Alkaline Phosphatase and the Differentiation of Gonads in the Albino Rat[1]

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Introduction

Present day investigations in developmental anatomy are rightly more and more concerned with the events of differentiation at the biochemical level. Actually this is a return to descriptive embryology, but now of a far more subtle kind than the conventional record of unfolding tissues, organs and systems. That is, the events of visible structural and functional differentiation are anticipated and accompanied by a host of biochemical transformations. Experimental manipulation having created as many problems as it solved, the new task of the embryologist has been to identify the kinds and locations of the biochemical ingredients within differentiating systems, the order of their appearance, their interactions, transformations and permutations, and the catalytic agents which accompany them. To this end the sensitive techniques of immunology and radio-active tracers play important roles as do also a variety of cyto- and histochemical methods. Among the more reliable of the latter are those for the demonstration of the phosphatases.

This is not the occasion for a review of the variety of the phosphatases and the functions they perform (vide Moog, '46). Suffice it to say that of all these, alkaline phosphatase is probably the best known and clearly appears to play a part in differentiation (e.g., Moog, '44, and McAlpine, '51) and regeneration (Karczmar and Berg, '51). The present study is intended to link the patterns of distribution and concentration of this enzyme to already established descriptive and experimental analyses of the developing gonads of the albino rat (Torrey, '45 and '50).

Materials and Methods

The materials and methods employed in the morphological studies just mentioned are recorded in the papers cited. The observations on alkaline phosphatase derive from a complete and closely spaced series of embryos and postpartum stages of the rat prepared with the ultimate aim of a full delineation of the developmental pattern of the enzyme in all the organ-systems. The bulk of the series was prepared by and for Dr. R. J. McAlpine, formerly of the Indiana University Department of Anatomy, to whom I am indebted for the loan of the slides pertaining to this study. An additional 19 embryos covering the critical developmental interval of 11 to 16 days were prepared personally.

The method of identification of the enzyme was one involving a slight modification of the Gomori ('39) technique and recorded in full by McAlpine ('51). It need only be added that all interpretations have been made in full awareness of the limitations of the method (Stafford and Atkinson, '48; Novikoff, '51 and '52; Goetsch et al., '52). It is realized, that is, that with a method which reveals only 25-30% of the in vivo phosphatase,

1. Contribution No. 570 from the zoological laboratories of Indiana University.

measurements have qualitative significance only; and with the potential error introduced by diffusion and adsorption identifications of precise intracellular distribution sites may be meaningless. However, the technique appears sufficiently reliable to validate determinations of relative amounts and distributions of the enzyme.

To envisage such relative amounts an arbitrary measuring scale has been set up as follows: "0" refers to complete absence of precipitate and thus of the enzyme whose presence and activity the precipitate indicates. "Minimal" refers to a minute amount of precipitate in the nuclear membrane and nucleolus of a cell with none in the cytoplasm. A small amount of indicator within nucleus and cytoplasm is noted as "1+". A dense precipitate in both nucleus and cytoplasm to a degree almost obscuring cell outlines is indicative of maximum amounts of phosphatase and is noted as "5+". Gradations between these extremes are given the values of "2+", "3+", and "4+".

Observations

The Coelomic Epithelium and Gonads

The developmental history of the gonads begins with the coelomic epithelium in embryos of 11 somites (10 days). At this time the cells comprising the epithelium form a continuous layer on the coelomic side only. Basally the cells exhibit long processes like those of the underlying mesenchyme and there is no basement membrane delimiting epithelial from mesenchymal cells. The epithelium and deeper mesenchyme are considered to be a unit all of which will ultimately be concerned in the establishment of a gonadal blastema (Torrey, '45). At this stage, and on into embryos with as many as 18 somites, the phosphatase level in epithelial and adjacent mesenchymal cells is essentially "0", but approaches the minimal level by reason of indicative precipitate in the nucleoli. In interesting contrast, however, those cells which may be interpreted as primordial germ cells exhibit phosphatase to a 3+ and 4+ degree (fig. 1). It may be noted in passing, since the germ cells represent a side issue not to be pursued in detail here, that in embryos of this age group (11-18 somites) the germ cells are found within the coelomic epithelium at anterior levels only. Caudally they lie in the yolk sac, yolk stalk and wall of the gut (fig. 1).

