulate fraction of the homogenates.

Reduced triphosphopyridine nucleotide oxidase. To a cuvette, containing 1 ml. of 0.05 M-tris, pH 7.4, and 0.06 ml. of extract at 35°, was added 0.02 ml. (0.116 mg.) of freshly prepared TPNH. The decrease in $E_{340\text{ m}\mu}$ during 10–20 min. was compared with the non-enzymic decrease in a parallel cuvette. Then 0.2 μmole of oxidized glutathione was added and the readings were continued for 10–20 min. further to determine TPNH-glutathione reductase. Both TPNH oxidases were slightly inhibited by either Mg^{2+} ions or Mn^{2+} ions at a concentration of 4 mM.

Other determinations. TPN nucleotidase was determined by the cyanide method of Kaplan *et al.* (1953).

Amperometric titrations of acid-soluble SH groups were performed by the method of Benesch, Lardy & Benesch (1955).

The protein content of the extracts was determined by the bromsulphalein method of Bonting & Jones (1957), with bovine-serum albumin as a standard.

RESULTS

Results are reported on 37 rats. Two rats died during the operation and they were counted as un-operated controls.

Table 1 presents data on the weights of the animals (c), the wet weight of the whole uterus (d) and the scraped uterus (e), the dry weight of the extracted scraped uterus (homogenate residue g) and the total soluble protein in the myometrial extracts (j). There was no correlation between the uterine weight and the weight of the whole rat. The wet weight of the uterus (d) was increased after 2 hr. after oestradiol, over the control ovariectomized rat. An increase was not evident in the dry weight (g). This is in line with the findings of others (Reynolds, 1949) that there is imbibition of water by the uterus of the ovariectomized rat, at about 2 hr. after the injection of oestrogens. That this increase of water content occurred in the myometrium as well as the endometrium is shown by column h, the percentage dry weight of the scraped uterus. The change in mass after scraping is calculated in column f. This value does not seem to correspond to the weight of endometrial tissue scraped off, for the sum of the dry weight of the endometrial residue plus the total protein of the extract ranged between 12 and 22 mg. Column f would seem to indicate that the control uteri imbibed water *in vitro* from the sucrose solution during scraping. Uteri, removed in the period

Fig. 1. Transhydrogenases of extracts of rat uterus. Rat no. 51 received 100 $\mu\text{g.}$ of oestradiol 10 days after ovariectomy and again 24 hr. later and was killed after a further 24 hr. Each 1.5 ml. cuvette contained 5 μmoles of glucose 6-phosphate (G 6-P) in tris buffer, 0.05 M, pH 7.4. Extracts, in 0.25 M-sucrose, were: $\blacktriangle$, 0.02 ml. of adrenal gland (40 $\mu\text{g.}$ of protein); $\circ$, 0.1 ml. of endometrial (116 $\mu\text{g.}$ of protein); $\bullet$, 0.05 ml. of myometrial (150 $\mu\text{g.}$ of protein). At 5 min. 0.02 ml. of DPN (10 mg./ml.) and at 8 min. 0.01 ml. of TPN (1 mg./ml.) were added to each cuvette. Total volume was 1 ml. Extinction $E_{340\text{ m}\mu}$ was read on a Beckman spectrophotometer at 35°. Complete reduction of the TPN added to the system would give an increase in $E_{340\text{ m}\mu}$ of about 0.08.

Table 1. *Effect of oestradiol on weights and soluble protein of uteri of ovariectomized rats*

(a) Animal no.	(b) Time of killing after oestradiol (hr.)	(c) Wt. of rat (g.)	(d) Whole uterus (mg. wet wt.)	(e) Scraped uterus (mg. wet wt.)	(f) Difference (e) - (d) (mg.)	(g) Homo- genate residue (mg. dry wt.)	(h) Percentage dry wt. [100 (g)/ (e)]	(j) Total soluble protein of myo- metrium (mg.)	(k) Percentage of soluble protein [100 (j)/ (g + j)]
43	0	180	146	159	+13	62	39	4.5	6.8
44	0	182	125	142	+17	61	43	4.9	7.3
48	0	184	172	179	+7	61	34	4.1	6.3
37	2	204	158	146	-12	45	31	5.5	10.9
31	2.2	224	213	203	-10	64	31	5.6	8.0
29	2.5	202	273	209	-64	64	30	5.9	8.4
39	4.2	200	275	240	-35	64	27	11.4	15.1
32	4.3	211	258	234	-24	75	32	5.9	7.3
33	6	187	253	216	-37	56	26	7.5	11.8
41	8.2	227	350	288	-62	77	27	9.0	10.5
34	8.2	231	322	263	-59	81	31	6.5	7.4
42	10.2	199	303	261	-42	59	23	11.0	15.7
26	12.4	191	—	203	—	59	29	5.4	8.4
49	15	180	321	281	-40	77	27	8.1	9.5
46	15.3	185	226	209	-17	64	31	4.1	6.0
38	18	242	206	198	-8	51	26	7.3	12.4
35	24	210	182	181	-1	60	33	8.2	12.0
47	36	181	214	203	-13	63	31	6.5	9.4
36	48	202	239	234	-5	69	29	9.6	12.2
30	44.3	237	322	303	-19	93	31	9.7	9.4
28	76	241	191	191	0	81	42	5.6	6.5
51	48*	188	269	267	-2	84	31	5.5	6.2
52	48*	186	256	244	-12	75	31	7.1	8.7

