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Experimental Studies on Sex-differentiation in Two Japanese Salamanders[1)]

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(With 2 Textfigures and 8 Plates)

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I. Introduction and review

The hermaphroditic tendency in the sex-differentiation of amphibians was first reported by Pflüger (1882), who found that the ratio of male and female was normally found to be 1:1 in adult frogs of *Rana temporaria*, but the tadpoles of some local races of the same species were all females. He noticed, also, the frequent occurrence of hermaphrodites among them and concluded that sex-differentiation in the tadpoles of such local races starts first in the female direction and that gonads of ovarian structure are uniformly developed. The genetical males manifest themselves by the transformation of young ovaries into testes at a certain time of larval development. This report attracted the attention of R. Hertwig and at the beginning of this century an extensive series of experiments were undertaken on the problem of sex-differentiation and sex-determination in amphibians. His cross-breeding experiments of different local races of *R. esculenta* yielded rather significant results and further investigations on natural as well as experimental sex transformation were carried out by his students Schmitt-Marcell (1908), Kuschakewitsch (1910) and Witschi (1914, ...). According to Witschi (1929) there are in *R. temporaria* three different sex races which are distinguished by the type of sex-differentiation, viz., undifferentiated, semi-differentiated and differentiated race. At the time of metamorphosis, the offspring of the first race consists of females only. At the corresponding stage of development, the semi-differentiated race consists, in various percentages, of females, hermaphrodites and males and only in the differentiated race, is the clear 1:1 ratio of males and females exhibited in the early larval period.

But it was not until the establishment of the theory of quantitative sex determination by Goldschmidt and Morgan, that a satisfactory interpretation could be offered for the results obtained by these early investigators. It was found that female as well as male individuals carry female determining and male determining genes. The importance of these genes in sex determination rests largely on their relative relationship. Witschi introduced this theory of genetics into the hypothesis for the sex-differentiation in amphibians, and applying various arbitrary values to the male determining and female determiing factors of different sex races, he could show that Hertwig's hybrid series as well as his own cross-breeding experiments on

R. temporaria could find a conforming interpretation. Thus it became evident that the analysis of sex-differentiation involves two large fields of experimental biology, viz., heredity and embryology.

From the embryological point of view it is generally accepted that in vertebrates, two separate histological components in the primordial gonad, the cortex and medulla, play an important rôle in the differentiation of sex. The development of the cortex produces the female differentiation and that of the medulla the male differentiation. Witschi (1913) confirmed in amphibians that genetically male germ cells, if retained in the cortex, differentiate into oocytes and he concluded that the cortex acts as an inductor of female differentiation. This idea was further elaborated in the following year (Witschi, 1914a, b) and it was realized that the medulla acts also as the inductor of male differentiation. According to him, both the inductors produce their effects by the elaboration and release of specific morphogenic substances, which he later (Witschi, 1931) called "cortexin" and "medullarin." The two substances act as antagonists to each other. Adequate evidence for this hypothesis was given by the high temperature experiments on frogs and toads; when their tadpoles are exposed over a certain period of development to high temperature of about 32°C, the cortical development of the ovary is suppressed which is soon followed by the compensatory development of medulla, resulting in the reversal of genetical female into male (Witschi 1929, Piquet 1930). These experiments have recently been repeated and extended to a salamander by Uchida (1937). His results corroborate, in general, the conclusion above stated. Obviously high temperature disturbs the balance of medulla-cortex antagonism.

So far only inductive effects within a single gonad had been taken into consideration. In 1916, however, the epochal work of Lillie on cattle twins appeared and distinct evidence was presented that the inductive effects are transmitted over a much wider range by the transport of effective substance in the blood stream. After him, extragonadal dispersion of effective substances has been proved on various forms of vertebrates by grafting experiments, but the most significant result in amphibians was first obtained by Burns (1925a, b) by the method of parabiosis. He united the embryos of *Amblystoma maculatum* (=*punctatum*) and *A. tigrium* in parabiotic twins at a stage shortly after the closure of the neural fold and kept

them until sex was distinguishable histologically. Since sex is not recognizable at the time of operation, male and female are combined by chance. The parabiotic pairs must, therefore, be combined in a 1 MM:2 MF:1 FF ratio. According to Burns, however, both members of a pair are invariably of the same sex and the development is apparently normal in every respect.

Humphrey (1928, 1929, 1938) performed similar experiments in a more skilful way. In his experiment one gonadal preprimordium of *Amblystoma* was replaced by that of another embryo and an ovary thus brought under the influence of testes throughout its development. In contrast to Burns, Humphrey found many cases in which the sex of the host proved to differ from that of the implant. The testis was always normal but the development of the ovary, in the majority of cases, suffered retardation of various degrees and a intersexual gonad was produced with a few germ cells in the cortical germ layer as well as in the medulla. In the meantime, the first report of Burns was submitted to a partial correction by Humphrey (1931) but the common conclusion obtained by these two investigators was that sex reversal is brought about in heterosexual pairs by the earlier differentiating member, especially if it can exert its influence over a long period.

This is, however, in unreconcilable conflict with the conclusion of Witschi, who made, in colloboration with his students, a more extensive series of experiments of parabiosis in various forms of amphibians. According to him the antagonistic influences exhibited in the process of sex differentiation in heterosexual parabiotic twins differ in various forms of experimental animals. In the toad, the gonads develop normally in both members of the twin without any interference. In this case the inductive action exerts its influence only within a single gonad. The same is true in the early larval stage of the frog but later a conflict between the testicular and ovarial development becomes gradually evident and the closer the twins are grafted together, the earlier it appears. The first sign of disturbance is always found in the inner gonads of the twin and in most cases in the ovary at the region nearest to the testis. This is expressed by the suppression of cortical development, which is immediately followed by the compensatory development of the medulla, resulting in a progressive transformation of sex. This process of sex reversal suggests that the inductive action is transmitted by diffusion of a

morphogenic substance through the tissue but the effect falls off rapidly with distance. The most extensive series of Witschi's experiments were performed on urodeles. In general here again a sharp mutual antagonistic influence is observed in the early development and almost sterile gonads are produced in both members of the co-twin, but about the time of metamorphosis the male gonads rapidly recover normal size while the female gonads are left in a reduced condition. According to Witschi, inhibition of the ovarial development in salamanders is not followed by testicular development. The male interference dominates almost completely over the female and the same effect is always observed irrespective of the mutual position of the twin mates. For instance, if the animals are connected only by a narrow bridge of gill arch, the male member completely disturbs the ovarial differentian of the female co-twin. Therefore one can assume that the inhibitive morphogenic substance is transmitted by the blood stream and is able to reach all parts of the twin system. Here, for the first time, the hormonal conception of the inductive substance in the strict sense is accepted and the stimulative and inhibitive substances become clearly distinguishable.

Corresponding to the sex races in frogs, there are also in salamanders specific or racial types of sex-differentiation; while some of them are purely gonochoristic, others exhibit marked hermaphroditic tendency during the process of sex-differentiation. It is of surprising interest to find that there is an intimate relationship between the intensity of inhibitory inductive activity and the type of sex-differentiation. It is high, in general, if produced by the male of differentiated form and less if caused by the male of semi-differentiated race. Only occasionally, when the development of the female proceeds ahead of the male partner, is the cortical development free from disturbance. These facts prove that the inductive dominance of the male is not absolute, but depends upon the quantitative ratio of the antagonistic substances and certain time relationship in their appearance and duration. For the problem of what at the end, may become of free-martin gonads Witschi's experiment in 1937 produced important results. He found that in heterosexual pairs of *A. tiginum*×*A. jeffersonianum* the ovaries of the small and slow differentiating *jeffersonianum* become perfectly sterile and "have a tendency to disappear or to become reduced into simple ligaments." In the reciprosal combination, on the other hand, the *jeffersonianum*

male produced hermaphrodite glands, which contained both matured spermatozoa and large pigmented egg cells. Hermaphrodite glands of similar structure were also obtained in a pair of *A. tigrinum*× *Axolotl*. In every case the sex reversal in the sense of Burns and Humphrey did not occur. One must here consider the fact that the constitution of the experimental animals is genetically as well as morphologically of bisexual nature.

Recent advance in the preparation and synthesis of hormonal substances has provided another method of attack on the problem of sex-differentiation and various experiments have been undertaken concerning the influence of sex hormone upon sex-differentiation in vertebrates. In amphibians, the first report on experiments of this type was published by Padoa (1936, 1938), who added the follicular hormone to aquarium water, in which the tadpoles of *R. esculenta* were reared. According to him, this induced the great majority of the experimental animals to differentiate into males. Witschi & Crown (1938), however, having repeated the similar experiment on *R. pipiens*, failed to observe the masculinizing effect of estrogen. Burns (1938, 1939) and Foote (1940) reported, on the contrary, that the injection of estrogenic substances in *A. maculatum* and *A. tigrinum* caused transformation of testes into ovaries. The masculinizing effect of androgen, on the other hand, was affirmed by Gallien (1937), Witschi & Crown (1938), Foote (1938), Witschi & Foote (1939) on several forms of frogs but in salamanders Burns (1939) reported that the injection of testosterone-propionate produced only intersexual gonad in genetical females and Foote (1940) also confirmed that testosterone exerts little influence on the differentiation of ovary. Puckett's (1939, 1940) experiments are of special interest. He claimed that in an undifferentiated race of *R. catesbiana* "there is no suggestion of a stimulation of gonads by the sex hormone alone," but "the injection of sex hormone in addition to a pituitary extract brings about striking modification in both male and female."

Thus the influence of sex hormone upon the sex-differentiation in amphibians is rather contradictorily reported. This is perhaps due partially to the different embryological characters of the experimental animals and partially to the different chemical nature of the hormonal substance administrated. Anyhow, it remains unsettled whether the sex hormones are essentially identical with the morphogenic substance of sex-differentiation or whether they are a separate

substance which indirectly disturbs the balance of medulla-cortex antagonism, in a similar way in principle to the different temperature levels in the experiment on *R. esculenta.*

Since several years ago the present writer could fortunately have an opportunity to carry on a series of experiments on the sex differentiation in two Japanese salamanders *Hynobius retardatus* and *H. lichenatus* which belong respectively to semi-differentiated and differentiated type in regard to sex differentiation. The conclusion was obtained that the inductive substances and sex hormones are distinctly separate substances which exert entirly different influences upon the sex differentiation. The following description is based on the observation of results obtained by the experiments just mentioned.

Before going further hearty thanks should expressed to Prof. Tohru Uchida under whose constant guidance and kind encouragement this work has been carried out. The expense of the present investigation was partly defrayed from the Scientific Research Expenditure of the Department of Education.

II. Normal sex differentiation in *H. retardatus* and *H. lichenatus*

(A) ***H. retardatus.*** *H. retardatus* is a salamander peculiar to Hokkaido and the fresh eggs are abundantly found in the environs of Sapporo in the early spring. The materials used in the present experiment were all collected from fields in their early developmental stages. The larvae are voracious and violently cannibalistic. In laboratory, they are easily fed with fresh liver and earth worms.

(*i*) *Formation of primordial gonad.* The primordial gonads in *H. retardatus* appear first in larvae about 30 days old, 21 mm in total length and with fore-limb buds. In cross section, the rudimentary gonad is a small ridge at the base of dorsal mesentry. It is composed of a small number of germ cells intermingled with small mesenchym cells and is covered by the peritoneal epithelium. In larvae of about 60 days and 28 mm in total length, the cells forming the gonad rudiment are arranged in the peripheral or cortical region with a cavity in the center which is filled with some fluid substance (the primary cavity). The germ cells, now increased in size, are sometimes characterized by the formation of pigments in them. In

the meantime, the fat-bodies make their appearance. At first they are small buds at the base of rudimentary mesogonium, but they increase rapidly in size soon surpassing the gonads. In the next stage of gonad development the primary cavities are caused to disappear by the proliferation of rete cells. These cells originate from mesonephric blastema, move towards and enter the central cavities of the gonad rudiments where they form the medulla. The germ cells are now arranged in the layer between the medulla and the peritoneal epithelium. Thus in larvae 70 days old and 35–40 mm in total length, the gonad consists of two distinct histological components of rather diverse origin, the cortex and medulla.

(*ii*) *Differentiation of the ovaries.* The differentiation of the ovary is characterized by the rapid growth of cortical germ layer. The proliferation of rete cells ceases and the medulla become gradually transformed into an epithelial sac, which is known as the ovarial sac. Along with the growth of larvae, the germ cells in the cortex increase rapidly in number, the ovarian cavity becomes widened and at the end of metamorphosis, which occurs about 3 months after fertilization, the gonads acquire the characteristic structure of ovaries. In the next spring, the gonad appears macroscopically as a thin thread hanging on the midventral surface of the mesonephros. In well fed animals the germ cells increase considerably in size. In the third spring the sexes are easily identified as the surface of the ovary becomes granulated by the growing follicles. In the fourth spring the ovaries grow into large lobeted organs, containing numerous pigmented eggs which seem now to be fertile.

(*iii*) *Differentiation of testes.* Up to the beginning of metamorphosis, the gonads in *H. retardatus* take, in both sexes, the structure of an ovary; the future testes are provided with a well differentiated cortical germ layer and a wide ovarian cavity. With the onset of metamorphosis, however, the gonads of genetical males gradually change their original structure. The ovarian cavities disappear with the subsequent migration of rete cells from the mesonephros and the gonads again acquire a solid structure. At the same time the germ cells which were arranged in the cortical layer move towards the medulla, and become intermingled evenly with rete cells. Thus the gonads take the characteristic structure of the embryonal testes, and at the end of metamorphosis the sex ratis of 50:50 is established but the differentiation of seminal tubules and efferent

ducts becomes complete in the second or third spring and the spermatozoa mature under laboratory conditions in the fourth spring.

In the field condition, the growth of the animals is semetimes conspicuously retarded, perhaps by the deficiency of foods. In the early spring, larvae of the preceding year are occasionally found in the pools together with fresh egg clusters. The behaviour of the young animals after metamorphosis, however, is not certain because the present salamander is rarely found in the fields except in the breeding season when they appear in groups.

(*B*) ***H. lichenatus.*** The eggs of *H. lichenatus* used in the present experiment were collected from Asamushi in Aomori prefecture. The eggs as well as the adult animals of this form are smaller in size than those of *H. retardatus*, and the larvae are feeble and slow in growth. After metamorphosis, however, the young animals become voracious and grow rapidly. In laboratory both male and female mature in the third spring.

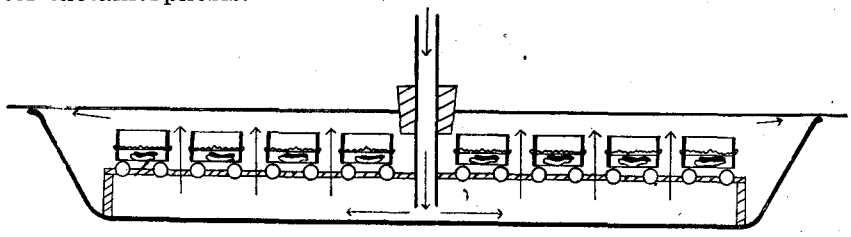
(*i*) *Differentiation of the male.* In regard to sex differentiation, the present species is a true gonochorist, free from hermaphroditic tendency. The primordial gonads appear about a month after fertilization, and the two sexes undergo early and complete sex differentiation. In the male the germinal epithelium of the indifferent gonad is reduced during early larval development and the germ cells become included within the compact medulla; neither formation of ovarial sac nor the development of cortical germ layer is detected during the whole process of testicular differentiation.

(*ii*) *Differentiation of the female.* In the female, the germ cells persist in the peripheral germ layer and the rete cells in the medulla transform into epithelial wall lining the ovarian cavity. In about 50 control animals of various developmental stages, nearly equal numbers of males and females were obtained. The metamorphosis occurs, as a rule, one or two weeks later than that of *H. retardatus*.

III. Experiment 1. Sex modification produced by the homogenous and heterogenous parabictic twins of *H. retardatus* and *H. lichenatus*

In the first experiment, the sex modification in homogenous as well as heterogenous parabiotic twins of two salamanders above

mentioned was examined. Parabiotic twins were made at tail bud stage by grafting the two embryos in usual left to right position. Generally severe mortality followed for several days after the operation but this was avoided to a considerable extent by placing the united embryos in a glass cylinder with a base of cellophane membrane. The cylinders were supported by glass holders in running tap water and the whole apparatus was covered with large glass plates. Text-figure 1 shows schematically the transverse section of the entire arrangement. When the united pairs begin to swim about they were removed to ordinary glass vessels containing about 1 liter of tap water. In animals that were kept beyond the feeding stage, the losses were very small. Although the two embryos are united side by side at the time of operation, they shift gradually to the ventral position in relation to each other. Consequently the united pairs cannot get to land and all of them were preserved immediately after metamorphosis.



Text-fig. 1. Cross section of apparatus used for rearing the united embryos. Explanation in the text.

(A) ***H. retardatus* × *H. retardatus*.** Among 51 pairs that survived up to the time of metamorphosis the following distribution of sex was observed;

$$14\,♀\,♀ + 20\,♀\,♂ + 17\,♂\,♂ = 51$$

In general the gonads of the experimental animals were a little smaller than those of single controls.

(i) ♀ ♀ *pairs*. In ♀ ♀ combination, the differentiation of ovaries at the time of metamorphosis is normal. As a rule they have a well developed cortical germ layer and a wide ovarian cavity (Fig. 1). In 5 pairs out of 14, the differentiation of germ cells proceeded considerably ahead of normal ones, and a pretty large number of egg cells which are provided with vesicular nuclei and dense protoplasmic substances are found in the cortex (Fig. 2). In the single

control females oocytes of this size are never observed during a few months after metamorphosis.

(*ii*) ♂♂ *pairs*. In 13 pairs out of 17 ♂♂ combination, the testes are well organized for the age. In general they show no essential difference from those of single males. In the remaining 4 pairs the fundamental structure of the gonads is testicular (Figs. 7, 8). Curiously enough, however, they contain varying numbers of female germ cells intermingling with well organized testicular tissue (Figs. 4, 6, 9, 10, 12). In three pairs they appeared in both members of the co-twin. These female germ cells show in every detail the characteristics of egg cells, provided with large vesicular nuclei and granular protoplasm. The gonads containing these egg cells are covered by greater or less amount of cortical tissue (Figs. 5, 6). Obviously the egg cells originate from these cortical germ cells, though most of the small germ cells in the periphery are in the process of degeneration. In the last one pair, the tetes are entirely normal in one of the co-twin, and in another, the gonads show equally well differentiated testicular structure in the anterior half (Fig. 11). The remaining part of the same gonads, however, show mixed structure in both sides and every section contains one or two of the large egg cells (Fig. 12). These egg cells are the largest ones obtained in the present experiment. Anterior and posterior halves of the gonad are connected by a narrow neck which is nearly or completely sterile. It must be noticed that the growth of the last mentioned pair was retarded conspicuously and their metamorphosis was not complete until the next spring.

There remains some doubt whether these four pairs are certainly of original double male combination, but they could be classified conveniently into double male pairs because the gonads are perfectly solid and the medulla show nearly normal testicular structure through considerable length. On the other hand, there is a conspicuous gap between the types of sex modification obtained in these four pairs and those observed in the heterosexual pairs which will be described in the following section.

(*iii*) ♀♂ *pairs*. In 16 pairs out of 20 of heterosexual combination, the development of both ovary and testis are always normal. Their differentiation proceeds as in single controls of male and female respectively; the medulla of the testis is well organized for the age

(Fig. 13) and the ovary is provided with well developed cortical germ layer and wide ovarian cavity (Fig. 14). In two cases, even large egg cells are found in the ovary of the female, while the male has normally differentiated testes. Sometimes the cavity of the ovary is closed through a considerable length, but this is not due to the influence of the male partner; the ovaries of similar structure are frequently observed in the double female pairs too (Fig. 3).

In 3 pairs, the testes of the male are normal (Figs. 15, 18, 21). The differentiation of the ovaries on the other hand, is retarded in various degrees and one can find, in certain limited stretches, a considerable amount of unorganized rete cells filling the ovarian sac (Figs. 16, 20). The degree of the retardation in ovarian development is not dependent upon the amount of testicular tissue in the male member. For instances, in the pair illustrated in Figs. 15–17 the testes of the male are conspicuously large and their differentiation has proceeded far ahead of onrmal ones, but the inhibition in the ovarial development in the female member is not severe; the rete cells occclude the vovarian cavity only for short stretches. On the contrary, the testes in the other two pairs are rather small and underdeveloped (Figs. 18, 21) but the retardation in the ovarial differentiation is more complete and the medulla contains a small number of germ cells (Figs. 22, 24).

In the remaining one pair, the ovaries of the female member are well differentiated, and contain a rather large number of large egg cells. The gonads of the male, however, show mosaic structure: in anterior half both the gonads take distinct testicular structure, while the posterior half of the same gonads contains many large eggs which are similar to those observed in the pairs of double male combination.

The results obtained observing the homogenous parabiotic twins of *H. retardatus*, show distinctly the fact that in semi-differentiated race the male member exerts a relatively slight influence upon the development of the ovary in the female co-twin. This forms a striking contrast to the results obtained in the combination of *H. lichenatus*.

(B) *H. lichenatus* × *H. lichenatus*. Sex distribution among homogenous parabiotic twins of *H. lichenatus* deviates considerably in favour of male combination as there were obtained;

$$4\,♀\,♀ + 6\,♂\,♂ + 23\,♂\,♂ = 33$$

The microscopical observation of the gonad was as follows.

(*i*) ♀♀ *pairs*. In 3 pairs of double female combination, the ovaries are provided with healthy growing cortical germ layer and wide ovarian cavities (Fig. 25). In the remaining one pair the growth of the germ cells considerably exceeds that of normal ones (Fig. 26) and the cavities are partially occluded by the enlarged egg cells.

(*ii*) ♀♂ *pairs*. In all 6 pairs of heterosexual combination, the male member has always normal testes of intermediate size (Figs. 27, 31, 36, 40). The retardation of ovarian differentiation in the female member is, in contrast with the cases of *H. retardatus*, far more pronounced. In 4 pairs the ovaries are provided with a thick cortical germ layer which contains many egg cells of considerable size (Figs. 28, 29, 34), but the central cavity is occluded through almost the entire length by the proliferation of various numbers of rete cells (Fig. 28). The open cavity is found only as several intervals of the section at the terminal region of the gonad (Fig. 30). In none of these 4 pairs are the medullary germ cells included in the medulla. In the remaining 2 pairs, the medulla of the affected ovaries included a pretty good number of germ cells and the entire gonad assumes a more complete testicular structure especially in the middle region. (Figs. 32, 33, 37). The gonad, however, is capped for a certain length by various amounts of cortical tissue (Fig. 38). It is interesting that some of the germ cells in the cortex have grown into large egg cells which surpass in size the oocytes of single females (Fig. 34). Only in the posterior one third, is the ovarian structure dominant although even in this region, the cavities are nearly or completely solid (Figs. 35, 39, 41). The suppression of growth of egg cells in the cortex of modified ovaries is not proportionally complete to the development of the medullary component. Large egg cells appear sometimes in the ovaries whose medullary differentiation shows a more advanced condition, while the cortex of less modified ovaries contains only small germ cells. This suggests that the morphological differentiation of the gonad and the differentiation of germ cells are not controlled by the same factor. The results of hormone injection offer more satisfactory evidence on this problem.