A definitive gonadal blastema is established in embryos of 25-29 somites (11 days). The manner of its buildup has been described previously

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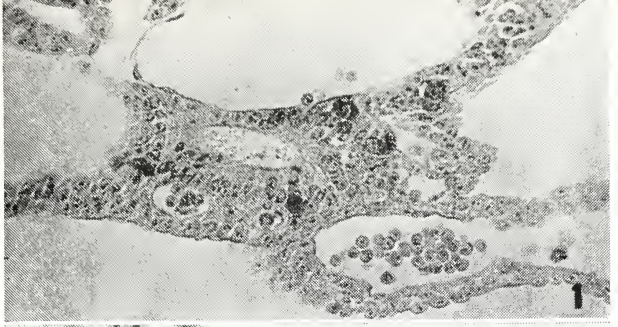
Fig. 1. Primordial germ cells, recognizable by phosphatase content, in gut and mesentery of 10 2/3 day embryo. x125.

Fig. 2. Blastema of 11-day embryo. Nuclei in "center" of blastema 3+ phosphatase, grading off to minimal levels in adjacent non-genital areas. x400.

Fig. 3. Blastema of 12-day embryo. Explanation in text. Arrow directed towards a germ cell. x400.

Fig. 4. Gonad of 13-day embryo. Explanation in text. Arrow directed toward a germ cell. x600.

Fig. 5. Testis of 14-day embryo. Cytoplasmic phosphatase up to 3+ level. Compare with figure 4. x600.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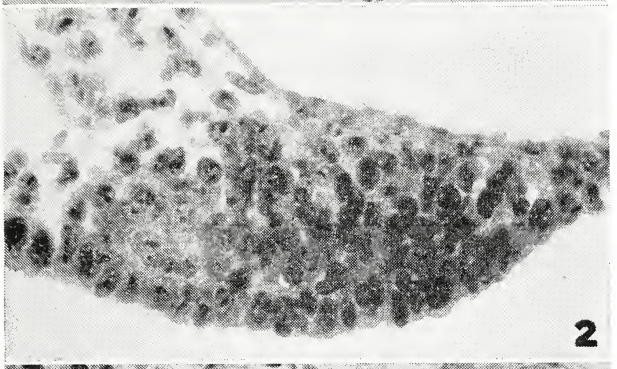
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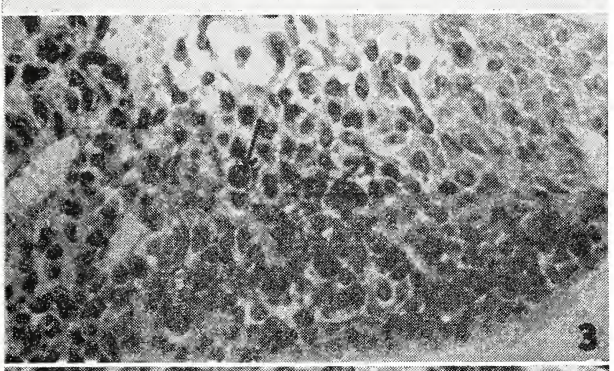
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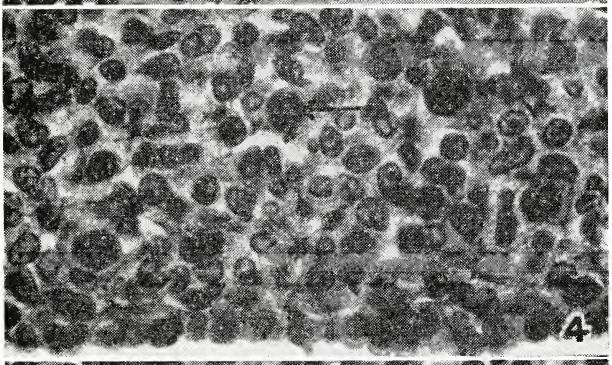
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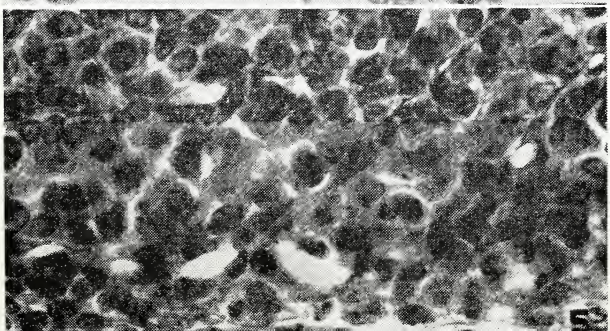
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(Torrey, op. cit.). Suffice it to say there now exists a primordium of sufficient bulk to be identified as a genital ridge. To the right and left of the ridge the coelomic epithelium is now clearly defined, whereas within the area of the ridge itself the epithelial cells blend with the general mesenchyme which in turn is not set off sharply from the adjacent nephrogenous tissue. The pattern of phosphatase distribution emphasizes these gradations (fig. 2). The nuclei of those cells comprising the "center" of the ridge show up to a 3+ concentration of enzyme with a gradual falling off to minimal levels in surrounding non-genital areas. The cytoplasm of all cells, however, appears to be devoid of phosphatase activity.