* Two injections.

2.5–15 hr. after oestradiol, lost water as well as endometrial tissue during scraping.

Column *k* is the percentage of soluble protein of the extracts of the myometrium, with the dry weight plus the soluble protein as 100 %. This value was below 7.5 % for control uteri, and except for rat no. 46, from 7.3 to 15 % for at least 48 hr. after one injection of oestradiol. Some or all of this increase may represent the uptake of serum proteins (Kalman & Lowenstein, 1958).

Dehydrogenases. Table 2 gives the dehydrogenase activities of extracts of the endometria of 31 ovariectomized rats, 26 of which had been injected with oestradiol and five of which were killed as controls. The calculated specific activities are charted in Fig. 2. A biphasic pattern of change in the enzyme activities is evident. Both enzyme activities were decreased from 2 to 10 hr. after oestradiol as compared with control. From this low point of enzyme activity, the glucose 6-phosphate-dehydrogenase activity started to rise at 12 hr., and at 36–48 hr. attained activity about three times that of the ovariectomized control uteri and then decreased. The phosphogluconate-dehydrogenase activity also fell at 2 hr., started to rise at 12 hr. and achieved a maximum with one injection of oestradiol between 36 and 48 hr. Two injections of oestradiol at 24 hr. intervals increased

the 48 hr. enzyme activities over those with one injection.

Extracts of myometrium exhibited no early decrease of dehydrogenase activity (Fig. 3). Increase in glucose 6-phosphate-dehydrogenase activity was evident at 15 hr.; there was a twofold increase by 36 hr. and the activity had not returned to normal in the one uterus analysed at 76 hr. With one injection of oestradiol, the change in the phosphogluconate-dehydrogenase activities was significant at the 5 % level at 15–24 hr. and at the 1 % level at 36 hr. With two injections, both enzymes were more markedly increased at 48 hr.; the phosphogluconate dehydrogenase was three and four times the mean control activity, and glucose 6-phosphate dehydrogenase was increased three- to six-fold.

Since these activities were calculated in terms of increase of absorption at 340 m μ due to the reduction of TPN/mg. of protein, the drop in activity in the first 2 hr. may have been caused by increased protein content due to serum protein leaking into the uterus. Therefore the total dehydrogenase activities of the extract were divided by the 'dry weight' of the uterine residue, that is of the homogenates from which the soluble protein had been extracted. Both series were analysed statistically. The values for the enzyme activities seemed to fall

Table 2. *Dehydrogenase activities of extract of uterus*G 6-P, glucose 6-phosphate; PG, phosphogluconate; ΔE , change in $E_{340\text{ m}\mu}$

