GLYCOGEN, WET WEIGHT, AND DRY WEIGHT CHANGES IN THE VAGINA OF THE MOUSE

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Summary

(i) The subcutaneous or intravaginal administration of oestrone to the ovariectomized mouse causes an increase in the dry weight of the vagina with a maximum at full keratinization. The water content of the tissue is increased in the pre-keratinization phase, and reduced in the keratinization phase. The true glycogen content of the vagina does not increase until keratinization commences.

(ii) The dry weight, wet weight, and total true glycogen content of the vaginae of intact mice in the various stages of the oestrous cycle are compared with those obtained in ovariectomized mice.

(iii) Studies have also been made on the dry weight, wet weight, and total glycogen content of the uterus of intact mice. The pattern of changes seen in this organ is quite different from the pattern of changes seen in the vagina.

(iv) A misleading picture of events is formed if the experimental results are expressed only in the form of ratios of either true glycogen content or wet weight to the dry weight. A full interpretation of the data requires direct reference to the total dry weight, wet weight, or true glycogen content of the organs.

(v) The significance of these findings is discussed in relation to the mode of action of oestrogens and the histochemical aspects of the vaginal response to oestrogens.

I. INTRODUCTION

Oestrogens cause rapid proliferation of the epithelium of the vagina of the rodent, the response being characterized by stratification and keratinization. Such changes may be produced in the ovariectomized animal by either systemic or local (intravaginal) injection of oestrogens (see Biggers (1953*a*) for a review of the literature). Few biochemical studies have been made of the vaginal response to oestrogens. The glycogen content of the vagina has been studied because of its relationship to the metabolism of mitosis (Bullough 1952) and to the cellular synthesis of keratin (Scothorne and Scothorne 1953), both phenomena being characteristic of the response of the vagina to oestrogens (Biggers and Claringbold 1954).

The presence of glycogen in the vagina has been demonstrated in man (Miura 1928; Gisbertz 1930; Cruikshank and Sharman 1934; Lison and Vokaer 1949), the monkey (Corner 1923; Miura 1928; van Dyke and Ch'en 1936*a*, 1936*b*), the guinea pig (Tribby 1943), and the mouse (Biggers 1953*a*). Also Raynaud (1941) has described the deposition of glycogen in the urogenital sinus of the mouse embryo. However, glycogen has been reported absent from the vagina of the pig, lamb, and squirrel (Bremicker 1927), and the cat, rat, dog, rabbit, and guinea pig (Miura 1928). All of these workers used histochemical techniques, and it seems that no quantitative chemical studies have been made. Several workers have reported cyclical changes in

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the glycogen of the vagina which may be correlated with the oestrous or menstrual cycles (Miura 1928; Tribby 1943; Lison and Vokaer 1949; Biggers 1953*a*). In studies of the action of oestrone on the vagina of the ovariectomized mouse, Biggers (1953*a*) reported the deposition of glycogen in the stratum spinosum after keratinization had begun.

Few studies have been made on the water content of the vagina. Van Dyke and Ch'en (1936*a*, 1936*b*) demonstrated changes in the monkey during the menstrual cycle. Zuckerman, Palmer, and Hanson (1950) studied the water content in the rat, but their results, although suggestive of changes, were not statistically significant.

In the work to be described below, the glycogen content, wet weight, and dry weight of the vagina of the mouse have been determined simultaneously. Both ovariectomized mice stimulated with oestrone and intact mice have been used.

II. Material and Methods

(*a*) *Animals*

A randomly-bred strain of albino mice was used in this work. In the ovariectomized animal, the response of the vagina was studied, while in the intact animal both the vagina and uterus were studied. The organs were removed as described by Balmain, Biggers, and Claringbold (1956). The vagina was cut into approximately equal parts by dividing it along the line of the urethra; in the case of the uterus each horn was treated as a half-organ.

Each half-organ was allotted at random to one of two determinations using tables of random numbers (Fisher and Yates 1953). In experiment 1 the two determinations were total glycogen and dry weight, while in experiment 2 they were total glycogen and glycogen "blank". Wet weight determinations were made on all pieces of tissue. Twice the mean value obtained in each determination thus yields a mean estimate for the whole organ.

