ON THE PRENATAL GROWTH OF THE MAMMARY GLAND RUDIMENT IN THE MOUSE

By B. I. BALINSKY*
Department of Zoology, University of the Witwatersrand, Johannesburg

The development of the mammary glands has attracted much attention from morphologists, embryologists and physiologists. For a review of the literature on the subject the reader is referred to Turner (1939) and Speert (1948). The postnatal development, especially, has been studied with great success and the factors controlling the growth and differentiation of the glands have been investigated in great detail. The prenatal development, on the other hand, has not been subjected to investigation by quantitative methods. In particular, the processes of cell proliferation, and the growth of the early rudiments of the mammary glands have not been studied, so far as I have been able to discover. The present work is an attempt to fill this gap.

MATERIAL AND METHODS

Mice of a heterogeneous population were used for this investigation, and no attempt was made to select embryos of a special genotype. Embryos of known age, from the 8th day to birth, were studied. The specimens were fixed in Bouin's fluid, embedded in paraffin, cut transversely and stained with haematoxylin alone or with haematoxylin and eosin.

The growth of the mammary glands was estimated from the volume, obtained by drawing every section of a number of glands with a camera lucida and then measuring the surface of the section with a planimeter. Adding up the surface area of all the sections gave the volume in arbitrary units. The rate of increase of the gland volume was then compared with the growth of the whole embryo. To determine the latter several embryos for every day of development were weighed, starting with 9-day-old embryos. The figures obtained give only a very crude approximation and no attempt has been made either to exclude the variability of the embryos, which seems to be very considerable, or to treat the results statistically.

For the investigation of the cellular proliferation in the mammary gland rudiments extensive use was made of the mitotic index method. The cellular proliferation is, of course, proportional to the growth rate only under certain conditions, especially if the size of the cells does not change to a great degree and if there is no loss of cells. Both these conditions apply to the mammary glands at least approximately. The mitotic index in early mammary gland rudiments was compared with the mitotic index in the epidermis, as a tissue from which the mammary glands are derived and which therefore is a convenient standard against which the mitotic activity of the glands can be tested. Accordingly, an approximately equal number of epidermis cells was counted in the immediate vicinity of each mammary gland used for mitosis counts. In the case of late mammary gland rudiments (age over 15 days) there was no point in comparing the mitotic activity of mammary gland cells and

* This work was done while the author was at the Animal Breeding and Genetics Research Organization at the Institute of Animal Genetics, Edinburgh University.

epidermis cells because of the obvious divergence in their differentiation. The mitotic index of these glands was therefore compared with the index in earlier mammary gland rudiments. Besides this, counts were made in distal and in proximal parts of the late mammary glands and the mitotic indices of these parts compared. All the cells of early mammary gland rudiments were counted, but in the very much larger glands of later stages this could not be done, and only a part of the sections of each gland was used for the counts. Only those cells were counted as mitoses in which there was no nuclear membrane, that is, metaphases, anaphases, and the earliest telophases with an irregular contour of the daughter nuclei. The subjective factor in the evaluation of the early prophases is thus excluded.

The method of determining the mitotic index has its defects and dangers which have not been fully realized by investigators using this method. One danger lies in the evaluation of the statistical significance which can be attached to the figures obtained. It has been pointed out by Pasteels (1940) that the method has been applied uncritically and that, in fact, conclusions have been drawn from material that is statistically inadequate. I have checked Pasteels' conclusions and found his criticism quite correct. The formula to be applied for the calculation of the standard error of the mean of the mitotic index of a single sample is the following:

$$s_m = \sqrt{\frac{pq}{N}},$$

where $p =$ percentage of dividing cells ('mitotic index'), $q = 1 - p$ and N is the total number of cells counted. Applying this formula, and taking into consideration the actual values of the mitotic indices observed, it was found that it is necessary to count several thousands of cells in order to discover a statistically significant difference in the mitotic indices of different tissues. This has a direct bearing on the determination of the mitotic index in mammary glands as the number of cells in the early mammary gland rudiments is less than a thousand. Even a complete count of all the cells in one mammary gland rudiment is therefore insufficient for the results to be statistically significant.

