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Artificial Parthenogenesis in Rana Pipiens

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ARTIFICIAL PARTHENOGENESIS IN RANA PIPIENS

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Brief Survey of Contents of Thesis

ARTIFICIAL PARTHENOGENESIS

The majority of eggs cannot develop unless they are fertilized, that is to say, unless a spermatozoon enters the egg. The question arises: How does the spermatozoon cause the egg to develop into a new organism? The spermatozoon is a living organism with a complicated structure and it is impossible to explain the development of the egg from the structure of the spermatozoon. No progress was possible in this field until ways were found to replace the action of the living spermatozoon by well-known physicochemical agencies. Various observers such as Tichomiroff, R. Hertwig, and T. H. Morgan had found that unfertilized eggs may begin to segment under certain conditions, but such eggs always disintegrated in their experiments without giving rise to larvae. In 1899 Loeb succeeded in causing the unfertilized eggs of the sea urchin Arbacia to develop into swimming larvae, blastulae, gastrulae, and plutei, by treating them with hypertonic sea water of a definite osmotic pressure for about two hours. When such eggs were then put back into normal sea water many segmented and a certain percentage developed into perfectly normal larvae, blastulae, gastrulae, and plutei. Soon afterward this was accomplished by other methods for the unfertilized eggs of a large number of marine animals, such as starfish, molluscs, and annelids. None of these eggs can develop under normal conditions unless a spermatozoon enters. These experiments furnished proof that the activating effect of the spermatozoon upon the egg can be replaced by a purely physicochemical agency.

The first method used in the production of larvae from the unfertilized eggs did not lend itself to an analysis of the activating effect of the spermatozoon upon the egg, since nothing was known about the action of a hypertonic solution, except that it withdraws water from the egg; and there was no indication that the entrance of the spermatozoon causes the egg to lose water. No further progress was possible until another method of arti-

ficial parthenogenesis was found. When a spermatozoon enters the egg of a sea urchin or starfish or certain annelids, the surface of the egg undergoes a change which is called membrane formation; and which consists in the appearence of a fine membrane around the egg, separated from the latter by a liquid. O. and R. Hertwig and Herbst had observed that such a membrance could be produced in an unfertilized egg if the latter was put into chloroform or xylol, but such eggs perished at once. It was generally assumed, moreover, that the process of membrane formation was of no significance in the phenomena of fertilization, except perhaps that the fertilization membrane guarded the fertilized egg against a further invasion by sperm. However, since the fertilized egg is protected against this possibility by other means the membrane is hardly needed for such a purpose.

In 1905 Loeb found that membrane formation, or rather the change of the surface of the egg underlying the membrane formation, is the essential feature in the activation of the egg by a spermatozoon. He observed that when unfertilized eggs of the Californian sea urchin Strongylocentrotus purpuratus are put for from one and a half to three minutes into a mixture of 50 c.c. of sea water plus 2.6 c.c N/10 acetic or propionic or butyric or valerianic acid and are then put into normal sea water all or the majority of the eggs form membrances; and that such eggs when the temperature is very low will segment once or repeatedly and may even - if the temperature is as low as 4° C. or less - develop into swimming blastulae; but they will then disintegrate. On the other hand, if they are kept at room temperature they will develop only as far as the aster formation and nuclear division and then begin to disintegrate. It should be mentioned that that the time which elapses between artificial membrane formation and nuclear division is greater than that between the entrance of a spermatozoon and nuclear division.

It was obvious, therefore, that artificial membrance formation induced by butyric acid initiates the processes underlying development of the egg

but that for some reason the egg is sickly and perishes rapidly.

When, however, such eggs were given a short treatment with hypertonic sea water or with lack of oxygen or with KCN they developed into normal larvae. This new or improved method of artificial parthenogenesis is as follows: The eggs are put for from two to four minutes into 50 c.c sea water containing a little amount of N/10 butyric acid (2.6 c.c in the case of S. purpuratus in California and 2.0 c.c. in the case of Arbacia in Woods Hole). Ten or fifteen minutes later the eggs are put into hypertonic sea water (50 c.c. sea water plus 8 c.c. 2½ m NaCl or Ringer solution or cane sugar) in which they remain, at 15° C. from thirty-five to sixty minutes in the case of purpuratus, and from 17½ minutes to 22½ minutes at 23° C. in the case of Arbacia at Woods Hole. If the eggs are then transferred to normal sea water they will develop. In making these experiments, which have been repeated and confirmed by many investigators, it should be remembered that this effect of the hypertonic solution has a high temperature coefficient (about two for 10° C.) and that a slight overexposure to the hypertonic sea water injures the eggs so that development is abnormal. By this method it is possible to imitate the activating effect of the living spermatozoon upon the egg in every detail and eggs treated in this way will develop in large numbers into perfectly normal larvae.