(*b*) *Glycogen Determinations*

The total glycogen and glycogen blank were determined by the methods described by Balmain, Biggers, and Claringbold (1956). The total glycogen represents all the anthrone-positive material present after precipitation with 60 per cent. ethanol and extraction of the precipitate with 5 per cent. trichloroacetic acid (TCA), and consists of the glycogen blank (non-glycogen substances which are extracted and are anthrone-positive), as well as the true glycogen.

(*c*) *Wet and Dry Weights*

The pieces of tissue were mopped roughly dry on filter paper, and weighed quickly on a torsion balance to 0·5 mg. For the dry weight determination the pieces were placed on weighed coverslips, left overnight in an oven at 100°C, cooled in a desiccator, and weighed on a microbalance to 0·1 mg. Observations on 100 vaginae, dissected out and weighed under these conditions, indicated that most of the water was driven off in the first 3 hr of drying. When the drying was continued for a further 21 hr less than 0·5 per cent. further change in the dry weight was found.

(*d*) *Administration of Oestrone to Ovariectomized Mice*

Groups of up to 70 ovariectomized mice were primed 1-6 weeks before use in an experiment by the subcutaneous injection of 1 μg oestrone in nut oil. Five animals were kept per box. One-half of the animals were stimulated by the intravaginal administration of 0·002 μg oestrone in 0·02 ml of 0·01 per cent. aqueous bovine plasma albumin (Biggers 1953*b*), and the other half were stimulated by the subcutaneous administration of 0·5 μg oestrone in 0·1 ml nut oil (Emmens 1950). In both cases the total dose was administered in two injections 24 hr apart. Both of these procedures are expected to elicit 100 per cent. responses within 96 hr, and are thus maximal doses.

TABLE 1

WET WEIGHT, DRY WEIGHT, AND GLYCOGEN CONTENT OF THE VAGINA OF THE OVARIECTOMIZED MOUSE FOLLOWING OESTROGENIC STIMULATION

Experiment No.	Route of Administration	Measurement	Time after First Injection (hr)					
			0	24	48	72	96	120
1	Intravaginal	Wet weight (mg)	16	27	34	32	30	—
		Dry weight (mg)	3·6	6·0	8·2	7·8	6·4	—
		Total glycogen (μg)	18	32	32	52	52	—
	Subcutaneous	Wet weight (mg)	19	27	40	44	40	—
		Dry weight (mg)	4·2	5·8	9·0	11·0	9·6	—
		Total glycogen (μg)	27	38	28	34	58	—
2	Intravaginal	Wet weight (mg)	20	26	36	30	34	22
		Total glycogen (μg)	44	46	46	74	96	72
		Glycogen blank (μg)	28	24	32	42	40	38
	Subcutaneous	Wet weight (mg)	16	27	44	41	30	31
		Total glycogen (μg)	35	37	41	56	73	54
		Glycogen blank (μg)	26	26	23	25	36	26

(*e*) *Intact Animals*

On the morning of each day on which analyses were made, vaginal smears were used to indicate the stage of the oestrous cycle for each of 100 adult female mice. The smears were stained for 8 min in 1 per cent. methylene blue, and dried in air. Four stages of the oestrous cycle were recognized—dioestrus, pro-oestrus, oestrus, and metoestrus—and these were identified following the original description of Allen (1922). Five animals from each stage were used on each day making 20 in all. This experimental unit will be called a replicate.

III. RESULTS

Since the data are extensive (involving approximately 2500 observations) only mean values will be tabulated, and the data reduced by means of the analysis of variance and covariance (Fisher 1948). Table 1 gives the mean values for ovariecto-

mized animals following oestrogenic stimulation, and Tables 2 and 3 the corresponding analyses of variance. Table 4 gives the mean values for intact animals. Preliminary examination of the data showed that it was reasonable to assume that the observations were log-normally distributed. Consequently, all analyses have been made by using logarithms, and the mean values shown in Tables 1 and 4 are obtained by taking antilogarithms. Since logarithms have been used, standard errors cannot be given on an arithmetic scale (cf. Cochran and Cox 1950; Hald 1952, for discussion of the transformation). In this case an appropriate measure of accuracy of a mean value are the 5 per cent. fiducial limits, and these are given in Table 4. They are not given in Table 1, since estimates of error are available from the analysis of variance tables.