(*iii*) ♂♂ *pairs*. In the majority of double male pairs, the testes show normal structure. The medullary tissue is, in general, well organized for the age and contains a good number of small germ

cells. In some animals, however, the testes are extremely small and are reduced to a nearly sterile condition; only a few germ cells are found scattered in the compact mass of rete cells (Fig. 42). In other ainmals of the double male pairs, on the other hand, one can occasionally find that some germ cells at the peripheral region are in the process of degeneration, although their medullary tissue is relatively well organized. The distribution as well as the morphological character of these degenerating germ cells show clearly that they are of cortical nature. In normal male differentiation of *H. lichenatus* the testes at the stage of metamorphosis are completely free from hermaphroditic tendency. Consequently there arises some doubt if the parabiotic twins which were classified into double male pairs in the present experiment contain, in fact, a certain number of original heterosexual pairs whose ovarial development of the female member is modified in male direction. The observation of free-martin ovaries in the heterosexual combination proves increasingly the validity of this idea. Practically, however, it was impossible to identify the original sex of these animals because the medullary tissue of their gonads was too well organized and the amount of cortical cells covering the crest was sometimes too small. It is not reasonable to classify certain animals into genetical females only because they have a few cortical germ cells attached to the surface of the gonads. These facts, however, do not mean that reversal of sex has occurred in the genetical females. The detail of this problem shall be discussed in a later chapter.

(C) ***H. retardatus* × *H. lichenatus*** The embryos as well as the larvae of *H. lichenatus* are a little smaller than those of *H. retardatus*. In color *H. lichenatus* is dark brown while *H. retardatus* is dark green. When united in parabiotic twins, the growth of the small *lichenatus* member is frequently retarded conspicuously. In such pairs the food is taken mainly by the large *retardatus* member which carries the *lichenatus* co-twin like a parasite hanging on the ventral abdomen. The metamorphosis occurs almost simultaneously in both members though its process requires a longer time than in single controls. The sex distribution among 55 pairs was as follows;

$$10\,♀\,♀ + 8\,(\textit{lichenatus}\,♀ \times \textit{retardatus}\,♂) + 7\,(\textit{lichenatus}\,♂ \times \textit{retardatus}\,♀) + 30\,♂\,♂ = 55$$

Again in this combination, the double male pairs appeared conspicuously in excess.

(*i*) ♀♀ *pairs.* The differentiation of ovaries in 10 pairs of double female combination is not different in each of both members from that of single controls. The growth of germ cells proceeds normally for the age.

(*ii*) *H. lichenatus* ♀ ×*H. retardatus* ♂. In 4 pairs out of 8 *lichenatus* ♀ ×*retardatus* ♂ combination, neither of the twinmates exhibits any sign of interference in sex differentiation. The ovaries of the *lichenatus* females are normal in every respect and are provided with wide ovarian cavities and healthy growing cortical germ cells of considerable size (Fig. 44). Equally well differentiated are the testes of the *retardatus* males (Fig. 43). In the other 4 pairs the retardation in the female differentiation is not severe. Though their central cavities are always closed by the proliferation of rete cells the ovaries contain a pretty good number of egg cells with large vesicular nuclei (Fig. 45). In the testes of the *retardatus* males, on the other hand, the medullary tissue is, in general, well organized but the crest of the gonad is covered, for various lengths, by cortical germ layer which is in the process of degeneration (Fig. 46). In some sections the degenerating cortical germ cells exceed the healthy medullary germ cells in number. Obviously the present animals are genetical males and the cortex which covers the testes originates from that which has developed in the rudimentary testes. In the normal development of male of *H. retardatus* the cortical germ layer disappears as a rule at the time of metamorphosis and the gonad takes distinct testicular structure. When united with *lichenatus* female, the cortex of its ovaries inhibits more or less the medullary differentiation of the *retardatus* male, which permits the further development of cortex. The degenerating cortical germ cells are the remnant of the testicular cortex which indicates the exaggerated characteristics of semi-differentiated sex-differentiation of the *retardatus* male.

(*iii*) *H. lichenatus* ♂ ×*H. retardatus* ♀. In the reciprocal heterosexual combination, the testes of the *lichenatus* male are rather small in size and the germ cells are rather rare, but the development of the ovaries in the *retardatus* female suffered severe modification. For instance, in pairs illustrated in Figs. 47–51 and 52–54, the central cavities are reduced to narrow slit-like spaces, which stretch only for a few intervals of sections at the anterior end of the gonad (Figs. 49, 53);

the major part of the medulla becomes solid by the proliferation of rete cells. The germ cells are limited in the anterior one-third strictly to the cortical layer (Figs. 48, 54), while in the middle region they are intermingled with medullary rete cells (Figs. 50, 51). In the posterior one-third, the female characteristics of the gonad are again dominantly expressed by the development of cortex. Compared to the free-martin ovaries of *lichenatus* female united with *lichenatus* male, the differentiation of the medullary tissue is, in general, less conspicuous and the number of the medullary germ cells is relatively small. In this respect the ovaries of *retardatus* are more resistant to the testes of *lichenatus*, but the cortical germ cells in the free-martin ovary of *retardatus* have never grown into large egg cells. Evidence is again given that the suppression of the egg cell formation in the cortex and the development of medullary component are controlled by separate factors. In 3 pairs out of 7 *lichenatus* ♂ × *retardatus* ♀ combination, the ovaries show the structure of this type. In 2 other pairs, the ovaries are completely solid through their entire length, and the proliferation of rete cells is more conspicuous than in the 3 pairs just described, but no germ cells are enveloped in the medulla. Only in the last 2 pairs is the ovarial structure of the gonad dominant for a considerable length.

(*iv*) ♂♂ *pairs*. For convenience in description, 30 pairs of double male combination of *lichenatus* × *retardatus* are classified into three groups as follows. (a) In the 4 pairs of the first group *lichenatus* has always normal testes of intermediate size, while the testes of *retardatus* are covered by various amounts of cortical tissue (Figs. 59, 60). The germ cells in the cortex always present more or less the degenerative appearance and sometimes the border of the gonad is fringed by irregulary scattered small germ cells (Fig. 59). The medulla in the other level of the same gonad, on the other hand, differentiates very well and in the major part the cross sections show normal testicular structure (Fig. 58). (b) In most of the 22 pairs comprising the second group both *lichenatus* and *retardatus* have normal testes. Sometimes a very few cortical germ cells are left attached to the crest of the gonad, but the medulla is well organized for the age and constins a considerable number of germ cells. In a certain number of pairs of this group, on the other hand, the gonads of either *lichenatus* or *retardatus* are conspicuously small in size and the germ cells are rather rare. In some animals the entire gonad is

reduced to a nearly sterile condition; only a small number of germ cells are found scattered in the compact mass of small rete cells. (c) The remaining 4 pairs comprise the extreme cases of the second group. In them one of the twin mates has become completely sterile and the gonads are represented by peritoneal folds hanging at both sides of the dorsal mesentry (Figs. 61, 62). In 2 cases the sterile member is *retardatus* and in the other 2 cases, *lichenatus*. In cross section the rete cells forming the vestigial gonad are more numerous in *retardatus* than in *lichenatus*.

It may be an important fact that the gonads of *retardatus* in double male pairs of *lichenatus*×*retardatus* combination show a continuous variation between hermaphroditic structure and complete sterile condition. The testes of *retardatus* of the first 4 pairs above mentioned show a conspicuous hermaphroditic tendency in respect to their rudimentary cortical tissue. Indeed there remains some doubt whether these 4 pairs are originally of heterosexual combinations. They were classified provisionally as males because in all of them the medulla are equally well differentiated as in normal males. Besides it has already been mentioned that the testes of *retardatus* male united with *lichenatus* female is capped with similar cortical covering. It is noticeable, on the other hand, that the fundamental structural difference is very slight between these hermaphroditic glands and the modified ovaries of *retardatus* female grafted with *lichenatus* male. The essential difference is only in the existence of the rudimentary central cavity and the amount of cortical tissue attached to the surface of the gonads. Therefore it is very difficult, in practise, to classify all experimental animals precisely as male and female. The classification was made only as a matter of convenience. The sterile gonads comprise other extreme cases of continuous variation. In these cases, too, the original sex of the animals is not certain. But as mentioned above the testes in unisexual pairs of *lichenatus*×*retardatus* are sometimes very small. They have but a few germ cells in the medulla and the cross sections occasionally resemble the sterile gonads. Consequently it was convenient to classify them as males.

The hermaphroditic tendency in the testes of *lichenatus* is far less conspicuous. In none of them are female characteristics observed; their structure varies only from normal condition to sterile.

(D) ***Summary of experiment I.***

The results obtained by observing heterogenous as well as homogenous parabiotic twins of *H. retardatus* and *H. lichenatus* prove clearly that the inductive influence exerted by the *lichenatus* male of the differentiated species, upon the development of varies in its female co-twin is far more severe than the influence of *retardatus* male of the semi-differentiated race. While the testes of *retardatus* male exert little or sometimes no influence at all, the testes of *lichenatus* inhibit the ovarial differentiation in its female co-twin in various degrees. The first step of retardation appears in the suppression of the formation of the ovarian cavity. This is followed by the subsequent migration of mesonephric blastema in the gonads and the more proximal germ cells become enveloped in the medulla. The development of medulla in the modified ovaries is not so prominent generally as in frogs, but it is far more pronounced than in the case of *Ambylstoma* and in some sections the gonad takes more or less the complete structure of young testes. This fact must be considered in intimate relation with the deviation of sex ratio in the pairs combined with *lichenatus*. Besides there is a continuous variation between the structure of free-martin ovaries and the testes with cortical germ layer, which are observed in some of the double male pairs. On the other hand, however, there are a certain number of nearly or completely sterile animals which are produced clearly by the elimination of germ cells. These variations of structure in the gonads of parabiotic twins suggest that the further development of the free-martin ovaries would take various courses according to the incidental condition of the experimental pairs. Some of them will become completely sterile and others hermaphrodite, and it is not always unreasonable to suppose that the gonads of certain females would take at least the structure of young testes. But a hasty conclusion upon the problem of sex reversal in parobiotic twins of salamanders should be avoided at the present stage of experiments.

In the second series of experiments the above described results were compared with the sex modification induced by the administration of postpuberol sex hormone of male and female.

IV. Experiment 2. Administration of sex hormone upon larvae of *H. retardatus*

The results obtained by the administration of sex hormones upon the larvae of *H. retardatus* have already been reported in the writer's

previous papers (Hanaoka, 1941, a, b). An outline of the experiments, however, will be repeated for comparison with the results with parabiotic twins.

The female sex hormone used in the present experiments was pelain (Tōkyo Zōkiyaku-kenkyusho). It is a preparation of crystalline oesterone containing 500 m. u. in every cubic centimeter. The male hormone was perandren (Ciba), which contains 5 m. g. of testosterone propionate dissolved in one cubic centimeter of sesame oil.

(A) ***Influence of follicular hormone added to the aquarium water.*** Padoa reported in 1936 and 1938 that the water solution of follicular hormone, in which the tadpoles of *Rana esculenta* were raised, induced male sex differentiation in the great majority of experimental animals. In the first group of the present experiment the same experiment was repeated to reexamine whether the sex reversal in *H. retardatus* will be actually induced by female sex hormone as it is reported in *R. esculenta.*

The larvae were raised in water solution of pelanin which contains 5 m. u. of follicular hormone per cubic centimeter. The experiment was begun with larvae of 21 mm in total length, in which the primordial gonads had just appeared. All of them were preserved a few days after metamorphosis. Among 18 animals which were available at the time of preservation the following sex ratio was obtained.

$$11\,♂ + 4\,♂\ \text{(with vestigial ovarian cavity)} + 3\,♀ = 18$$

(*i*) The microscopic observation of the gonads in 11 males proved that they are normal in general structure as well as in size. Compared to the testes of control animals, however, the rete cells in some of these testes are extremely rare and the whole gonads consist of a large number of germ cells enveloped in peritoneal epithelium. In the normal development of the testes at the time of metamorphosis, the germ cells are evenly intermingled with a pretty large number of rete cells and rudimentary spermatic tubules appear in the medulla.

(*ii*) In 4 animals the gonads have narrow slit-like cavities at several intervals of the section. These gonads, however, cannot be considered as ovaries merely because they have central cavities; there

are continuous variations of structure between these gonads and some of the testes described in the preceding section.

(*iii*) The gonads of the remaining 3 animals have central cavities of considerable width, but these cavities never exceed one-third of that of the normal ones.

Sorry to say, the number of animals used in the present experiment was not large enough to yield any final conclusion. Microscopical observation, however, shows that the follicular hormone added to the aquarium water obviously interfers with the normal differentiation of cortex in the ovaries. But this is not followed by the development of medullary component; the gonad become mechanically solid as a result of the rapid multiplication of germ cells. This calls for caution against the conclusion that the reversal of sex may be actually induced by the follicular hormone.

(B) *Influence of injection of follicular hormone.* In the second experiment the animals received injections of 5 m. u. of pelanin at intervals of 5–7 days over periods of 3–5 months. They are divided into three groups. In the first group the experiment was begun with larvae of 21 mm in total length. In the second group the larvae were 35 mm or more. At this stage of development the gonads of the genetical males are in their female phase of semi-differentiated sex differentiation and the gonads of both male and female take ovarian structure with a cavity in the center. In the third group the injection was begun about 2 weeks after metamorphosis. The sex differentiation in these animals is always complete. At autopsy it was found that the sex modification produced by the injection of follicular hormone differs according to the different age of the animals at the beginning of the experiment, and the longer the period of injection continues, the more pronounced is the influence upon the development of the gonads.

(*i*) Sex modification in 120 animals which received the injection from the beginning of sex differentiation was as follows.

(*a*) *Before or during metamorphosis.* In 30 animals which were preserved before or during the progress of metamorphosis, the gonads are a little smaller than normal ones. Microscopical observation confirmed that they are normal in every detail; the ovaries have wide ovarian cavities in the center and a healthy growing cortical germ layer, containing many germ cells of small size. In

the testes the medulla is well organized for the age. The sex distribution was as follows.

$$16♂ + 14♀ = 30$$

These results clearly show that in *H. retardatus* the follicular hormones exert little influence upon the morphological differentiation of gonads before the stage of metamorphosis. The injection cannot inhibit the development of the medulla of the genetical males or accelerate the differentiation of female germ cells in the ovaries.

(*b*) *One or one and half months after metamorphosis.* 34 animals were preserved one or one and half months after metamorphosis. Among them 23 were female which have normal ovaries with wide central cavities. Germ cell differentiation in these ovaries, however, had proceeded considerably ahead of normal ones; they are always provided with large vesicular nuclei and granular protoplasmic substance. 8 animals had testes normally differentiated for the age. In the remaining 3, which are preseumably genetical males, the medullary tissue is well organized and the gonad is completely solid through the entire length, but intermingling with normal male germ cells of small size, one can find various numbers of abnormally large germ cells, which morphologically resemble in every detail the female germ cells in the ovaries. These gonads take, therefore, mixed structure of testis and ovary.

(*c*) *Two or more months after metamorphosis.* In 56 animals, the injection was continued until two or four months after metamorphosis. Sex distribution among them was as follows.

2 months after metamorphosis .	2♂ + 5⚥ (with mosaic gonads)	+12♀ = 19
3 months after metamorphosis .	3♂ + 2⚥ (with mosaic gonads)	+ 7♀ = 12
4 months after metamorphosis .	7♂ + 4⚥ (with mosaic gonads)	+14♀ = 25
Total	12♂ + 11⚥	+33♀ = 56

The injection exerts the more conspicuous influence upon the development of the ovaries, and the older the animals become, the more pronounced is the acceleration of germ cell differentiatinn observed in the ovaries. At the end of four months after metamorphosis, the oocytes become as large as the eggs observed in the females of the third spring.

In the mosaic gonads the egg cells have increased in size as well as in number. It is interesting to note that these mosaic gonads are occasionally covered by a greater or less amount of ovarial cortex and apparently many of the egg cells originate from the cortical germ cells. In other animals the cortical layer covering the mosaic gonad becomes rather thick and the cross sections occasionally show the structure of an ovary. The medulla in these cases is reduced to a nearly sterile condition, containing only a few germ cells or none at all. In extreme cases when a large number of egg cells are crowded, their cross sections appear like those of an ovary, whose cavity is occluded by the enlarged egg cells. But in none of the mosaic gonads is an ovarian cavity ever formed, and in the major portion of the gonads the development of the medullary component is dominant.

(*ii*) In the second group, the animals were 35 mm or more in total length when the injection was begun. The dosage and the intervals of the injection are similar to those of the first group. Sex distribution among 25 animals is as follows.

1 month after metamorphosis .	3♂	+ 8♀ = 11
2 months after metamorphosis .	3♂ +1⚥ (with mosaic gonads)	+10♀ = 14
Total	6♂ +1⚥	+18♀ = 25

Microscopical observation confirmed that the sex-differentiation in the female is accelerated to the same degree as in the first group. The ovaries are always provided with distinct ovaran cavities and well differentiated cortex containing many large egg cells. In the male, on the contrary, the sex modification is found only in the development of secondary sex characters; the testes are nearly normal except in one only, in which a small number of egg cells are formed.

(*iii*) In the third group, the animals received injections only after metamorphosis. The sex distribution was as follows.

2 months after metamorphosis	6♂+ 7♀ = 13
4 months after metamorphosis	10♂+ 7♀ = 17
Total	16♂ +14♀ = 30

Again the ovaries in the injected females show pronounced acceleration in germ cell differentiation as in the two preceding groups.

In the male on the other hand, no influence is detectable on the development of the testes, although they are slightly smaller in size than normal ones.

The results obtained by the injection of follicular hormone may be summarized as follows: (1) Before the stage of metamorphosis, the injection of oestrogenic substance exerts no influence upon the sex differentiation. The development of the gonads proceeds in both sexes as in normal cases. (2) After metamorphosis, however, similar injection stimulates the ovaries to precocious differenciation and large egg cells are formed in the young ovaries. (3) In the male, on the other hand, the injection produces mixed gonad if the treatment is begun from the beginning of the gonad development. Egg cells which appear in the mixed gonad grow nearly as large as those in the female. (4) After the morphological differentiation of the gonad is complete the injections exert no influence on the development of the testes at least in the doses used in the present experiment.

(C) ***Influence of injection of male sex hormone.*** In the last series of the present experiments, sex modification produced by the injection of male sex hormone was examined in comparison with the results obtained with the heterosexual parabiotic twins. At the beginning of the experiment, 58 animals of the first group averaged 21 mm of overall length. In 16 animals of the second group, the average length was 34 mm. The injections, each of which consists of 50 γ of synthetic testoterone-propionate dissolved in 0.01 cc of sesame oil, are made at intervals of 10 days. The oil drops which accumulate by the successive injections, remain in the body cavity for a long time and more or less retard the growth of the experimental animals. The first autopsy was made immediately after metamorphosis, and the last one about 3 months thereafter. Since, however, no fundamental difference is detected among the groups of different ages, all will be described together.

The injection of testosterone-propionate produced in both male and female diverse modification of sex differentiation and in many cases the gonads became abnormal to so conspicuous a degree that it was impossible to identify the sex of the experimental animals in the usual manner. Consequently the sex distribution could not be ascertained in the present experiment. Microscopical observation of the modified gonads yielded the following information.

(*i*) *Elimination of germ cells.* In the previous experiment on *H. retardatus* (Hanaoka, 1939), in which the pieces of adult testes were successively transplanted in addition to hyophyses, it was observed that certain germ cells at the peripheral region become degenerated and were ultimately eliminated from the gonad. The injection of testosterone-propionate causes similar elimination of germ cells in a more conspicuous degree and in many animals the gonads are reduced to mere folds of peritoneal epithelium. In animals with gonads of this type, the sex cannot be distinguished. In general the gonads of both sides are similarly modified.

(*ii*) *The differentiation of vasa efferentia in relation to the development of the testes.* The differentiation of testis in amphibians is always preceded by the rapid migration of a large amount of mesonephric blastema or rete cells which enter the medulla of the gonad and envelop the germ cells of the more proximal region. In *H. retardatus* when injected with testicular hormone, the migration of rete cells occurs in many cases rather violently. Curiously enough, however, these rete cells never enter the medulla of the gonad, but only form the vasa efferentia which connect the germinal ridges and mesonephros. In this respect, the findings obtained by the injection of male hormone are in remarkable contrast with those observed in parabiotic twins. The modification of ovarial development, exhibited by the female member of the heterosexual parabiotic twins is characterized by the proliferation of rete cells, which occlude the ovarian cavity. Under favourable condition these rete cells induce the migration of germs cells from the cortex into the medulla where they are organized into rudimental spermatic tubules. But no sign of precocious development of accessory sex organs is observable, at least at the time of metamorphosis. When injected with testosterone-propionate the rete cells, which form loose cellular strands between the gonads and mesonephros, differentiate precociously into vasa efferentia, but this process proceeds quite independently of the development of testicular medulla. In some animals a few germ cells are found strayed amongst the rete cells and the cross section at the end of rudimentary vasa efferentia gives the appearance of a small testis. But this is always limited to a small area and the major part of the gonad remains sterile.

In other animals the rudimentary vasa efferentia acquire tubular structure and form a distinct duct. But this tubular structure cannot

be traced within the confines of the mesonephros and the connection of the gonads and wolfian duct is always incomplete. In still other animals the lumen of the tubular vasa efferentia becomes enormously wide. In such cases, the wall of the duct is extremely thin and consists of one layer of flat cells. In less completely modified animals in which the ovarian structure of the gonad is maintained, the lumen of the duct continues to the cavity of the ovarian sac. When the elimination of germ cells occurs in a conspicuous degree in a gonad of this type, the gonad is represented only by a small number of degenerative germ cells which are scattered on the thin wall of the efferential duct.

(*iii*) *Differentiation of female germ cells.* While the majority of the germ cells are degenerated and cast off from the gonad by the influence of male sex hormone, some germ cells, which survived one month or longer after metamorphosis, grow into egg cells of considerable size. These egg cells resemble in size as well as in structure those observed in the female treated with follicular hormone; they are always provided with granular plasmic substance and large vesicular nuclei. The gonads, which contain such large egg cells, in many cases take distinct ovarian structure with a wide ovarian cavity in the center, but the number of germ cells is rather small and many of them are in the process of degeneration. The male sex hormone, therefore exerts stimulative influences of opposite direction upon the differentiation of germ cells and the accessory sex organ in one and the same individual. The appearance of large egg cells is not always limited to the gonads with ovarial structure. Equally large germ cells appeared often in the gonad with testicular structure intermingling with small ones.

As described above the injection of testosterone-propionate produces diverse modifications of sex differentiation and the abnormality of the gonad becomes so conspicuous that it is practically impossible to classify the affected gonad according to the morphological structure. The injection exerts great influence, but obviously, the sex modification does not proceed in the direction of male differentiation. In this respect the results of the present experiment differ very much from the findings of the previous investigators obtained with frogs and salamanders. The detailed discussion of the problem, however, well be postponed to the next chapter.

V. Discussion

(*i*) *Quantiative difference of inductive activity in H. retardatus and H. lichenatus.* In regard to the quantitative difference of inhibitory inductive activity in *Amblystoma tigrinum* and *A. maculatum*, the latter belonging to distinct sex races, Witschi (1933, 1936, 1937) obtained a clear conclusion in his experiments with heterogenous parabiotic twins. According to him: "all three types of the male used in these experiments send into the twin female a principle, which interfers with the ovarial development. However, inhibition shows great qauntitative differences. It is high if produced by the *tigrinum* male, which reduces the gonad of *maculatum* female into mere sterile or nearly sterile rudiment. It is less if caused by a *maculatum* male of differentiated race which prevents ovarial differentiation completely in *maculatum* females and incompletely in *tigrinum* females. And it is least if coming from a *maculatum* male of semidifferentiated race, which interferes with the ovarial development only moderately if the twin is a *maculatum* female and not appreciably at all, if the twin is a *tigrinum* female." He illustrated these quantitative relations of inductive activities by curves which have different hights of abscissae.