A gonadal blastema of considerably greater thickness is attained by embryos of 32-40 somites (12 days). The cells are now more closely packed and cell boundaries have become obscure. The gonad is essentially syncytial. There is still no basement membrane delimiting the coelomic epithelium and in routinely stained preparations the gonadal areas grade imperceptibly into the deeper non-gonadal mesenchyme. But the pattern of phosphatase distribution now makes the limits of the gonad considerably more obvious. It is not too difficult to determine the dorsal limits of the gonad in figure 3, for example, although the lateral boundary, grading as it does into the nephrogenous cells abutting the nephric duct, is still indistinguishable. A few primordial germ cells with large spherical heavily blackened nuclei are self-evident (fig. 3). The far more numerous somatic cells have smaller, irregular nuclei. These nuclei average about a 3+ concentration of enzyme; the somatic cytoplasm has now attained concentrations of 1+ to 2+.

Further thickening of the gonad is seen in embryos of 42-48 somites (13 days). Even in conventionally stained material the blastema is obviously sharply marked off from the deeper tissues and on the coelomic side the epithelium is becoming established. However, the matrix of the gonad is essentially like that of the immediately preceding stage: fairly compact with numerous germ cells with large nuclei and somatic cells whose nuclei are smaller and irregular. The phosphatase concentration in the large conspicuous nuclei of the germ cells is in the order of 4+ and that in the cytoplasm now more intense than before, about 3+ (fig. 4). The somatic cells show slight change, the nuclei presenting on the average a concentration of 3+ and the cytoplasm not less than 2+. This is the morphological and enzymatic status of the indifferent gonad. From this

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Fig. 6. Testis of 15½-day embryo. Explanation in text. x600.

Fig. 7. Testis of 16½-day embryo. Note 5+ phosphatase level in tunica albuginea. x600.

Fig. 8. Testis of 19½-day embryo. Explanation in text. x400.

Fig. 9. Three-day postpartum testis. x400.

Fig. 10. Ovary of 14-day embryo. Figure oriented with coelomic epithelium to right. Compare with figure 4. Arrow directed towards a germ cell. Note low reactivity of cells along coelomic boundary. x500.

Fig. 11. Ovary of 15-day embryo. Figure oriented with coelomic epithelium to right. Explanation in text. x500.

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time on sexual differences arise and it is thus convenient to consider separately the development of the testis and ovary.

The Testis

Gonads recognizable as testes are found toward the end of the 13th and beginning of the 14th day, varying with individual embryos. Primary sex cords are set off by the appearance of chromophylic membranes which serve to subdivide the original blastema (Torrey, '45). The coelomic epithelium is now complete and a prospective tunica albuginea lies between the epithelium and the peripheral ends of the primary cords. The essential modification of the phosphatase pattern concerns the primordial germ cells (fig. 5) which can no longer be distinguished so readily. Their nuclei are now reduced to a 3+ phosphatase concentration, putting them on a par with the somatic nuclei, and the cytoplasm is only slightly less. At the same time the cytoplasmic phosphatase of the somatic areas shows a rise to a 3+ level, thus the testis appears to possess a more uniform distribution of phosphatase than in the immediately preceding stage (compare figs. 4 and 5).