TABLE 2

ANALYSES OF VARIANCE FOR THE DATA OBTAINED IN EXPERIMENT 1

The degrees of freedom corresponding to times have been partitioned by orthogonal polynomial coefficients as described by Fisher and Yates (1953)

Source of Variation	D.F.	Mean Squares		
		Wet Weight	Dry Weight	Glycogen
Replicates (A)	2	6·5*	4·5*	54·6**
Routes of administration (B)	1	60·9***	29·4***	1·5
Times (C)	(4)			
Linear	1	429·4***	229·8***	275·6***
Quadratic	1	178·4***	101·2***	1·1
Cubic	1	0·2	3·3	20·1**
Quartic	1	2·7	1·6	30·9***
First order interactions				
A×B	2	0·3	1·8	0·1
A×C	8	3·1	2·2	4·5
B×C	4	4·6*	5·9**	16·8***
Second order interactions				
A×B×C	8	1·2	0·9	0·8
Error	150	1·81	1·16	2·65

* $0{\cdot}05 > P > 0{\cdot}01$.
** $0{\cdot}01 > P > 0{\cdot}001$.
*** $P < 0{\cdot}001$.

(*a*) *Ovariectomized Mice*

(i) *Wet Weight.*—The analyses of variance (Table 2) show that in both experiments large changes in wet weight occurred in the vagina after the administration of oestrone by both subcutaneous and intravaginal routes. In both analyses there was a significant interaction between the effects of route of administration and the time after the first injection, showing that the changes are different after each route of administration. Examination of the mean values shows that subcutaneous injection produced a greater increase in wet weight than intravaginal injection. In the first experiment, maximum wet weight was reached 48 hr after intravaginal administration and 72 hr after subcutaneous administration, after which the wet weight decreased.

In the second experiment, the maximum wet weight was the same for both routes and was reached between 48 and 72 hr; the significant quintic mean square is due to fluctuations in the falling of wet weight, of unknown cause. The exact description of this phase must await further investigation.

(ii) *Dry Weight.*—The analysis of variance (Table 2) indicates that dry weight changes are of a similar form to wet weight changes, although the mean values show that the changes are not proportional. Subcutaneous injection produced a greater increase in dry weight than intravaginal injection.

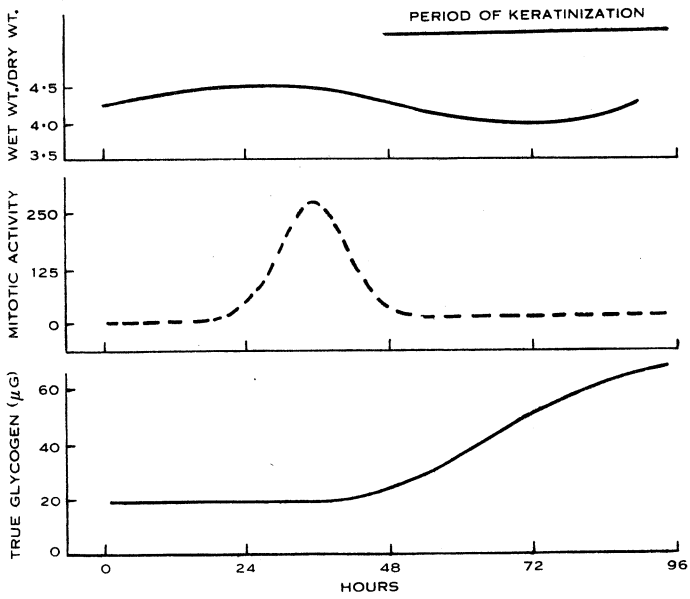

Fig. 1.—Schematic account of the variables studied against the same time-axis. In addition, the mitotic activity (taken from Biggers and Claringbold 1954) is indicated. The period of keratinization was determined by Biggers (1953*a*).