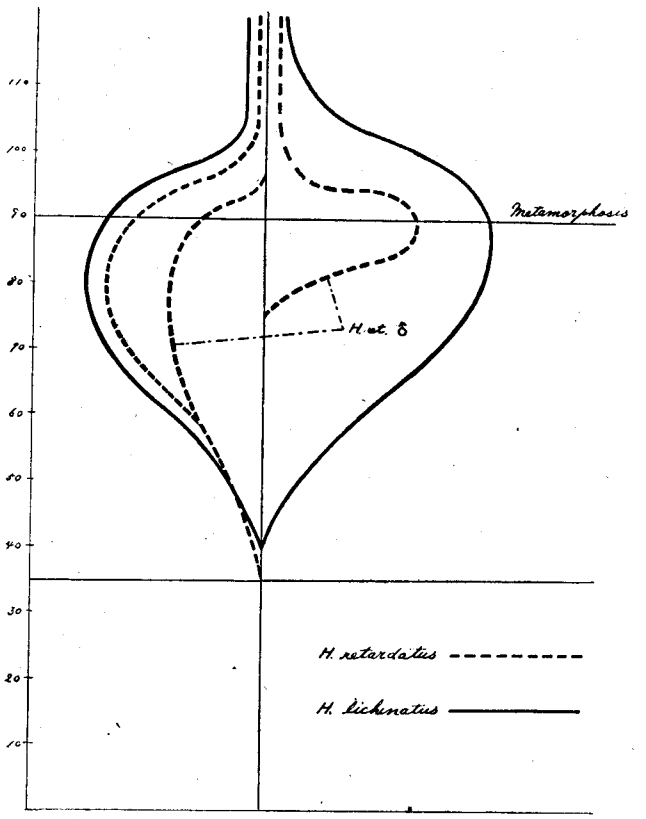
The results obtained in the present experiment on *H. retardatus* and *H. lichenatus* agree in general with the findings of Witschi on American salamanders. The male of both *retardatus* and *lichenatus* interfers with the ovarial development of the female co-twin, but the inhibition is high if caused by the *lichenatus* male and less if produced by the *retardatus* male. In most of the *retardatus* females grafted with the male of *retardatus*, the development of the ovaries proceeds as in single controls. Only in 3 cases out of 20 pairs is a moderate retardation of ovarial development exhibited. In the similar homogenous combination of *lichenatus*, on the contrary, the retardation of ovarial development is more complete. Although the ovaries are provided with thick cortical germ layer containing a considerable number of healthy growing oocytes, their central cavities are in most cases occluded through almost the entire length by the proliferation of various numbers of unorganized rete cells, and a pretty good number of germ cells are included in the medulla. In heterogenous pairs of *retardatus* ♂ ×*lichenatus* ♀ , the occlusion of the cavities in *lichenatus* ovaries, is more complete than in *retardatus* female of

homogenous combination. In this respect the ovary of *retardatus* female is more antagonistic to the medulla than that of *lichenatus*. On the other hand, the differentiation of germ cells in the ovary of *lichenatus* proceeds occasionally far ahead of normal ones. The structural differentiation of the ovary and the differentiation of germ cells are controlled, therefore, by different factors. In the reciprocal combination of *retardatus* ♀ × *lichenatus* ♂, the modification of the ovarial development is the largest. In none of the *retardatus* females are large egg cells observed in the ovary and the larger number of germ cells are included in the medulla.

These relationships between the different inductive activities in *H. retardatus* and *H. lichenatus* can be illustrated, as in *Amblystoma*, by the diagramatic curves in text-figure 2. The male curves have in general higher abscissae than those of females which indicate the dominant inhibitive action of medullary induction. The curve of *retardatus* male is twofold; during the early period of larval development it is mainly of cortical nature. This disappears shortly before the time of metamorphosis and medullary induction then becomes predominant. The stronger inhibitory action of *lichenatus* male is expressed by the abscissa higher than that of *retardatus* male. The heights of female curves are determined indirectly from the intensity of resistance to the inhibitive action of *retardatus* as well as that of *lichenatus* males. In the present forms the quantitative difference of cortical inductive activities seems to be very small.

(*ii*) *Will sex reversal occur in free-martin ovaries?* In 1925 Burns reported on *A. punctatum* (=*maculatum*) that members of a pair of parabiotic twins were invariably of the same sex; the development of the gonad was apparently normal and no indication of intersexual condition was observed. The absence of heterosexual combination, he proposed to interpret by the assumption that complete sex reversal occurs in the slower developing member of the twin-mates. The faster developing member dominates the sex differentiation in "all or none" type of reaction and there is no prepotency constantly favouring one or the other sex. This report of Burns, however, was later submitted to a partial correction by Humphrey (1932), who had been given the opportunity to reexamine a part of Burns' slides and found among 57 pairs, 6 that should be classified as undifferentiated and 8 that were heterosexual. In the succeeding experiment on *A. tigrinum*, Burns (1930, 1931, 1935) himself found

that there were a certain number of heterosexual pairs, but he still maintained the interpretation that the modified ovaries in the female member were in the process of sex reversal and "the most advanced



Text-fig. 2. Diagramm representing the difference of quantity in the release of inductive substance in *H. retardatus* and *H. lichenatus*. Explanation in the text.

cases approximate or attain virtual transformation into rudimentary testes, which are believed capable of regeneration into functional organs."

Humphrey (1936, a, b) also published two reports on homogenous twins of *A. tigrinum* and *A. maculatum*. His experiments were

undertaken to ascertain the correctness of his conclusion regarding sex reversal which he obtained previously by the transplantation of preprimordia of one gonad into another embryo. But his conclusion was that "the writers (Humphrey 1936a) previous conclusion that ovaries of *Amblystoma tigrinum* kept continuously under the influence of a testis from embryonic life onward may undergo reversal to testes is fully confirmed."

The interpretation of these two authors, however, shows unreconcilable conflict with the opinion of Witschi. He claimed that in the female of heterosexual parabiotic twins of newts and salamanders "only feeble attempts at sex reversal are made which never proceed beyond the first steps of testicular development" (Witschi, 1934). For instances in the pairs of *A. tigrinum* and *axolotl* he observed that "even at the age of 13 months, the medulla is still found in a extremely rudimentary condition, containing few if any germ cells and no distinct organization of seminal tubules," and "while these developments of modified ovaries represent unmistakably a step toward testicular differentiation, they do not reach any one of the stages seen in normal testicular development." He, therefore, hesitated to apply there the term sex reversal because "it is not at all certain, and in fact even probable that all free martin gonads of *Amblystoma* twins, which contain some gonia in medullary position would eventually differentiate into testes, if the animals were kept alive long enough." (Witschi, 1937). Consequently the problem is reduced to the question "of what becomes of the free-martin gonad at the approach and during the stage of maturity." Regarding this problem Witschi (1937) offered interesting data. He found in heterosexual pairs of *A. tigrinum*×*A. jeffersonianum* that the ovaries of the small and slow differentiating *jeffersonianum* female become perfectly sterile and have a tendency to disappear or to become reduced into simple ligaments. In the reciprocal heterosexual combination, on the other hand, the medullary induction in the testis of *jeffersonianum* male is suppressed by the cortical induction of the fast developing *tigrinum* female, which is immediately followed by the cortical development. The gonad assumes therefore partially ovarial structure. With the onset of sexual maturity, the cortical element as well as the medullary rudiment surviving in this gonad are stimulated to further differentiation and hermaphrodite glands are produced with matured spermatozoa and large eggs. Later on,

however, this morphological female (genetical male) transforms into a male; the ovarial structures degenerate and testicular ones develop instead. (Witschi 1937). In this case no ovary developed in the genetical male, but only transitory hermaphodite gland. Similar hermaphrodite gland with mature dspermatozoa and large pigmented eggs were observed in the female of *A. tigrinum*×*axolotl* twin at the age of 2½ years. In regard to the development of the hermaphrodite gland, however, one must consider, as Witschi pointed out, the fact that the constitution of the animals is a bisexual and not a purely male.

The problem of sex reversal in parabiotic twins must be considered from statistical as well as embryological point of view. In the present experiment the sex ratio in homogeneous parabiotic twins of *retardatus* was nearly normal but males in both heterogeneous and homogeneous twins of *lichenatus* appeared in considerable excess, i.e., the sex ratio in homogeneous pairs of *lichenatus* is 52♂ : 14♀, and in heterogeneous pairs 75♀ : 35♂. In single control of *retardatus* and *lichenatus*, nearly equal number of male and female always appear at the end of metamorphosis. It must be noticed further that although the mortality in the animals in the present experiment is pretty high in the several days following the operation, no evidence could be found that the deviation of the sex ratio should be ascribed to selective mortality. The losses occur only in the early period of the embryonal development before the primordial gonads become visible in cross sections. The deviation of sex ratio just mentioned gives, at first glance, the impression that in *Hynobius* the reversal of sex from female to male has been induced by the strong inductive action of *lichenatus* male. But for the final conclusion the embryological examination of the modified gonad must be taken into consideration.

The microscopical observation shows that the testes of some double male combination are covered by varying amount of cortical germ cell layer. In some animals the cortex is represented only by a few germ cells attached to the crest of the gonads but in others nearly half of the entire gonad is capped by the cortex. On the other hand, in certain females of both *retardatus* and *lichenatus*, which are combined with *lichenatus* males, the medullary component of the modified ovaries well differentiated and the cross section of the severely affected ovaries assume occasionally similar structure to the

testis. These ovaries are distinguished from the testes only by the presence of rudimentary ovarian cavity, and there is a continuous morphological variation among the modified ovaries with solid medulla and the testes with cortical germ cell layer, which frequently appear in some of the double male pairs. Therefore the critical point by which the experimental animals are divided distinctly into male and female cannot be determined and the classification was made only provisionally. The double male pairs in the present experiment may have contained a certain number of pairs which are originally of heterosexual combination and the excess of the males is mainly due to the appearance of intersexual animals which were classified into males. Consequently the conclusion of the problem depends to the question of what becomes of these intersexual gonads. Sorry, however, the observation in the present experiment was made only for a few weeks after metamorphosis. It is not impossible that in some of these intersexual gonads the cortical germ cells are completely eliminated and the medullary component differentiates into purely testicular structure. But it is more probable that, by the action of post puberal sex hormone, both the cortical and the medullary components of the intersexual gonad are stimulated to further differentiation and ultimately hermaphrodite glands are produced as in the case of *Amblystoma*. The results obtained by the injection of sex hormone, suggest the more probable occurrence of the latter case. In regard to the final development of the free-martin ovaries, it must be also noticed that in some animals germ cells are eliminated and the gonads are reduced to nearly or completely sterile folds, and when male sex hormone is administrated this elimination of germ cells occurs to an extreme degree. Anyhow, sex reversal in the sense that normal testes are produced in genetical females does not seem to take place in *Hynobius*.

Of the occurrence of sex reversal in opposite direction, i.e. from male to female, there is no probability. In some males of *lichenatus* ×*reardatus* the differentiation of testes is slightly modified and a small amount of cortical germ layer develops on the surface of embryonal testes, but the structure of the entire gonad is far different from that of an ovary.

(*iii*) *Appearance of female germ cells in double male pairs of H. retardatus.* In some males of *H. retardatus* it was found that the testes contain varying numbers of female germ cells intermingled

with normal small germ cells. In 3 cases they appeared in both members of double male twins and in 1 case in the male of heterosexual combination. The differentiation of these female germ cells proceeded always ahead of normal ones in the ovary. They were provided with large vesicular nuclei and granular protoplasmic substances. The gonads containing these germ cells take the structure of a mixed gonad. The gonads of this type were previously observed in the present writer's experiment on *H. retardatus*, in which testicular pieces were transplanted in addition to hypophyses. At that time they were inferred to be ovaries, whose differentiation was modified by the influence of maturation hormone of testis and hypophysis. In the present experiment, the female germ cells appeared in genetical males. Embryological observation as well as the experimental studies on *H. retardatus* shows obviously that they have a strong tendency toward hermaphroditism. But just now the writer finds himself unable to offer any proper explanation why female germ cells appear in the testes of double male pairs of parabiotic twin. Some unknown physiological condition which is produced by the parabiotic combination must have disturbed the balance of cortico-medullary antagonism of the male differentiation. But no further information was elicited by the present experiment.

(*iv*) *Sex modification produced by the administration of sex hormone.* (A) Masculinizing effect of follicular hormone. The incompatibility of Padoa's hypothesis for the masculinizing effect of follicular hormone was pointed out in the writer's previous paper (Hanaoka 1941a). In *H. retardatus* no masculinizing effect is observed when the follicular hormone is added to aquarium water. Obviously the ovarian cavities show a tendency to be occluded, but that is due mainly to the abnormal multiplication of germ cells and no definite differentiation of testicular tissue such as seminal tubules are observed. Normal differentiation of testis is always preceded by the migration of rete cells into the ovarian cavity and the germ cells become enveloped within them. Thus the result obtained by the present experiment is distinctly different from that of Padoa. Probably this is not due to the different materials of the two experiments. A similar experiments was performed adding follicular hormone to aquarium water on a semi-differentiated race of *Rana temporaria* (Unpublished). The dose of the hormone administrated was exactly the same as in the case of *H. retardatus*. Microscopical observation of the resultant

animals, however, showed that the hermaphroditic tendency of the male differentiation becomes more pronounced and the female germ cells in the larval ovary of the genetical male grow larger than in control animals, but the transformation of ovary into testis begins as in normal ones with the onset of metamorphosis. Horié (Unpublished) also, obtained similar results by the method of injection of the same substance. So far as the present writer is aware, the masculinizing effect of follicular hormone has been observed by no investigator except Padoa. Would he find it worth while to try again the similar experiment by the method of injection?

(B) Feminizing effect of follicular hormone. According to Burns (1938) repeated injection of small doses of esterone into larval *A. punctatum* causes transformation of testes into ovaries and the process of reversal takes the normal histological course of ovarial development. Foote (1940) also reported that estrogen appears to have a feminizing action upon testis. In *H. retardatus,* the injection of follicular hormone causes no transformation of testes into ovaries, but only the formation of intersexual gonads with a mixed structure of male and female characteristics.

Concerning the development of mixed gonads, two important facts must be noticed. First, while the medullary component is, in general, well organized for the age, the gonads are occasionally covered by varying amounts of cortical germ layer. In some gonads the development of the cortical tissue exceeds locally that of the medullary component and the cross sections through the affected part show the structure of modified ovaries with solid medulla. But in other animals only a small number of cortical cells are left attached to the terminal crest. At any rate, however, the cortical component in genetical males is stimulated by the influence of follicular hormone to differentiate excessively. The fact that the cortical development is favoured by the influence of follicular hormone is evidenced also by the experiments on *R. temporaria* mentioned in a preceding paragraph. Microscopical observation yields evidence that the majority of the egg cells in the mixed gonads are produced by these cortical germ cells whose differentiation is precociously accelerated. The appearance of the egg cells in the mixed gonads, however, is not restricted to the cortical germ layer. At times they are observed in the medullary region. In connection with this problem Witschi's comment about the occurrence of egg cells in the testes of adult frogs

and newts is very interesting. He says that "their unmistakable ovarial character may be due to a reactivation of female inductive properties in follicular cells which, as we recall, are of cortical origin. We have to consider that the sex cords carry not only germ cells but also their follicular and adhering stroma cells into the medulla" (Witschi, 1936). It is questionable in fact, whether the female inductive properties of follicular cells are reactivated by the treatment of follicular hormone, because if the injections are made only after metamorphosis when the embryonal follicular cells have completely lost their inductive properties and are organized into distinct medulla, the formation of egg cells in the testes is never induced. But if the injection is continued from the beginning of sex differentiation, the follicular cells may keep their inductive properties for a certain period after they have migrated into the medulla. Consequently some germ cells are maintained in the fields of female inductive activity and differentiate into large egg cells. Once the follicular cells have lost their female inductive properties, it seems to be difficult to reactivate them by the action of follicular hormone, at least by the dosage used in the present experiment.

The second important fact in the development of mixed gonads is the time relationship of the appearance of egg cells. Before the time of metamorphosis the injection of follicular hormone exerts no appreciable influence on the differentiation of testes nor of ovaries. The growth of egg cells in the ovaries proceeds as in control females and the medulla of the testes are in general normally organized. The acceleration of growth of egg cells becomes remarkable only one or two weeks after metamorphosis and in parallel with this, the structure of the mixed gonad become distinct. Before metamorphosis the germ cells have not yet entered a stage capable of reaction to the follicular hormone. In this respect the action of follicular hormone differs very much from that of Witschi's cortexin. Cortexin acts as an inductive substance which inhibits the development of medulla during the course of morphological differentiation of the gonads. It disappears gradually as the animals approach maturity. The follicular hormone, on the other hand, does not act directly as an inductor for the morphogenesis of the gonads, but as an accelerator of female germ cell differentiation. It is certain, however, that the cortical development is favored in some indirect way by the administration of follicular hormone. Therefore, if a higher dose of hormone is

given, it is not impossible to reverse the balance of the medulla-cortex antagonism, and as in the case of Burns (1938) and Foote (1940), the development of the medulla might be inhibited by favouring the development of the cortex.

(C) Influence of male sex hormone. Gallien (1937), Witschi & Crown (1938), Foote (1938), Foote & Witschi (1939) reported regarding several forms of frogs that the administration of male sex hormone caused transformation of ovaries into testes, and according to the personal information of Horié, a similar result is, also, observable in *R. temporaria* and *Bufo vulgaris*. In urodelans, however, Burns (1939) obtained a somewhat different result; the injection of male hormone in *A. punctatum* produced no sex transformation, but only intersexuality. Puckett (1939, 1940), on the other hand, repeatedly reported that in *R. catesbiana* the sex hormone alone had no morphological effect in sex differentiation, but the injection of sex hormone together with pituitary factor uniformly brought about the development of testes or ovaries in all of the experimental animals.

In *H. retardatus*, the injection of testosterone-propionate produces striking modification of sex differentiation, but the structure of the modified gonads is rather malformed and irregular in various individuals. The important characteristics of modification may be summarized in three points as follows; (a) precocious differentiation of female germ cells, (b) migration of rete cells and hypertrophy of the gonoducts and (c) elimination of germ cells from the gonad. Complexity of the gonads observed in the animals treated with male hormone is due mainly to the different combinations of these morphological changes.

At the present state of experiment the writer cannot find proper comment for the stimulative action of male sex hormone upon the differentiation of female germ cells. As a matter of fact, however, the acceleration of egg differentiation induced by the male hormone is obserxed in *H. retardatus* (Hanaoka, 1939) as well as in *H. lichenatus* (Uchida and Hanaoka, 1942. In press), which received the transplantation of testicular pieces. In both cases, the germ cells in the ovaries became occasionally as large as those found in the ovary treated with follicular hormone. In the present experiment, the injection of testosterone-propionate in most cases caused the elimination of germ cells, resulting in the reduction of gonads to nearly or completely sterile condition, but in certain animals the

germ cells were accelerated to differentiate into large egg cells. It is certain, therefore, that the male hormone exerts in *Hynobius* some influences which favors the differentiation of female germ cells. Witschi (1937), also, reported that "under the influence of maturity hormone both cortical and medullary rudiment were stimulated to further development and differentiation." The appearance of female germ cells in the presumably genetical male treated with male sex hormone may be due to the embryological characteristic that the larval testes take the structure of ovaries and the germ cells are kept in the field of cortical induction.

The elimination of cortical germ cells observed in the present injection experiment is not due to the peculiar toxic action of synthetic male hormone. It seems that this elimination of germ cells is caused by the disharmony of hormonal stimulus and inductive activity to which the germ cells are exposed. One has always to bear in mind that, when the injection of testosterone-propionate was begun, the gonads of *H. retardatus* were in the female phase of sex differentiation. By the injection of male sex hormone, the germ cells were exposed to the stimulations of opposite direction, in which case they were compelled to degenerate.

The third important feature of sex modification produced by the injection of male hormone is the rapid migration of rete cells. It is an interesting fact that these rete cells never enter the medulla of the gonad but produce only hypertrophy of the rudimentary efferential ducts, in both male and female. In other words, the rete cell migration, induced by the stimulation of male sex hormone, causes precocious differentiation of the male accessory sex organs, but it is not directly concerned with the morphological differentiation of gonads. There is an intimate relationship between the migration of rete cells and the reversal of sex from female to male. In frogs and toads, the degeneration of cortical germ layer is immediately followed by the compensatory immigration of rete cells into the medulla which in turn induces the migration of germ cells from cortex into medulla. In urodelans, on the other hand, the degeneration of cortex causes only the reduction of the entire gonad. The migration of rete cells in the medulla is induced only when some other stimulative influence is given. In this respect, the action of hypophysis plays an important rôle. Witschi claimed that the embryonal hypophysis is not concerned with the development of the gonads, but the matter seems to

be different with adult hypophysis. The fact that the hypophysial hormone induces the early migration of rete cells in the male and consequently the precocious differentiation of testes in *H. retardatus* is clearly demonstrated by the earlier experiments already mentioned (Hanaoka, 1939). In genetical females, however, the rete cell migration is not induced by the pituitary hormone alone but if the hypophysis is transplanted in addition to testis, the migration occurs and although many of the germ cells are eliminated from the gonad, some more proximal germ cells become included in the medulla and rudimentary testicular tissue is formed. Therefore, the development of medulla in genetical female is the result of cooperation of testicular and hypophyseal hormones. In addition it must be noticed that to induce the complete sex reversal in salamanders, the pituitary and testicular stimulation must be kept in a certain balanced condition. If they lose their balance, the medullary development becomes incomplete or the entire gonad becomes sterile by the elimination of germ cells. Therefore, the experiment by the method of injection of chemically extracted substances will produce more clearcut results because the stimulation given by the implantation of the secretory tissue is very irregular in its quantity and duration. In this respect Puckett's experiment on *R. catesbiana* is brilliant. Contrary to other forms of anurans, sex reveral in the bull-frog is induced only when the testicular hormone is given in addition to pituritary factor. It is obvious that in the present experiment on *H. retardatus*, the hormonal condition is extremely out of balance. Consequently the majority of the germ cells become eliminated and only the accessory sexual organs such as vasa efferentia are stimulated to further differentiation. The results of the present experiment must be always considered in comparison with the those of the transplantation experiment.

VI. Summary and Conclusion

(1) *Hynobius retardatus* belongs to a semi-differentiated form, and the male exhibits a conspicuous hermaphroditic tendency during the course of sex differentiation, while *H. lichenatus* is a true gonochorist free from hermaphroditic tendency.

(2) In the first series of the present experiment, the relative intensities of inductive action of the two forms above mentioned were

studied by the method of homogenous as well as heterogenous parabiosis. Microscopical observation proves that the medulla of *H. lichenatus* is more antagonistic to the cortex than the medulla of *H. retardatus*. While the testes of *H. retardatus* exerts little or no influence upon the development of the ovaries in its female co-twin, the testes of *H. lichenatus* prevent the ovarial differentiation in both *retardatus* and *lichenatus* in various degrees.

(3) The development of medullary tissue in the free-martin ovaries seems to be more advanced than in the case of *Amblystoma*, but no observation was made upon the final course of development of these hermaphrodite glands.

(4) In the second series, the influences of male and female sex hormone upon the sex differentiation of *H. retardatus* was examined in comparison with the results of parabiosis.

(5) The female sex hormone exerts certain influences upon the development of cortex but no appreciable morphological changes are observable before the stage of metamorphosis. After metamorphosis, however, the egg cell differentiation in the ovaries is accelerated far ahead of normal cases while in the testes, various numbers of egg cells appear intermingled with well differentiated medullary tissue.

(6) The male sex hormone in most cases causes elimination of germ cells reducing the entire gonad to nearly or entirely sterile condition. On the other hand. the accessory sex organs such as efferential duct, are stimulated to precocious differentiation by the rapid migration of rete cells.

