source of high-energy phosphate. The particles were separated by centrifuging and serum albumin was released by ultrasonic vibrations. The albumin was purified finally by zone electrophoresis on cellulose acetate.

3. A rat was intravenously injected with [^{14}C]-leucine and after 30 min. the animal was killed. Albumin was isolated from the microsome fraction of the liver.

4. The radioactive albumin samples obtained under conditions *in vitro* and *in vivo* were denatured, oxidized and hydrolysed with trypsin. The peptides resulting were fractionated by ionophoresis on paper and the [^{14}C]leucine-containing peptides located by radioautography.

5. Comparison of the radioautographs indicated about 20 bands of active peptides in each case. The peptides labelled were in general similar under the two conditions but the degree of labelling showed marked differences.

6. The results are consistent with the synthesis of serum albumin by the isolated microsome fraction. They appear to exclude the incorporation of [^{14}C]leucine into serum albumin by attachment to the residues of the basic or acidic amino acids or by a limited exchange with leucine residues. The extent to which entirely new polypeptide chains are synthesized under the conditions *in vitro* is not at present known.

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samples and the ionophoresis of the peptides. We are also grateful for their interest and valuable criticisms. We should also like to thank Professor F. Dickens, F.R.S., for his interest, Dr J. K. Whitehead for assistance with the purification of the [^{14}C]leucine and Mr H. E. H. Jones for performing the partial hepatectomies and the intravenous injections. This work was made possible by a grant to the Medical School from the British Empire Cancer Campaign.

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Sulphydryl Groups of the Uterus of the Ovariectomized Rat after Treatment with Oestradiol

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Scott & Lisi (1960) reported the changes in activities of some enzymes of extracts of the uteri of ovariectomized rats after injection of oestradiol- 17β . In order to be sure that the changes noted could not have been affected by the oxidation of TPNH_2 by oxidized glutathione in the extracts, a few extracts were titrated for acid-soluble sulphydryl-group content. The concentration of sulphur reducible to sulphydryl groups was found not to be sufficient to affect the enzyme determinations. However, to be sure this was a valid deduction at all times considered in the enzyme study, another series of animals were investigated

to determine any changes in the sulphydryl-group concentration and content of the uterus after injection of oestradiol. Changes in sulphydryl groups which were parallel to the changes in enzyme activities are reported.

MATERIALS AND METHODS

Chemicals. TPN, monosodium salt, and glucose 6-phosphate, disodium salt, were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. GSH and GSSG were obtained from Schwarz Laboratories Inc., Mount Vernon, N.Y., U.S.A. All water was doubly distilled, the second time in an all-glass still.

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

Operation. Wistar-strain rats (170–230 g.) were ovariectomized as described by Scott & Lisi (1960). Oestradiol-17 β (100 μ g.) was injected into the peritoneum 10 days after the operation.

Preparation of extracts. At selected times after the injection, the animals were killed and extracts were prepared as described previously, except that the cut pieces of the scraped myometrium were not homogenized but finely minced with scissors and then were ground with Al_2O_3 and extracted with a volume of 0.25 M-mannitol equal to 5.0 ml./g. of myometrial wet wt. The suspensions of both endometrial scrapings (1 ml.) and of ground myometrium were centrifuged at 10 000g for 1 hr. in an International refrigerated centrifuge at 4°.

To a part of the clear supernatant fluid was added 0.1 ml. of a mixture of glucose 6-phosphate (4 μ moles), TPN (0.5 μ mole) and nicotinamide (200 μ g.). After 15–20 min. at room temperature, the mixture was again chilled. To it, and to an untreated sample of the extract, was added $\frac{1}{2}$ vol. of 20% sulphosalicylic acid solution. After 20 min. in ice the proteins were separated by centrifuging.