A set of readings of wet weight and dry weight from the same half-vagina was available for further analysis. The difference between the logarithms of the wet and dry weights is the logarithm of the wet weight/dry weight ratio. Since the two measurements are highly correlated ($r_{(149)} = 0{\cdot}892$, $P<0{\cdot}001$) an analysis of covariance was made. From this analysis a common estimate of the variances of the log wet weight/dry weight ratio was computed. This estimate was used to test the homogeneity of the ratios obtained on different days, and between different routes of administration. The test of significance showed extremely highly significant changes in the ratio from day to day ($\chi^2_{(4)} = 34{\cdot}0$, $P<0{\cdot}001$), there being no significant difference between routes. The result is shown graphically in Figure 1. It can be seen that the water content of the vagina was raised above the control level over the first 48 hr and was lowered in the subsequent 48 hr.

(iii) *Total Glycogen.*—The analyses of variance (Table 3) for both experiments indicate highly significant differences in the glycogen content of the vagina at different times after the injection of oestrone. In both analyses, a significant routes $\times$times interaction shows that the pattern of change of total glycogen depends on the route of administration. When subcutaneous administration was used there was a sudden rise which commenced between 48 and 72 hr, reached a peak at 96 hr, and then fell off. After intravaginal administration this rise was slightly in advance of that observed with subcutaneous administration. Although the curves follow different patterns in the later stages, the results clearly demonstrate a large rise in total glycogen after 48 hr from the first injection in both cases.

TABLE 3

ANALYSES OF VARIANCE OF THE DATA OBTAINED IN EXPERIMENT 2

Source of Variation	D.F.	Mean Square		
		Total Glycogen	Glycogen Blank	Wet Weight
Replicates (A)	1	30·9***	28·0***	0·5
Routes of administration (B)	1	2·4	4·0	22·2***
Times (C)	(5)			
Linear	1	123·0***	26·3***	106·9***
Quadratic	1	4·1*	0·1	247·0***
Cubic	1	36·0***	18·5***	0·4
Quartic	1	8·8**	0·9	0·9
Quintic	1	1·6	0·0	7·7***
First order interactions				
A×B	1	3·1	1·7	1·9
A×C	5	1·2	4·3	2·4
B×C	5	3·7*	0·4	5·3**
Second order interactions				
A×B×C	5	0·0	2·0	1·9
Error	96	0·82	1·32	1·32

* $0{\cdot}05 > P > 0{\cdot}01$.
** $0{\cdot}01 > P > 0{\cdot}001$.
*** $P < 0{\cdot}001$.

(iv) *Glycogen Blank.*—The mean values and the analysis of variance (Table 3) show that a significant rise in the blank occurred corresponding to the rise in total glycogen, and that no differences were observed between routes of administration.

The difference between the logarithms of total glycogen and glycogen blank is a measure of the amount of true glycogen, and this was computed from the data of experiment 2. The variance of this difference must take account of any correlation of these two measurements, since they are made on the one organ. The correlation coefficient is $r_{(95)} = -0{\cdot}04$, $P > 0{\cdot}05$. From the analyses of variance and covariance, the variance of the difference was estimated for each time and route of administration. Subsequent analysis, as used in analysing the wet weight/dry weight ratio, indicates that only the time differences are significant ($\chi^2_{(4)} = 21{\cdot}4$, $P < 0{\cdot}001$). The result is

shown graphically in Figure 1, where it is seen that there was a considerable increase in the true glycogen 48 hr after the first injection.

(*b*) *Intact Mice*

Preliminary examination of the data indicated that the variance of the measurements depends on the stage of the oestrous cycle. Therefore, individual analyses were made within each stage, after removing sums of squares attributable to differences between replicates. From the error term in each analysis the 5 per cent. fiducial limits for the mean values were calculated (Table 4).