Rat no.	Time after oestradiol (hr.)	Myometrium				Endometrium			
		Homogenate vol. (ml.)	Extract protein (mg./ml.)	Dehydrogenase activities		Extract Vol. (ml.)	Protein (mg./ml.)	Dehydrogenase activities	
				G 6-P (ΔE /min./ml.)	PG			G 6-P (ΔE /min./ml.)	PG
16	0	—	2.3	0.55	0.26	—	0.50	0.25	0.103
17	0	—	1.6	0.53	0.18	—	0.88	0.30	0.106
40	0	2.0	4.40	0.85	0.40	1.2	1.03	0.44	0.20
43	0	1.8	2.72	0.90	0.54	1.3	0.37	0.32	0.16
44	0	1.8	2.96	0.84	0.48	1.6	0.35	0.25	0.11
37	2	2.4	2.38	0.58	0.30	1.5	0.68	0.06	0.09
31	2.2	2.1	2.96	0.96	0.56	0.9	1.00	0.38	0.15
29	2.5	2.2	2.96	1.00	0.60	1.2	1.00	0.25	0.10
39	4.1	2.0	6.46	0.90	0.50	1.4	2.67	0.48	0.22
32	4.3	2.0	3.26	0.92	0.50	1.2	1.05	0.29	0.11
25	6	1.9	1.95	0.70	0.20	1.3	0.68	0.24	0.08
33	6	2.3	3.60	0.80	0.44	1.2	1.22	0.23	0.12
34	8.2	2.0	3.80	1.34	0.78	1.2	2.50	0.58	0.25
41	8.2	2.2	4.76	1.20	0.60	2.4	2.68	0.78	0.35
42	10.1	2.1	5.94	1.60	0.80	1.4	2.29	0.50	0.25
26	12.4	1.8	3.39	1.00	0.48	—	0.92	0.52	0.19
49	15.0	2.1	4.46	2.10	1.00	1.2	1.68	1.20	0.55
46	15.2	1.6	2.97	1.56	0.60	1.5	0.99	0.73	0.29
22	18	—	2.12	0.96	0.50	—	0.41	0.20	0.06
23	18	—	2.60	1.34	0.67	—	0.72	0.60	0.18
38	18	2.1	3.84	1.14	0.52	1.7	0.95	0.50	0.20
35	24	2.3	3.93	1.20	0.52	1.3	0.56	0.13	0.06
45	24.3	—	—	—	—	1.2	1.25	1.35	0.50
47	36	1.7	4.30	3.20	1.30	0.9	1.14	2.46	0.90
50	39	2.0	4.40	2.55	0.93	1.0	1.46	2.45	0.68
24	40	—	3.60	2.00	0.92	—	1.05	1.70	0.60
30	44.3	2.5	4.43	2.60	1.25	1.1	1.03	2.20	0.65
36	48	2.2	4.81	2.40	1.00	1.2	0.93	1.25	0.45
28	76	2.3	2.68	1.35	0.40	1.3	0.65	0.49	0.20
51	48 (2 ×)	2.1	2.98	2.86	1.42	1.1	1.16	2.40	0.95
20	48 (2 ×)	—	2.18	2.55	1.50	—	0.62	1.56	0.52
21	48 (2 ×)	—	1.70	3.0	0.80	—	0.65	2.2	0.63

in periods for which means were determined and the significance of the differences between them were calculated as shown in Table 3. In Table 3 the drop in specific activity of glucose 6-phosphate dehydrogenase of endometrial extracts in the period 2–10 hr. after oestradiol was seen to be significant. However, when calculated in terms of total activity per 'dry weight' the decrease was not significant (Table 4).

Hexokinase. Activators, or inhibitors of other reactions, such as nicotinamide, ethylenediamine-tetra-acetate (EDTA) or potassium fluoride have been found not to affect the activity of the dehydrogenases in fresh extracts of two other mammalian tissues which have been studied subsequent to the work on the uterus, i.e. hamster cheek pouch and cultured rabbit-kidney cells. Hexokinase activity was increased by EDTA and potassium fluoride and these effects were not additive. Also for the hexokinase determination, where the reactions continue over a longer time, nicotinamide was found necessary to protect the

TPN from TPN nucleotidase. Samples of extracts of endometrium, myometrium and adrenal gland were tested for TPN nucleotidase and destruction of TPN was found to be not greater than 2% in 10 min. at 37°. None of the other activators were used in the determination of uterine hexokinase, because the uterine extracts available were not sufficient for an adequate study of their effects. These results are so incomplete for this series that they are presented only as suggesting that the activity of this enzyme may also be affected by injection of oestradiol and that the effect deserves further investigation.

The series of hexokinase determinations on the extracts of both the endometrium and the myometrium suggested a biphasic response of the endometrial enzyme, as shown in Table 5. In the endometrial extract the increase with two injections of oestradiol was only 100% at 48 hr. The hexokinase of myometrial extracts increased 100% at 36 hr. after one injection and 200% at 48 hr. after two injections of oestradiol.

Fig. 2. Dehydrogenase activities of extracts of endometrium. Each cuvette contained 0.05M-tris buffer, pH 7.4, and 1μmole of glucose 6-phosphate (●) or 0.5 μmole of phosphogluconate (○) and 0.1 or 0.2 ml. of extract in a total volume of 1 ml. The extract was the supernatant of the scrapings of the endometrial surface of the uterus in 0.25M-sucrose and contained 0.5–3.0 mg. of protein/ml. TPN, 0.02 ml. (200 μg.), was added and $E_{340\text{ m}\mu}$ was read at 0.5 min. intervals for 5 min. The enzyme activity was calculated from the change in $E_{340\text{ m}\mu}/\text{min.}$ from the first to fourth minutes to μmoles of TPN reduced/min./mg. of protein. Each symbol represents the enzyme activity of the extract of one rat. Ten days after ovariectomy, controls were killed and treated rats were injected with 100 μg. of oestradiol and killed at a time later as shown on the abscissa. Δ and $\blacktriangle$, the rat had received a second injection of 100 μg. of oestradiol at 24 hr. and was killed at 48 hr.