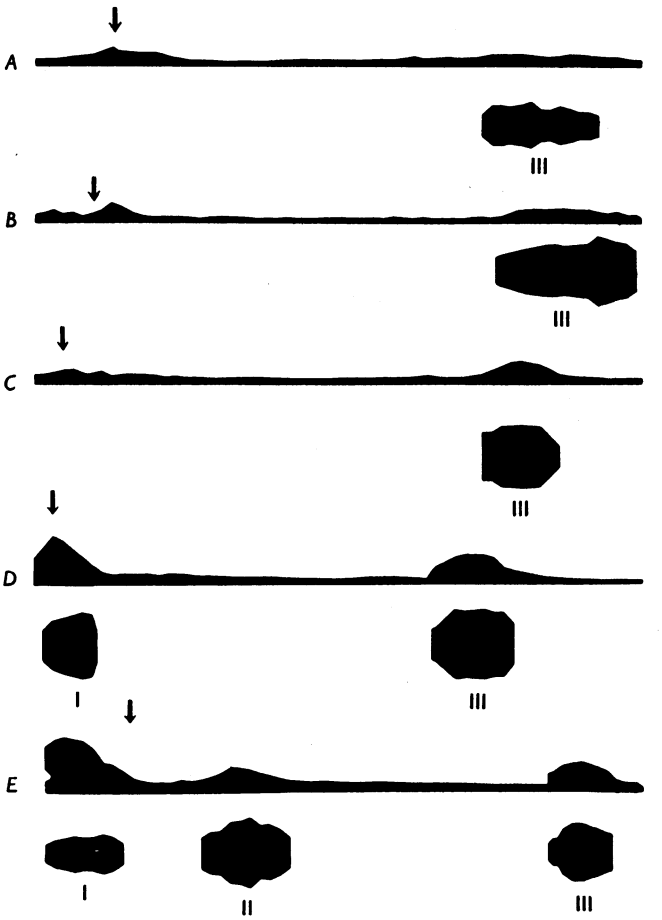
If the mitosis counts are made in several different embryos or in several different glands of the same embryo the question arises whether it is legitimate to treat such data as samples of one and the same binomial distribution. The significance test to be applied to such data depends on whether the difference between the tissues which are to be compared is the same for all samples (in statistical terms, whether there is interaction as regards the mitotic index between sample and tissue). Such data may be analysed by a treatment of the differences within samples, with appropriate weighting. This method as applied here is the same as that given by Snedecor (1946, p. 291), but for enumeration rather than measurement data.

DATA ON THE MORPHOLOGY OF THE DEVELOPING GLANDS

It has been necessary to re-investigate the morphological changes in the mammary gland rudiments as a background for the investigation of growth and proliferation. The claim by Turner & Gomez (1933) to have found a mammary line in 13-day-old mouse embryos could not be confirmed. No such line could be discovered in the embryos studied. The mammary gland rudiments seemed to make their appearance

independently of one another. There is some variation in the time when the first mammary gland rudiments can be discerned, but usually they could be found in 11-day-old embryos.

As it is sometimes difficult to visualize the form and exact extent of structures from serial sections, graphic reconstructions were prepared with the aid of a camera lucida of the epidermal thickenings of several embryos at the time when the mammary



Text-fig. 1. Graphic reconstruction of epidermal thickenings on the body side during the formation of the mammary gland rudiments. Continuous black line—greatest thickness of skin epidermis. Black spots—multilayered thickenings (mammary gland rudiments) in surface view. I, II, III—numbers of the glands. Arrowheads show the posterior margin of the forelimb. The five cases (*A–E*) represented are arranged in order of increasing differentiation. The irregular broadening of the line representing the thickness of the epidermis in the region of the forelimb (on left) is partly due to the sections going oblique to the surface of the epidermis.

gland rudiments first become visible. These reconstructions are presented in Text-fig. 1. The maximal thickness of the epidermis along the side of the body in each section is represented in the drawing by a continuous line. The thickened parts of the epidermis where it has become multilayered are represented as black areas. The

latter are shown in surface view. Both have been drawn to the same scale. Only the region of the three pectoral glands has been shown, and the arrows indicate the position of the posterior border of the forelimb in each case. The uppermost reconstruction (*A*) represents an advanced 10-day-old embryo, the earliest in which a distinct epidermal thickening has been found. The rest represent 11-day-old embryos arranged in order of increasing differentiation. The individual glands here and later will be referred to by numbers, I, II and III.

The reconstructions show that there is no continuous longitudinal thickening from which the individual glands are derived. It is remarkable that the glands do not appear simultaneously; in the first four embryos gland III is already distinct, but gland II appears only in embryo *E*. The reconstructions show further that the rudiment of gland III, when first discernible, has a distinctly elongated form, but this is the only feature suggestive of a mammary line that could be found. In a few hours the rudiment ceases to be elongated and becomes oval or round. In the development of gland II, as clearly seen from the reconstruction, there is no elongated thickening preceding the formation of the rudiment or linking it up with the rudiments I and III.