(7) Rete cells which are induced to migrate from the mesonephros by the influence of male hormone, never enter the medulla of the gonad and consequently do not effect the morphological differentiation of the gonads. The medullary development in *Hynobius* is induced by some other factors. In this matter the cooperative action of adult hypophysis and testis seems to play an important rôle.

(8) Occasionally male sex hormone stimulates the germ cells of both made and female to differentiate into large egg cells.

(9) The data above described show clearly that the medullary and cortical inductive actions are quite different from those of sex hormones; the influences of the sex hormones on the development of the germ glands are always indirect.

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Explanation of Plates

PLATE IV

H. retardatus ♀ ×*H. retardatus* ♀

1. Normally differentiated ovary.
2. Ovary with large egg cells in the germ layer.
3. Ovary with closed cavity.

H. retardatus ♂ ×*H. retardatus* ♂ (a)

4. Mosaic gonad containing large egg cell.

H. retardatus ♂ ×*H. retardatus* ♂ (b)

5–6. Two sections through a mosaic gonad with cortical covering. In 6 one of the germ cells is differentiated into an egg cell.

PLATE V

7. Section through testicular part of the same gonad illustrated in 5–6.
8–10. Three sections of a mosaic gonad of the co-twin, showing normal testicular structure and egg cells.

H. retardatus ♂ ×*H. retardatus* ♂ (c)

11–12. Two sections of other mosaic gonad, showing normal testicular structure and egg cell. The egg cell illustrated in 12 is the largest one obtained in the present experiments.

H. retardatus ♂ ×*H. retardatus* ♀ (a)

13. Normally differentiated testis of the male.

PLATE VI

14. Normally differentiated ovary of the female co-twin.

H. retardatus ♂ ×*H. retardatus* ♀ (b)

15. Well differentiated testis of the male.
16–17. Two sections of the free-martin ovary of the female co-twin.

H. retardatus ♂ ×*H. retardatus* ♀ (c)

18. Testis of the male.
19–20. Two sections of the free-martin ovary of the female co-twin.

PLATE VII

H. retardatus ♂ ×*H. retardatus* ♀ (d)

21. Testis of the male.
22–24. Three sections of the free martin ovary of the female co-twin.

H. lichenatus ♀ ×*H. lichenatus* ♀

25. Normally differentiated ovary.
26. Ovary with precociously differentiated egg cells.

H. lichenatus ♂ ×*H. lichenatus* ♀ (a)

27. Testis of the male.
28. Section of the free-martin ovary of the female co-twin. The cortex is well developed but the medulla is occluded by rete cells.

PLATE VIII

29–30. Two sections of the same gonad illustrated in 28 showing large egg cells and rudimentary ovarian cavity.

H. lichenatus ♂ ×*H. lichenatus* ♀ (b)

31. Testis of the male.
32–35. Four sections of the free-martin ovary of the female co-twin. The medullary development shows a more advanced condition than in (a).

PLATE IX

H. lichenatus ♂ ×*H. lichnatus* ♀ (c)

36. Testis of the male.
37–39. Three sections of the free-martin ovary of the female co-twin. The figure in 37 shows nearly complete testicular structure but in 38 the medullary tissue is covered by degenerating cortical germ cells. In 39 the remnant of the cavity is noticeable in the terminal region.

H. lichenatus ♂ ×*H. lichenatus* ♀ (d)

40. Testis of the male.
41. Free-martin ovary with solid medulla.

H. lichenatus ♂ ×*H. lichenatus* ♂

42. Testis which is reduced to nearly sterile condition.

PLATE X

H. lichenatus ♀ ×*H. retardatus* ♂ (a)

43. Normally differentiated testis of *retardatus* male.
44. Ovary of *lichenatus* female with well differentiated egg cells.

H. lichenatus ♀ ×*H. retardatus* ♂ (b)

45. Ovary of *lichenatus* female with solid medulla.
46. Testis of *retardatus* male covered with cortical germ cells.

H. lichenatus ♂ ×*H. retardatus* ♀ (a)

47. Normally differentiated testis of *lichenatus*.
48–51. Four sections of free-martin ovary of retardatus female. 48 and 49 show the ovarial structure, while 50 and 51 the predominant development of medulla.

PLATE XI

H. lichenatus ♂ ×*H. retardatus* ♀ (b)

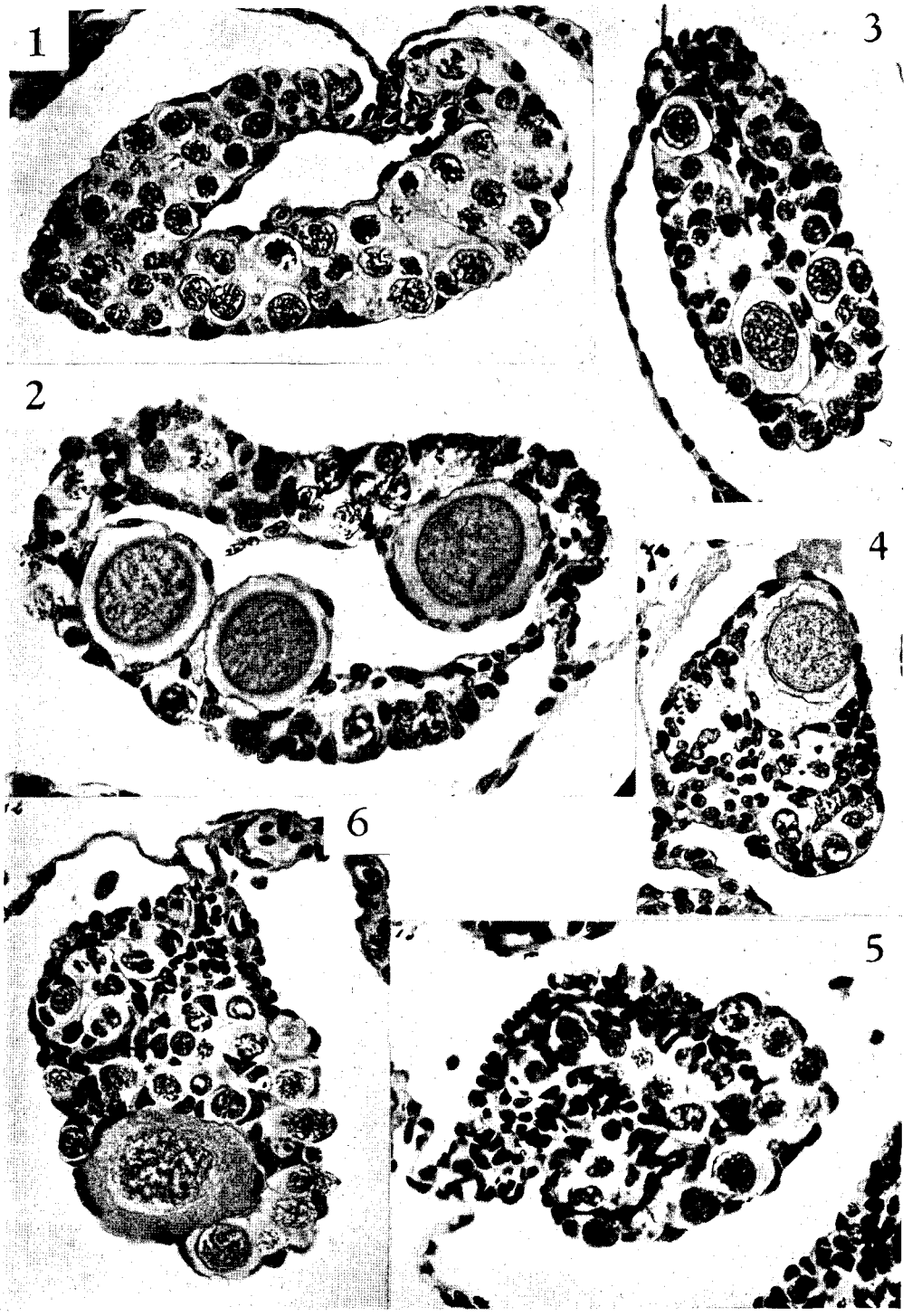
52. Testis of *lichenatus* male, germ cells rather rare.
53–54. Two sections of free-martin ovary of *retardatus* female. In 54 the cavity is completely occluded by a large amount of rete cells.

H. lichenatus ♂ ×*H. retardatus* ♀ (c)

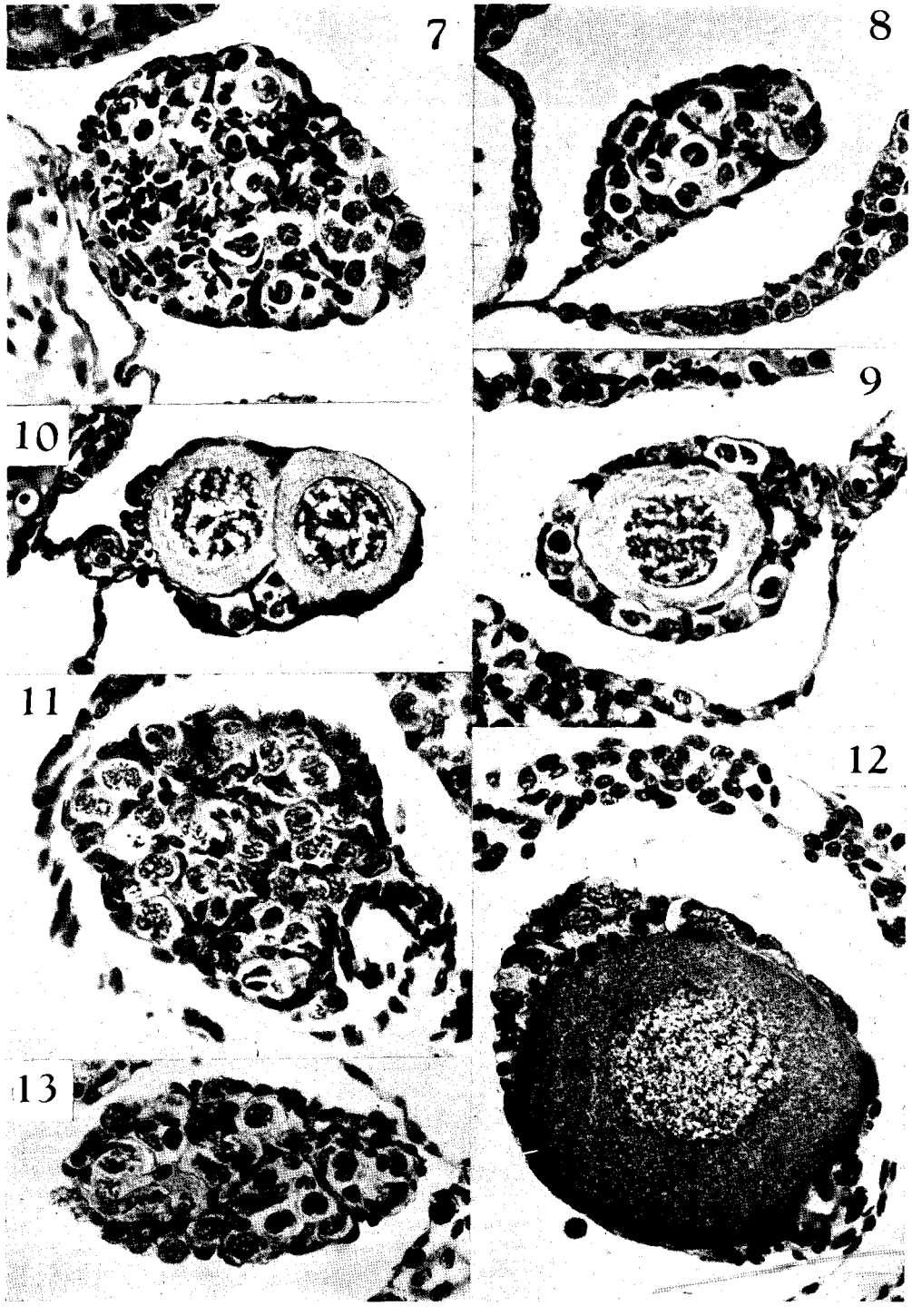
55. Testis of the *lichenatus* male.
56–57. Two sections of the free-martin ovary of *retardatus* female.

H. lichenatus ♂ ×*H retardatus* ♂ (a, b, c)

58–60. Three sections of testis of *retardatus*, showing the covering of irregularly arranged cortical germ cells.
61. Sterile gonad of *retardatus*.
62. Sterile gonad of *lichenatus*.



K. I. Hanaoka: Sex-differentiation in two Japanese salamanders



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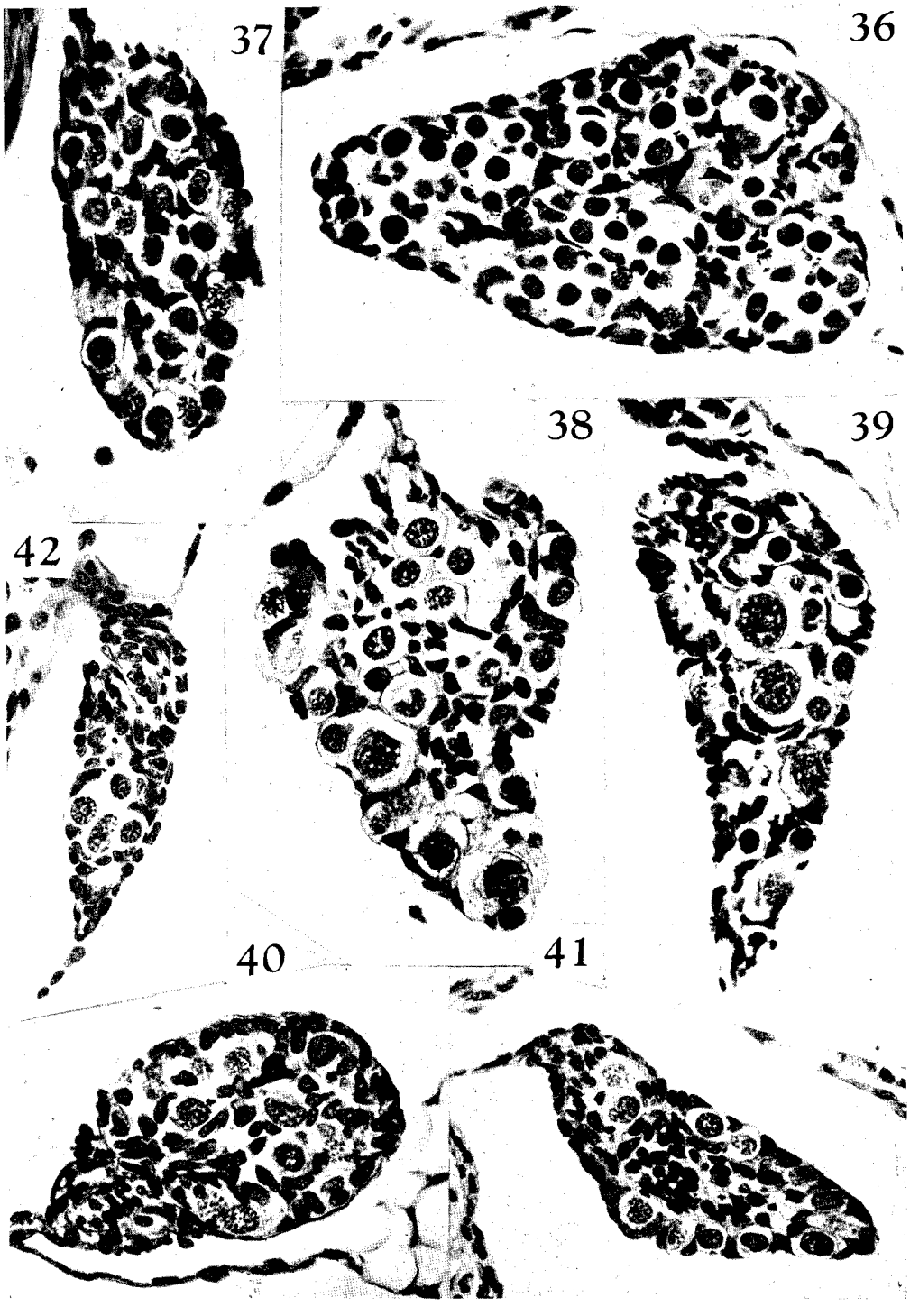

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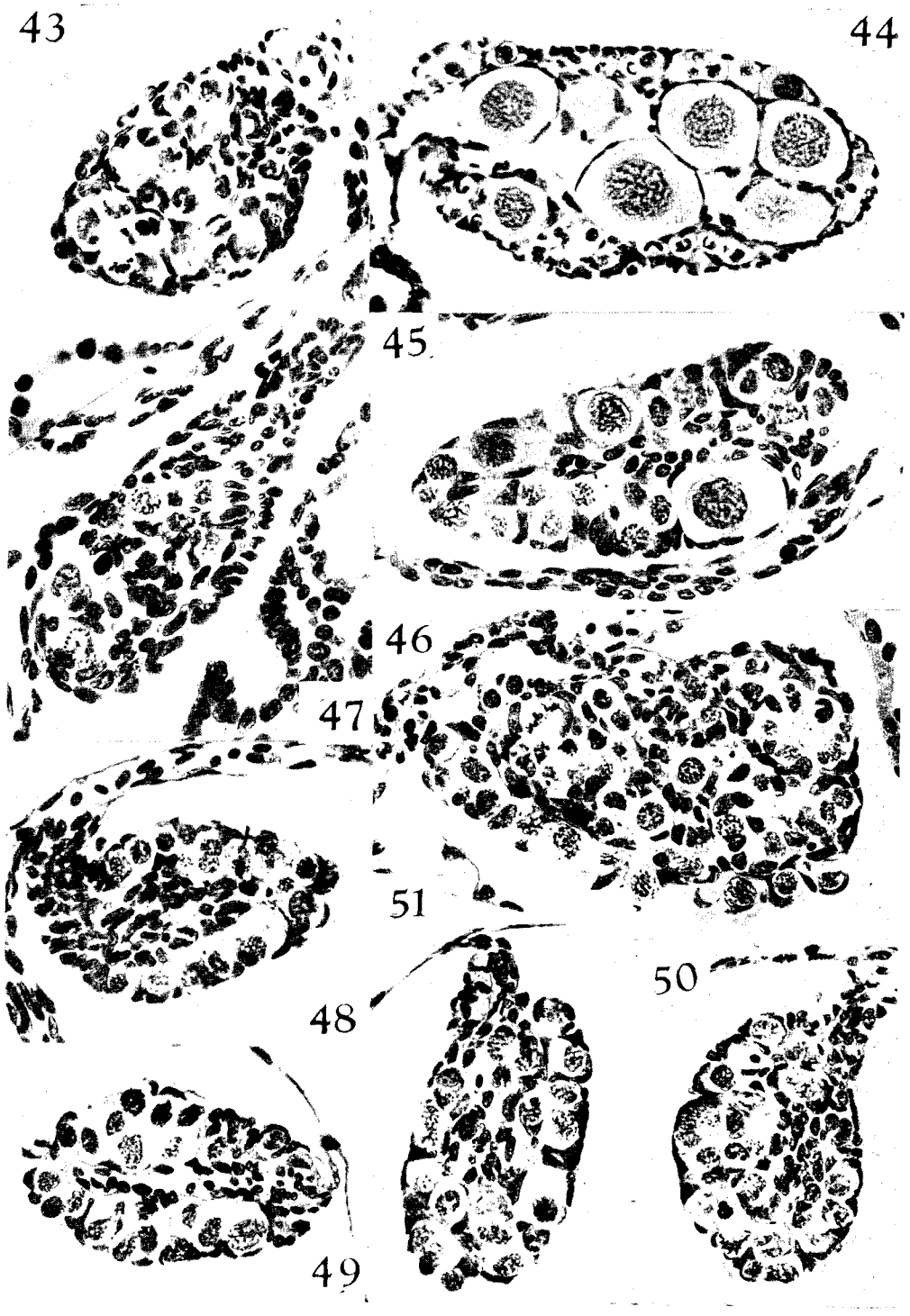

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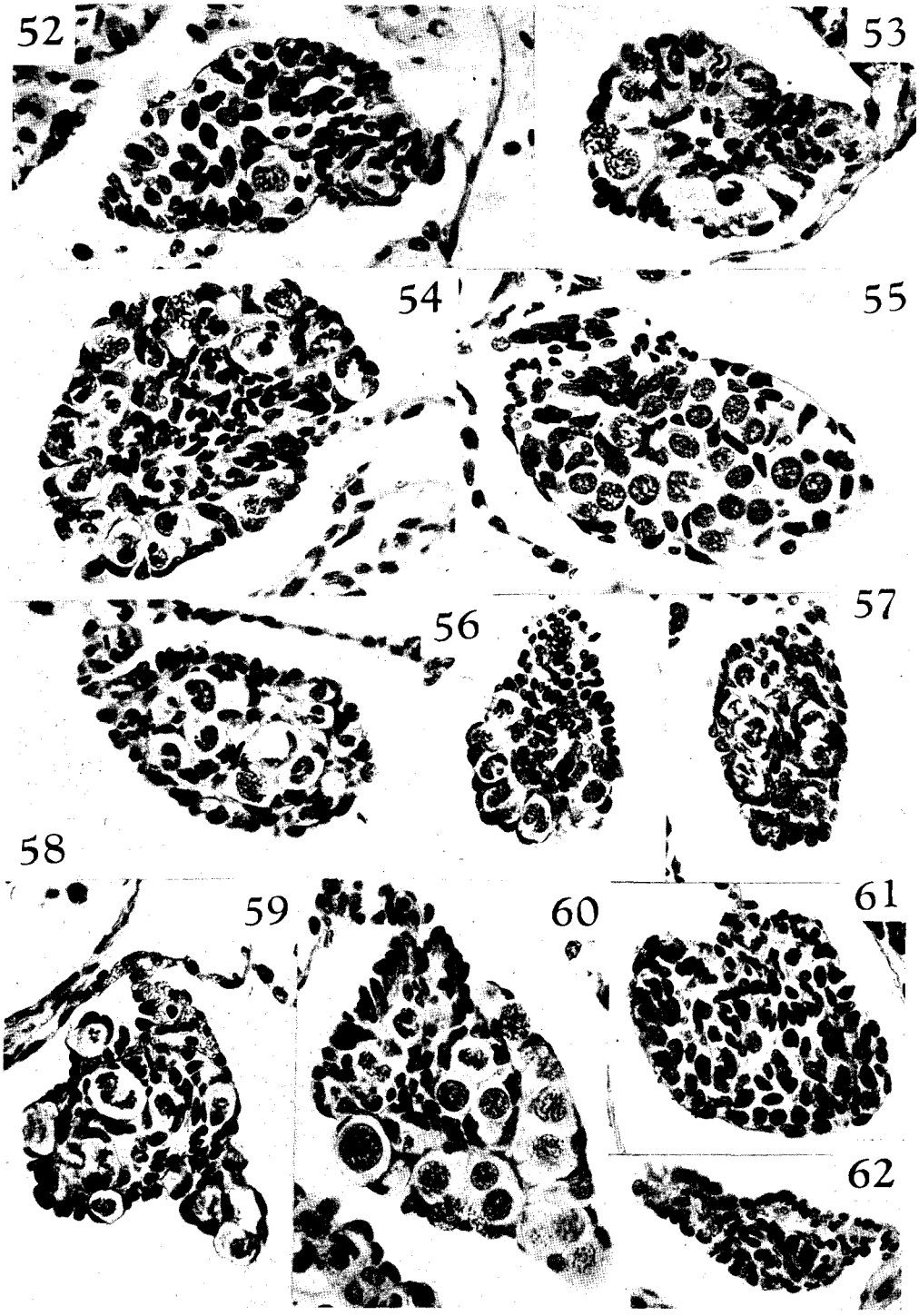

K. I. Hanaoka : Sex-differentiation in two Japanese salamanders

Experimental Studies on Sex-differentiation in Two Japanese Salamanders[1)]

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(With 2 Textfigures and 8 Plates)

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1) Contribution No. 168 from the Zoological Institute, Faculty of Science, Hokkaido Imperial University, Sapporo.