Fig. 3. Dehydrogenase activities of extracts of myometrium. Enzyme activities were determined as in Fig. 2, with 0.05 or 0.1 ml. of extract of the scraped homogenized uterus, containing 100–200 μg. of protein. ●, Glucose 6-phosphate dehydrogenase; ○, phosphogluconate dehydrogenase. Δ and $\blacktriangle$, See legend to Fig. 2.

Transhydrogenase. Determination of transhydrogenase on the extracts of the uterus revealed no activity in the endometrial extracts and moderate transhydrogenase in the myometrial extracts. Values for the transhydrogenase of myometrial extract were decreased after oestradiol injection

and recovered after 10 hr. (Table 5). These values may be compared with the active transhydrogenase in extracts of adrenal gland (Table 7). Addition of oestradiol to the myometrial extracts of ovariectomized controls did not increase the activity.

Endogenous substrates. The reduction of TPN by endogenous substrates during the first minute is shown in Table 6. The most definite pattern in the endometrium is evident in the endogenous substrate calculated as μmole/mg. of protein in the extract. The amounts decreased at 2 hr. compared with the control and increased at 12.5–24 hr., decreasing to the control level later. The myometrial extracts from control rats contained very little endogenous substrate/mg. of protein. From 2 to 48 hr. after oestradiol there was a steady increase and at 76 hr. the values had not declined to levels as low as the controls. We have not obtained sufficient material to enable us to determine the identity of this endogenous substrate which reacts with TPN.

Reduced triphosphopyridine nucleotide oxidases. Samples of extracts of the uteri of two intact rats were analysed for glutathione. Direct titration of the extracts from which protein had been precipitated by 5% sulphosalicylic acid gave mean values of 0.050 and 0.049 μmole of soluble SH group/mg. of protein for the endometrium and the corresponding values of 0.045 and 0.036 for the myometrium. When the extracts were treated with TPN and glucose 6-phosphate before the protein was precipitated, the titrations indicated 0.062 and 0.055 μmole/mg. of protein for the endometrium and myometrium respectively. Thus in preparation of endometrium extract 21% of the SH groups were oxidized. In the homogenized myometrial extract 35% of the SH groups were oxidized. If all of the glutathione were to be oxidized, the amount in a sample of aged extract, containing 0.05–0.2 mg. of endometrial protein, would contain not more than 0.01 μmole of oxidized glutathione. Samples of freshly prepared endometrial extracts used for enzyme assay might be expected to contain up to 0.002 μmole of oxidized glutathione, which in oxidizing TPNH might cause a total decrease in extinction of 0.012, which is negligible. Similar calculations for oxidized glutathione of samples of myometrial extracts might cause a total change of 0.036 in extinction. The rate of oxidation of TPNH by glutathione would extend this reaction over a longer period than that taken for dehydrogenase measurement and therefore its effect would be negligible.

Freshly prepared extracts which had been kept cold did not reoxidize TPNH after the substrate was exhausted unless oxidized glutathione was added to the reaction mixture. Tests performed with extracts of endometrium and myometrium

from operated rats at 12 hr. and 48 hr. after injection of oestradiol oxidized no TPNH even in the presence of oxidized glutathione.

Fig. 4 is the graph of an experiment on the oxidation of added TPNH by fresh extracts of the endometrium and the myometrium of the uterus of an unoperated rat and the control oxidation in the tris buffer without extract. The oxidation of TPNH by extracts is only slightly greater than without them and is not much increased by addition of $0.2 \mu\text{mole}$ of oxidized glutathione, an amount greater than is present in the tissue extracts. These rates are too low to have any influence on the rates of reduction of TPN by the dehydrogenases.

With limiting concentration of TPN, either *in vitro* or *in vivo*, TPNH oxidation by a dye (Dickens & McIlwain, 1938) or some other hydrogen acceptor (Kinoshita, 1957) is necessary for the continued availability of TPN for the dehydrogenase reactions. In our assay with substrate concentrations of TPN the rate is not limited by TPN concentration nor inhibited by the TPNH formed, in the first few minutes.

Adrenal-gland enzymes. The enzyme activities of the extracts of the adrenals (Table 7) varied considerably, with no correlation with time after oestradiol injection. All extracts tested had high transhydrogenase activity. No endogenous substrate was found that reduced TPN.

DISCUSSION

The dehydrogenase, but not the hexokinase, activities as measured by the assays represent the near maximum potential activity of the enzymes *in vitro*. This potential would not be expected to be fully expressed *in vivo* unless conditions were such that both the substrate was present in optimum concentration and sufficient TPN was present in the oxidized state. Analyses of other normal tissues have indicated that glucose 6-phosphate concentration is suboptimum, phosphogluconate has not been detected and, according to Glock & McLean (1955a), TPN is more than 90 % reduced except in muscle and the total in liver is about equal to the Michaelis constant (10^{-6}M). Calculations of the

Table 3. *Specific dehydrogenase activities*

For G 6-P, PG and ΔE see Table 2. Numbers of rats are given in parentheses.