At 15½ days the primary sex cords are much more sharply defined and by the same token the interstitial areas and tunica are more conspicuous (fig. 6). The cords contain cells exhibiting considerable variation in nuclear size, shape, and phosphatase activity (fig. 6). The phosphatase concentrations of the nuclei range from 2+ to 4+ and it is now difficult to recognize germ cells. The gradations in nuclear size and the irregular granular appearance of some of the nuclei suggest the original primordial germ cells may be disintegrating and are destined to be replaced by a new generation somatic in origin. The cytoplasm within the cords has declined to levels ranging from 1+ to 2+. The cavitation of the cords in the case pictured in fig. 6 must be an artifact, for cavitation normally does not occur until the 15th day postpartum.

The general reactivity of the interstitial areas is higher than that of the sex cords (fig. 6). Many of the nuclei are blackened to a 4+ degree with the cytoplasm averaging 2+. The tunica shows an even more intense activity with the whole area approaching a 4+ level, and in an embryo just one day older (16½ days) the tunica and membranes delimiting the sex cords show a maximal phosphatase activity as evidenced by an intense 5+ blackening (fig. 7).

The changes in phosphatase content in the days immediately following are so gradual that to make them stand out let us pass abruptly to an embryo of 19½ days (fig. 8). Within the sex cords the nuclei show a 2+ concentration. There is still some gradation in size and shape of these nuclei, but the variations seem less than in embryos three days younger. The cytoplasm of these cells has now returned to a minimum level and only rarely can germ cells be identified with certainty. The interstitial cells, in contrast to the situation at 16½ days, now show a greatly reduced phosphatase concentration. Many of the nuclei are hardly more than 1+ with some approaching a 2+ level; the cytoplasm is 0 to minimal. The limiting membranes of the sex cords, however, range from 3+ to 5+ and the tunica is blackened to a 5+ level (fig. 8).

Testes of 3-day postpartum individuals present an organization which foreshadows that of the fully-formed gonads. The somatic cells of the testes cords are assembled as a regular epithelium and germ cells are once again conspicuous (fig. 9). The nuclei of both types of cells show a 2+ to 3+ phosphatase level while the cytoplasm is minimal. Interstitial tissues are now very diffuse with the nuclei at 1+ or less. The tunica albuginea, however, remains at 5+. In subsequent days only the limiting membranes of the cords and tubules and the tunica will show a high level of activity; the nuclei of the somatic and germ cells will show a decline to 1+ and below as will also the interstitial elements.

The Ovary

At the 14th and 15th days postcoitum ovaries are recognizable only in a negative way. That is, testes at this time exhibit characteristic structural features, thus gonads of this age which lack these features, appearing instead as indifferent gonads, may be presumed to be ovaries. They differ from the equivalent indifferent percursors of testes only in that they are somewhat more vascular (fig. 10). The primordial germ cells again stand out by reason of their larger nuclei with a 5+ concentration of phosphatase. The cytoplasm of these cells is 3+. The somatic cells, except near the coelomic border, show 2+ to 3+ for both their smaller, irregular nuclei and cytoplasm. The cells along the coelomic boundary are the least reactive, with no more than 1+ nuclei and 0 to minimal cytoplasm; they also lack a basement membrane setting them off as an epithelium and are devoid of associated germ cells (fig. 10).

At 15 days (fig. 11) germ cells are still numerous, but their nuclei are less reactive (4+) and their cytoplasm down to 0. The somatic cells show a greater range of nuclear size, so while some are obviously distinct from the germ cells, others cannot be distinguished readily. Their nuclear reactivity ranges from 2+ to 3+ and the cytoplasm from 1+ to 2+. The lowest phosphatase concentration is again in the prospective but still unlimited coelomic epithelium.

In ovaries of 16 to 16½ days primary sex cords are clearly set off by intervening connective tissue showing a 4+ reactivity (fig. 12). These primary cords consist of cells with 3+ nuclei and 1+ to 2+ cytoplasm. As before, some of the cells are obviously germ cells, but the gradations in sizes make a precise distinction between germinal and indifferent elements difficult. The coelomic epithelium is now quite distinct by reason of a generally low phosphatase reactivity, minimal to 1+ on the average for the nuclei and 0 to minimal for the cytoplasm (fig. 12). A basement membrane of about 3+ activity is just beginning to form. Where the membrane is absent, the cells of the mesothelium are continuous with and blend with the adjacent primary cords. Occasional germ cells with 3+ nuclei lie in the epithelium.