The next task was to find out the nature of the action of the two agencies upon the development of the egg. It soon became obvious that the membrane formation (or the alteration underlying membrane formation) was the more important of the two, since in the eggs of starfish and annelids this was sufficient for the production of larvae; and that the second treatment had only the corrective effect, of overcoming the sickly condition in which mere membrane formation had left the eggs. It was, therefore, of great interest to ascertain what substances or agencies caused membrane formation in the egg, since it now became clear that the spermatozoon could only cause membrane

formation by carrying one such substance into the egg. These investigations led Loeb to the result that all those substances and agencies which are known to cause cytolysis or hemolysis will also induce membrane formation, and that the essential feature in the causation of development is a cytolysis of the superficial or cortical layer of the egg. As soon as this layer is destroyed the development of the egg can begin.

The substances and agencies which cause cytolysis and hence, if their action is restricted to the surface of the egg, will induce development are, besides fatty acids: (1) saponin or solanin or bile salts; (2) the solvents of lipoids, benzol, toluol, amylene, chloroform, aldehyde, ether, alcohols, etc.; (3) bases; (4) hypertonic or hypotonic solutions; (5) rise in temperature, and (6) certain salts, e. g., $BaCl_2$ and $SrCl_2$ in the case of the egg of purpuratus, and according to R. Lillie, NaI or NaCNS in the egg of Arbacia. Whenever we submit an unfertilized sea-urchin egg to any of these agencies and restrict the cytolysis to the superficial or cortical layer of the egg (i. e., if we transfer the egg to normal sea water before the cytolytic agent has had time to diffuse into the main egg) the egg will form a membrane and behave as if the membrane formation had been called forth by a fatty acid, with this difference only, that the various agencies are not all equally harmless for the egg.

If the idea was correct that the change underlying membrane formation was essentially a cytolysis of the cortical layer of the egg,it was to be expected that the blood serum or the cell extracts of foreign species would also cause membrane formation, and thus induce the development of the unfertilized egg, while the serum of animals of the same species or genus would have no such effects. This was found to be correct. In 1907 Loeb showed that the blood serum of a Gephyrean worm, Dendrostoma, was able to cause membrane formation in the egg of the sea urchin. When added in a dilution of 1 c.c. of serum to 500 or 1000 c.c of sea water to eggs of purpuratus a certain number

formed fertilization membranes. It was found later that the serum and tissue extracts of a large number of animals, especially of mammals had the same effect, though it was necessary to use higher concentrations, one-half sea water and one-half isotonic blood serum. The eggs of every female sea urchin, however, did not give the reaction and not all the eggs even of sensitive females formed membranes. Loeb found, however, that it was possible to increase the susceptibility of the eggs against foreign blood serum by putting them into a 2/8 m solution of $SrCl_2$ for from five to 10 minutes before exposing them to the foreign blood serum. $BaCl_2$ acts similarly. The fact that $SrCl_2$ alone can cause membrane formation in unfertilized eggs if they are left long enough in the solution suggests that the sensitizing effect of the substance consists in a modification of the cortical layer similar to that underlying membrane formation; and that the subliminal effect of a short treatment with $SrCl_2$ and the subliminal effect of the foreign serum when combined suffice to bring about the membrane formation.

Not only the watery extract of foreign cells but also that of foreign sperm, induces membrane formation in the sea-urchin egg. The watery extract ofsperm of starfish is especially active, but the degree of activity varies considerably with the species of starfish from which the sperm is taken. The eggs of different species of sea urchins also show a different degree of susceptibility for the sperm of foreign species. Thus the eggs of Strongylocentrotus purpuratus require a higher concentration of sperm extract than the eggs of S. franciscanus. For the latter the amount of foreign cell constituents which suffices to call forth membrane formation is so small that contact with almost any foreign living spermatozoon produces this effect; and as a rule no previous sensitizing action of $SrCl_2$ is required. When we bring the unfertilized eggs of S. franciscanus into contact with the living sperm of starfishor shark or even of fowl, the eggs form a fertilization membrane without previous sensitization. A specific substance from the for-

eign spermatozoon causes membrane formation before the spermatozoon has time to enter the egg. The effect is the same as if artificial membrane formation had been called forth with butyric acid, i. e., they begin to develop and then disintegrate unless they receive a second short treatment.

When, however, we treat the eggs with the watery extracts from the cells of their own or closely related species we find that these extracts are utterly inactive, even if used in comparatively strong concentrations.

These phenomena lead to a very paradoxical result; namely that while in the case of foreign sperm we can cause membrane formation by both the living and the dead spermatozoon, only the living spermatozoon of the same species can induce membrane formation. This might find its explanation on the assumption that the active substance contained in the foreign sperm or serum is water-soluble and a protein, while the activating or membrane -forming substance in the spermatozoon is insoluble in water but soluble in the egg. If this assumption is correct the two substances are essentially different.

Robertson has succeeded in extracting a substance from the sperm of the sea urchin which causes membrane formation of the sea urchin egg after the latter has been sensitized by a treatment with $SrCl_2$. It seemed to Loeb that if the substance extracted by Robertson were the real fertilizing agent contained in the spermatozoon it should fertilize the egg without a previous sensitization of the egg with $SrCl_2$ being required.