Titration for SH group content were performed by the amperometric method of Benesch, Lardy & Benesch (1955). Samples (0.1–0.5 ml.) of the supernatant fluid or the whole pellet of precipitated protein were added to a beaker containing 10 ml. of tris-nitrate buffer, pH 7.38, with a Pt electrode rotating at approximately 600 rev./min. in the liquid. The half-cell was Hg–HgO–Ba(OH) $_2$ (Samuelson & Brown, 1935). Portions (5 μ l.) of 1 mM-AgNO $_3$ were added from a microburette dipping below the liquid surface. At the end point of the titration, single additions produced deflexions of 5–10 mm. on the galvanometer (Rubicon with Ayrton shunt). This method has been described in detail and carefully evaluated by Stern (1961). Before each series of titrations, the apparatus was tested by titration of glutathione. The values reported for acid-soluble SH groups are the means of duplicates. The average variations from the means were 10, 8.6, 8.1 and 6.8% for the direct and reduced endometrial and the direct and reduced myometrial extracts respectively. Sometimes the volume of myometrial extract was insufficient to determine both direct and reduced SH groups. Each total pellet of precipitated protein was titrated to avoid errors of sampling, so there were no duplicate determinations of acid-insoluble SH groups.

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

Chromatography. Intact female rats were injected with 100 μ g. of oestradiol twice, the second injection 24 hr. after the first. The rats were killed 24 hr. after the second injection, and the uteri removed and extracts prepared as usual. Tissue extracts were treated with 2.4 vol. of cold 95% ethanol and the precipitated proteins were separated by centrifuging. In one experiment the cut uterus was scraped and washed with water and the supernatant fluid was treated with ethanol. After grinding, the myometrial tissue was extracted with 70% ethanol. In one experiment scrapings in water were pooled and the finely cut uteri from four rats were pooled. The pooled endometrial extract was divided into two portions, one of which was treated with ethanol directly and the other after incubation with TPN,

glucose 6-phosphate and nicotinamide. One portion of the myometrial mince was extracted with ethanol and the other with water. One half of the water extract was treated immediately with alcohol and the other after incubation with TPN, glucose 6-phosphate and nicotinamide. The supernatant fluids were treated with *N*-ethylmaleimide (Hanes, Hird & Isherwood, 1950) and evaporated at room temperature. The residue was extracted with small volumes of 70% ethanol, and the extract placed on strips of Whatman no. 1 filter paper. Solvents for chromatography were either butan-1-ol-formic acid-water (7:2:1, by vol.) or propan-2-ol-water (7:3, v/v). The papers were treated with *N*-ethylmaleimide-propan-2-ol and then KOH in propan-2-ol (Benesch, Benesch, Gutcho & Laufer, 1956). Pink spots developed and were marked immediately as fading was rapid (Stern, 1961).

RESULTS

The weights of the rats, the changes in the wet weight of the excised uterus at times after injection of oestradiol, and the protein concentrations of the endometrial and myometrial extracts were similar to those reported by Scott & Lisi (1960). The total myometrial protein started to rise at 2 hr. and reached the peak at 4 hr. with a mean concentration twice that of controls. From 4 to 48 hr. protein concentrations were above the control range. The endometrial total protein showed less variation, with an increase in some samples between 6 and 21 hr.

Fig. 1 shows the mean concentrations of SH groups in the acid-soluble fractions of the extracts of one, two or three uteri at each time period. A decreased concentration of SH groups in the

Fig. 1. Concentration of acid-soluble sulphhydryl groups in uterine extracts. Each symbol represents the mean of experiments at the time indicated after injection of oestradiol. $\circ$ and $\bullet$, Endometrium; $\triangle$ and $\blacktriangle$, myometrium; $\bullet$ and $\blacktriangle$, by direct titration; $\circ$ and $\triangle$, after incubation with TPNH $_2$. The large symbols indicate uteri of rats injected with oestradiol a second time at 24 hr. after the first injection. The lines are drawn by eye.

Table 1. *Student's t test of significance of differences of acid-soluble sulphydryl groups*