The earliest rudiments, as mentioned above, are represented by very slight thickenings. Pl. 1, fig. 1, shows a section of the rudiment of gland II in the youngest embryo (*A*) which was used for the graphic reconstruction. The cells of the epidermis have scarcely begun to arrange themselves into a multilayered thickening. Pl. 1, fig. 2, shows a section of the next youngest embryo (*B*). In this case the thickening is still very slight though already clearly multilayered. This rudiment, as seen from the reconstruction, is a distinct gland rudiment (gland III) although still of an elongated form. The rudiments soon become thicker and more sharply delimited, as shown in Pl. 1, fig. 3, representing also a section of an 11-day-old embryo. In 12 day-old embryos the epidermal thickening is already transformed into a hemisphere (Pl. 1, fig. 4) sometimes called the 'mammary hillock' and soon after the gland rudiment becomes a spherical body connected with the epidermis only by a narrow neck (Pl. 1, fig. 5). The glands retain this spherical form up to the age of 15 days, and sometimes even to 16 days, but then there begins a new phase in their development: the rudiment begins to elongate and produces the primary sprout (Pl. 1, fig. 6). The single (in the mouse) primary sprout soon begins to produce secondary sprouts, so that by the time of birth (which in my material occurred 19–20 days after fertilization) each gland is represented by a ramifying system of ducts.

DATA ON THE GROWTH AND PROLIFERATION IN THE GLANDS

Table 1 presents a comparison of the growth of the mammary glands with the growth of the whole embryo.

It is evident that for several days after the formation of the mammary gland rudiments their growth lags behind that of the body as a whole. Between the 11th and the 15th day the mammary gland rudiments increase about four and a half times. In the same period the whole embryo increases more than ten-fold. But in the last days of pregnancy the relations are reversed. Between the 16th and the 19th day of development there is at least a four-fold increase in the volume of the mammary

Table 1. *Growth of mammary gland compared with the growth of the whole embryo*

(Mammary glands in arbitrary units (surface of sections), whole embryo in milligrams.)

Age in days	Mammary glands (single observations)	Whole embryo (average)
10	—	11·3
11	530 829 1055	17·95
12	788 828 1791	50·5
13	1027 1804 1944	105·3
14	3548 3680	139·5
15	3354	206·2
16	7497	415·5
17	5559 7806	544·2
18	19020 20950	758·9
19	36174	862·0

Table 2. *The mitotic index of the mammary glands and the epidermis*

Age in days	Epidermis		Mammary glands	
	Counts*	Mitotic index	Counts*	Mitotic index
10	1472 (21)	1·42	—	—
	875 (11)	1·25	—	—
11	510 (4)	0·78	609 (2)	0·32
	507 (2)	0·39	614 (2)	0·32
	772 (7)	0·90	1031 (1)	0·09
12	622 (3)	0·48	940 (3)	0·31
13	915 (8)	0·87	1580 (2)	0·12
14	935 (7)	0·74	2311 (2)	0·08
	1270 (16)	1·26	2068 (6)	0·29

	Proximal part of gland		Distal part of gland		Mitotic index for proximal and distal parts together
	Counts*	Mitotic index	Counts*	Mitotic index	
16	2180 (21)	0·96	2165 (36)	1·66	1·30
	1804 (5)	0·28	1670 (13)	0·78	0·52
17	1935 (21)	1·08	1850 (42)	2·27	1·66
	2649 (21)	0·79	1523 (22)	1·44	1·03
	1424 (12)	0·84	1253 (19)	1·51	1·16
	974 (8)	0·82	1641 (9)	0·55	0·65
	1908 (22)	1·15	1818 (24)	1·32	1·23
19	608 (11)	1·81	1850 (26)	1·40	1·50

* The figures in brackets show the number of mitoses.

gland rudiments, whereas the weight of the whole embryo is only doubled. This change coincides with the beginning of sprouting.

Table 2 presents the data on the mitotic activity in the mammary gland rudiments and in the epidermis.

It will be seen that the mitotic activity shows the same difference between the early (non-sprouting) mammary glands and the late (sprouting) mammary glands, as was found for the increase in volume of the glands. A comparison between the mitotic indices of early and late mammary glands cannot be made by the method of weighted differences (see below), as the data cannot be grouped in pairs; it will be necessary, therefore, to compare the average mitotic indices for both groups in the usual, though not very exact, way.