The action of acids in the mechanism of artificial parthenogenesis provides some interesting physiological problems. When unfertilized sea-urchin eggs are left in sea water containing any of the lower fatty acids up to capronic, the eggs will form no membranes, while in such sea water, and they will show no outer signs of cytolysis (swelling). When, however, the eggs are left in sea water containing any of the fatty acids from heptylic upward the eggs will form membranes while in the acid sea water and soon afterward will cytolyze completely and swell enormously. In solutions of the mineral acids

no membranes are formed and none are formed as a rule when the eggs are transferred back to sea water. When both a mineral and a fatty acid (lower) e. g., butyric, are added to sea water the mineral acid acts as if were not present, i.e., the eggs form membranes when transferred back to sea water if the concentration of the butyric acid is high enough. All these data are comprehensible if we assume that only that part of the acid causes membrane formation which is lipoid soluble, while the water soluble part is not involved in the process of membrane formation; and that the cytolysis or swelling of the whole egg can only take place in the higher fatty acids, whose water solubility is comparatively low and whose lipoid solubility is high, while the lower fatty acids, which are very soluble in water, can only bring about a cytolysis and swelling in the cortical layer but not in the rest of the egg. This makes it appear as though the part undergoing an alteration in membrane formation was a lipoid and this would harmonize with the assumption that the specific membrane-inducing substance in the spermatozoon is not soluble in water, but soluble in fat.

These and other observations led Loeb to the view that the essential process which causes development might be an alteration of the surface of the egg, in all probability an alteration of the superficial layer probably of the nature of a superficial cytolysis. The question remains: What could be the physicochemical nature of this cytolysis? Loeb had suggested in former papers that in the cytolysis underlying membrane formation lipoids were dissolved, and he supposed that the substance to be dissolved might be a calcium-lipoid compound which mightform a continuous layer under the surface of the egg. V. Knaffl, working on the cytolysis of eggs in Loeb's laboratory, gave the following idea of the process.

Protoplasm is rich in lipoids: probably it is mainly an emulsion of these and proteins. Any physical or chemical stimulus which can liquefy the lipoids causes cytolysis of the egg. The protein of the egg can really only swell or

be dissolved if the condition of aggregation of the lipoid is altered by chemical or physical agencies. The mechanism of cytolysis consists in the liquefaction of the lipoids and thereupon the lipoid-free protein swells or is dissolved by taking up water....Hence this supports Loeb's view that membrane formation is induced by the liquefaction of lipoids.

Loeb suggested that the destruction of an emulsion in the cortical layer might possibly be the essential feature of the alteration leading to membrane formation and development. It has been long observed that unfertilized starfish eggs may begin to develop apparently without any outside "stimulus," and A. P. Mathews found that slight mechanical agitation of these eggs in sea water increased the number which developed. It has been shown in numerous experiments by Delage, R. S. Lillie, and Loeb, that the substances causing development in the starfish egg are identical or closely related to those which bring about this effect in the egg of the sea urchin and in both cases the development is preceded by a membrane formation.

But how can membrane formation be produced by mere agitation? It seems to me that this can be understood if we suppose that it depends upon the destruction of an emulsion in the cortical layer of the egg. It is conceivable that in the egg of certain forms the stability of the emulsion is so small that mere shaking would be enough to destroy it and thus induce membrane formation and development.

The durability of emulsions varies, and where an emulsion is very durable shaking has no effect, while where it is at the critical point of separating into two continuous phases a slight shaking will bring about the separation, and where the emulsion is still less durable we observe the phenomena of a "spontaneous" parthenogenesis. Eggs like those of most sea urchins belong to the former, eggs like those of some starfish and annelids belong to the second or third type.

It is impossible to state at present whether the fertilization membrane is preformed in the fertilized egg and merely lifted off from the egg or

whether its formation is due to the hardening of a colloidal substance separated from the emulsion (or excreted) and hardened in touch with sea water. But we can be sure of one thing, namely, that the liquid between egg and membrane contains some colloidal substance which determines the tension and shperical shape of the membrane. The membrane is obviously permeable not only to water but also to dissolved crystalloids, while it is impermeable to colloids. When we add some colloidal solution to the sea water containing fertilized eggs of purpuratus, the membrane collapses and lies close around the egg;while if the eggs are put back into sea water or a sugar solution the membrane soon assumes its spherical shape. This is intellibible on the assumption that in the process of membrane formation (or in the destruction of the emulsion in the cortical layer) a colloidal substance goes into solution which cannot diffuse into the sea water since the membrane is impermeable to the colloidal particles. The membrane is, however, permeable to the constituents of sea water or to sugar. Consequently sea water will diffuse into the space between membrane and egg until the tension of the membrane equals the osmotic pressure of the colloid dissolved in the space between egg and the membrane. If we add enough colloid to the outside solution so that its osmotic pressure is higher than that of the colloidal solution inside the membrane the latter will collapse.