In the interval between 16½ and 19 days the germ cells, somatic cells, and connective tissue of the primary (medullary) area of the ovary become increasingly reactive. Thus in a 19½ day ovary (fig. 13) the interior of the gonad shows phosphatase concentrations in both nuclei and cytoplasm from 3+ to 5+. Many nuclei appear irregular and pycnotic

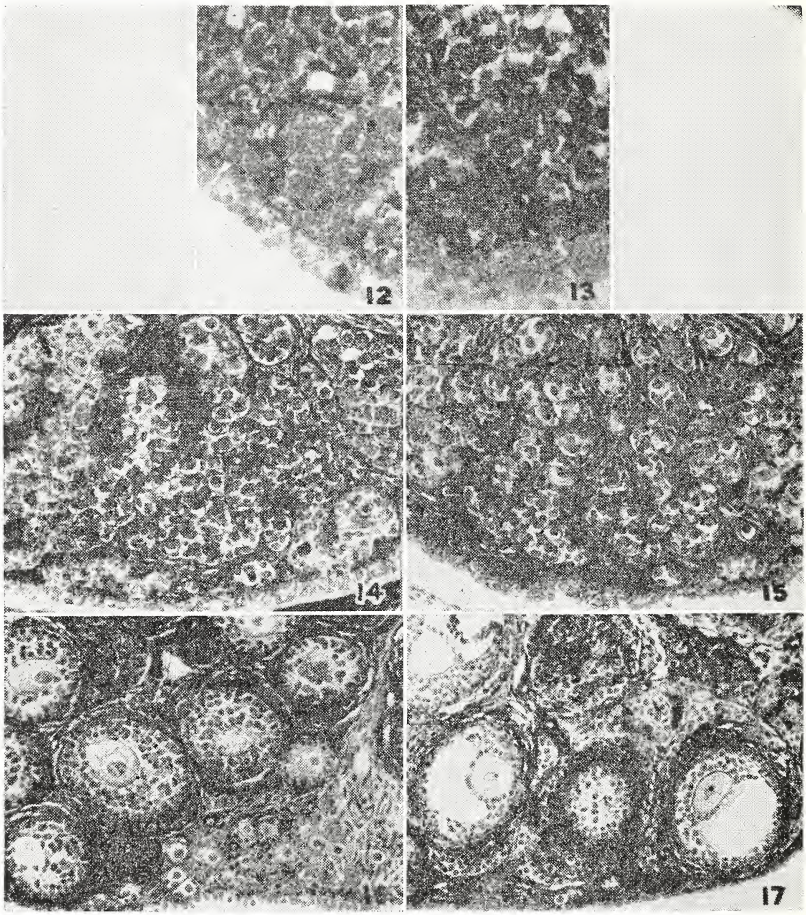

Fig. 12. Ovary of 16½-day embryo. Note low phosphatase content of coelomic epithelium (bottom) compared with that of primary cords. x500.

Fig. 13. Ovary of 19½-day embryo. Concentration of phosphatase in coelomic epithelium (bottom) low; in primary cords very high. x500.

Fig. 14. Two-day postpartum ovary. Explanation in text. x400.

Fig. 15. Five-day postpartum ovary. x400.

Fig. 16. Twelve-day postpartum ovary. x400.

Fig. 17. Sixteen-day postpartum ovary. Stratum granulosum shows low phosphatase level; that of theca very high. x400.

and one gets the impression of the start of a general breakdown of the interior. The basement membrane setting off the germinal (coelomic) epithelium is now complete and it, too, along with the septa separating the primary cords, is blackened to a 5+ degree. The germinal epithelium

itself, however, shows a minimal reaction except at those sites where secondary (cortical cords) are being inaugurated (fig. 13). Both the nuclei and cytoplasm of the secondary somatic cells react to a 2+ to 3+ degree and germ cells with large 5+ nuclei may occasionally be seen.