Average mitotic index for glands of 11- to 14-day-old embryos	$0{\cdot}218 \pm 0{\cdot}043$
Average mitotic index for glands of 16- to 19-day-old embryos	$1{\cdot}132 \pm 0{\cdot}138$
Difference	$0{\cdot}914 \pm 0{\cdot}145$
Ratio: difference/standard error of same	6·30
Considering 13 degrees of freedom	$P < 0{\cdot}001$

The difference is therefore fully significant statistically.

A comparison of the mitotic index of early mammary glands and of the epidermis can be carried through by the method of weighted differences. Table 3 presents the

Table 3. *Comparison of the mitotic indices of mammary glands and of the epidermis in 11- to 14-day-old embryos by the method of weighted differences*

Epidermis	Mammary gland	Difference	Weight $\left(\frac{N_1 N_2}{N_1 + N_2}\right)$
0·78	0·32	0·46	277·6
0·39	0·32	0·07	277·7
0·90	0·09	0·81	454·0
0·48	0·31	0·17	374·3
0·87	0·12	0·75	579·4
0·74	0·08	0·66	665·7
1·26	0·29	0·97	786·8

Interaction $\chi^2 = 6{\cdot}34$, therefore for 6 degrees of freedom $P = 0{\cdot}30$–$0{\cdot}50$. Weighted difference $= 0{\cdot}6487$. Weighted standard error $= 0{\cdot}1185$. $t = 5{\cdot}47$, therefore for 6 degrees of freedom $P = 0{\cdot}001$.

respective data. The comparison, carried through by this very strict method, proves that there is a fully significant difference between the mitotic indices of the mammary glands and of the epidermis, from which the mammary glands have been derived. It follows that the formation of the mammary glands is accompanied by a considerable decrease in the mitotic activity of the cells partaking in this morphogenetic process.

The method of weighted differences may be further applied to the comparison of the proximal and distal parts of the glands* in stages when the sprouting has already begun (16- to 19-day-old embryos). The data are presented in Table 4. The calculations support the conclusion that the mitotic activity is higher at the distal ends of the glands, which corresponds to the impression one gets when studying sections of sprouting glands. The degree of significance is in this case very much less than in the case of the comparison of the mitotic indices of mammary glands and epidermis, or of mammary glands of early and late embryos. Still, the probability is well beyond the 5 % margin.

* By 'proximal' is meant the part of the gland connected with the epidermis; by 'distal'—the sprouts growing into the subcutaneous connective tissue.

Table 4. *Comparison of the mitotic index in the proximal and distal parts of sprouting mammary glands by the method of weighted differences*

Proximal parts of glands	Distal parts of glands	Difference	Weight $\left(\frac{N_1 N_2}{N_1+N_2}\right)$
0·96	1·66	0·70	1086
0·28	0·78	0·50	867
1·09	2·27	1·18	945
0·79	1·44	0·65	967
0·84	1·51	0·67	666
0·82	0·55	−0·27	611
1·15	1·32	0·17	930
1·81	1·40	−0·41	457

Interaction $\chi^2 = 11{\cdot}84$, therefore for 7 degrees of freedom $P = 0{\cdot}10$–$0{\cdot}20$. Weighted difference = 0·489. Weighted standard error = 0·173. $t = 2{\cdot}83$, therefore for 7 degrees of freedom $P = 0{\cdot}02$–$0{\cdot}05$.

CONCLUSIONS

From what has been said it appears that in the embryonic development of the mammary glands three distinct phases may be distinguished, each with its characteristic processes of growth and differentiation.

The first phase is the formation of epithelial thickenings, the mammary buds. During this stage the growth rate of the rudiments decreases from the high level intrinsic for the epidermis to the low level found later in the gland rudiments. This decrease of growth rate may well be the result of a differentiation process taking place in the gland rudiments (compare Schmalhausen, 1927).

There follows a second phase during which the gland rudiments are rather passive in every respect. They show no progressive differentiation and their growth is comparatively small. The glands are negatively heterogonic in respect of the whole embryo. This phase of a relative standstill in development continues from the age of 11 days to the age of 15 or even 16 days.