TABLE 4

WET WEIGHT, DRY WEIGHT, AND GLYCOGEN CONTENT OF THE VAGINA OF THE MOUSE AT VARIOUS STAGES OF THE OESTROUS CYCLE. MEANS AND 5% FIDUCIAL LIMITS ARE TABULATED

Experiment No.	Stage of Oestrous Cycle	Vagina		Uterus	
		Wet Weight (mg)	Dry Weight (mg)	Wet Weight (mg)	Dry Weight (mg)
1	Dioestrus	27·1 (25·1-29·2)	6·08 (5·56-6·65)	26·4 (21·8-31·8)	5·50 (4·57-6·61)
	Pro-oestrus	64·9 (60·4-69·7)	15·7 (14·5-16·9)	112 (96·4-130)	19·3 (17·0-22·0)
	Oestrus	55·6 (51·1-60·5)	13·5 (12·2-15·0)	70·7 (63·5-78·5)	13·5 (12·3-14·7)
	Metoestrus	32·7 (30·3-35·4)	7·02 (6·41-7·68)	39·9 (34·8-45·7)	8·18 (7·30-9·19)
		Total Glycogen (μg)	Glycogen Blank (μg)	Total Glycogen (μg)	Glycogen Blank (μg)
2	Dioestrus	52·1 (47·0-57·8)	23·0 (21·3-24·9)	29·5 (23·9-36·5)	22·4 (18·0-27·9)
	Pro-oestrus	46·5 (40·2-53·7)	20·6 (17·9-23·7)	94·9 (76·2-118)	33·7 (30·1-37·8)
	Oestrus	41·3 (36·8-46·3)	24·2 (21·5-27·2)	85·3 (75·5-96·4)	39·9 (38·2-41·7)
	Metoestrus	62·5 (52·5-74·5)	30·5 (27·5-33·8)	50·9 (44·5-58·4)	36·7 (31·9-42·3)

(i) *Wet Weight.*—In both the vagina and uterus, the wet weight changes were parallel, with a maximum at pro-oestrus, but the changes in the uterus were very much greater.

(ii) *Dry Weight.*—Dry weight changes were similar to the wet weight changes for both organs. The wet weight/dry weight ratio was calculated for each stage in each organ, and is illustrated graphically in Figure 2, where two differences between each organ may be seen. First, the uterus contained more water at all stages than the vagina. Secondly, at the pro-oestrous and oestrous stages the vagina was dehydrated and the uterus hydrated, relative to the metoestrous and dioestrous stages.

(iii) *Total Glycogen.*—The changes in total glycogen were entirely different in the two organs. In the vagina the minimum level was at oestrus and the maximum was at metoestrus. In the uterus total glycogen was minimal at dioestrus and maximal at pro-oestrus.

(iv) *Glycogen Blank.*—These changes were similar to the changes of total glycogen although smaller in degree. The differences between total glycogen and glycogen blank, i.e. true glycogen, have been plotted in Figure 3 for both organs, where large differences in the distribution of true glycogen are seen. In the vagina the maximum amount of glycogen is present at metoestrus and the minimum amount at oestrus, whilst in the uterus the maximum amount is at pro-oestrus and the minimum at dioestrus.