It should be stated also that the unfertilized eggs of many marine animals are surrounded by a jelly (chorion) which is dissolved when the egg is fertil-Loeb has shown that the same chemical substances which will induce membrane formation and artificial parthenogenesis will as a rule also cause a swelling and liquefaction of the chorion.

Much space has been devoted to the mechanism of membrane formation since it is likely to give a clearer insight into the physicochemical nature of physiological processes than the phenomena of muscular stimulation and contraction or nerve stimulation, upon which the majority of physiologists base their conclusions concerning the mechanism of life phenomena.

Before we come to the discussion of the second factorin the activation of the egg it should be stated more definitely that for the eggs of some forms the first factor, the process underlying membrane formation, suffices for the development of the egg into a larva and that no second factor is required in these cases. This is true for the eggs of starfish and certain annelids. Thus in 1901 Loeb and Neilson showed that a short treatment with HCl and HNO_3 sufficed to cause some eggs of Asterias in Woods Hole to develop into larvae without a second treatment being needed, and Delage showed the same for CO_2; and in 1905 Loeb found that the eggs of the Californian starfish Asternia can be induced to form a membrane by butyric acid treatment and that ten per cent. of these eggs developed into normal larvae. Quite recently R. S. Lillie observed that the eggs of Asterias at Woods Hole can be caused to form membranes and develop into larvae by a treatment with butyric acid and that the time of exposure required to get a maximal number of larvae varies approximately inversely with the concentration of the acid, within a range of 0.0005 to 0.006 N butyric acid.If the exposure is too short membrane formation will occur without normal development.

All this leads us to the conclusion that the main effect of the spermatozoon in inducing the development of the egg consists in an alteration of the surface of the latter which is apparently of the nature of a cytolysis of the cortical layer. Anything which causes this alteration without endangering the rest of the egg may induce its development. The spermatozoon, therefore, causes the development of the egg by carrying a substance into the latter which effects an alteration of its surface layer.

We will now discuss the action of the second,corrective factor, in the inducement of development. When we cause membrane formation in a sea-urchin egg by the proper treatment with butyric acid it will commence to develop and segment but will disintegrate rapidly if kept at room temperature and the more rapidly the higher the temperature. If, however, the eggs are treated after-

ward for a certain length of time(from thirty-five to sixty minutes at 15° C. for purpuratus and 17½ to 22½ minutes for Arbacia at 23° C.) in a solution which is isosmotic with 50 c.c. sea water plus 8 c.c. 2½ m NaCl, they will develop into larvae, many of which may be normal. Any hypertonic solution of this osmotic pressure, sea water, sugar, or a single salt, will suffice provided the solution does not contain substances that are too destructive for living matter. The hypertonic solution produces its corrective effect only if the egg contains free oxygen; and in a slightly alkaline medium more rapidly than in a neutral medium. The time of exposure in the hypertonic solution diminishes in certain limits with the concentration of OH ions in the solution.

It is strange that in the eggs of purpuratus the corrective effect can also be brought about by exposing the eggs after the artificial membrane formation for about three hours to normal sea water free from oxygen; or to sea water in which the oxidations have been retarded by the addition of KCN. This method is not so reliable as the treatment with hypertonic solution.

What does the hypertonic solution do to prevent the disintegration of the egg after the artificial membrane formation. Loeb suggested in 1905 that the artificial membrane formation alone starts the development but leaves the eggs usually in a sickly condition. The second factor is, according to this view, merely a corrective or curative factor - the hypertonic solution allows the eggs to recuperate from their sickly condition. The following observations will explain the reasons for such an assumption.

Loeb found that if we keep the unfertilized eggs after artificial membrane formation in sea water deprived of oxygen the disintegration of the egg following artificial membrane formation is prevented for a day at least. The same result can be obtained by adding ten drops of 1/10 per cent. KCN to 50 c.c. of sea water, and certain narcotics, e. g., chloral hydrate act in the same way. Wasteneys and Loeb found that chloral hydrate (and other narcotics) in the concentration required do not suppress or even lower the oxidations in the egg to any considerable extent, but they prevent the processes of cell division.

Hence it seems that the egg disintegrates so rapidly after artificial membrane formationbecause it is killed by those processes leading to nuclear division or cell division which are induced by the artificial membrane formation. If we suppress these phenomena of development (for not too long a time) we give the egg a chance to recover and if now the impulseto develop is still active we notice a perfectly normal development. If the egg is kept too long without oxygen it suffers for other reasons and cannot develop; Loeb has shown that if eggs fertilized by sperm are kept for too long a time without oxygen they also will no longer be able to develop normally. The short treatment with a hypertonic solution supplies the corrective factor required, so that the egg can then undergo cell division at room temperaturewithout disintegrating.