Time (hr.)		SH (μ m-moles/ mg. of protein) (mean $\pm$ s.d.)	Numbers of animals are given in square brackets.		SH (μ m-moles/ uterus) (mean $\pm$ s.d.)	P
			Time (hr.)			
			P			
			Endometrial extracts by direct titration			
(1)	0-1 [5]	52 $\pm$ 16.5	—	(1) 0-18 [15]	32 $\pm$ 9.7	—
(2)	2-18 [9]	30 $\pm$ 7.8	(2) v. (1) < 0.001	(2) 18-72 [9]	60 $\pm$ 16	(2) v. (1) < 0.001
(3)	18-73 [10]	67 $\pm$ 20	(3) v. (1) > 0.1*	(3) 48 (2 injections) [1]	86	(3) v. (1) < 0.001
(4)	—	—	(3) v. (2) < 0.001			
	48 (2 injections) [1]	120	(4) v. (3) < 0.05			
			Endometrial extracts titrated after reduction			
(1)	0-1 [5]	52 $\pm$ 14.9	—	(1) 0-18 [16]	36 $\pm$ 8.6	—
(2)	2-12 [8]	31 $\pm$ 6.2	(2) v. (1) < 0.01	(2) 18-72 [10]	66 $\pm$ 18	(2) v. (1) < 0.001
(3)	12-21 [4]	49 $\pm$ 6.2	(3) v. (2) 0.1*	(3) 48 (2 injections) [1]	105	(3) v. (1) < 0.001
(4)	24-73 [9]	74 $\pm$ 23	(4) v. (1) 0.05			
	—	—	(4) v. (2) < 0.001			
(5)	48 (2 injections) [1]	134	(5) v. (4) < 0.05			
			Myometrial extracts by direct titration			
(1)	0-1 [3]	14 $\pm$ 1	—	(1) 0-3 [5]	20 $\pm$ 3	—
(2)	2-21 [11]	8 $\pm$ 2.9	(2) v. (1) 0.001	(2) 4-21 [9]	48 $\pm$ 23	(2) v. (1) < 0.05
(3)	24-72 [6]	12 $\pm$ 6.8	(3) v. (2) 0.1*	(3) 24-72 [6]	57 $\pm$ 31	(3) v. (1) < 0.05
						(3) v. (2) > 0.05*
(4)	48 (2 injections) [1]	16	(4) v. (2) 0.02	(4) 48 (2 injections) [1]	216	(4) v. (3) < 0.01
			Myometrial extracts titrated after reduction			
(1)	0-73 [21]	20 $\pm$ 5.7	—	(1) 0-3 [6]	57 $\pm$ 23	—
(2)	48 (2 injections) [1]	36	(2) v. (1) 0.01	(2) 4-21 [10]	104 $\pm$ 37	(2) v. (1) < 0.02
				(3) 30-73 [6]	127 $\pm$ 48	(3) v. (1) < 0.02
						(3) v. (2) > 0.05*
				(4) 48 (2 injections) [1]	490	(4) v. (3) < 0.001

* Difference is not significant.

endometrial extracts was evident from 2 to 6 hr. and thereafter there was a rise. The highest concentrations were found at 48 hr. after two injections of oestradiol.

Changes in SH-group concentrations of the myometrial extracts were not as striking as those of the endometrium. A decrease in the SH-group concentration with direct titration was found between 2 and 21 hr. An increase in the concentration after reduction was found only after two injections of oestradiol. A greater difference between concentrations before and after incubation with TPN and glucose 6-phosphate is evident in the myometrial extracts than in the endometrial extracts. Statistical analyses of these values for significant differences are shown in Table 1.

When the results were calculated as total SH groups of the extracts (Table 1), the early decrease was not apparent and the later increase was more striking. Thus the endometrial protein that was increased from 4 to 18 or 21 hr. was not accompanied by titratable SH groups as was the later protein. On the other hand, the increased protein in the myometrial extracts during that time was accompanied by titratable SH groups.

The results of the titrations of the acid-insoluble protein pellets of the extracts were calculated as $\mu\text{m-moles/mg. of protein}$ and are shown in Table 2.

The total titratable SH-group content of the myometrial extracts of individual uteri, both the acid-soluble and acid-insoluble protein SH groups before and after incubation are tabulated in Table 3. The SH-group contents of the acid-soluble fractions of the extracts were higher after incubation, whereas the SH-group contents of the protein fraction were lower after incubation. This was true of extracts of uteri taken at 2 hr. or later after oestradiol. In the experiments where all parameters were determined, the mean difference between incubated and untreated SH-group contents was $+46 \mu\text{m-moles}$ for the acid-soluble fraction and $-56 \mu\text{m-moles}$ for the protein fraction of the extracts.