I. Introduction and review

The hermaphroditic tendency in the sex-differentiation of amphibians was first reported by Pflüger (1882), who found that the ratio of male and female was normally found to be 1:1 in adult frogs of *Rana temporaria*, but the tadpoles of some local races of the same species were all females. He noticed, also, the frequent occurrence of hermaphrodites among them and concluded that sex-differentiation in the tadpoles of such local races starts first in the female direction and that gonads of ovarian structure are uniformly developed. The genetical males manifest themselves by the transformation of young ovaries into testes at a certain time of larval development. This report attracted the attention of R. Hertwig and at the beginning of this century an extensive series of experiments were undertaken on the problem of sex-differentiation and sex-determination in amphibians. His cross-breeding experiments of different local races of *R. esculenta* yielded rather significant results and further investigations on natural as well as experimental sex transformation were carried out by his students Schmitt-Marcell (1908), Kuschakewitsch (1910) and Witschi (1914, ...). According to Witschi (1929) there are in *R. temporaria* three different sex races which are distinguished by the type of sex-differentiation, viz., undifferentiated, semi-differentiated and differentiated race. At the time of metamorphosis, the offspring of the first race consists of females only. At the corresponding stage of development, the semi-differentiated race consists, in various percentages, of females, hermaphrodites and males and only in the differentiated race, is the clear 1:1 ratio of males and females exhibited in the early larval period.

But it was not until the establishment of the theory of quantitative sex determination by Goldschmidt and Morgan, that a satisfactory interpretation could be offered for the results obtained by these early investigators. It was found that female as well as male individuals carry female determining and male determining genes. The importance of these genes in sex determination rests largely on their relative relationship. Witschi introduced this theory of genetics into the hypothesis for the sex-differentiation in amphibians, and applying various arbitrary values to the male determining and female determiing factors of different sex races, he could show that Hertwig's hybrid series as well as his own cross-breeding experiments on

R. temporaria could find a conforming interpretation. Thus it became evident that the analysis of sex-differentiation involves two large fields of experimental biology, viz., heredity and embryology.

From the embryological point of view it is generally accepted that in vertebrates, two separate histological components in the primordial gonad, the cortex and medulla, play an important rôle in the differentiation of sex. The development of the cortex produces the female differentiation and that of the medulla the male differentiation. Witschi (1913) confirmed in amphibians that genetically male germ cells, if retained in the cortex, differentiate into oocytes and he concluded that the cortex acts as an inductor of female differentiation. This idea was further elaborated in the following year (Witschi, 1914a, b) and it was realized that the medulla acts also as the inductor of male differentiation. According to him, both the inductors produce their effects by the elaboration and release of specific morphogenic substances, which he later (Witschi, 1931) called "cortexin" and "medullarin." The two substances act as antagonists to each other. Adequate evidence for this hypothesis was given by the high temperature experiments on frogs and toads; when their tadpoles are exposed over a certain period of development to high temperature of about 32°C, the cortical development of the ovary is suppressed which is soon followed by the compensatory development of medulla, resulting in the reversal of genetical female into male (Witschi 1929, Piquet 1930). These experiments have recently been repeated and extended to a salamander by Uchida (1937). His results corroborate, in general, the conclusion above stated. Obviously high temperature disturbs the balance of medulla-cortex antagonism.

So far only inductive effects within a single gonad had been taken into consideration. In 1916, however, the epochal work of Lillie on cattle twins appeared and distinct evidence was presented that the inductive effects are transmitted over a much wider range by the transport of effective substance in the blood stream. After him, extragonadal dispersion of effective substances has been proved on various forms of vertebrates by grafting experiments, but the most significant result in amphibians was first obtained by Burns (1925a, b) by the method of parabiosis. He united the embryos of *Amblystoma maculatum* (=*punctatum*) and *A. tigrium* in parabiotic twins at a stage shortly after the closure of the neural fold and kept

them until sex was distinguishable histologically. Since sex is not recognizable at the time of operation, male and female are combined by chance. The parabiotic pairs must, therefore, be combined in a 1 MM:2 MF:1 FF ratio. According to Burns, however, both members of a pair are invariably of the same sex and the development is apparently normal in every respect.

Humphrey (1928, 1929, 1938) performed similar experiments in a more skilful way. In his experiment one gonadal preprimordium of *Amblystoma* was replaced by that of another embryo and an ovary thus brought under the influence of testes throughout its development. In contrast to Burns, Humphrey found many cases in which the sex of the host proved to differ from that of the implant. The testis was always normal but the development of the ovary, in the majority of cases, suffered retardation of various degrees and a intersexual gonad was produced with a few germ cells in the cortical germ layer as well as in the medulla. In the meantime, the first report of Burns was submitted to a partial correction by Humphrey (1931) but the common conclusion obtained by these two investigators was that sex reversal is brought about in heterosexual pairs by the earlier differentiating member, especially if it can exert its influence over a long period.

This is, however, in unreconcilable conflict with the conclusion of Witschi, who made, in colloboration with his students, a more extensive series of experiments of parabiosis in various forms of amphibians. According to him the antagonistic influences exhibited in the process of sex differentiation in heterosexual parabiotic twins differ in various forms of experimental animals. In the toad, the gonads develop normally in both members of the twin without any interference. In this case the inductive action exerts its influence only within a single gonad. The same is true in the early larval stage of the frog but later a conflict between the testicular and ovarial development becomes gradually evident and the closer the twins are grafted together, the earlier it appears. The first sign of disturbance is always found in the inner gonads of the twin and in most cases in the ovary at the region nearest to the testis. This is expressed by the suppression of cortical development, which is immediately followed by the compensatory development of the medulla, resulting in a progressive transformation of sex. This process of sex reversal suggests that the inductive action is transmitted by diffusion of a

morphogenic substance through the tissue but the effect falls off rapidly with distance. The most extensive series of Witschi's experiments were performed on urodeles. In general here again a sharp mutual antagonistic influence is observed in the early development and almost sterile gonads are produced in both members of the co-twin, but about the time of metamorphosis the male gonads rapidly recover normal size while the female gonads are left in a reduced condition. According to Witschi, inhibition of the ovarial development in salamanders is not followed by testicular development. The male interference dominates almost completely over the female and the same effect is always observed irrespective of the mutual position of the twin mates. For instance, if the animals are connected only by a narrow bridge of gill arch, the male member completely disturbs the ovarial differentian of the female co-twin. Therefore one can assume that the inhibitive morphogenic substance is transmitted by the blood stream and is able to reach all parts of the twin system. Here, for the first time, the hormonal conception of the inductive substance in the strict sense is accepted and the stimulative and inhibitive substances become clearly distinguishable.

Corresponding to the sex races in frogs, there are also in salamanders specific or racial types of sex-differentiation; while some of them are purely gonochoristic, others exhibit marked hermaphroditic tendency during the process of sex-differentiation. It is of surprising interest to find that there is an intimate relationship between the intensity of inhibitory inductive activity and the type of sex-differentiation. It is high, in general, if produced by the male of differentiated form and less if caused by the male of semi-differentiated race. Only occasionally, when the development of the female proceeds ahead of the male partner, is the cortical development free from disturbance. These facts prove that the inductive dominance of the male is not absolute, but depends upon the quantitative ratio of the antagonistic substances and certain time relationship in their appearance and duration. For the problem of what at the end, may become of free-martin gonads Witschi's experiment in 1937 produced important results. He found that in heterosexual pairs of *A. tiginum*×*A. jeffersonianum* the ovaries of the small and slow differentiating *jeffersonianum* become perfectly sterile and "have a tendency to disappear or to become reduced into simple ligaments." In the reciprosal combination, on the other hand, the *jeffersonianum*

male produced hermaphrodite glands, which contained both matured spermatozoa and large pigmented egg cells. Hermaphrodite glands of similar structure were also obtained in a pair of *A. tigrinum*× *Axolotl*. In every case the sex reversal in the sense of Burns and Humphrey did not occur. One must here consider the fact that the constitution of the experimental animals is genetically as well as morphologically of bisexual nature.

Recent advance in the preparation and synthesis of hormonal substances has provided another method of attack on the problem of sex-differentiation and various experiments have been undertaken concerning the influence of sex hormone upon sex-differentiation in vertebrates. In amphibians, the first report on experiments of this type was published by Padoa (1936, 1938), who added the follicular hormone to aquarium water, in which the tadpoles of *R. esculenta* were reared. According to him, this induced the great majority of the experimental animals to differentiate into males. Witschi & Crown (1938), however, having repeated the similar experiment on *R. pipiens*, failed to observe the masculinizing effect of estrogen. Burns (1938, 1939) and Foote (1940) reported, on the contrary, that the injection of estrogenic substances in *A. maculatum* and *A. tigrinum* caused transformation of testes into ovaries. The masculinizing effect of androgen, on the other hand, was affirmed by Gallien (1937), Witschi & Crown (1938), Foote (1938), Witschi & Foote (1939) on several forms of frogs but in salamanders Burns (1939) reported that the injection of testosterone-propionate produced only intersexual gonad in genetical females and Foote (1940) also confirmed that testosterone exerts little influence on the differentiation of ovary. Puckett's (1939, 1940) experiments are of special interest. He claimed that in an undifferentiated race of *R. catesbiana* "there is no suggestion of a stimulation of gonads by the sex hormone alone," but "the injection of sex hormone in addition to a pituitary extract brings about striking modification in both male and female."

Thus the influence of sex hormone upon the sex-differentiation in amphibians is rather contradictorily reported. This is perhaps due partially to the different embryological characters of the experimental animals and partially to the different chemical nature of the hormonal substance administrated. Anyhow, it remains unsettled whether the sex hormones are essentially identical with the morphogenic substance of sex-differentiation or whether they are a separate

substance which indirectly disturbs the balance of medulla-cortex antagonism, in a similar way in principle to the different temperature levels in the experiment on *R. esculenta.*

Since several years ago the present writer could fortunately have an opportunity to carry on a series of experiments on the sex differentiation in two Japanese salamanders *Hynobius retardatus* and *H. lichenatus* which belong respectively to semi-differentiated and differentiated type in regard to sex differentiation. The conclusion was obtained that the inductive substances and sex hormones are distinctly separate substances which exert entirly different influences upon the sex differentiation. The following description is based on the observation of results obtained by the experiments just mentioned.

Before going further hearty thanks should expressed to Prof. Tohru Uchida under whose constant guidance and kind encouragement this work has been carried out. The expense of the present investigation was partly defrayed from the Scientific Research Expenditure of the Department of Education.

II. Normal sex differentiation in *H. retardatus* and *H. lichenatus*

(A) ***H. retardatus.*** *H. retardatus* is a salamander peculiar to Hokkaido and the fresh eggs are abundantly found in the environs of Sapporo in the early spring. The materials used in the present experiment were all collected from fields in their early developmental stages. The larvae are voracious and violently cannibalistic. In laboratory, they are easily fed with fresh liver and earth worms.

(*i*) *Formation of primordial gonad.* The primordial gonads in *H. retardatus* appear first in larvae about 30 days old, 21 mm in total length and with fore-limb buds. In cross section, the rudimentary gonad is a small ridge at the base of dorsal mesentry. It is composed of a small number of germ cells intermingled with small mesenchym cells and is covered by the peritoneal epithelium. In larvae of about 60 days and 28 mm in total length, the cells forming the gonad rudiment are arranged in the peripheral or cortical region with a cavity in the center which is filled with some fluid substance (the primary cavity). The germ cells, now increased in size, are sometimes characterized by the formation of pigments in them. In

the meantime, the fat-bodies make their appearance. At first they are small buds at the base of rudimentary mesogonium, but they increase rapidly in size soon surpassing the gonads. In the next stage of gonad development the primary cavities are caused to disappear by the proliferation of rete cells. These cells originate from mesonephric blastema, move towards and enter the central cavities of the gonad rudiments where they form the medulla. The germ cells are now arranged in the layer between the medulla and the peritoneal epithelium. Thus in larvae 70 days old and 35–40 mm in total length, the gonad consists of two distinct histological components of rather diverse origin, the cortex and medulla.

(*ii*) *Differentiation of the ovaries.* The differentiation of the ovary is characterized by the rapid growth of cortical germ layer. The proliferation of rete cells ceases and the medulla become gradually transformed into an epithelial sac, which is known as the ovarial sac. Along with the growth of larvae, the germ cells in the cortex increase rapidly in number, the ovarian cavity becomes widened and at the end of metamorphosis, which occurs about 3 months after fertilization, the gonads acquire the characteristic structure of ovaries. In the next spring, the gonad appears macroscopically as a thin thread hanging on the midventral surface of the mesonephros. In well fed animals the germ cells increase considerably in size. In the third spring the sexes are easily identified as the surface of the ovary becomes granulated by the growing follicles. In the fourth spring the ovaries grow into large lobeted organs, containing numerous pigmented eggs which seem now to be fertile.

(*iii*) *Differentiation of testes.* Up to the beginning of metamorphosis, the gonads in *H. retardatus* take, in both sexes, the structure of an ovary; the future testes are provided with a well differentiated cortical germ layer and a wide ovarian cavity. With the onset of metamorphosis, however, the gonads of genetical males gradually change their original structure. The ovarian cavities disappear with the subsequent migration of rete cells from the mesonephros and the gonads again acquire a solid structure. At the same time the germ cells which were arranged in the cortical layer move towards the medulla, and become intermingled evenly with rete cells. Thus the gonads take the characteristic structure of the embryonal testes, and at the end of metamorphosis the sex ratis of 50:50 is established but the differentiation of seminal tubules and efferent

ducts becomes complete in the second or third spring and the spermatozoa mature under laboratory conditions in the fourth spring.

In the field condition, the growth of the animals is semetimes conspicuously retarded, perhaps by the deficiency of foods. In the early spring, larvae of the preceding year are occasionally found in the pools together with fresh egg clusters. The behaviour of the young animals after metamorphosis, however, is not certain because the present salamander is rarely found in the fields except in the breeding season when they appear in groups.

(*B*) ***H. lichenatus.*** The eggs of *H. lichenatus* used in the present experiment were collected from Asamushi in Aomori prefecture. The eggs as well as the adult animals of this form are smaller in size than those of *H. retardatus*, and the larvae are feeble and slow in growth. After metamorphosis, however, the young animals become voracious and grow rapidly. In laboratory both male and female mature in the third spring.

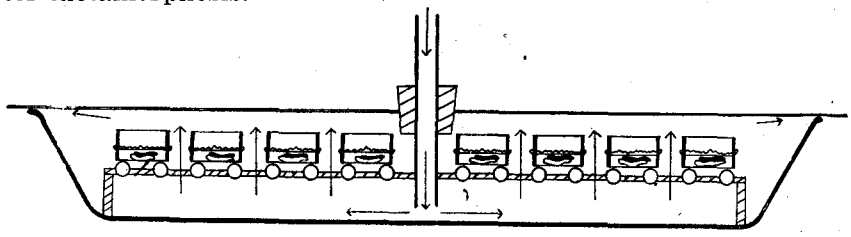
(*i*) *Differentiation of the male.* In regard to sex differentiation, the present species is a true gonochorist, free from hermaphroditic tendency. The primordial gonads appear about a month after fertilization, and the two sexes undergo early and complete sex differentiation. In the male the germinal epithelium of the indifferent gonad is reduced during early larval development and the germ cells become included within the compact medulla; neither formation of ovarial sac nor the development of cortical germ layer is detected during the whole process of testicular differentiation.

(*ii*) *Differentiation of the female.* In the female, the germ cells persist in the peripheral germ layer and the rete cells in the medulla transform into epithelial wall lining the ovarian cavity. In about 50 control animals of various developmental stages, nearly equal numbers of males and females were obtained. The metamorphosis occurs, as a rule, one or two weeks later than that of *H. retardatus*.

III. Experiment 1. Sex modification produced by the homogenous and heterogenous parabictic twins of *H. retardatus* and *H. lichenatus*

In the first experiment, the sex modification in homogenous as well as heterogenous parabiotic twins of two salamanders above

mentioned was examined. Parabiotic twins were made at tail bud stage by grafting the two embryos in usual left to right position. Generally severe mortality followed for several days after the operation but this was avoided to a considerable extent by placing the united embryos in a glass cylinder with a base of cellophane membrane. The cylinders were supported by glass holders in running tap water and the whole apparatus was covered with large glass plates. Text-figure 1 shows schematically the transverse section of the entire arrangement. When the united pairs begin to swim about they were removed to ordinary glass vessels containing about 1 liter of tap water. In animals that were kept beyond the feeding stage, the losses were very small. Although the two embryos are united side by side at the time of operation, they shift gradually to the ventral position in relation to each other. Consequently the united pairs cannot get to land and all of them were preserved immediately after metamorphosis.



Text-fig. 1. Cross section of apparatus used for rearing the united embryos. Explanation in the text.

(A) ***H. retardatus* × *H. retardatus*.** Among 51 pairs that survived up to the time of metamorphosis the following distribution of sex was observed;

$$14\,♀\,♀ + 20\,♀\,♂ + 17\,♂\,♂ = 51$$

In general the gonads of the experimental animals were a little smaller than those of single controls.

(i) ♀ ♀ *pairs*. In ♀ ♀ combination, the differentiation of ovaries at the time of metamorphosis is normal. As a rule they have a well developed cortical germ layer and a wide ovarian cavity (Fig. 1). In 5 pairs out of 14, the differentiation of germ cells proceeded considerably ahead of normal ones, and a pretty large number of egg cells which are provided with vesicular nuclei and dense protoplasmic substances are found in the cortex (Fig. 2). In the single

control females oocytes of this size are never observed during a few months after metamorphosis.

(*ii*) ♂♂ *pairs*. In 13 pairs out of 17 ♂♂ combination, the testes are well organized for the age. In general they show no essential difference from those of single males. In the remaining 4 pairs the fundamental structure of the gonads is testicular (Figs. 7, 8). Curiously enough, however, they contain varying numbers of female germ cells intermingling with well organized testicular tissue (Figs. 4, 6, 9, 10, 12). In three pairs they appeared in both members of the co-twin. These female germ cells show in every detail the characteristics of egg cells, provided with large vesicular nuclei and granular protoplasm. The gonads containing these egg cells are covered by greater or less amount of cortical tissue (Figs. 5, 6). Obviously the egg cells originate from these cortical germ cells, though most of the small germ cells in the periphery are in the process of degeneration. In the last one pair, the tetes are entirely normal in one of the co-twin, and in another, the gonads show equally well differentiated testicular structure in the anterior half (Fig. 11). The remaining part of the same gonads, however, show mixed structure in both sides and every section contains one or two of the large egg cells (Fig. 12). These egg cells are the largest ones obtained in the present experiment. Anterior and posterior halves of the gonad are connected by a narrow neck which is nearly or completely sterile. It must be noticed that the growth of the last mentioned pair was retarded conspicuously and their metamorphosis was not complete until the next spring.

There remains some doubt whether these four pairs are certainly of original double male combination, but they could be classified conveniently into double male pairs because the gonads are perfectly solid and the medulla show nearly normal testicular structure through considerable length. On the other hand, there is a conspicuous gap between the types of sex modification obtained in these four pairs and those observed in the heterosexual pairs which will be described in the following section.

(*iii*) ♀♂ *pairs*. In 16 pairs out of 20 of heterosexual combination, the development of both ovary and testis are always normal. Their differentiation proceeds as in single controls of male and female respectively; the medulla of the testis is well organized for the age

(Fig. 13) and the ovary is provided with well developed cortical germ layer and wide ovarian cavity (Fig. 14). In two cases, even large egg cells are found in the ovary of the female, while the male has normally differentiated testes. Sometimes the cavity of the ovary is closed through a considerable length, but this is not due to the influence of the male partner; the ovaries of similar structure are frequently observed in the double female pairs too (Fig. 3).

In 3 pairs, the testes of the male are normal (Figs. 15, 18, 21). The differentiation of the ovaries on the other hand, is retarded in various degrees and one can find, in certain limited stretches, a considerable amount of unorganized rete cells filling the ovarian sac (Figs. 16, 20). The degree of the retardation in ovarian development is not dependent upon the amount of testicular tissue in the male member. For instances, in the pair illustrated in Figs. 15–17 the testes of the male are conspicuously large and their differentiation has proceeded far ahead of onrmal ones, but the inhibition in the ovarial development in the female member is not severe; the rete cells occclude the vovarian cavity only for short stretches. On the contrary, the testes in the other two pairs are rather small and underdeveloped (Figs. 18, 21) but the retardation in the ovarial differentiation is more complete and the medulla contains a small number of germ cells (Figs. 22, 24).

In the remaining one pair, the ovaries of the female member are well differentiated, and contain a rather large number of large egg cells. The gonads of the male, however, show mosaic structure: in anterior half both the gonads take distinct testicular structure, while the posterior half of the same gonads contains many large eggs which are similar to those observed in the pairs of double male combination.

The results obtained observing the homogenous parabiotic twins of *H. retardatus*, show distinctly the fact that in semi-differentiated race the male member exerts a relatively slight influence upon the development of the ovary in the female co-twin. This forms a striking contrast to the results obtained in the combination of *H. lichenatus*.

(B) *H. lichenatus* × *H. lichenatus*. Sex distribution among homogenous parabiotic twins of *H. lichenatus* deviates considerably in favour of male combination as there were obtained;

$$4\,♀\,♀ + 6\,♂\,♂ + 23\,♂\,♂ = 33$$

The microscopical observation of the gonad was as follows.

(*i*) ♀♀ *pairs*. In 3 pairs of double female combination, the ovaries are provided with healthy growing cortical germ layer and wide ovarian cavities (Fig. 25). In the remaining one pair the growth of the germ cells considerably exceeds that of normal ones (Fig. 26) and the cavities are partially occluded by the enlarged egg cells.

(*ii*) ♀♂ *pairs*. In all 6 pairs of heterosexual combination, the male member has always normal testes of intermediate size (Figs. 27, 31, 36, 40). The retardation of ovarian differentiation in the female member is, in contrast with the cases of *H. retardatus*, far more pronounced. In 4 pairs the ovaries are provided with a thick cortical germ layer which contains many egg cells of considerable size (Figs. 28, 29, 34), but the central cavity is occluded through almost the entire length by the proliferation of various numbers of rete cells (Fig. 28). The open cavity is found only as several intervals of the section at the terminal region of the gonad (Fig. 30). In none of these 4 pairs are the medullary germ cells included in the medulla. In the remaining 2 pairs, the medulla of the affected ovaries included a pretty good number of germ cells and the entire gonad assumes a more complete testicular structure especially in the middle region. (Figs. 32, 33, 37). The gonad, however, is capped for a certain length by various amounts of cortical tissue (Fig. 38). It is interesting that some of the germ cells in the cortex have grown into large egg cells which surpass in size the oocytes of single females (Fig. 34). Only in the posterior one third, is the ovarian structure dominant although even in this region, the cavities are nearly or completely solid (Figs. 35, 39, 41). The suppression of growth of egg cells in the cortex of modified ovaries is not proportionally complete to the development of the medullary component. Large egg cells appear sometimes in the ovaries whose medullary differentiation shows a more advanced condition, while the cortex of less modified ovaries contains only small germ cells. This suggests that the morphological differentiation of the gonad and the differentiation of germ cells are not controlled by the same factor. The results of hormone injection offer more satisfactory evidence on this problem.