The correctness of this interpretation, which is in reality mainly a statement of observations, is proved by the following groups of facts. The older observers had already noticed that the unfertilized eggs of the sea urchinwhen lying in sea water will die after a day or more, and that occasionally such eggs show nuclear division or even the beginning of cell division shortly before disintegration sets in. Loeb has studied this phenomena in the unfertilized eggs of purpuratus and found that only the eggs of certain females show this cell division before disintegration and that the cell division is preceded by an atypical form of membrane formation; the eggs surrounding themselves by a fine gelatinous film comparable to that produced in the egg of Arbacia by a treatment withbutyric acid. It is difficult to say what induces the alteration of the surface of the eggs that lie so long in sea water. It may be due to the CO_2 formed by the eggs - since we know that CO_2 may induce membrane formation - or it may be due to the alkalinity of the sea water or to a substance originating from the jelly surrounding the eggs. It was found that if such eggs are kept without oxygen their disintegration (and cell division) will be delayed considerable. The presumable explanation for

this is that the lack of oxygen prevents the internal changes underlying cell division and thus prevents the disintegration of the egg. The direct proof that an egg in the process of cell division is more endangered by abnormal solutions than an egg at rest has been furnished by numerous observations of Loeb. He showed in 1906 that the fertilized egg of purpuratus dies rather rapidly in a pure m/2 NaCl or any other abnormal isotonic solution, while the unfertilized egg can live for days in such solutions. In a series of papers, beginning in 1905, he showed that the fertilized egg will live longer in hypertonic, hypotonic, and otherwise abnormally constituted solutions when the cell divisions are suppressed by lack of oxygen or by the addition of KCn or of chloral hydrate. It is thus obvious that coincident with the changes underlying nuclear division or cell division alterations occur in the sensitiveness of the egg to salt solutions of abnormal concentration or constitution, e. g., NaCl plus $CaCl_2$ isotonic with sea water, hypertonic, or hypotonic solutions.

We must, therefore, conclude that artificial membrane formation induces development but that it leaves the egg in a sickly condition in which the very processes leading to cell division bring about its destruction; that if it is given time it can recover from this condition and that the treatment with the hypertonic solution also brings about this recovery rapidly and reliably.

Herlant suggested that the corrective effect of the hypertonic solution consisted in the proper development of the astrospheres required for cell division. According to Loeb mere membrane formation does not lead to the formation of sufficiently large astrospheres and hence cell division may remain impossible. Loeb has no a priori objection to this suggestion which agrees with earlier observations by Morgan except that it is at present difficult to harmonize it with all the facts. Why should it be possible to replace the treatment with the hypertonic solution by a suspension of the oxidations in the egg for three hours while we know that lack of oxygen suppresses the formation of astrospheres in the fertilized eggs? What becomes of the astrospheres if the

treatment with the hypertonic solution precedes the membrane formation by a number of hours or a day, and why do they not induce cell division, if Herlant's idea is correct. Nevertheless the suggestion of Herlant deserves to be taken into serious consideration.

How can an alteration of the surface of the egg - e. g., a cytolytic or other destruction of the cortical layer - lead to a beginning of development? The answer is possibly given in the relation of oxidation to development. Loeb found in 1895 that if oxygen is withdrawn from the fertilized sea-urchin egg it can not segment and this seems to be the case for eggs in general. In 1906 he found that the rapid disintegration of the eggs of the sea urchin which follows artificial membrane formation could be prevented when the eggs were deprived of oxygen or when the oxidations were suppressed in the eggs by KCN. This suggested a connection between the disintegration of the egg after artificial membrane formation and the increase in the rate of oxidations; and he found further that the formation of acid is greater in the fertilized than in the unfertilized egg. He, therefore, expressed the view in 1906 that the essential feature (or possibly one of the essential features) of the process of fertilization was the increase of the rate of oxidations in the egg and that this increase was caused by the membrane formation alone. These conclusions have been since amply confirmed by the measurements of O. Warburg as well as those of Loeb and Wasteneys, both showing that the entrance of the spermatozoon into the egg raises the rate of oxidations from 400 to 600 per cent., and that membrane formation alone brings about an increase of similar magnitude. Loeb and Wasteneys found that the hypertonic solution does not increase the rate of oxidations in a fertilized egg. It does do so, however, in an unfertilized egg without membrane formation, but merely for the reason that in such an egg the hypertonic solution brings about the cytolytic change in the cortex of the egg underlying membrane formation. According to Warburg it is probable that the oxidations occur mainly if not exclusively at the surface of the egg since NaOH, which does not diffuse into the egg, raises the rate of

oxidations more than NH_4OH which does diffuse into the egg. And finally, the same author showed that the oxidations in the sea-urchin egg are due to a catalytic process in which iron acts as a catalyzer. In view of all these facts and their harmony with the methods of artificial parthenogenesis the suggestion is justifiable that the alteration or cytolysis of the cortical layer of the egg is in some way connected with the increased rate of oxidations.

The question remains then: How can membrane formation or the alteration of the cortical layer underlying membrane formation cause an increase in the rate of oxidations? One possibility is that the iron (or whatever the nature of the catalyzer may be) exists in the cortex of the egg in a masked condition - or in a condition in which it is not able to act - while the alteration of the cortical layer makes the iron active. It might be that either the iron or the oxidizable substrate is contained in the lipoid layer in the unfertilized condition of the egg and that the destruction or cytolysis of the cortical layer brings both the iron and the oxidizable substrate into the watery phase in which they can interact.