		Myometrial extracts	
		G 6-P ($\Delta E/\text{min./mg. of protein}$)	PG
Controls	Mean	(6) 0.30	(6) 0.145
	S.D.	0.080	0.045
2-12 hr.	Mean	(11) 0.29	(10) 0.147
	S.D.	0.069	0.035
Combined 0-12 hr.	Mean	(17) 0.29	(16) 0.146
	S.D.	0.067	0.040
15-24 hr.	Mean	(6) 0.43	(6) 0.20
	S.D.	0.10	0.052
	<i>P</i> (vs. control)	<0.01	<0.05
36-48 hr.	Mean	(5) 0.60	(5) 0.25
	S.D.	<0.094	0.041
	<i>P</i> (vs. control)	<0.01	<0.01
		Endometrial extracts	
		G 6-P ($\Delta E/\text{min./mg. of protein}$)	PG
Controls	Mean	(5) 0.57	(5) 0.24
	S.D.	0.22	0.12
2-10 hr.	Mean	(10) 0.23	(9) 0.137
	S.D.	0.096	0.08
	<i>P</i> (vs. control)	<0.01	>0.05*
12-24 hr.	Mean	(8) 0.63	(8) 0.25
	S.D.	0.24	0.095
	<i>P</i> (vs. control)	>0.05*	>0.05*
	<i>P</i> (vs. 2-10 hr.)	<0.01	<0.05
36-48 hr.	Mean	(5) 1.79	(5) 0.58
	S.D.	0.37	0.42
	<i>P</i> (vs. control)	<0.01	<0.01

* Difference is not significant.

Table 4. *Dehydrogenase activities calculated as total units/g. dry wt. of insoluble residue*

Numbers of rats are given in parentheses. For G 6-P, PG and ΔE see Table 2.

		Myometrial extracts	
		G 6-P (total ΔE /min./g. dry wt.)	PG
Controls	Mean	(3) 32.2	(3) 17.3
	S.D.	3.8	4.2
2-8 hr.	Mean	(9) 30.3	(9) 16.1
	S.D.	3.6	4.1
	<i>P</i> (vs. control)	>0.05*	>0.05*
10-24 hr.	Mean	(7) 40.0	(6) 23.0
	S.D.	11.2	6.2
	<i>P</i> (vs. control)	>0.05*	>0.05*
	<i>P</i> (vs. 2-8 hr.)	<0.05	<0.05
36-48 hr.	<i>P</i> (vs. combined 0-8 hr.)	<0.05	<0.05
	Mean	(4) 78.0	(4) 33.0
	S.D.	6.6	1.8
	<i>P</i> (vs. control)	<0.01	<0.01

		Endometrial extracts	
		G 6-P (total ΔE /min./g. dry wt.)	PG
Controls	Mean	(3) 9.2	(3) 4.2
	S.D.	4.5	2.0
2-6 hr.	Mean	(6) 6.0	(7) 2.5
	S.D.	2.2	1.05
	<i>P</i> (vs. control)	>0.05*	>0.05*
8-24 hr.	Mean	(8) 15.6	(8) 6.0
	S.D.	7.5	3.1
	<i>P</i> (vs. control)	>0.05*	>0.05*
	<i>P</i> (vs. 2-6 hr.)	0.01	<0.05
36-48 hr.	Mean	(4) 30.8	(4) 9.7
	S.D.	2.6	2.4
	<i>P</i> (vs. control)	<0.01	<0.05
	<i>P</i> (vs. 2-6 hr.)	<0.01	<0.01
	<i>P</i> (vs. 8-24 hr.)	<0.01	<0.05

* Difference is not significant.

Table 5. *Hexokinase and transhydrogenase activities of uterus extracts*

ΔE is change in $E_{340\text{ m}\mu}$.					
Rat no.	Time after oestradiol (hr.)	Hexokinase (ΔE /min./mg. of protein)		Transhydrogenase (ΔE /min./mg. of protein)	
		Endometrium	Myometrium	Endometrium	Myometrium
40	0	—	0.03	—	0.010
48	0	—	—	—	0.030
43	0	0.04	0.04	0	0.022*
31	2	—	—	0	0
39	4	—	0.017	0	0.002
41	8	0.017	0.025	0	0.005
42	10	0.010	0.04	0	0
46	15	0.05	0.05	—	0.017
49	15	0.024	0.05	0	0.022
47	36	0.035	0.075	0	0.022
50	39	—	—	0	0.013
51	48†	0.09	0.12	0	0.013

* Activity was the same when 10 $\mu\text{g.}$ of oestradiol was added to the reaction mixture.