(*iii*) ♂♂ *pairs*. In the majority of double male pairs, the testes show normal structure. The medullary tissue is, in general, well organized for the age and contains a good number of small germ

cells. In some animals, however, the testes are extremely small and are reduced to a nearly sterile condition; only a few germ cells are found scattered in the compact mass of rete cells (Fig. 42). In other ainmals of the double male pairs, on the other hand, one can occasionally find that some germ cells at the peripheral region are in the process of degeneration, although their medullary tissue is relatively well organized. The distribution as well as the morphological character of these degenerating germ cells show clearly that they are of cortical nature. In normal male differentiation of *H. lichenatus* the testes at the stage of metamorphosis are completely free from hermaphroditic tendency. Consequently there arises some doubt if the parabiotic twins which were classified into double male pairs in the present experiment contain, in fact, a certain number of original heterosexual pairs whose ovarial development of the female member is modified in male direction. The observation of free-martin ovaries in the heterosexual combination proves increasingly the validity of this idea. Practically, however, it was impossible to identify the original sex of these animals because the medullary tissue of their gonads was too well organized and the amount of cortical cells covering the crest was sometimes too small. It is not reasonable to classify certain animals into genetical females only because they have a few cortical germ cells attached to the surface of the gonads. These facts, however, do not mean that reversal of sex has occurred in the genetical females. The detail of this problem shall be discussed in a later chapter.

(C) ***H. retardatus* × *H. lichenatus*** The embryos as well as the larvae of *H. lichenatus* are a little smaller than those of *H. retardatus*. In color *H. lichenatus* is dark brown while *H. retardatus* is dark green. When united in parabiotic twins, the growth of the small *lichenatus* member is frequently retarded conspicuously. In such pairs the food is taken mainly by the large *retardatus* member which carries the *lichenatus* co-twin like a parasite hanging on the ventral abdomen. The metamorphosis occurs almost simultaneously in both members though its process requires a longer time than in single controls. The sex distribution among 55 pairs was as follows;

$$10\,♀\,♀ + 8\,(\textit{lichenatus}\,♀ \times \textit{retardatus}\,♂) + 7\,(\textit{lichenatus}\,♂ \times \textit{retardatus}\,♀) + 30\,♂\,♂ = 55$$

Again in this combination, the double male pairs appeared conspicuously in excess.

(*i*) ♀♀ *pairs.* The differentiation of ovaries in 10 pairs of double female combination is not different in each of both members from that of single controls. The growth of germ cells proceeds normally for the age.

(*ii*) *H. lichenatus* ♀ ×*H. retardatus* ♂. In 4 pairs out of 8 *lichenatus* ♀ ×*retardatus* ♂ combination, neither of the twinmates exhibits any sign of interference in sex differentiation. The ovaries of the *lichenatus* females are normal in every respect and are provided with wide ovarian cavities and healthy growing cortical germ cells of considerable size (Fig. 44). Equally well differentiated are the testes of the *retardatus* males (Fig. 43). In the other 4 pairs the retardation in the female differentiation is not severe. Though their central cavities are always closed by the proliferation of rete cells the ovaries contain a pretty good number of egg cells with large vesicular nuclei (Fig. 45). In the testes of the *retardatus* males, on the other hand, the medullary tissue is, in general, well organized but the crest of the gonad is covered, for various lengths, by cortical germ layer which is in the process of degeneration (Fig. 46). In some sections the degenerating cortical germ cells exceed the healthy medullary germ cells in number. Obviously the present animals are genetical males and the cortex which covers the testes originates from that which has developed in the rudimentary testes. In the normal development of male of *H. retardatus* the cortical germ layer disappears as a rule at the time of metamorphosis and the gonad takes distinct testicular structure. When united with *lichenatus* female, the cortex of its ovaries inhibits more or less the medullary differentiation of the *retardatus* male, which permits the further development of cortex. The degenerating cortical germ cells are the remnant of the testicular cortex which indicates the exaggerated characteristics of semi-differentiated sex-differentiation of the *retardatus* male.

(*iii*) *H. lichenatus* ♂ ×*H. retardatus* ♀. In the reciprocal heterosexual combination, the testes of the *lichenatus* male are rather small in size and the germ cells are rather rare, but the development of the ovaries in the *retardatus* female suffered severe modification. For instance, in pairs illustrated in Figs. 47–51 and 52–54, the central cavities are reduced to narrow slit-like spaces, which stretch only for a few intervals of sections at the anterior end of the gonad (Figs. 49, 53);

the major part of the medulla becomes solid by the proliferation of rete cells. The germ cells are limited in the anterior one-third strictly to the cortical layer (Figs. 48, 54), while in the middle region they are intermingled with medullary rete cells (Figs. 50, 51). In the posterior one-third, the female characteristics of the gonad are again dominantly expressed by the development of cortex. Compared to the free-martin ovaries of *lichenatus* female united with *lichenatus* male, the differentiation of the medullary tissue is, in general, less conspicuous and the number of the medullary germ cells is relatively small. In this respect the ovaries of *retardatus* are more resistant to the testes of *lichenatus*, but the cortical germ cells in the free-martin ovary of *retardatus* have never grown into large egg cells. Evidence is again given that the suppression of the egg cell formation in the cortex and the development of medullary component are controlled by separate factors. In 3 pairs out of 7 *lichenatus* ♂ × *retardatus* ♀ combination, the ovaries show the structure of this type. In 2 other pairs, the ovaries are completely solid through their entire length, and the proliferation of rete cells is more conspicuous than in the 3 pairs just described, but no germ cells are enveloped in the medulla. Only in the last 2 pairs is the ovarial structure of the gonad dominant for a considerable length.

(*iv*) ♂♂ *pairs*. For convenience in description, 30 pairs of double male combination of *lichenatus* × *retardatus* are classified into three groups as follows. (a) In the 4 pairs of the first group *lichenatus* has always normal testes of intermediate size, while the testes of *retardatus* are covered by various amounts of cortical tissue (Figs. 59, 60). The germ cells in the cortex always present more or less the degenerative appearance and sometimes the border of the gonad is fringed by irregulary scattered small germ cells (Fig. 59). The medulla in the other level of the same gonad, on the other hand, differentiates very well and in the major part the cross sections show normal testicular structure (Fig. 58). (b) In most of the 22 pairs comprising the second group both *lichenatus* and *retardatus* have normal testes. Sometimes a very few cortical germ cells are left attached to the crest of the gonad, but the medulla is well organized for the age and constins a considerable number of germ cells. In a certain number of pairs of this group, on the other hand, the gonads of either *lichenatus* or *retardatus* are conspicuously small in size and the germ cells are rather rare. In some animals the entire gonad is

reduced to a nearly sterile condition; only a small number of germ cells are found scattered in the compact mass of small rete cells. (c) The remaining 4 pairs comprise the extreme cases of the second group. In them one of the twin mates has become completely sterile and the gonads are represented by peritoneal folds hanging at both sides of the dorsal mesentry (Figs. 61, 62). In 2 cases the sterile member is *retardatus* and in the other 2 cases, *lichenatus*. In cross section the rete cells forming the vestigial gonad are more numerous in *retardatus* than in *lichenatus*.

It may be an important fact that the gonads of *retardatus* in double male pairs of *lichenatus*×*retardatus* combination show a continuous variation between hermaphroditic structure and complete sterile condition. The testes of *retardatus* of the first 4 pairs above mentioned show a conspicuous hermaphroditic tendency in respect to their rudimentary cortical tissue. Indeed there remains some doubt whether these 4 pairs are originally of heterosexual combinations. They were classified provisionally as males because in all of them the medulla are equally well differentiated as in normal males. Besides it has already been mentioned that the testes of *retardatus* male united with *lichenatus* female is capped with similar cortical covering. It is noticeable, on the other hand, that the fundamental structural difference is very slight between these hermaphroditic glands and the modified ovaries of *retardatus* female grafted with *lichenatus* male. The essential difference is only in the existence of the rudimentary central cavity and the amount of cortical tissue attached to the surface of the gonads. Therefore it is very difficult, in practise, to classify all experimental animals precisely as male and female. The classification was made only as a matter of convenience. The sterile gonads comprise other extreme cases of continuous variation. In these cases, too, the original sex of the animals is not certain. But as mentioned above the testes in unisexual pairs of *lichenatus*×*retardatus* are sometimes very small. They have but a few germ cells in the medulla and the cross sections occasionally resemble the sterile gonads. Consequently it was convenient to classify them as males.

The hermaphroditic tendency in the testes of *lichenatus* is far less conspicuous. In none of them are female characteristics observed; their structure varies only from normal condition to sterile.

(D) ***Summary of experiment I.***

The results obtained by observing heterogenous as well as homogenous parabiotic twins of *H. retardatus* and *H. lichenatus* prove clearly that the inductive influence exerted by the *lichenatus* male of the differentiated species, upon the development of varies in its female co-twin is far more severe than the influence of *retardatus* male of the semi-differentiated race. While the testes of *retardatus* male exert little or sometimes no influence at all, the testes of *lichenatus* inhibit the ovarial differentiation in its female co-twin in various degrees. The first step of retardation appears in the suppression of the formation of the ovarian cavity. This is followed by the subsequent migration of mesonephric blastema in the gonads and the more proximal germ cells become enveloped in the medulla. The development of medulla in the modified ovaries is not so prominent generally as in frogs, but it is far more pronounced than in the case of *Ambylstoma* and in some sections the gonad takes more or less the complete structure of young testes. This fact must be considered in intimate relation with the deviation of sex ratio in the pairs combined with *lichenatus*. Besides there is a continuous variation between the structure of free-martin ovaries and the testes with cortical germ layer, which are observed in some of the double male pairs. On the other hand, however, there are a certain number of nearly or completely sterile animals which are produced clearly by the elimination of germ cells. These variations of structure in the gonads of parabiotic twins suggest that the further development of the free-martin ovaries would take various courses according to the incidental condition of the experimental pairs. Some of them will become completely sterile and others hermaphrodite, and it is not always unreasonable to suppose that the gonads of certain females would take at least the structure of young testes. But a hasty conclusion upon the problem of sex reversal in parobiotic twins of salamanders should be avoided at the present stage of experiments.

In the second series of experiments the above described results were compared with the sex modification induced by the administration of postpuberol sex hormone of male and female.

IV. Experiment 2. Administration of sex hormone upon larvae of *H. retardatus*

The results obtained by the administration of sex hormones upon the larvae of *H. retardatus* have already been reported in the writer's

previous papers (Hanaoka, 1941, a, b). An outline of the experiments, however, will be repeated for comparison with the results with parabiotic twins.

The female sex hormone used in the present experiments was pelain (Tōkyo Zōkiyaku-kenkyusho). It is a preparation of crystalline oesterone containing 500 m. u. in every cubic centimeter. The male hormone was perandren (Ciba), which contains 5 m. g. of testosterone propionate dissolved in one cubic centimeter of sesame oil.

(A) ***Influence of follicular hormone added to the aquarium water.*** Padoa reported in 1936 and 1938 that the water solution of follicular hormone, in which the tadpoles of *Rana esculenta* were raised, induced male sex differentiation in the great majority of experimental animals. In the first group of the present experiment the same experiment was repeated to reexamine whether the sex reversal in *H. retardatus* will be actually induced by female sex hormone as it is reported in *R. esculenta.*

The larvae were raised in water solution of pelanin which contains 5 m. u. of follicular hormone per cubic centimeter. The experiment was begun with larvae of 21 mm in total length, in which the primordial gonads had just appeared. All of them were preserved a few days after metamorphosis. Among 18 animals which were available at the time of preservation the following sex ratio was obtained.

$$11\,♂ + 4\,♂\ \text{(with vestigial ovarian cavity)} + 3\,♀ = 18$$

(*i*) The microscopic observation of the gonads in 11 males proved that they are normal in general structure as well as in size. Compared to the testes of control animals, however, the rete cells in some of these testes are extremely rare and the whole gonads consist of a large number of germ cells enveloped in peritoneal epithelium. In the normal development of the testes at the time of metamorphosis, the germ cells are evenly intermingled with a pretty large number of rete cells and rudimentary spermatic tubules appear in the medulla.

(*ii*) In 4 animals the gonads have narrow slit-like cavities at several intervals of the section. These gonads, however, cannot be considered as ovaries merely because they have central cavities; there

are continuous variations of structure between these gonads and some of the testes described in the preceding section.

(*iii*) The gonads of the remaining 3 animals have central cavities of considerable width, but these cavities never exceed one-third of that of the normal ones.

Sorry to say, the number of animals used in the present experiment was not large enough to yield any final conclusion. Microscopical observation, however, shows that the follicular hormone added to the aquarium water obviously interfers with the normal differentiation of cortex in the ovaries. But this is not followed by the development of medullary component; the gonad become mechanically solid as a result of the rapid multiplication of germ cells. This calls for caution against the conclusion that the reversal of sex may be actually induced by the follicular hormone.

(B) *Influence of injection of follicular hormone.* In the second experiment the animals received injections of 5 m. u. of pelanin at intervals of 5–7 days over periods of 3–5 months. They are divided into three groups. In the first group the experiment was begun with larvae of 21 mm in total length. In the second group the larvae were 35 mm or more. At this stage of development the gonads of the genetical males are in their female phase of semi-differentiated sex differentiation and the gonads of both male and female take ovarian structure with a cavity in the center. In the third group the injection was begun about 2 weeks after metamorphosis. The sex differentiation in these animals is always complete. At autopsy it was found that the sex modification produced by the injection of follicular hormone differs according to the different age of the animals at the beginning of the experiment, and the longer the period of injection continues, the more pronounced is the influence upon the development of the gonads.

(*i*) Sex modification in 120 animals which received the injection from the beginning of sex differentiation was as follows.

(*a*) *Before or during metamorphosis.* In 30 animals which were preserved before or during the progress of metamorphosis, the gonads are a little smaller than normal ones. Microscopical observation confirmed that they are normal in every detail; the ovaries have wide ovarian cavities in the center and a healthy growing cortical germ layer, containing many germ cells of small size. In

the testes the medulla is well organized for the age. The sex distribution was as follows.

$$16♂ + 14♀ = 30$$

These results clearly show that in *H. retardatus* the follicular hormones exert little influence upon the morphological differentiation of gonads before the stage of metamorphosis. The injection cannot inhibit the development of the medulla of the genetical males or accelerate the differentiation of female germ cells in the ovaries.

(*b*) *One or one and half months after metamorphosis.* 34 animals were preserved one or one and half months after metamorphosis. Among them 23 were female which have normal ovaries with wide central cavities. Germ cell differentiation in these ovaries, however, had proceeded considerably ahead of normal ones; they are always provided with large vesicular nuclei and granular protoplasmic substance. 8 animals had testes normally differentiated for the age. In the remaining 3, which are preseumably genetical males, the medullary tissue is well organized and the gonad is completely solid through the entire length, but intermingling with normal male germ cells of small size, one can find various numbers of abnormally large germ cells, which morphologically resemble in every detail the female germ cells in the ovaries. These gonads take, therefore, mixed structure of testis and ovary.

(*c*) *Two or more months after metamorphosis.* In 56 animals, the injection was continued until two or four months after metamorphosis. Sex distribution among them was as follows.

2 months after metamorphosis .	2♂ + 5⚥ (with mosaic gonads)	+12♀ = 19
3 months after metamorphosis .	3♂ + 2⚥ (with mosaic gonads)	+ 7♀ = 12
4 months after metamorphosis .	7♂ + 4⚥ (with mosaic gonads)	+14♀ = 25
Total	12♂ + 11⚥	+33♀ = 56

The injection exerts the more conspicuous influence upon the development of the ovaries, and the older the animals become, the more pronounced is the acceleration of germ cell differentiatinn observed in the ovaries. At the end of four months after metamorphosis, the oocytes become as large as the eggs observed in the females of the third spring.

In the mosaic gonads the egg cells have increased in size as well as in number. It is interesting to note that these mosaic gonads are occasionally covered by a greater or less amount of ovarial cortex and apparently many of the egg cells originate from the cortical germ cells. In other animals the cortical layer covering the mosaic gonad becomes rather thick and the cross sections occasionally show the structure of an ovary. The medulla in these cases is reduced to a nearly sterile condition, containing only a few germ cells or none at all. In extreme cases when a large number of egg cells are crowded, their cross sections appear like those of an ovary, whose cavity is occluded by the enlarged egg cells. But in none of the mosaic gonads is an ovarian cavity ever formed, and in the major portion of the gonads the development of the medullary component is dominant.

(*ii*) In the second group, the animals were 35 mm or more in total length when the injection was begun. The dosage and the intervals of the injection are similar to those of the first group. Sex distribution among 25 animals is as follows.

1 month after metamorphosis .	3♂	+ 8♀ = 11
2 months after metamorphosis .	3♂ +1⚥ (with mosaic gonads)	+10♀ = 14
Total	6♂ +1⚥	+18♀ = 25

Microscopical observation confirmed that the sex-differentiation in the female is accelerated to the same degree as in the first group. The ovaries are always provided with distinct ovaran cavities and well differentiated cortex containing many large egg cells. In the male, on the contrary, the sex modification is found only in the development of secondary sex characters; the testes are nearly normal except in one only, in which a small number of egg cells are formed.

(*iii*) In the third group, the animals received injections only after metamorphosis. The sex distribution was as follows.

2 months after metamorphosis	6♂+ 7♀ = 13
4 months after metamorphosis	10♂+ 7♀ = 17
Total	16♂ +14♀ = 30

Again the ovaries in the injected females show pronounced acceleration in germ cell differentiation as in the two preceding groups.

In the male on the other hand, no influence is detectable on the development of the testes, although they are slightly smaller in size than normal ones.

The results obtained by the injection of follicular hormone may be summarized as follows: (1) Before the stage of metamorphosis, the injection of oestrogenic substance exerts no influence upon the sex differentiation. The development of the gonads proceeds in both sexes as in normal cases. (2) After metamorphosis, however, similar injection stimulates the ovaries to precocious differenciation and large egg cells are formed in the young ovaries. (3) In the male, on the other hand, the injection produces mixed gonad if the treatment is begun from the beginning of the gonad development. Egg cells which appear in the mixed gonad grow nearly as large as those in the female. (4) After the morphological differentiation of the gonad is complete the injections exert no influence on the development of the testes at least in the doses used in the present experiment.

(C) ***Influence of injection of male sex hormone.*** In the last series of the present experiments, sex modification produced by the injection of male sex hormone was examined in comparison with the results obtained with the heterosexual parabiotic twins. At the beginning of the experiment, 58 animals of the first group averaged 21 mm of overall length. In 16 animals of the second group, the average length was 34 mm. The injections, each of which consists of 50 γ of synthetic testoterone-propionate dissolved in 0.01 cc of sesame oil, are made at intervals of 10 days. The oil drops which accumulate by the successive injections, remain in the body cavity for a long time and more or less retard the growth of the experimental animals. The first autopsy was made immediately after metamorphosis, and the last one about 3 months thereafter. Since, however, no fundamental difference is detected among the groups of different ages, all will be described together.

The injection of testosterone-propionate produced in both male and female diverse modification of sex differentiation and in many cases the gonads became abnormal to so conspicuous a degree that it was impossible to identify the sex of the experimental animals in the usual manner. Consequently the sex distribution could not be ascertained in the present experiment. Microscopical observation of the modified gonads yielded the following information.

(*i*) *Elimination of germ cells.* In the previous experiment on *H. retardatus* (Hanaoka, 1939), in which the pieces of adult testes were successively transplanted in addition to hyophyses, it was observed that certain germ cells at the peripheral region become degenerated and were ultimately eliminated from the gonad. The injection of testosterone-propionate causes similar elimination of germ cells in a more conspicuous degree and in many animals the gonads are reduced to mere folds of peritoneal epithelium. In animals with gonads of this type, the sex cannot be distinguished. In general the gonads of both sides are similarly modified.

(*ii*) *The differentiation of vasa efferentia in relation to the development of the testes.* The differentiation of testis in amphibians is always preceded by the rapid migration of a large amount of mesonephric blastema or rete cells which enter the medulla of the gonad and envelop the germ cells of the more proximal region. In *H. retardatus* when injected with testicular hormone, the migration of rete cells occurs in many cases rather violently. Curiously enough, however, these rete cells never enter the medulla of the gonad, but only form the vasa efferentia which connect the germinal ridges and mesonephros. In this respect, the findings obtained by the injection of male hormone are in remarkable contrast with those observed in parabiotic twins. The modification of ovarial development, exhibited by the female member of the heterosexual parabiotic twins is characterized by the proliferation of rete cells, which occlude the ovarian cavity. Under favourable condition these rete cells induce the migration of germs cells from the cortex into the medulla where they are organized into rudimental spermatic tubules. But no sign of precocious development of accessory sex organs is observable, at least at the time of metamorphosis. When injected with testosterone-propionate the rete cells, which form loose cellular strands between the gonads and mesonephros, differentiate precociously into vasa efferentia, but this process proceeds quite independently of the development of testicular medulla. In some animals a few germ cells are found strayed amongst the rete cells and the cross section at the end of rudimentary vasa efferentia gives the appearance of a small testis. But this is always limited to a small area and the major part of the gonad remains sterile.

In other animals the rudimentary vasa efferentia acquire tubular structure and form a distinct duct. But this tubular structure cannot

be traced within the confines of the mesonephros and the connection of the gonads and wolfian duct is always incomplete. In still other animals the lumen of the tubular vasa efferentia becomes enormously wide. In such cases, the wall of the duct is extremely thin and consists of one layer of flat cells. In less completely modified animals in which the ovarian structure of the gonad is maintained, the lumen of the duct continues to the cavity of the ovarian sac. When the elimination of germ cells occurs in a conspicuous degree in a gonad of this type, the gonad is represented only by a small number of degenerative germ cells which are scattered on the thin wall of the efferential duct.

(*iii*) *Differentiation of female germ cells.* While the majority of the germ cells are degenerated and cast off from the gonad by the influence of male sex hormone, some germ cells, which survived one month or longer after metamorphosis, grow into egg cells of considerable size. These egg cells resemble in size as well as in structure those observed in the female treated with follicular hormone; they are always provided with granular plasmic substance and large vesicular nuclei. The gonads, which contain such large egg cells, in many cases take distinct ovarian structure with a wide ovarian cavity in the center, but the number of germ cells is rather small and many of them are in the process of degeneration. The male sex hormone, therefore exerts stimulative influences of opposite direction upon the differentiation of germ cells and the accessory sex organ in one and the same individual. The appearance of large egg cells is not always limited to the gonads with ovarial structure. Equally large germ cells appeared often in the gonad with testicular structure intermingling with small ones.

As described above the injection of testosterone-propionate produces diverse modifications of sex differentiation and the abnormality of the gonad becomes so conspicuous that it is practically impossible to classify the affected gonad according to the morphological structure. The injection exerts great influence, but obviously, the sex modification does not proceed in the direction of male differentiation. In this respect the results of the present experiment differ very much from the findings of the previous investigators obtained with frogs and salamanders. The detailed discussion of the problem, however, well be postponed to the next chapter.

V. Discussion

(*i*) *Quantiative difference of inductive activity in H. retardatus and H. lichenatus.* In regard to the quantitative difference of inhibitory inductive activity in *Amblystoma tigrinum* and *A. maculatum*, the latter belonging to distinct sex races, Witschi (1933, 1936, 1937) obtained a clear conclusion in his experiments with heterogenous parabiotic twins. According to him: "all three types of the male used in these experiments send into the twin female a principle, which interfers with the ovarial development. However, inhibition shows great qauntitative differences. It is high if produced by the *tigrinum* male, which reduces the gonad of *maculatum* female into mere sterile or nearly sterile rudiment. It is less if caused by a *maculatum* male of differentiated race which prevents ovarial differentiation completely in *maculatum* females and incompletely in *tigrinum* females. And it is least if coming from a *maculatum* male of semidifferentiated race, which interferes with the ovarial development only moderately if the twin is a *maculatum* female and not appreciably at all, if the twin is a *tigrinum* female." He illustrated these quantitative relations of inductive activities by curves which have different hights of abscissae.