The third phase is the period of sprouting. The formation and the elongation of the sprouts is due to a growth process and not to a pure change of form and though the growth is particularly rapid at the ends of the sprouts it is not exclusively terminal. The rate of proliferation (mitotic activity) is increased roughly five-fold above the level of the preceding phase. There is a period when the growth activity of the glands exceeds that of the whole embryo—the gland becomes positively heterogonic. This is the more remarkable since after birth the growth of the mammary glands is not positively heterogonic. Cowie & Folley (1949) report that in the rat the growth of the mammary glands is isometric for about 3 weeks after birth, and only then becomes positively heterogonic, in connexion with approaching puberty.

There is good reason for believing that the second phase in the development of the mammary glands—the phase of retarded growth and differentiation—is of widespread occurrence in mammals. In his investigation on the development of the mammary glands of the cow, Turner (1930) states that after the formation of the spherical mammary gland rudiment the same does not increase appreciably in size for about 3 weeks (it is almost the same in 2·4 cm. embryos as in 8·4 cm. embryos). Bresslau (1920) finds that in the Opossum, *Didelphys*, the spherical mammary gland rudiments, although formed in early embryonic stages, persist in this form without

further change until after the marsupial period of postnatal development. I have found the same phase of retarded growth and differentiation in the rabbit, where it continues from the age of 13 days to shortly before the age of 25 days.

SUMMARY

1. The intra-uterine development of the mammary gland rudiments in the mouse was studied by preserving and sectioning embryos of known age. The rate of growth of the mammary gland rudiments was determined by calculating the mitotic index and by measuring the surface of each section drawn with the aid of a camera lucida. The rate of growth of the mammary gland was compared with that of the epidermis and with the growth of the whole embryo.

2. There is no mammary line in the mouse, each gland making its appearance independently of the others. Epidermal thickenings representing individual mammary glands were found in advanced 11-day-old embryos.

3. In newly formed mammary gland rudiments (mammary buds) the rate of growth and cell proliferation decreases far below the level intrinsic for the epidermis from which the mammary gland rudiments have been derived. There follows a period of relatively very slow growth accompanied by an absence of progressive differentiation, except that the original lenticular thickening is transformed into a hemisphere and then into a sphere connected with the epidermis by a narrow neck. In this state the rudiments persist for several days.

4. The phase of arrested development is followed by a phase of more rapid growth and cellular proliferation. This is the period of sprouting when first the primary sprout is formed and then the ramifying duct system of the gland. During this period the growth rate of the gland rises above the growth rate of the whole embryo. Growth is especially rapid in the distal parts of the sprouts although it is not exclusively terminal.

5. It appears that a period of arrested development, with very low rate of growth and absence of changes of form, occurs in the embryonic development of most if not all mammals.

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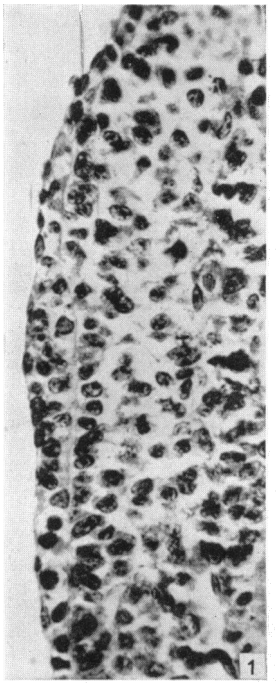
REFERENCES

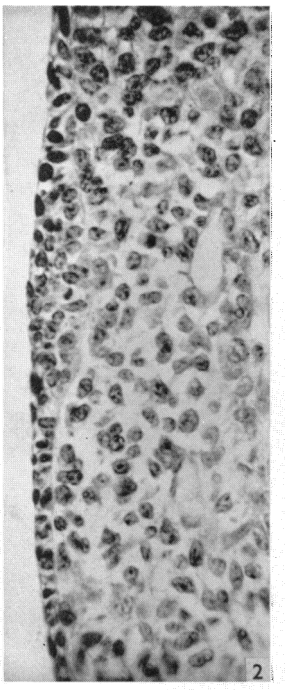
Bresslau, E. (1920). *The Mammary Apparatus of the Mammalia.* London: Methuen and Co.

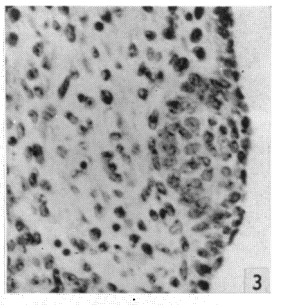
Cowie, A. T. & Folley, S. J. (1949). Relative growth of the mammary gland in the normal, gonadectomized and adrenalectomized rat. *J. Endocrinol.* **6**, *Proc.* vi–vii.