† Two injections of oestradiol.

Table 6. *Endogenous substrate for triphosphopyridine nucleotide in extracts of uterus*

The change in $E_{340\text{ m}\mu}$ (ΔE) was measured during the first minute under the conditions described in the text.

Time after oestradiol (hr.)	Endometrium			Myometrium		
	Initial rate ($\Delta E/\text{min.}/\text{mg.}$)	Amount of TPN reduced		Initial rate ($\Delta E/\text{min.}/\text{mg.}$)	Amount of TPN reduced	
		$\mu\text{mole}/\text{mg.}$ of protein	Total μmole		$\mu\text{mole}/\text{mg.}$ of protein	Total μmole
0	0.09	0.060	0.074	>0	0.007	0.057
0	0.04	0.040	0.026	>0	0.009	0.044
0	0.08	—	—	>0	—	—
2	0.01	0.016	0.014	0.01	0.024	0.135
2.5	0.02	0.022	0.035	0.01	0.012	0.070
8	0.06	0.028	0.180	0.02	0.014	0.125
8.5	—	—	—	0.03	—	—
10	0.01	0.014	0.045	0.03	0.022	0.240
12.5	0.08	0.078	0.086	0.03	0.017	0.091
15	0.05	0.045	0.067	0.04	0.035	0.144
15	0.11	0.062	0.124	0.007	0.038	0.307
18	0.035	—	—	0.038	0.032	0.234
24	0.20	0.10	0.15	0.02	0.016	0.131
36	0.14	0.056	0.058	0.10	0.056	0.363
39	0.10	0.056	0.082	0.05	0.040	0.303
48	0.13	0.056	0.063	0.11	0.061	0.590
48*	0.21	0.113	0.145	0.13	0.069	0.380
76	0.046	0.040	—	0.041	0.023	0.129

* Two injections.

Fig. 4. Reduced triphosphopyridine nucleotide oxidase of extracts of rat uterus. Each cuvette contained 0.2 ml. of 0.05M-tris, pH 7.4, and 0.02 ml. of TPNH (0.116 mg.). Total volume was 1 ml. O, Non-enzymic control; to E was added 0.06 ml. of extract of endometrium containing 66 $\mu\text{g.}$ of protein; to M was added 0.06 ml. of extract of myometrium containing 132 $\mu\text{g.}$ of protein. The uterus was taken from an intact rat. At 21 min. 0.01 ml. of oxidized glutathione (GSSG; 0.2 μmole of glutathione) and at 41 min. 0.01 ml. of cysteine (0.1 μmole) were added to all cuvettes. At 54 min. a further 0.06 ml. of endometrial extract was added to E and 0.12 ml. of myometrial extract was added to M.

total glucose 6-phosphate-dehydrogenase activity of extracts of *E. coli* have shown it to be sufficient to account for up to 80 % of the glucose used by a bacterial culture and the phosphogluconate dehydrogenase could account for about 40 % of the glucose (Scott & Cohen, 1953); isotope experiments have indicated that approx. 15–40 % of the glucose is so used by *E. coli* (Cohen, 1951; Roberts, Abelson, Cowie, Bolton & Britten, 1955). For other organisms and tissues, isotopic data have indicated similar or much smaller proportions of glucose metabolized via the hexose monophosphate shunt. Even if one considers the possibility that some of the carbon dioxide released from phosphogluconate may be recaptured by synthetic reactions, it is obvious that in *E. coli*, and probably in mammalian tissues (Beck, 1958), these dehydrogenases, especially the glucose 6-phosphate dehydrogenase, are not operating at full capacity.

When the normal or steady state is interrupted by a change in the environment that will increase the substrate, such as an increase in permeability or of hexokinase activity or the addition of a hydrogen acceptor, which will increase the TPN:TPNH ratio, then one may visualize a state in which the enzyme is operating at capacity. In such a state all the enzyme would be bound to substrate and if the substrate were still in excess one could expect new enzyme formation to be induced. Whether glucose 6-phosphate or TPN or both are the substrates which are necessarily bound is not clear. In the oestradiol-treated uterus, either in-

Table 7. *Activities of some enzymes in adrenal-gland extracts*For G 6-P, PG and ΔE see Table 2.