Three animals died under ether and the SH-group values by direct titration of the extracts of the uteri were very low, and the values after reduction were low or normal. Direct titration of the extracts of the uterus of an animal that had tumours of the ovaries showed no SH groups. After treatment with TPN and glucose 6-phosphate, the SH-group content ($\mu\text{m-moles/mg. of protein}$) of endometrium was 29 and of myometrium 16. A series of experiments, the extracts of which were frozen at -22° for 2-6 weeks before acidification and titration, have not been included in the results. The values by direct titration were low whereas those after reduction were either low or within the range of others at similar times after oestradiol.

Table 2. *Sulphydryl-group concentration in protein pellets*

Values are given as $\mu\text{m-moles of SH/mg. of protein}$.

Time after oestradiol (hr.)	Endometrium		Myometrium	
	Direct	Incubated	Direct	Incubated
0*	49	27	27	24
2	23	—	70	65
4	40	26	39	36
6	20	25	42	32
9	44	44	50	39
12	104	—	48	—
18	39	39	50	39
21	19	42	—	22
36	—	45	—	46
48	58	66	68	52
60	76	81	74	57
72	72	34	62	54
48 (2 injections)	81	89	69	55
Mean	52	46	54 ± 15	44 ± 13

* Uninjected operated control.

Table 3. *Total titratable sulphydryl-group content of myometrial extracts*

Values are given as $\mu\text{m-moles/uterus}$.

Time (hr.)	Acid-soluble SH		Acid-insoluble protein SH	
	Direct	Incubated	Direct	Incubated
0*	26	19	100	90
0	18	—	—	—
0	—	62	—	—
1	—	37	—	—
1	23	—	—	—
2	17	62	150	140
2	20	73	—	—
3	—	58	—	—
4	111	170	394	356
4	68	136	—	—
6	32	58	165	130
9	64	98	228	177
12	24	89	213	—
12	—	152	—	—
18	71	117	350	272
18	22	91	—	—
21	27	96	—	116
24	56	—	—	—
24	106	185	—	—
30	32	114	—	—
36	24	36	—	62
48	17	112	344	226
60	74	91	410	316
72	41	75	185	161
73	—	131	—	—
48 (2 injections)	218	490	950	700

* Uninjected operated control.

The chromatograms of ethanolic supernatant fluids of the mannitol or water extracts or of ethanol extracts of both endometrium and myometrium developed either only one strong spot corresponding to the glutathione-*N*-ethylmaleimide complex or an additional small, faint spot corresponding to the cysteine-*N*-ethylmaleimide complex. The chromatograms of the combined endometrial scrapings in water of four uteri, either untreated or incubated with TPN, glucose 6-phosphate and nicotinamide, had strong spots corresponding to glutathione, faint spots corresponding to cysteine and very faint spots half way between, trailing from the glutathione. The chromatograms from the pooled myometria each showed only one spot corresponding to glutathione, faint for the water extract, and strong for the alcohol extract and the incubated water extract. Glutathione-*N*-ethylmaleimide usually showed only one spot on chromatograms. However, occasionally an additional one or even two faint spots were discernible when glutathione was carried through the procedure alongside the extracts. Therefore we cannot be sure that the spot corresponding to cysteine and the extra spot were not artifacts of the method, perhaps representing hydrolysis during evaporation.

DISCUSSION

The pattern of change of concentration of SH groups by direct titration of the uterine extracts paralleled the changes in uterine enzymes (Scott & Lisi, 1960). Table 4 presents the changes in enzymes and acid-soluble SH groups of myometrial extracts as percentages of control values. The early decreases in both parameters can be attributed to an influx of protein (Kalman, 1958) that does not contribute to the enzymic activity or to the sulphhydryl-group content. In the later period the extent of increase of glucose 6-phosphate dehydrogenase was greater than that of phosphogluconate dehydrogenase and both were greater than the increases of SH groups. The increases of enzyme activities started at an earlier time than the increases of the SH-group concentrations. The later increase of protein which occurs 12-18 hr.

after oestradiol injection may be regarded as newly formed protein which contributed to the enzyme activities and which contained SH groups and was accompanied by acid-soluble SH groups.