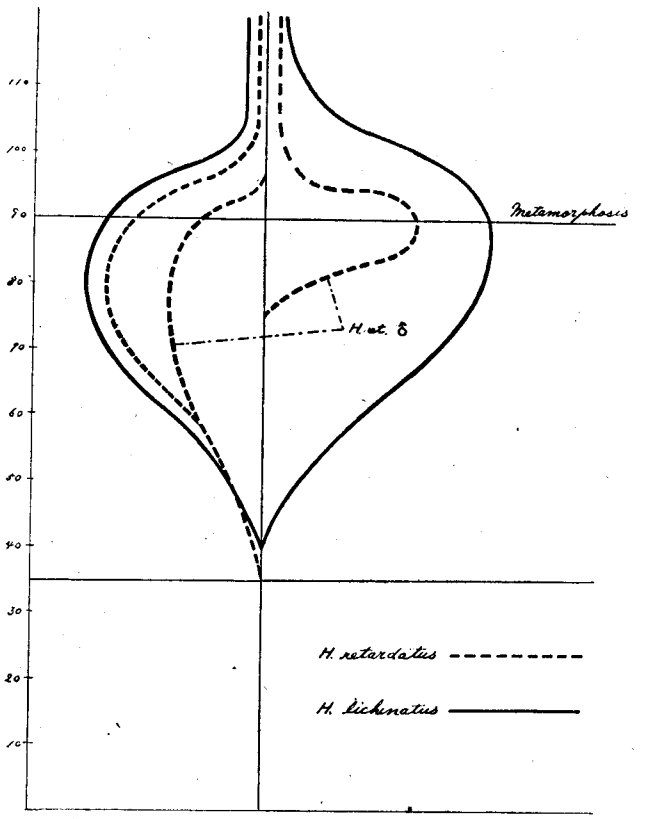
The results obtained in the present experiment on *H. retardatus* and *H. lichenatus* agree in general with the findings of Witschi on American salamanders. The male of both *retardatus* and *lichenatus* interfers with the ovarial development of the female co-twin, but the inhibition is high if caused by the *lichenatus* male and less if produced by the *retardatus* male. In most of the *retardatus* females grafted with the male of *retardatus*, the development of the ovaries proceeds as in single controls. Only in 3 cases out of 20 pairs is a moderate retardation of ovarial development exhibited. In the similar homogenous combination of *lichenatus*, on the contrary, the retardation of ovarial development is more complete. Although the ovaries are provided with thick cortical germ layer containing a considerable number of healthy growing oocytes, their central cavities are in most cases occluded through almost the entire length by the proliferation of various numbers of unorganized rete cells, and a pretty good number of germ cells are included in the medulla. In heterogenous pairs of *retardatus* ♂ ×*lichenatus* ♀ , the occlusion of the cavities in *lichenatus* ovaries, is more complete than in *retardatus* female of

homogenous combination. In this respect the ovary of *retardatus* female is more antagonistic to the medulla than that of *lichenatus*. On the other hand, the differentiation of germ cells in the ovary of *lichenatus* proceeds occasionally far ahead of normal ones. The structural differentiation of the ovary and the differentiation of germ cells are controlled, therefore, by different factors. In the reciprocal combination of *retardatus* ♀ × *lichenatus* ♂, the modification of the ovarial development is the largest. In none of the *retardatus* females are large egg cells observed in the ovary and the larger number of germ cells are included in the medulla.

These relationships between the different inductive activities in *H. retardatus* and *H. lichenatus* can be illustrated, as in *Amblystoma*, by the diagramatic curves in text-figure 2. The male curves have in general higher abscissae than those of females which indicate the dominant inhibitive action of medullary induction. The curve of *retardatus* male is twofold; during the early period of larval development it is mainly of cortical nature. This disappears shortly before the time of metamorphosis and medullary induction then becomes predominant. The stronger inhibitory action of *lichenatus* male is expressed by the abscissa higher than that of *retardatus* male. The heights of female curves are determined indirectly from the intensity of resistance to the inhibitive action of *retardatus* as well as that of *lichenatus* males. In the present forms the quantitative difference of cortical inductive activities seems to be very small.

(*ii*) *Will sex reversal occur in free-martin ovaries?* In 1925 Burns reported on *A. punctatum* (=*maculatum*) that members of a pair of parabiotic twins were invariably of the same sex; the development of the gonad was apparently normal and no indication of intersexual condition was observed. The absence of heterosexual combination, he proposed to interpret by the assumption that complete sex reversal occurs in the slower developing member of the twin-mates. The faster developing member dominates the sex differentiation in "all or none" type of reaction and there is no prepotency constantly favouring one or the other sex. This report of Burns, however, was later submitted to a partial correction by Humphrey (1932), who had been given the opportunity to reexamine a part of Burns' slides and found among 57 pairs, 6 that should be classified as undifferentiated and 8 that were heterosexual. In the succeeding experiment on *A. tigrinum*, Burns (1930, 1931, 1935) himself found

that there were a certain number of heterosexual pairs, but he still maintained the interpretation that the modified ovaries in the female member were in the process of sex reversal and "the most advanced



Text-fig. 2. Diagramm representing the difference of quantity in the release of inductive substance in *H. retardatus* and *H. lichenatus*. Explanation in the text.

cases approximate or attain virtual transformation into rudimentary testes, which are believed capable of regeneration into functional organs."

Humphrey (1936, a, b) also published two reports on homogenous twins of *A. tigrinum* and *A. maculatum*. His experiments were

undertaken to ascertain the correctness of his conclusion regarding sex reversal which he obtained previously by the transplantation of preprimordia of one gonad into another embryo. But his conclusion was that "the writers (Humphrey 1936a) previous conclusion that ovaries of *Amblystoma tigrinum* kept continuously under the influence of a testis from embryonic life onward may undergo reversal to testes is fully confirmed."

The interpretation of these two authors, however, shows unreconcilable conflict with the opinion of Witschi. He claimed that in the female of heterosexual parabiotic twins of newts and salamanders "only feeble attempts at sex reversal are made which never proceed beyond the first steps of testicular development" (Witschi, 1934). For instances in the pairs of *A. tigrinum* and *axolotl* he observed that "even at the age of 13 months, the medulla is still found in a extremely rudimentary condition, containing few if any germ cells and no distinct organization of seminal tubules," and "while these developments of modified ovaries represent unmistakably a step toward testicular differentiation, they do not reach any one of the stages seen in normal testicular development." He, therefore, hesitated to apply there the term sex reversal because "it is not at all certain, and in fact even probable that all free martin gonads of *Amblystoma* twins, which contain some gonia in medullary position would eventually differentiate into testes, if the animals were kept alive long enough." (Witschi, 1937). Consequently the problem is reduced to the question "of what becomes of the free-martin gonad at the approach and during the stage of maturity." Regarding this problem Witschi (1937) offered interesting data. He found in heterosexual pairs of *A. tigrinum*×*A. jeffersonianum* that the ovaries of the small and slow differentiating *jeffersonianum* female become perfectly sterile and have a tendency to disappear or to become reduced into simple ligaments. In the reciprocal heterosexual combination, on the other hand, the medullary induction in the testis of *jeffersonianum* male is suppressed by the cortical induction of the fast developing *tigrinum* female, which is immediately followed by the cortical development. The gonad assumes therefore partially ovarial structure. With the onset of sexual maturity, the cortical element as well as the medullary rudiment surviving in this gonad are stimulated to further differentiation and hermaphrodite glands are produced with matured spermatozoa and large eggs. Later on,

however, this morphological female (genetical male) transforms into a male; the ovarial structures degenerate and testicular ones develop instead. (Witschi 1937). In this case no ovary developed in the genetical male, but only transitory hermaphodite gland. Similar hermaphrodite gland with mature dspermatozoa and large pigmented eggs were observed in the female of *A. tigrinum*×*axolotl* twin at the age of 2½ years. In regard to the development of the hermaphrodite gland, however, one must consider, as Witschi pointed out, the fact that the constitution of the animals is a bisexual and not a purely male.

The problem of sex reversal in parabiotic twins must be considered from statistical as well as embryological point of view. In the present experiment the sex ratio in homogeneous parabiotic twins of *retardatus* was nearly normal but males in both heterogeneous and homogeneous twins of *lichenatus* appeared in considerable excess, i.e., the sex ratio in homogeneous pairs of *lichenatus* is 52♂ : 14♀, and in heterogeneous pairs 75♀ : 35♂. In single control of *retardatus* and *lichenatus*, nearly equal number of male and female always appear at the end of metamorphosis. It must be noticed further that although the mortality in the animals in the present experiment is pretty high in the several days following the operation, no evidence could be found that the deviation of the sex ratio should be ascribed to selective mortality. The losses occur only in the early period of the embryonal development before the primordial gonads become visible in cross sections. The deviation of sex ratio just mentioned gives, at first glance, the impression that in *Hynobius* the reversal of sex from female to male has been induced by the strong inductive action of *lichenatus* male. But for the final conclusion the embryological examination of the modified gonad must be taken into consideration.

The microscopical observation shows that the testes of some double male combination are covered by varying amount of cortical germ cell layer. In some animals the cortex is represented only by a few germ cells attached to the crest of the gonads but in others nearly half of the entire gonad is capped by the cortex. On the other hand, in certain females of both *retardatus* and *lichenatus*, which are combined with *lichenatus* males, the medullary component of the modified ovaries well differentiated and the cross section of the severely affected ovaries assume occasionally similar structure to the

testis. These ovaries are distinguished from the testes only by the presence of rudimentary ovarian cavity, and there is a continuous morphological variation among the modified ovaries with solid medulla and the testes with cortical germ cell layer, which frequently appear in some of the double male pairs. Therefore the critical point by which the experimental animals are divided distinctly into male and female cannot be determined and the classification was made only provisionally. The double male pairs in the present experiment may have contained a certain number of pairs which are originally of heterosexual combination and the excess of the males is mainly due to the appearance of intersexual animals which were classified into males. Consequently the conclusion of the problem depends to the question of what becomes of these intersexual gonads. Sorry, however, the observation in the present experiment was made only for a few weeks after metamorphosis. It is not impossible that in some of these intersexual gonads the cortical germ cells are completely eliminated and the medullary component differentiates into purely testicular structure. But it is more probable that, by the action of post puberal sex hormone, both the cortical and the medullary components of the intersexual gonad are stimulated to further differentiation and ultimately hermaphrodite glands are produced as in the case of *Amblystoma*. The results obtained by the injection of sex hormone, suggest the more probable occurrence of the latter case. In regard to the final development of the free-martin ovaries, it must be also noticed that in some animals germ cells are eliminated and the gonads are reduced to nearly or completely sterile folds, and when male sex hormone is administrated this elimination of germ cells occurs to an extreme degree. Anyhow, sex reversal in the sense that normal testes are produced in genetical females does not seem to take place in *Hynobius*.

Of the occurrence of sex reversal in opposite direction, i.e. from male to female, there is no probability. In some males of *lichenatus* ×*reardatus* the differentiation of testes is slightly modified and a small amount of cortical germ layer develops on the surface of embryonal testes, but the structure of the entire gonad is far different from that of an ovary.

(*iii*) *Appearance of female germ cells in double male pairs of H. retardatus.* In some males of *H. retardatus* it was found that the testes contain varying numbers of female germ cells intermingled

with normal small germ cells. In 3 cases they appeared in both members of double male twins and in 1 case in the male of heterosexual combination. The differentiation of these female germ cells proceeded always ahead of normal ones in the ovary. They were provided with large vesicular nuclei and granular protoplasmic substances. The gonads containing these germ cells take the structure of a mixed gonad. The gonads of this type were previously observed in the present writer's experiment on *H. retardatus*, in which testicular pieces were transplanted in addition to hypophyses. At that time they were inferred to be ovaries, whose differentiation was modified by the influence of maturation hormone of testis and hypophysis. In the present experiment, the female germ cells appeared in genetical males. Embryological observation as well as the experimental studies on *H. retardatus* shows obviously that they have a strong tendency toward hermaphroditism. But just now the writer finds himself unable to offer any proper explanation why female germ cells appear in the testes of double male pairs of parabiotic twin. Some unknown physiological condition which is produced by the parabiotic combination must have disturbed the balance of cortico-medullary antagonism of the male differentiation. But no further information was elicited by the present experiment.

(*iv*) *Sex modification produced by the administration of sex hormone.* (A) Masculinizing effect of follicular hormone. The incompatibility of Padoa's hypothesis for the masculinizing effect of follicular hormone was pointed out in the writer's previous paper (Hanaoka 1941a). In *H. retardatus* no masculinizing effect is observed when the follicular hormone is added to aquarium water. Obviously the ovarian cavities show a tendency to be occluded, but that is due mainly to the abnormal multiplication of germ cells and no definite differentiation of testicular tissue such as seminal tubules are observed. Normal differentiation of testis is always preceded by the migration of rete cells into the ovarian cavity and the germ cells become enveloped within them. Thus the result obtained by the present experiment is distinctly different from that of Padoa. Probably this is not due to the different materials of the two experiments. A similar experiments was performed adding follicular hormone to aquarium water on a semi-differentiated race of *Rana temporaria* (Unpublished). The dose of the hormone administrated was exactly the same as in the case of *H. retardatus*. Microscopical observation of the resultant

animals, however, showed that the hermaphroditic tendency of the male differentiation becomes more pronounced and the female germ cells in the larval ovary of the genetical male grow larger than in control animals, but the transformation of ovary into testis begins as in normal ones with the onset of metamorphosis. Horié (Unpublished) also, obtained similar results by the method of injection of the same substance. So far as the present writer is aware, the masculinizing effect of follicular hormone has been observed by no investigator except Padoa. Would he find it worth while to try again the similar experiment by the method of injection?

(B) Feminizing effect of follicular hormone. According to Burns (1938) repeated injection of small doses of esterone into larval *A. punctatum* causes transformation of testes into ovaries and the process of reversal takes the normal histological course of ovarial development. Foote (1940) also reported that estrogen appears to have a feminizing action upon testis. In *H. retardatus,* the injection of follicular hormone causes no transformation of testes into ovaries, but only the formation of intersexual gonads with a mixed structure of male and female characteristics.

Concerning the development of mixed gonads, two important facts must be noticed. First, while the medullary component is, in general, well organized for the age, the gonads are occasionally covered by varying amounts of cortical germ layer. In some gonads the development of the cortical tissue exceeds locally that of the medullary component and the cross sections through the affected part show the structure of modified ovaries with solid medulla. But in other animals only a small number of cortical cells are left attached to the terminal crest. At any rate, however, the cortical component in genetical males is stimulated by the influence of follicular hormone to differentiate excessively. The fact that the cortical development is favoured by the influence of follicular hormone is evidenced also by the experiments on *R. temporaria* mentioned in a preceding paragraph. Microscopical observation yields evidence that the majority of the egg cells in the mixed gonads are produced by these cortical germ cells whose differentiation is precociously accelerated. The appearance of the egg cells in the mixed gonads, however, is not restricted to the cortical germ layer. At times they are observed in the medullary region. In connection with this problem Witschi's comment about the occurrence of egg cells in the testes of adult frogs

and newts is very interesting. He says that "their unmistakable ovarial character may be due to a reactivation of female inductive properties in follicular cells which, as we recall, are of cortical origin. We have to consider that the sex cords carry not only germ cells but also their follicular and adhering stroma cells into the medulla" (Witschi, 1936). It is questionable in fact, whether the female inductive properties of follicular cells are reactivated by the treatment of follicular hormone, because if the injections are made only after metamorphosis when the embryonal follicular cells have completely lost their inductive properties and are organized into distinct medulla, the formation of egg cells in the testes is never induced. But if the injection is continued from the beginning of sex differentiation, the follicular cells may keep their inductive properties for a certain period after they have migrated into the medulla. Consequently some germ cells are maintained in the fields of female inductive activity and differentiate into large egg cells. Once the follicular cells have lost their female inductive properties, it seems to be difficult to reactivate them by the action of follicular hormone, at least by the dosage used in the present experiment.

The second important fact in the development of mixed gonads is the time relationship of the appearance of egg cells. Before the time of metamorphosis the injection of follicular hormone exerts no appreciable influence on the differentiation of testes nor of ovaries. The growth of egg cells in the ovaries proceeds as in control females and the medulla of the testes are in general normally organized. The acceleration of growth of egg cells becomes remarkable only one or two weeks after metamorphosis and in parallel with this, the structure of the mixed gonad become distinct. Before metamorphosis the germ cells have not yet entered a stage capable of reaction to the follicular hormone. In this respect the action of follicular hormone differs very much from that of Witschi's cortexin. Cortexin acts as an inductive substance which inhibits the development of medulla during the course of morphological differentiation of the gonads. It disappears gradually as the animals approach maturity. The follicular hormone, on the other hand, does not act directly as an inductor for the morphogenesis of the gonads, but as an accelerator of female germ cell differentiation. It is certain, however, that the cortical development is favored in some indirect way by the administration of follicular hormone. Therefore, if a higher dose of hormone is

given, it is not impossible to reverse the balance of the medulla-cortex antagonism, and as in the case of Burns (1938) and Foote (1940), the development of the medulla might be inhibited by favouring the development of the cortex.

(C) Influence of male sex hormone. Gallien (1937), Witschi & Crown (1938), Foote (1938), Foote & Witschi (1939) reported regarding several forms of frogs that the administration of male sex hormone caused transformation of ovaries into testes, and according to the personal information of Horié, a similar result is, also, observable in *R. temporaria* and *Bufo vulgaris*. In urodelans, however, Burns (1939) obtained a somewhat different result; the injection of male hormone in *A. punctatum* produced no sex transformation, but only intersexuality. Puckett (1939, 1940), on the other hand, repeatedly reported that in *R. catesbiana* the sex hormone alone had no morphological effect in sex differentiation, but the injection of sex hormone together with pituitary factor uniformly brought about the development of testes or ovaries in all of the experimental animals.

In *H. retardatus*, the injection of testosterone-propionate produces striking modification of sex differentiation, but the structure of the modified gonads is rather malformed and irregular in various individuals. The important characteristics of modification may be summarized in three points as follows; (a) precocious differentiation of female germ cells, (b) migration of rete cells and hypertrophy of the gonoducts and (c) elimination of germ cells from the gonad. Complexity of the gonads observed in the animals treated with male hormone is due mainly to the different combinations of these morphological changes.

At the present state of experiment the writer cannot find proper comment for the stimulative action of male sex hormone upon the differentiation of female germ cells. As a matter of fact, however, the acceleration of egg differentiation induced by the male hormone is obserxed in *H. retardatus* (Hanaoka, 1939) as well as in *H. lichenatus* (Uchida and Hanaoka, 1942. In press), which received the transplantation of testicular pieces. In both cases, the germ cells in the ovaries became occasionally as large as those found in the ovary treated with follicular hormone. In the present experiment, the injection of testosterone-propionate in most cases caused the elimination of germ cells, resulting in the reduction of gonads to nearly or completely sterile condition, but in certain animals the

germ cells were accelerated to differentiate into large egg cells. It is certain, therefore, that the male hormone exerts in *Hynobius* some influences which favors the differentiation of female germ cells. Witschi (1937), also, reported that "under the influence of maturity hormone both cortical and medullary rudiment were stimulated to further development and differentiation." The appearance of female germ cells in the presumably genetical male treated with male sex hormone may be due to the embryological characteristic that the larval testes take the structure of ovaries and the germ cells are kept in the field of cortical induction.

The elimination of cortical germ cells observed in the present injection experiment is not due to the peculiar toxic action of synthetic male hormone. It seems that this elimination of germ cells is caused by the disharmony of hormonal stimulus and inductive activity to which the germ cells are exposed. One has always to bear in mind that, when the injection of testosterone-propionate was begun, the gonads of *H. retardatus* were in the female phase of sex differentiation. By the injection of male sex hormone, the germ cells were exposed to the stimulations of opposite direction, in which case they were compelled to degenerate.

The third important feature of sex modification produced by the injection of male hormone is the rapid migration of rete cells. It is an interesting fact that these rete cells never enter the medulla of the gonad but produce only hypertrophy of the rudimentary efferential ducts, in both male and female. In other words, the rete cell migration, induced by the stimulation of male sex hormone, causes precocious differentiation of the male accessory sex organs, but it is not directly concerned with the morphological differentiation of gonads. There is an intimate relationship between the migration of rete cells and the reversal of sex from female to male. In frogs and toads, the degeneration of cortical germ layer is immediately followed by the compensatory immigration of rete cells into the medulla which in turn induces the migration of germ cells from cortex into medulla. In urodelans, on the other hand, the degeneration of cortex causes only the reduction of the entire gonad. The migration of rete cells in the medulla is induced only when some other stimulative influence is given. In this respect, the action of hypophysis plays an important rôle. Witschi claimed that the embryonal hypophysis is not concerned with the development of the gonads, but the matter seems to

be different with adult hypophysis. The fact that the hypophysial hormone induces the early migration of rete cells in the male and consequently the precocious differentiation of testes in *H. retardatus* is clearly demonstrated by the earlier experiments already mentioned (Hanaoka, 1939). In genetical females, however, the rete cell migration is not induced by the pituitary hormone alone but if the hypophysis is transplanted in addition to testis, the migration occurs and although many of the germ cells are eliminated from the gonad, some more proximal germ cells become included in the medulla and rudimentary testicular tissue is formed. Therefore, the development of medulla in genetical female is the result of cooperation of testicular and hypophyseal hormones. In addition it must be noticed that to induce the complete sex reversal in salamanders, the pituitary and testicular stimulation must be kept in a certain balanced condition. If they lose their balance, the medullary development becomes incomplete or the entire gonad becomes sterile by the elimination of germ cells. Therefore, the experiment by the method of injection of chemically extracted substances will produce more clearcut results because the stimulation given by the implantation of the secretory tissue is very irregular in its quantity and duration. In this respect Puckett's experiment on *R. catesbiana* is brilliant. Contrary to other forms of anurans, sex reveral in the bull-frog is induced only when the testicular hormone is given in addition to pituritary factor. It is obvious that in the present experiment on *H. retardatus*, the hormonal condition is extremely out of balance. Consequently the majority of the germ cells become eliminated and only the accessory sexual organs such as vasa efferentia are stimulated to further differentiation. The results of the present experiment must be always considered in comparison with the those of the transplantation experiment.

VI. Summary and Conclusion

(1) *Hynobius retardatus* belongs to a semi-differentiated form, and the male exhibits a conspicuous hermaphroditic tendency during the course of sex differentiation, while *H. lichenatus* is a true gonochorist free from hermaphroditic tendency.

(2) In the first series of the present experiment, the relative intensities of inductive action of the two forms above mentioned were

studied by the method of homogenous as well as heterogenous parabiosis. Microscopical observation proves that the medulla of *H. lichenatus* is more antagonistic to the cortex than the medulla of *H. retardatus*. While the testes of *H. retardatus* exerts little or no influence upon the development of the ovaries in its female co-twin, the testes of *H. lichenatus* prevent the ovarial differentiation in both *retardatus* and *lichenatus* in various degrees.

(3) The development of medullary tissue in the free-martin ovaries seems to be more advanced than in the case of *Amblystoma*, but no observation was made upon the final course of development of these hermaphrodite glands.

(4) In the second series, the influences of male and female sex hormone upon the sex differentiation of *H. retardatus* was examined in comparison with the results of parabiosis.

(5) The female sex hormone exerts certain influences upon the development of cortex but no appreciable morphological changes are observable before the stage of metamorphosis. After metamorphosis, however, the egg cell differentiation in the ovaries is accelerated far ahead of normal cases while in the testes, various numbers of egg cells appear intermingled with well differentiated medullary tissue.

(6) The male sex hormone in most cases causes elimination of germ cells reducing the entire gonad to nearly or entirely sterile condition. On the other hand. the accessory sex organs such as efferential duct, are stimulated to precocious differentiation by the rapid migration of rete cells.