Another possibility is that the act of fertilization increases the permeability of the egg. This idea, which seems attractive, was first suggested and discussed by Loeb in 1906. He had found that when fertilized and unfertilized eggs were put in into abnormal salt solutions, e. g., pure solutions of NaCl, the fertilized eggs died more rapidly than the unfertilized and he pointed out that these experiments suggested the possibility that fertilization increases the permeability of the egg for salts. The reason for his hesitation to accept this interpretation was, that the fertilized egg is also more easily injured by lack of oxygen than the unfertilized egg and in this case the greater sensitiveness of the fertilized egg was obviously due to its greater rate of metabolism. Later experiments by Loeb showed that the fertilized egg can be made more resistant to abnormal salt solutions if its development is suppressed by lack of oxygen or by KCN or by certain narcotics. With our pre-

sent knowledge it does not seem very probable that lack of oxygen dimishes the permeability of the egg, but we know that it inhibits the developmental processes. Warburg has made it appear very probable that the fertilized egg is impermeable for NaOH and if this is the case it should also be impermeable for NaCl.

The idea that fertilization and membrane formation cause an increase in permeability of the egg was later accepted and elaborated by R. Lillie. This author assumes that the unfertilized egg cannot develop because it contains too much CO_2 but that the CO_2 can escape from the egg as soon as its permeability is increased through the destruction of the cortical layer of the egg. After the CO_2 has escaped, the excessive permeability must be restored to its former value and this is the role of the hypertonic treatment. It is, however, difficult to harmonize the assumption of an impermeability of the unfertilized egg for CO_2 with the fact that if the unfertilized egg is cut into two, as is done in merogony, no development takes place, while such pieces will develop when a spermatozoon enters. The cortical layer is removed along the cut surface and there is no reason why the CO_2 should not escape. Besides, the experiments of Godlewski and Loeb prove that the cortical layer of the unfertilized sea-urchin egg is apparently very permeable for CO_2 since the latter causes membrane formation if contained in the sea water in sufficiently high concentration.

Lillie assumes that the hypertonic treatment restores the permeability raised to excess by the butyric acid treatment, but this assumption is not in harmony with the following facts. Loeb has shown that it is immaterial whether the eggs are treated first with the hypertonic solution and then with the butyric acid or the reverse, if only the eggs remain longer in the hypertonic solution when the hypertonic treatment precedes the butyric acid treatment. It has been stated above that the development of the egg can be induced by hypertonic sea water, and we know the reason since hypertonic sea water is a

cytolytic agency. Loeb has foundthat when we expose unfertilized eggs of purpuratus for from two to two and a half-hours to hypertonic sea water they will often not develop and only a few eggs will undergo the first cell divisions, then going into a condition of rest. When these eggs, both the segmented and unsegmented, were treated twenty-four or thirty-six hours later with butyric acid, so that they formed a membrane, they all developed into larvae without further treatment. It is impossible to apply Lillie's theory to these facts, for the simple reason that the treatment with hypertonic sea water was just long enough to induce development in some eggs and hence according to Lillie's ideas must have increased the permeability of these eggs. Yet these same eggs were induced to develop normally when subsequently treated with butyric acid, while according to Lillie also acts by increasing the permeability. Nothing indicates that the treatment of the eggs with a hypertonic solution diminishes their permeability; the reverse would be much more probable.

Lillie's theory also fails to explain that mere treatment of the eggs with a hypertonic solution can bring about their development into larvae. This, however, is intellibible on the assumption that the hypertonic solution in this case has two different effects, first a cytolysis of the cortical layer of the egg and second an entirely different effect, possibly upon the interior of the egg, which represents the second or corrective effect.

McClendon has shown that the electrical conductivity of the egg is increased after fertilization, and J. Gray has found that this increase in conductivity is only transitory and disappears in fifteen minutes. This might indicate that the egg becomes transitorily more permeable for salts after the entrance of the spermatozoon or after membrane formation; alhough an increase in conductivity might be caused by other changes than a mere increase in permeability of the egg. Loeb is of the opinion that it is necessary to meet all these and other difficulties before we can state that the alteration of the

cortical layer, which is the essential feature of development, acts chiefly or exclusively by an increase in the permeability of the egg.