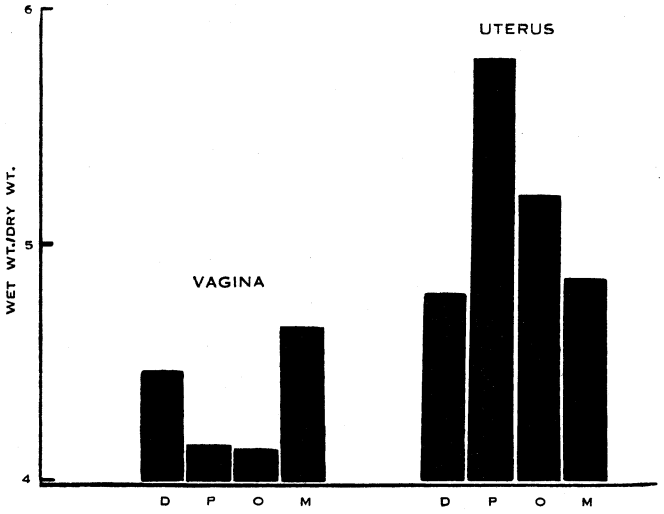

Fig. 2.—Comparison of wet weight/dry weight of the vagina and uterus at the four stages of the oestrous cycle. *D*, Dioestrus; *P*, pro-oestrus; *O*, oestrus; *M*, metoestrus. Whilst at every stage the uterus is more saturated than the vagina, the relative pattern of change is quite different.

IV. Discussion

(*a*) *Growth and Water Content*

The results show that both subcutaneous and intravaginal injection of oestrone cause growth of the vagina, manifested by an increase in dry weight. Maximum growth is attained at the time of full keratinization, and is reached earlier with the intravaginal route of administration. The wet weight changes in the organ do not follow proportionately the dry weight changes, and this fact implies that differential shifts in water occur during the response (Fig. 1). The water content is increased in the early pre-keratinization phase, the period of high mitotic activity and active growth (Allen, Smith, and Gardner 1937; Biggers and Claringbold 1954) while after keratinization the tissue is relatively dry in comparison with the resting organ. These results do not agree with those described by Zuckerman, Palmer, and Hanson (1950), who studied

the ovariectomized rat following the subcutaneous injection of oestradiol-3,17β. In all of our experiments significant secular changes occurred in all variables (Tables 2 and 3), and were also observed in the studies on intact mice. These large shifts make it imperative that all comparisons be made simultaneously. This requirement was not fulfilled in the work of Zuckerman *et al.*, and may thus account for the non-significance of their findings. It should also be noted that these changes are different from the changes in the uterus following oestrogen administration (see Reynolds (1949) for a review of the literature).

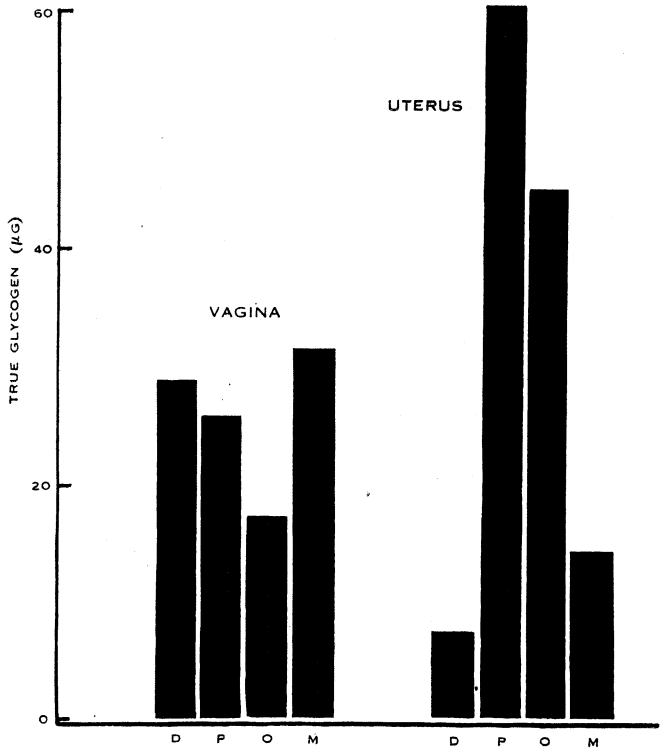

Fig. 3.—True glycogen content of the uterus and vagina at the four stages of the oestrous cycle. *D*, Dioestrus; *P*, pro-oestrus; *O*, oestrus; *M*, metoestrus. The pattern of change in the uterus is quite different to that seen in the vagina.

In intact animals there is a striking difference in water content between the uterus and vagina (Fig. 2). In the vagina the changes are similar to those following artificial stimulation by either intravaginal or subcutaneous administration. In both cases reduced water content is associated with the process of keratinization. In the uterus there is a large increase in water content at the time of vaginal cornification, and this is associated with the distension of the uterus with intra-uterine fluid at this stage.