Rat no.	Time (hr.)	Wet wt. (mg.)	G 6-P dehydrogenase (ΔE /min./mg. of protein)	PG dehydrogenase (ΔE /min./mg. of protein)	Hexokinase (ΔE /min./mg. of protein)	Transhydrogenase (ΔE /min./mg. of protein)
40	0	43	0.27	0.61	0.14	0.14
43	0	22	2.5	2.6	0.10	0.20
44	0	23	1.82	1.32	—	—
37	2	32	0.75	0.73	—	—
31	2.2	54	0.65	0.65	—	0.16
29	2.5	53	0.95	0.93	—	—
39	4.2	—	0.90	0.90	0.033	0.24
32	4.3	55	1.3	1.0	—	—
34	8.2	32	1.07	1.07	—	—
41	8.2	53	0.70	0.92	—	—
42	10.2	58	1.27	1.07	0.04	—
26	12.4	42	0.90	0.76	—	0.15
46	15.3	53	1.19	1.31	—	—
49	15	43	1.31	1.54	0.06	0.20
38	18	46	0.74	0.85	—	—
35	24	56	1.14	0.91	—	—
45	24.3	47	2.02	3.14	0.032	—
47	36	43	0.78	0.85	0	0.30
50	39	60	1.62	1.67	—	0.25
24	40	41	0.55	0.65	—	—
30	44.3	64	0.86	0.92	—	—
36	48	47	1.16	1.07	—	—
51	48 (2 ×)*	43	0.73	1.0	0.07	0.20
52	48 (2 ×)*	41	1.85	1.63	—	—
28	76	52	1.0	0.89	—	—

* Second injection of oestradiol at 24 hr.

creased permeability to glucose or oestrone oxidation of TPNH (Talalay & Williams-Ashman, 1958) or both may be involved.

Our attempts to find an effect of oestradiol added *in vitro* to any of the enzymes in the extracts investigated have had negative results, with either DPN or TPN as coenzyme. Hollander & Stephens (1959) were unable to show any effect of oestradiol in oxidizing DPNH by homogenates of rat uterus which were very active in oxidizing it when various phenols and chlorophenols were added in the presence of oxygen. This activity was found in particles which sedimented at 5000 *g* for 10 min. and 100 000 *g* for 1 hr. and none in the supernatant. Our extracts would contain some of the latter particles. The same authors found that homogenates of the uteri of ovariectomized rats did not oxidize DPNH in the presence of 2:4-dichlorophenol. Homogenates of uteri of ovariectomized animals which had received injections of 0.2 mg. of oestradiol 72 hr. previously had activities which were higher than the mean values from uteri of intact rats. The relationship of this enzyme to the enzymes we have studied has not been investigated.

The question of what happens to the increased phosphogluconate that is probably formed when glucose 6-phosphate dehydrogenase has increased and the phosphogluconate dehydrogenase has not,

is not yet solved. Growth of *E. coli* in the presence of dinitrophenol is accompanied by a great increase in pyruvic acid in the medium and the extracts show increased glucose 6-phosphate dehydrogenase and no change in phosphogluconate dehydrogenase. This might indicate the operation of the Entner & Doudoroff (1952) pathway for splitting of oxidized phosphogluconate. However, we have been unable so far to find any degradation of phosphogluconate in the absence of TPN in extracts of the uterus.

Another parameter which may affect the activities of the TPN dehydrogenases is the presence of the TPNH oxidases, especially glutathione reductase. The analyses reported for reduced and total sulphydryl groups would indicate that insufficient glutathione was present to affect the measurement of dehydrogenase activities. There was insufficient material for an analysis of glutathione in the uteri of the present series. It may be during the period 2–8 hr. after oestradiol injection, when the dehydrogenases are low and there is increased protein in the tissue, that glutathione may be increased, especially the oxidized form. Glutathione analyses for another series will be reported later.

The time course of the response in enzyme activity may be compared with various other parameters of oestrogen-induced reaction of the ovariectomized rat uterus, as reported in the literature

Table 8. *Changes in the rat uterus after oestradiol*

Time (hr.)	Parameter	Change	Reference
1	Serum albumin ¹³¹ I	+	Kalman & Lowenstein (1958)
1	Uptake of O ₂ (no glucose)	+	Szego & Roberts (1953)
2	Soluble protein	+	This paper
2	H ₂ O	+	Reynolds (1949)
2	²² Na	+	Kalman & Lowenstein (1958)
2	³² P	++	Kalman & Lowenstein (1958)
2	¹³¹ I	+	Kalman & Lowenstein (1958)
2	Glucose 6-phosphate and phosphogluconate dehydrogenase (endometrium)	-	This paper
4	Glucose uptake	+	Szego & Roberts (1953)
4	Lactate accumulation	+	Szego & Roberts (1953)
5	Incorporation of amino acids into protein	+	Mueller (1957)
6	Serine aldolase	+	Mueller (1957)
6	Incorporation of ¹⁴ CO ₂ into adenine	+	Mueller (1957)
6	Protein content	+	Telfer (1953)
6	Incorporation of acetate into lipid	+	Mueller (1957)
6	Ribonucleic acid content	-	Telfer (1953)
10	Uptake of O ₂ (glucose)	+	Szego & Roberts (1953)
12	Glucose 6-phosphate dehydrogenase and phosphogluconate dehydrogenase (endometrium)	+	This paper
12	Glycogen formation	+	Walaas (1952)
12	Uridine into ribonucleic acid	+	Mueller (1957)
13.5	Mitosis (mouse endometrium)	+	Allen, Smith & Gardner (1937)
15	Uptake of O ₂ (glucose)	+	Szego & Roberts (1953)
16	Glucose 6-phosphate dehydrogenase (myometrium)	+	This paper
20	Lactate accumulation	-	Szego & Roberts (1953)
21	Deoxyribonucleic acid content	+	Telfer (1953)
21	Ribonucleic acid content	+	Telfer (1953)