When the experiments on artificial parthenogenesis were first published they aroused a good deal of antagonism not only among reactionaries in general but also among a certain group of biologists. O. Hertwig had defined fertilization as consisting in the fusion of two nuclei, the egg nucleus and the sperm nucleus. No such fusion of two nuclei takes place in artificial parthenogenesis since no spermatozoon enters the egg, and it became necessary, therefore, to abandon Hertwig's definition as wrong. The objection raised that the phenomena are limited to a few species soon became untenable since it has been possible to produce artificial parthenogenesis in the egg of plants as well as of animals, from echinoderms up to the frog; and it may possibly one day be accomplished also in warm-blooded animals. A second objection was that the eggs caused to develop by the methods of artificial parthenogenesis could never reach the adult stage and that hence the phenomenon was merely pathological. There was no basis for such a statement, except that it is extremely difficult to raise marine invertebrates. Delage was courageous enough to make an attempt to raise parthenogenetic larvae of the sea urchin beyond the larval stage and he succeeded in one case in carrying the animal to the mature stage. It proved to be a male.

Better opportunities were offered when a method was discovered which induced the development of the unfertilized eggs of the frog. In 1907, Guyer made the surprising observation that if he injected lymph or blood into the unfertilized eggs of frogs he succeeded in starting development and he even obtained two free-swimming tadpoles. "Apparently the white rather than the red corpuscles are the stimulating agents which bring about the development, because injections of lymph which contain only white corpuscles produce the same effects as injections of blood." Curiously enough, Guyer thought that probably the cells which he introduced and not the egg were developing. In

1910, Bataillon showed that a mere puncture of the egg with a needle could induce development but he believes that for the full development the introduction of a fragment of a leucocyte is required. Bataillon has called attention to the analogy with Loeb's results on lower forms, the puncturing of the egg corresponding to the cytolysis of the surface layer of the egg and the introduction of a leucocyte as the analogue of the second or corrective factor. The method of producing artificial parthenogenesis by puncturing the egg has thur far been successful only in the egg of the frog. Loeb has tried it in vain on the eggs of many other forms. He has at present seven parthenogenetic frogs over a year old, produced by merely puncturing the eggs with a fine needle. These frogs have reached over half the size of the adult frog. They can in no way be distinguised from the frogs produced by fertilization with a spermatozoon. This makes the proof conclusive that the methods of artificial parthenogenesis can result in the production of normal organisms which can reach the adult stage.

Bancroft and Loeb tried to determine the sex of a parthenogenetic tadpole and of a frog just carried through metamorphosis. Since in early life the sex glands of both sexes in the frog contain eggs it is not quite easy to determine the sex, except that in the male the eggs gradually disappear and from this and other criteria they came to the conclusion that both parthenogenetic specimens, which were four months old, were males.

Loeb has recently examined the gonads of a ten months old parthenogenetic frog. Here no doubt concerning the sex was possible since the gonads were well-developed testicles containing a large number of spermatozoa of normal appearence, and no eggs. This would indicate that the frog belongs to those animals in which the male is heterozygous for sex.

The fact that the egg of so high a form as the frog can be made to develop into a perfect and normal animal without a spermatozoon - although normally the egg of this form does not develop unless a spermatozoon enters - corrob-

orates the idea that the egg is the future embryo and animal; and that the spermatozoon, aside from its activating effect, only transmits Mendelian characters to the egg. The question arises: Is it possible to cause a spermatozoon to develop into an embryo? The idea has been expressed that the egg was only the nutritive medium on which the spermatozoon developed into an embryo, but this idea has been rendered untenable by the experiments on artificial parthenogenesis. Nevertheless the question whether or not the spermatozoon can develop into an embryo on a suitable culture medium remains, and it can only be decided by direct experiments. It was shown by Boveri, Morgan, Delage, Godlewski, and others, that if a spermatozoon enters an enucleated egg or piece of egg it can develop into an embryo, but since the cytoplasm of the egg is the future embryo this experiment proves only that the egg nucleus may be replaced by the sperm nucleus; and also that the sperm nucleus carries into the egg the substances which induce development. Incidentally these experiments on merogony also prove that the mere tearing of the cortical layer, which must happen in the separation of the unfertilized egg into parts with and without a nucleus,- by dissection or by shaking, is not sufficient to start development in the sea-urchin egg.

J. de Meyer put the spermatozoa of sea urchins into sea water containing an extract of the eggs of the same species but found only that the spermatozoa swell in such a solution. Loeb and Bancroft made extensive experiments in cultivating spermatozoa of fowl in vitro on suitable culture media. In yolk and white of egg the head of the spermatozoon underwent transformation into a nucleus, but no mitosis or aster formation was observed.

Artificial Parthenogenesis of the Frog Egg

I. Materials needed:

A frog with eggs in uteri and a female frog for blood.

Glassware: 6-8 large sized slides, 2-3 pipettes and a large mouthed pipette, a scalpel, 6-8 petri dishes, a moist chamber, 4-5 syracuse watch glasses, 2-3 liters of chlorine-free spring, pond or tap water heated to 55-70° C (for sperm sterility), cooled and aerated (kept in closed containers).

Needles: May be made from glass or from platinum or manganese wire about 30 mu in diameter. Glass needles are made by heating the ends of glass rods (1/8" diameter) together in a micro-burner flame until soft enough to stick together and be pulled apart. A short stout sharp point (which may be as small as 10 mu in diameter) should be produced. A long slender point is too flexible. Successful procedures are only acquired by experience. Examine them with a 16 mm. objective. Micropipette needles may also be used. Platinum needles may be prepared tapered nearly to the diameter of the wire and then sealed in by heat, or they may first be sealed into capillary needles and then sealed or fastened into a tapered tube.