(7) Rete cells which are induced to migrate from the mesonephros by the influence of male hormone, never enter the medulla of the gonad and consequently do not effect the morphological differentiation of the gonads. The medullary development in *Hynobius* is induced by some other factors. In this matter the cooperative action of adult hypophysis and testis seems to play an important rôle.

(8) Occasionally male sex hormone stimulates the germ cells of both made and female to differentiate into large egg cells.

(9) The data above described show clearly that the medullary and cortical inductive actions are quite different from those of sex hormones; the influences of the sex hormones on the development of the germ glands are always indirect.

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Explanation of Plates

PLATE IV

H. retardatus ♀ ×*H. retardatus* ♀

1. Normally differentiated ovary.
2. Ovary with large egg cells in the germ layer.
3. Ovary with closed cavity.

H. retardatus ♂ ×*H. retardatus* ♂ (a)

4. Mosaic gonad containing large egg cell.

H. retardatus ♂ ×*H. retardatus* ♂ (b)

5–6. Two sections through a mosaic gonad with cortical covering. In 6 one of the germ cells is differentiated into an egg cell.

PLATE V

7. Section through testicular part of the same gonad illustrated in 5–6.
8–10. Three sections of a mosaic gonad of the co-twin, showing normal testicular structure and egg cells.

H. retardatus ♂ ×*H. retardatus* ♂ (c)

11–12. Two sections of other mosaic gonad, showing normal testicular structure and egg cell. The egg cell illustrated in 12 is the largest one obtained in the present experiments.

H. retardatus ♂ ×*H. retardatus* ♀ (a)

13. Normally differentiated testis of the male.

PLATE VI

14. Normally differentiated ovary of the female co-twin.

H. retardatus ♂ ×*H. retardatus* ♀ (b)

15. Well differentiated testis of the male.
16–17. Two sections of the free-martin ovary of the female co-twin.

H. retardatus ♂ ×*H. retardatus* ♀ (c)

18. Testis of the male.
19–20. Two sections of the free-martin ovary of the female co-twin.

PLATE VII

H. retardatus ♂ ×*H. retardatus* ♀ (d)

21. Testis of the male.
22–24. Three sections of the free martin ovary of the female co-twin.

H. lichenatus ♀ ×*H. lichenatus* ♀

25. Normally differentiated ovary.
26. Ovary with precociously differentiated egg cells.

H. lichenatus ♂ ×*H. lichenatus* ♀ (a)

27. Testis of the male.
28. Section of the free-martin ovary of the female co-twin. The cortex is well developed but the medulla is occluded by rete cells.

PLATE VIII

29–30. Two sections of the same gonad illustrated in 28 showing large egg cells and rudimentary ovarian cavity.

H. lichenatus ♂ ×*H. lichenatus* ♀ (b)

31. Testis of the male.
32–35. Four sections of the free-martin ovary of the female co-twin. The medullary development shows a more advanced condition than in (a).

PLATE IX

H. lichenatus ♂ ×*H. lichnatus* ♀ (c)

36. Testis of the male.
37–39. Three sections of the free-martin ovary of the female co-twin. The figure in 37 shows nearly complete testicular structure but in 38 the medullary tissue is covered by degenerating cortical germ cells. In 39 the remnant of the cavity is noticeable in the terminal region.

H. lichenatus ♂ ×*H. lichenatus* ♀ (d)

40. Testis of the male.
41. Free-martin ovary with solid medulla.

H. lichenatus ♂ ×*H. lichenatus* ♂

42. Testis which is reduced to nearly sterile condition.

PLATE X

H. lichenatus ♀ ×*H. retardatus* ♂ (a)

43. Normally differentiated testis of *retardatus* male.
44. Ovary of *lichenatus* female with well differentiated egg cells.

H. lichenatus ♀ ×*H. retardatus* ♂ (b)

45. Ovary of *lichenatus* female with solid medulla.
46. Testis of *retardatus* male covered with cortical germ cells.

H. lichenatus ♂ ×*H. retardatus* ♀ (a)

47. Normally differentiated testis of *lichenatus*.
48–51. Four sections of free-martin ovary of retardatus female. 48 and 49 show the ovarial structure, while 50 and 51 the predominant development of medulla.

PLATE XI

H. lichenatus ♂ ×*H. retardatus* ♀ (b)

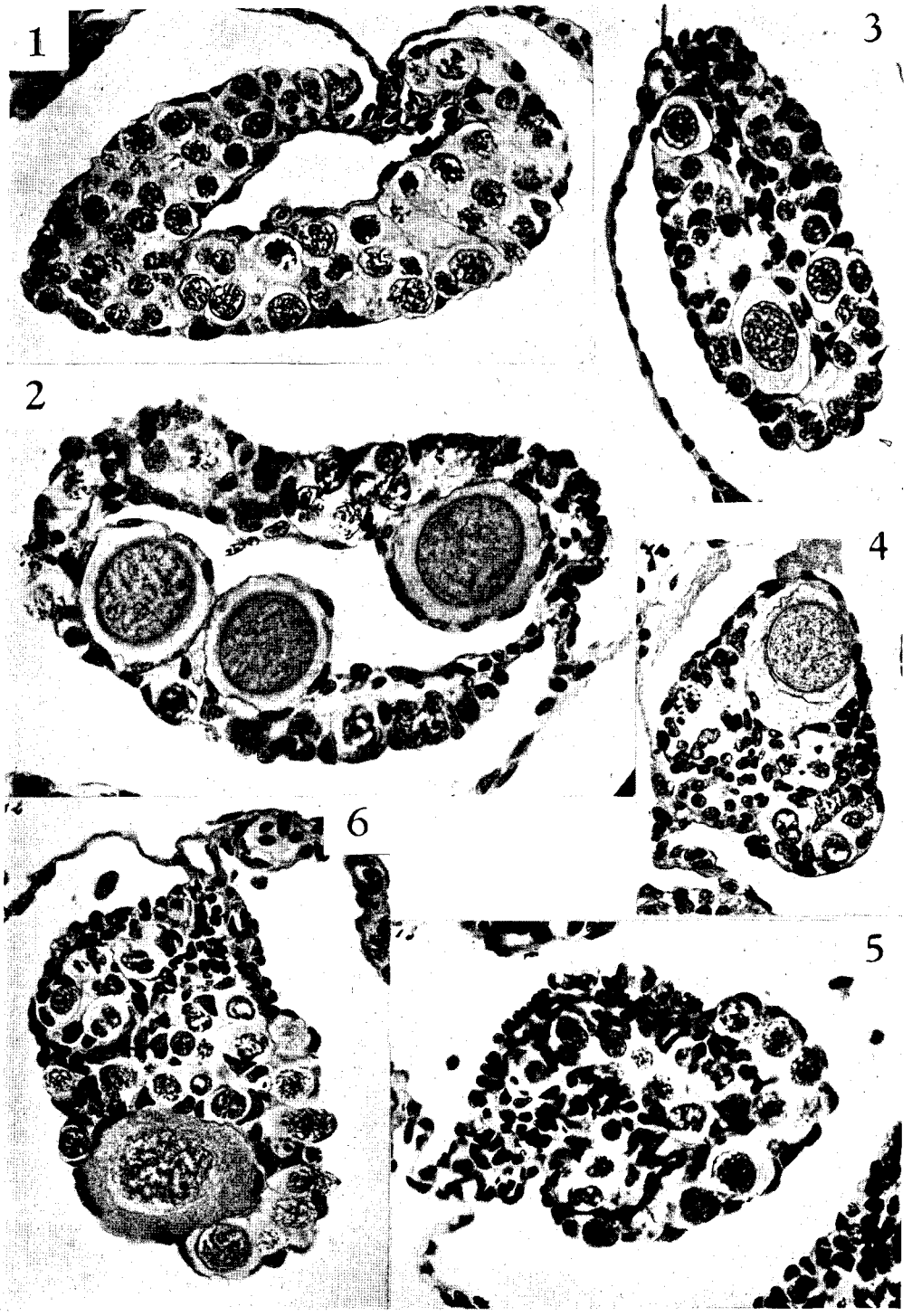
52. Testis of *lichenatus* male, germ cells rather rare.
53–54. Two sections of free-martin ovary of *retardatus* female. In 54 the cavity is completely occluded by a large amount of rete cells.

H. lichenatus ♂ ×*H. retardatus* ♀ (c)

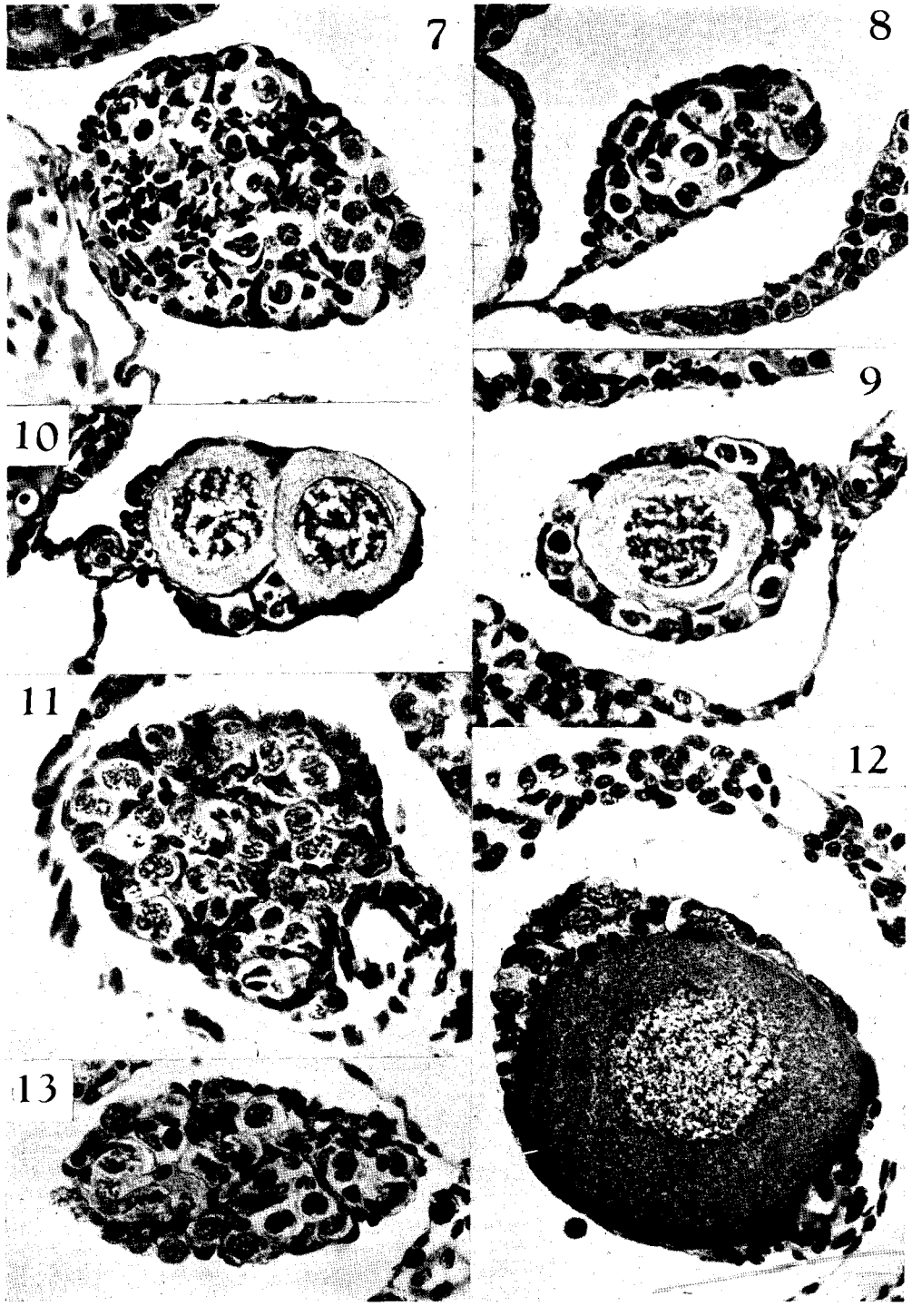
55. Testis of the *lichenatus* male.
56–57. Two sections of the free-martin ovary of *retardatus* female.

H. lichenatus ♂ ×*H retardatus* ♂ (a, b, c)

58–60. Three sections of testis of *retardatus*, showing the covering of irregularly arranged cortical germ cells.
61. Sterile gonad of *retardatus*.
62. Sterile gonad of *lichenatus*.



K. I. Hanaoka: Sex-differentiation in two Japanese salamanders



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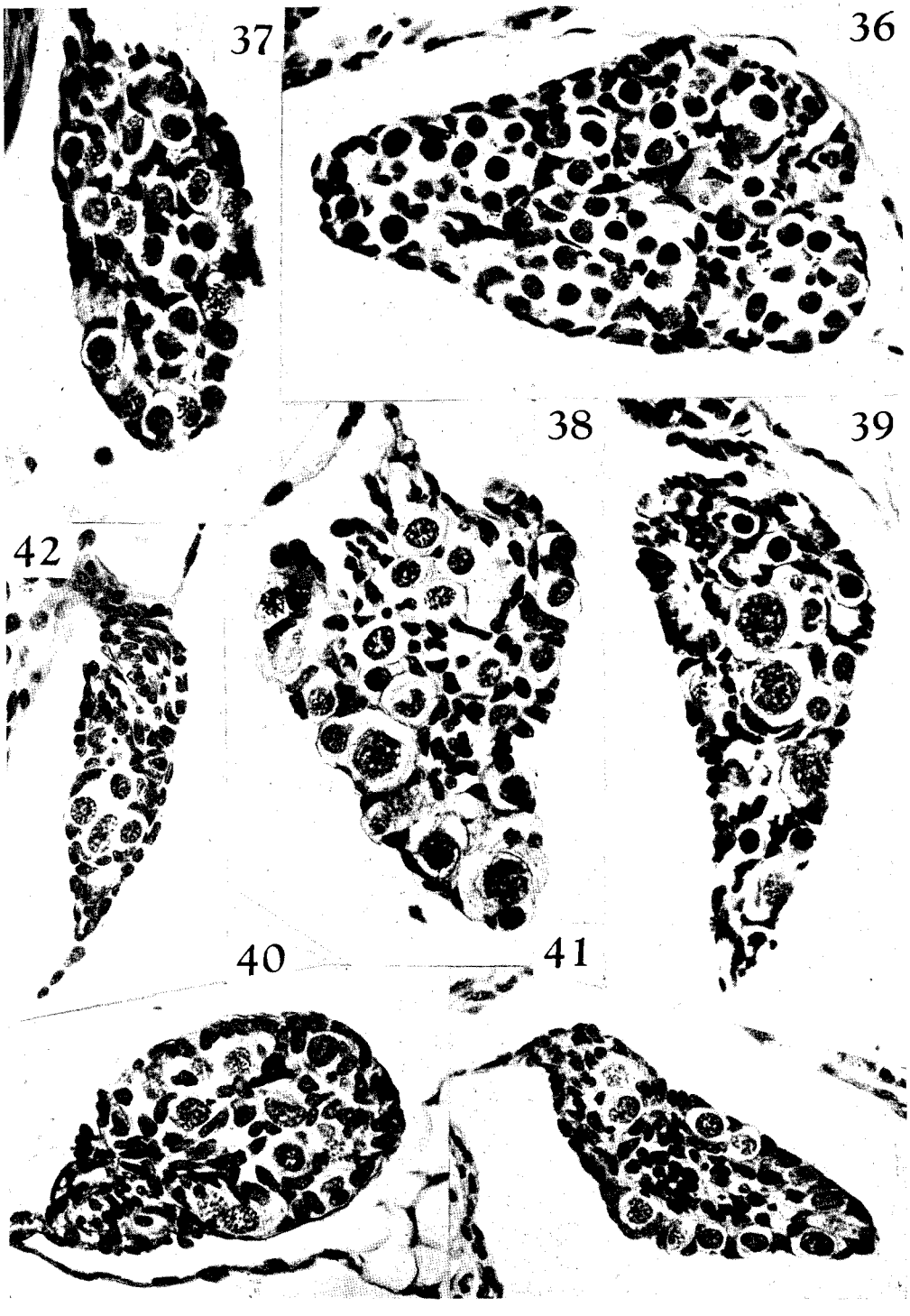

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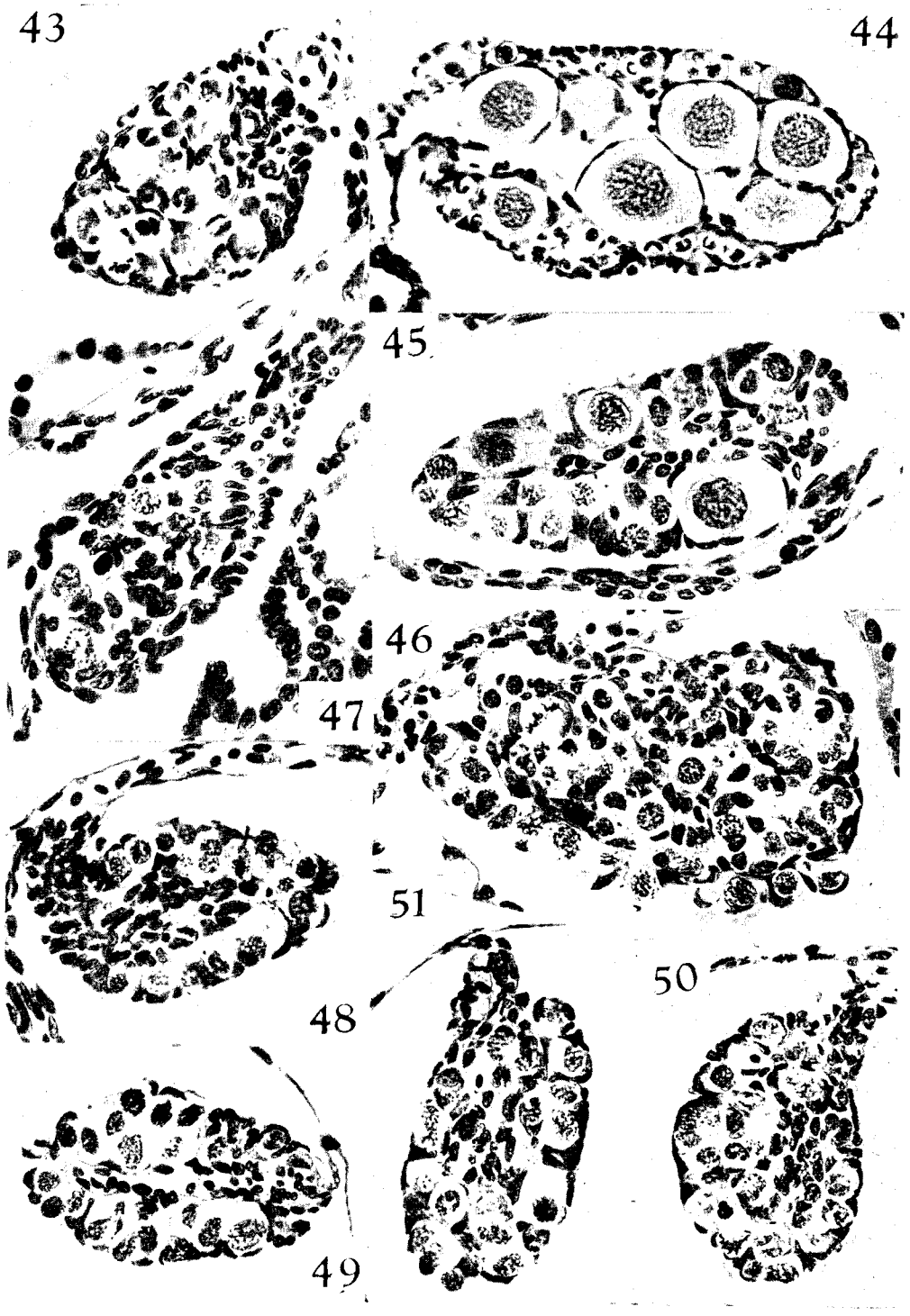
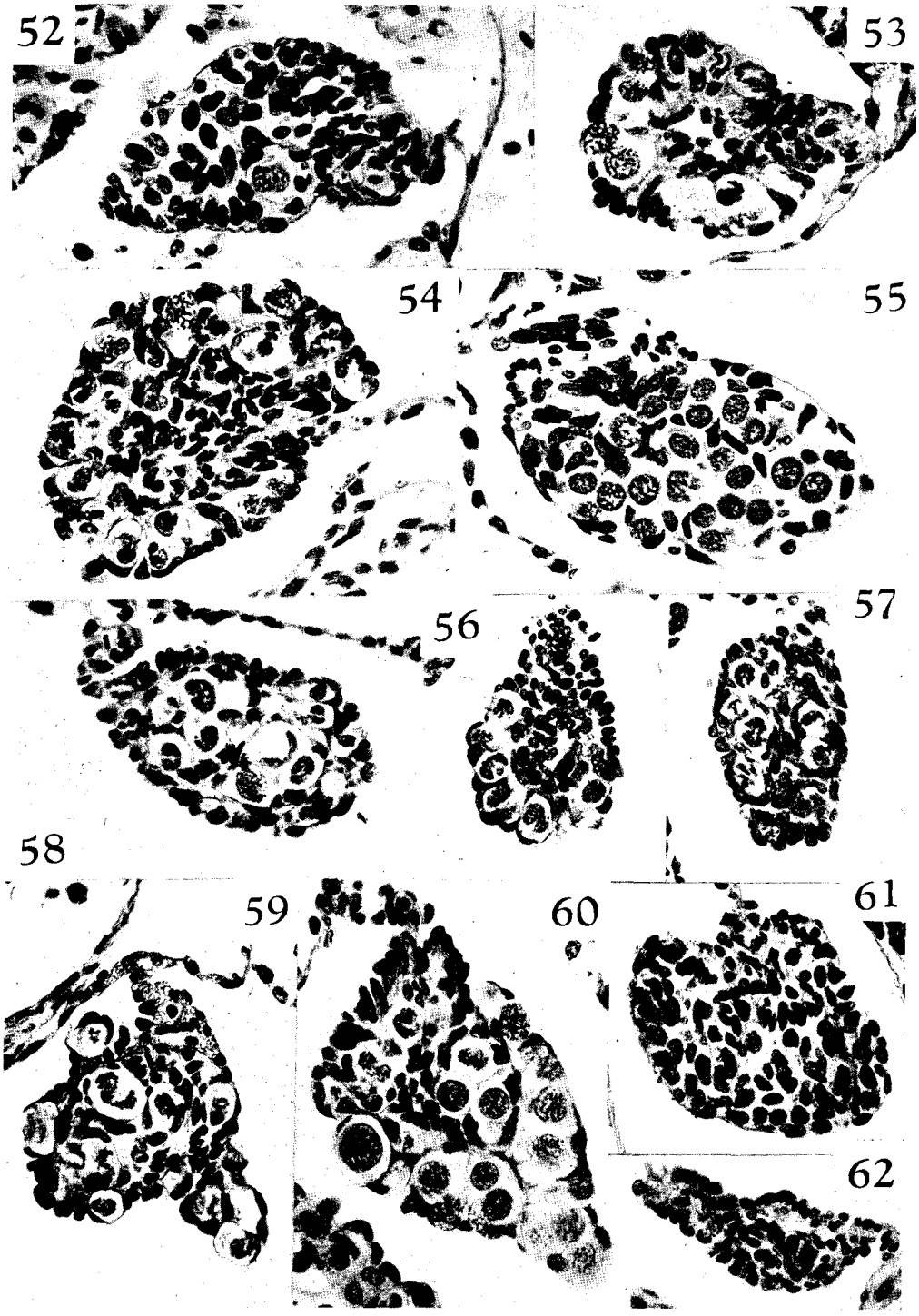


K. I. Hanaoka: Sex-differentiation in two Japanese salamanders



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