(Table 8). These various experimental results, although performed in different laboratories with all the variability of procedure, animals and even dosage, do seem to fall into a logical sequence. The early changes suggest an effect of the oestrogens and other steroids on permeability, as has been noted before (Kalman & Lowenstein, 1958; Elliott & Yrarrazaval, 1952). There is evidence that the primary effect of oestradiol is on the capillaries, causing permeability to protein but not to red cells (Kalman, 1958; Zierler, 1958). We may note that the decrease in enzyme specific activity in the oestrogen-stimulated endometrium occurred at 2 hr., the time when Kalman showed that increased permeability of the capillaries allowed serum albumin to pass into the tissue and ²²Na and ¹³¹I increased along with imbibition of water. Szego & Roberts (1953) found at 4 hr. after oestradiol injection a concomitant increase in glucose uptake and lactate accumulation, whereas the increase in oxygen uptake due to glucose occurred at 10 and 15 hr., at about the same times as the glucose 6-phosphate dehydrogenase started to increase. At 20 hr. glycolysis decreased. It would seem that the first reaction to increased glucose was glycolytic breakdown. It would be interesting to know whether this increased glycolysis was intracellular or extracellular or both (Shaw & Stadie, 1957), how much of the increased glucose uptake is accounted for by glycolysis and whether there was

also an induced increase of glycolytic enzymes. It is to be noted that the increased glycolysis occurs before increased protein synthesis and decreases after it. The first synthetic enzymic activities to be increased are those at 5 hr., incorporating amino acids into protein (Mueller, 1957). An increase in protein content was noted at 6 hr. by Telfer (1953). Mueller found increased incorporation of acetate at the same time into lipid and carbon dioxide into adenine, and Telfer found a decreased ribonucleic acid:protein ratio. At 10 hr. oxygen uptake in the presence of glucose was increased (Szego & Roberts, 1953). At 12 hr. the dehydrogenases for glucose 6-phosphate and phosphogluconate started to show increased activity in the endometrium, glycogen was formed (Walaas, 1952) and uridine was incorporated into ribonucleic acid (Mueller, 1957). At 15 hr. the oxygen uptake again increased, and glucose 6-phosphate-dehydrogenase activity started to increase in the myometrium. At 20 hr. glycolysis was decreased. At 21 hr. the nucleic acid content was increased (Telfer, 1953). Allen, Smith & Gardner (1937) reported increased mitosis in the myometrium of the mouse 13.5 hr. after oestradiol but we have been unable to find comparable observations in the rat.

Thus we can see a logical sequence of metabolic events leading to, or accompanying, an increase in size of the uterus. However, the cause and effect of relationships are not necessarily evident from the

sequence. A study such as Beck's (1958) on the pacemakers of glycolysis in leucocytes would be desirable, as would be a more complete picture of the initial reactions on which Kalman (1958) has done such interesting work.

SUMMARY

1. The sequence of changes in the activities of glucose 6-phosphate and phosphogluconate dehydrogenases has been determined in extracts of the endometrium and myometrium of the uteri of ovariectomized rats, untreated and at selected times after treatment with oestradiol-17 β .

2. The activity of the glucose 6-phosphate dehydrogenase in units/mg. of soluble protein was significantly less than that of controls from 2 to 10 hr. after oestradiol, but no difference was shown if the activity was calculated in terms of the insoluble residue of the uterus.

3. By both methods of calculation a significant increase in the activities of both enzymes of both extracts was found at later times up to 48 hr. and a return to control values at 76 hr.

4. The results of the determination of the specific activities of hexokinase of some of the extracts are reported.

5. Transhydrogenase was determinable in extracts of myometrium but not of endometrium.

6. Extracts of the adrenal glands of the same animals were analysed in the same way to serve as control tissues. The enzyme activities exhibited no changes correlated with oestradiol injection.

7. These data are discussed in relation to other changes reported in uteri of ovariectomized rats after injection of oestradiol.

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