The results described above show that the vaginal response, as measured by wet weight or dry weight, was larger after subcutaneous administration than after the

intravaginal administration. This may have been due to non-equivalence of the subcutaneous and intravaginal doses. As the relevant dose-response lines are unknown, this point cannot as yet be decided. However, Claringbold and Biggers (1955) have shown that fundamental differences exist in the action of oestrone given by both routes of administration, possibly due to changes in the connective tissue beneath the vaginal epithelium. The large increase in wet and dry weight, after subcutaneous administration, may well be due to connective tissue changes which are not elicited by the intravaginal route of administration.

(*b*) *Glycogen Content*

Since there are changes in wet and dry weight, the observations on glycogen content have not been placed on a wet weight or dry weight basis; to do so automatically—a very frequent practice—would obscure the phenomena which take place. Instead, the figures refer to absolute amounts in the whole organ. Such considerations apply to any biochemical measurements made on this organ.

The results show that glycogen is formed in considerable amounts in the vagina after keratinization has commenced, this being observed in intact mice undergoing normal oestrous cycles and also in ovariectomized mice artificially stimulated with oestrone. In the control animals, and in those in which the early stage of the response had been experimentally induced, only small amounts of glycogen were present. These changes have been studied histochemically in the mouse by Biggers (1953*a*) using the periodic acid-Schiff technique. In the early stages no reaction was obtained in the stratum spinosum, but after keratinization had commenced, considerable amounts of glycogen were detected in this region of the epithelium. Thus the histochemical results are consonant with the more sensitive biochemical results. The findings, however, seem to be disconsonant with the histochemical studies of Tribby (1943) who, working with guinea pigs, described the deposition of glycogen in the stratum spinosum during pro-oestrus. Since the work of Tribby was done on intact animals it is difficult to decide whether this finding indicates a species difference or is due to difficulties in the exact diagnosis of the stages of the oestrous cycle. For example, in the stage designated pro-oestrous by Tribby, keratinization is well developed, and therefore the differences between his and our results may be illusory. This problem of the diagnosis of the stages of the oestrous cycle is only avoided by the artificial production of cornification.

The observations reported above show that completely different phenomena occur in the vagina and in the uterus during oestrogenic stimulation, and show that each organ responds in a characteristic manner to the hormone. Cognisance of this fact should be taken in studies on the action of oestrogens or other hormones, since it challenges the validity of generalizations from one organ to another.

The presence of glycogen in growing and keratinizing epidermis and its derivatives is a widespread phenomenon, e.g. foetal epidermis (Bernard 1859), healing wounds (Bradfield 1951), skin autografts (Scothorne and Scothorne 1953), and hair follicles (Hardy 1953). In several papers Bullough and his colleagues have referred to unpublished work by Bullough and Eisa on the glycogen content of the skin of the female mouse following the injection of oestrone. They claim that the high glycogen

level coincides with maximum mitosis. The same workers have also mentioned the deposition of glycogen in the vagina and uterus of the mouse during the oestrous cycle, and have described in the vagina a biphasic rise in glycogen and lactic acid. Recently several workers have speculated on the function of the glycogen in epithelial tissues (Bullough 1950, 1952; Bullough and van Bordt 1950; Bradfield 1951; Carruthers and Suntzeff 1953; Scothorne and Scothorne 1953), and it has been suggested that the glycogen is concerned with the metabolism of mitosis and also with the synthesis of keratin.

Our work has shown that the period of accumulation of glycogen in the vagina does not coincide with the period of great mitotic activity, but that it appears after most cell divisions are complete. Thus the suggestion of Bullough (1952) that the mobilization of glycogen stimulates cell division, is not supported by our work. The appearance of glycogen in the vagina coincides with the formation of keratin, and Biggers (1953*a*) showed histochemically that the glycogen is in the stratum spinosum just beneath the region undergoing keratinization. As yet its function remains obscure.

V. Acknowledgments

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