From this point onward attention is centered on the secondary cords if for no other reason than the pattern of retrogression of the medullary elements of the rat ovary remains largely unknown. In the absence of this information, the pattern of phosphatase concentration and distribution would be meaningless.

A 2-day postpartum ovary is illustrative of the next events. The coelomic epithelium again shows a 0 to minimum phosphatase concentration and it is most interesting that the most recently formed cortical cords, namely those still connected with the epithelium, likewise show a minimal reaction (fig. 14). At deeper levels, however, the nuclei give a 3+ reaction although the cytoplasm has dropped to a minimum to 1+ level from the earlier high of 3+. Occasional cells with especially blackened nuclei may well be germ cells, but this identification is not certain. It is also difficult, at the deepest levels of the ovary, to determine where the secondary cords leave off and the primary cords begin. The membranes which bound the cords and delimit the germinal epithelium except at those points where it is continuous with newly forming cortical cords, are uniformly blackened to a 5+ degree (fig. 14).

The secondary cords of the 5-day postpartum ovary are beginning to be subdivided into nests of cells, the forerunners of the follicles. Such a fully delimited cluster consists of a central ovum enveloped by indifferent cells and separated from neighboring clusters by vascular and connective tissue (fig. 15). The nuclei of all the cells, germinal and indifferent, average about 3+ with the cytoplasm minimal. The septa range from 4+ to 5+; the germinal epithelium shows a minimal concentration.

The history of the ovary may be concluded with a brief consideration of 12-day and 16-day postpartum stages (figs. 16 and 17). The ova themselves show a uniformly 0 to minimal level of phosphatase. As for the developing follicles, the follicular cells at 12 days possess 3+ nuclei and minimal cytoplasm, but the surrounding tissues are 5+. So it is in those more mature follicles at 16 days (fig. 17). The ovum shows little or no phosphatase and likewise the cytoplasm of the stratum granulosum. The follicular theca, however, shows a phosphatase concentration of 5+. This is a pattern much like that reported by Corner ('48) for the mature follicles of human and pig ovaries.

Discussion

The observations just presented have pertained to a number of related but still somewhat distinct developmental events. Among these are the histories of the primordial germ cells, the tunica albuginea, and the interstitial cells as well as the somatic elements of the gonads. Except for recording the impression that the original stock of primordial germ cells disappears and is replaced by new generations of somatic origin, let us concentrate our attention on the sex cords and their precursors.

The essential facts with respect to phosphatase concentrations are summarized in table 1. Both nuclear and cytoplasmic values are recorded,

but in the light of the strong possibility that nuclear "staining" may be an artifact (Novikoff, '52), the burden of the argument to be developed will be placed on the cytoplasmic values. These facts may be linked with profit to a previously acquired set of observations on the behavior of embryonic gonads in intraocular grafts (Torrey, '50). These observations and the conclusions derived therefrom are recorded in detail in the publication cited; the essential facts are likewise summarized in table 1.

There appear to be two principal correlations that obtain as one examines the record. First, acquisition of the ability of primordial materials to differentiate into testes or ovaries is accompanied by the rise of cytoplasmic phosphatase to a 3+ level. This is evidenced by: (a) the coelomic epithelium in embryos of 11-20 somites (10 days) with its 0-level phosphatase shows no ability whatsoever to differentiate in grafts; (b) the modest ability of 11-12 day primordia, showing a gradual rise to a 2+ phosphatase content, to form testes reaches a maximum in 13 and 14 day primordia whose phosphatase content reaches a 3+ level; and (c) determination of secondary cords as manifested by the consistent appearance of ovaries in grafts is accompanied by a phosphatase concentration which approaches the 3+ level. Second, an earlier suggestion (Torrey, '50, pp. 46-47) that every gonad, no matter what its genetic predisposition, shows an initial bias towards maleness is supported by the developing pattern of phosphatase. That pattern is as follows: The initial rise in phosphatase, occurring in the interval between 12 and 14 days, involves the primary cords and it is at this time that determination in zygotic testes, as manifested by their performance in grafts, occurs. The same predilection towards maleness accompanying the 3+ level of phosphatase in the primary cords appears in zygotic ovaries, a predilection which if favored by a suitable environment such as the anterior chamber of the eye is carried through as an actual sex reversal. Not until the rise of secondary cords from the 16th day on and the buildup of their phosphatase content to a 3+ level does the developmental direction of zygotic ovaries shift towards femaleness. Prior thereto the germinal epithelium with its minimal enzyme content shows little or no ability to produce ovarian tissues.