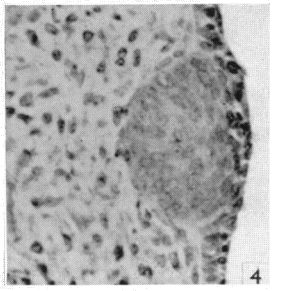
Pasteels, J. (1940). Un aperçu comparativ de la gastrulation chez les Chordes. *Biol. Rev.* **15**, 59–106.

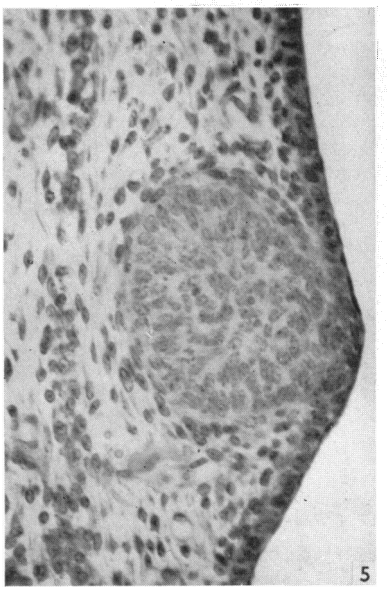
Schmalhausen, I. (1927). Beiträge zur quantitativen Analyse der Formbildung. I. Ueber die Gesetzmässigkeiten des embryonalen Wachstums. *Roux Archw. EntwMech. Organ.* **109**, 455–512.

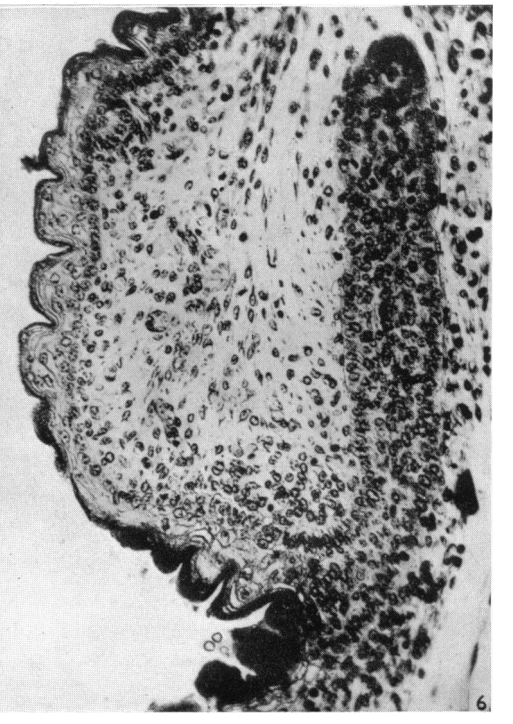












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SNEDECOR, G. W. (1946). *Statistical Methods*, 4th ed. Iowa: Iowa State College Press.

SPEERT, H. (1948). The normal and experimental development of the mammary gland of the rhesus monkey, with some pathological correlations. *Contr. Embryol. Carneg. Instn*, **32** (208), 11–66.

TURNER, C. W. (1930). The anatomy of the mammary gland of cattle. I. Embryonic development. *Res. Bull. Mo. agric. Exp. Sta.* no. 140, 3–34.

TURNER, C. W. (1939). The mammary glands. In *Sex and Internal Secretions*. Ed. by E. Allen. Pp. 740–803. London: Baillière, Tindall and Cox.

TURNER, C. W. & GOMEZ, E. T. (1933). The normal development of the mammary gland of the male and female albino mouse. I. Intra-uterine. *Res. Bull. Mo. agric. Exp. Sta.* no. 182, 3–20.

EXPLANATION OF PLATE

Fig. 1. Earliest epidermal thickening (rudiment of mammary gland) in an advanced 10-day-old embryo. ×400.

Fig. 2. Epidermal thickening (rudiment of mammary gland) in an 11-day-old embryo. ×400.

Fig. 3. Mammary gland rudiment of an advanced 11-day-old embryo. ×310.

Fig. 4. Mammary gland rudiment (mammary hillock) of a 12-day-old embryo. ×310.

Fig. 5. Fully developed spherical mammary gland rudiment. ×310.

Fig. 6. Mammary gland rudiment of a 17-day-old embryo: the primary sprout. ×200.