Scott & Lisi (1960) reported that the glutathione-reductase activity of both extracts was quite low. However, after incubation of the extracts with TPNH₂ produced by glucose 6-phosphate and the endogenous dehydrogenase, there was an increase of titratable SH groups, particularly in the myometrial acid-soluble fractions. By analysis of the results it is evident that very little reduction of soluble oxidized-sulphur compounds had occurred. The source of the increased SH groups in the acid-soluble fraction after reduction was the acid-insoluble protein of the myometrial extracts. During the incubation with TPNH₂ some part of the protein had been detached from the larger molecule and had been changed to an acid- or ethanol-soluble state. Chromatography of the ethanol-soluble fraction gave evidence that this substance was mainly glutathione.

Szgo & Roberts (1953) have reported that the oxygen uptake of rat uterus was increased as early as 1 hr. after injection of oestradiol. This extra oxygen uptake was not accompanied by increased glucose oxidation until about 14 hr. later, at which time the dehydrogenases for glucose 6-phosphate and phosphogluconate were increased (Scott & Lisi, 1960) as well as other dehydrogenases (Longwell & Reif, 1955).

The early oxygen uptake may be associated with the uterine phenol-peroxidase system (Martin, Neufeld, Lucas & Stotz, 1959) for which oestradiol-17 β can act as the phenol. The oestradiol is regenerated by reaction with reduced pyridine nucleotides. This reaction was reported by Williams-Ashman, Liao & Talalay (1960) to be increased in rat uterus after oestradiol treatment. Perhaps the hydrogen peroxide generated during this reaction could react also with the SH group of glutathione. The oxidized glutathione might then react with protein to form a mixed protein-glutathione compound of the sort postulated by Ungar & Romano (1958), Jocelyn (1960) and Eagle, Oyama & Piez (1960):

Table 4. *Changes in endometrium as percentage of controls*

Numbers of animals are given in parentheses.

Time after oestradiol (hr.)	Dehydrogenases		Acid-soluble SH groups			
	Glucose 6-phosphate	Phospho- gluconate	Time (hr.)	Untreated	Time (hr.)	Incubated
2-10	(8) - 54	(8) - 54	2-6	(5) - 46	2-12	(8) - 41
12-24	(8) + 10	(8) + 4	9-21	(5) - 21	12-21	(4) - 6
36-48	(5) + 214	(5) + 150	24-72	(7) + 50	24-72	(9) + 40
48 (2 injections)	(3) + 433	(3) + 265		(1) + 130		(1) + 158

On reduction with TPNH_2 , glutathione would be released:

The first reaction could also explain the loss of glutathione when it was added to sucrose extracts of hamster cheek pouches, whereas there was complete recovery of glutathione added to sulphosalicylic acid supernatant fluids of the extracts (Morris, Scott & Reiskin, 1961).

The production of TPN by reaction (2) as well as by the phenol-peroxidase system then might be responsible for induction of increased enzymes for which TPN is the coenzyme. After the enzyme increase, TPNH_2 would be available to release glutathione from the protein complex by reaction (2).

Since both the increases of the dehydrogenases and the glutathione occurred only 10–12 hr. after oestradiol, these effects must be regarded as secondary effects and not direct effects on the enzymes, such as the disaggregation of glutamic dehydrogenase by oestradiol reported by Tomkins, Yielding & Curran (1961).

SUMMARY

1. The sulphydryl-group content has been determined by amperometric titration of extracts of the endometrium and myometrium of uteri of ovariectomized rats, untreated, and at selected times after treatment with oestradiol-17 β .

2. The acid-soluble sulphydryl groups of endometrial extracts, either by direct titration or after reduction with reduced triphosphopyridine nucleotide, when calculated per mg. of protein, was decreased from 2 to 18 hr. after injection of oestradiol, as compared with uninjected controls. After 18 hr. this value increased to control levels.

3. The same values in terms of total content of the endometrium were not decreased before 18 hr. and were significantly increased after 18 hr.

4. Concentration of acid-soluble sulphydryl groups of the myometrium was significantly low from 2 to 21 hr. after injection of oestradiol and then increased. The concentration after reduction did not vary significantly from the controls. There

was therefore a significant increase in reducible sulphur during this period.

5. The total acid-soluble sulphydryl-group content of the myometrium, both direct and after reduction, was significantly higher than the ovariectomized controls at 4 hr. after injection of oestradiol and thereafter.

6. During incubation of the extracts with reduced triphosphopyridine nucleotide, some titratable sulphydryl groups were transferred from the acid-insoluble to the acid-soluble fraction.

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