Thus is the normal developmental order of appearance of primary and secondary cords and the time and degree of their determination as measured through grafts accompanied by a pattern of phosphatase development. Viewing that pattern, one sees a strong suggestion that the events of determination are accompanied by a pronounced increase in the cytoplasmic content of alkaline phosphatase. For the moment one can only speculate on the role played by the enzyme in these events of biochemical differentiation. There is, of course, the frequently mentioned possibility of a link between phosphatase and the nucleic acids RNA and DNA in the mechanisms of phosphate transfer related to the variety of metabolic operations probably involved (e.g., Kutsky, '50). That this is not unlikely is suggested by the steady rise of these three agents during the development of Amblystoma (Krugelis, Nicholas, and Vosgian, '52). For the time being, however, it seems wiser only to record the correlation between high levels of cytoplasmic alkaline phosphatase and the occasion of determination in the primary and secondary primordia of the gonad,

TABLE 1

Age in Days	Primordium		Nucleus	Cytoplasm	Behavior in Grafts (From Torrey, 1950)
10	Coelomic Epith.		0	0	No gonad differentiation
11	Blastema		2-3	0	Few scattered testis cords (3 of 21 grafts)
12	Blastema		3	1-2	Frequent testes (18 of 29 grafts) Rare ovaries (2 of 29 grafts)
13	Indifferent Gonad		3	2	Testes (47 of 79 grafts: 13 "converted medullary tubules" of genetic ovaries and 34 genetic testes) Ovotestis (1 of 79 grafts) Ovaries (3 of 79 grafts)
14	Testis		3	3	
	Ovary	Primary Cords	3	3	
		Coelomic Epith.	1	min.	
15	Testis		2-4	1-2	
	Ovary	Primary Cords	2-3	1-2	Ovotestes (4 of 17 grafts) Ovaries (5 of 17 grafts) Testes (4 of 17 grafts: "converted medullary tubules")
		Coelomic Epith.	1	min.	
16	Ovary	Primary Cords	3	1-2	
		Coelomic Epith.	min.-1	0-min.	
	Testis		2	min.	
19	Ovary	Primary Cords	3-5	3-5	Ovaries only (10 of 11 grafts)
		Secondary Cords	2	2-3	
		Coelomic Epith.	min.-1	min.-1	
2PP	Ovary	Secondary Cords	3	min.-1	
		Coelomic Epith.	0-min.	0-min.	
3PP	Testis		2	min.	
5PP	Ovary	Primary Follicle	3	min.	
		Coelomic Epith.	min.	min.	
12-16PP	Ovary	Stratum gran.	3	min.	
		Theca	5	5	

leaving the answer to the ultimate meaning of this to subsequent studies.

Whatever that answer turns out to be, the enzyme appears to play only a transitory role, for its concentrations drop to minimal levels once visible differentiation sets in. What new patterns of concentration and distribution may rise to accompany the later functional anatomy of the maturing gonads is beyond the scope of this study, except to take note of a high-level concentration of the enzyme which appears to presage the degeneration of the ovarian medulla.

Summary

1. The patterns of concentration and distribution of alkaline phosphatase are linked to a previously established descriptive and experimental analysis of the development of the gonads of the albino rat.

2. Acquisition by the primordial materials of the ability to differentiate into testes or ovaries is accompanied by a pronounced increase in the enzyme content of the cytoplasm. Once visible structural differentiation sets in, the concentration of phosphatase declines.

3. Every gonad, no matter what its genetic predisposition, first acquires testis-forming potentialities as evidenced by a phosphatase rise in the primary cords. Prospective ovaries develop as such only after a phosphatase rise in the later appearing secondary cords.

4. Incidental observations suggest: (a) that the original stock of primordial germ cells is replaced by new generations of somatic origin; and (b) that a very high-level concentration of phosphatase presages degeneration of the ovarian medulla.

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