Miscellaneous: paper towelling, thermometer, glass marking pencil, a Grenough binocular, with about 20X magnification, illumination preferably from a heatless spotlight, complete dissection kit.

II. Sterilization: All glassware, instruments, water, frog surfaces, hands, must be sperm-sterile. 55-70° C. or 70% alcohol is sufficient for this.

III. Procedure: 1) A slide was placed upon an inverted watch glass

and the spotlight focused to fall upon the eggs which were to be placed on slides at this level after stripping.

2) The eggs had to be smeared with blood before they were pricked. The blood was obtained from a female frog to avoid sperm contamination. It was first incapacitated by vigorously bumping its head two or three times upon the table top. Then it was immersed in 70% alcohol for 2-3 minutes and placed on its back on sperm-sterile wet towelling in a small tray which when covered formed a moist chamber. The abdomen was dried and opened by means of sterile instruments. Lateral incisions were made in the body wall sufficient to allow the abdominal wall to lie open. The tip of the heart was cut off and the blood allowed to escape into the coelom. Previous to opening the abdominal cavity the gastrocnemius was carefully excised so as to prevent an excessive loss of blood. If the frogs are rather small it would be best to use two, using the gastrocnemius of one and the blood of the other because it is very important that an adequate supply of blood be available. The gastrocnemius is used to smear the eggs prior to pricking. The blood may be oxilated but should be diluted as little as possible for diluted blood gives fewer cleavages. The frog should be kept covered (with a glass plate) at all times in its moist chamber so as to prevent the blood from coagulating in so far as possible.

3) Using forceps 2 slides were placed on separate inverted watch glasses. Onto these the eggs were stripped. In time after one has acquired sufficient technique it is possible to strip 5 or 6 slides at a time.

4) Stripping: From the frog with eggs in uteri all dripping water was removed with a towel and the hands and fingers were likewise thoroughly dried. The dry frog was placed in the palm of the right hand with its head pointing toward the wrist, dorsal surface uppermost. The left hind leg was held between the 2nd and 3rd fingers of the right hand, and they were curled around on the ventral surface of the frog extending over the abdomen just behind the posterior tip of the sternum, one directly behind the other. In so far as

I know this is a new technique. The technique advocated and employed by other investigators in the field is slightly different and didn't seem to be as satisfactory to me as did my own. Their method is to hold one hind leg between the 4th and 5th fingers with the first finger extending over the abdomen just behind the posterior tip of the sternum and the middle finger next behind it. The frog's other hind leg (right) is held with the left hand. With gentle pressure of the two fingers on the abdomen and with the thumb or index finger of the left hand on the side of the abdomen eggs emerged from the anus in a double row, one from each uterus. Excessive pressure must be guarded against lest the eggs emerge in large clumps making successful pricking virtually impossible. As the eggs emerged they were allowed to fall upon the above two sperm-sterile slides forming on each slide two rows, first along one side of the slide and then along the opposite edge. Care was taken to keep the frog 1/2-3/4" away from the slide nor were the fingers allowed to touch the slide. One slide was then placed in a moist chamber while the eggs on the other were pricked. (Eggs that happened to become attached to the frog's anal region or to the fingers were easily removed by touching with a piece of paper towel to which they adhered).

5) Pricking:

a) The eggs on the remaining slide was smeared with blood from the female prepared above, using the gastrocnemius dipped in the blood in the pericardial cavity.

b) With the aid of the binocular and the spotlight each egg was pricked as shallowly as possible with a minimum of injury. The eggs may be pricked at any point but the largest number of cleavages was obtained from eggs pricked on the animal pole. Injury to the pro-nucleus of the egg was avoided by pricking it slightly off the center of the animal pole.

c) As soon as the eggs had been pricked the slide was immersed in a petri dish 3/4 filled with sperm-sterile water. The other slide was then similarly treated. Two slides of eggs at a time were used to avoid desiccation

of the eggs while waiting to be pricked.

d) After the eggs were pricked they were counted and the number and time of pricking recorded.

e) To prevent pressure upon the eggs, after 10-15 minutes the swelling jelly was carefully loosened from the slide with a scalpel. The two ends of each row were left attached to the slide but the row was cut midway between either end to permit the jelly to expand without buckling the row as it imbibed water.

f) The water was changed after the first 30 mins. and when turbidity appeared later.

V. Preliminary Observations and Records:

About four hours after pricking the eggs began to cleave. It was necessary to distinguish between superficial furrows and puckering of the eggs and true cleavages. The true cleavage furrows were quite deep and clear cut. As soon as the living eggs reached the many celled stage they were separated from the others with a sharp scalpel and teasing needles and transferred with a wide mouthed pipette to another petri dish with as little water as possible being carried over from the first petri dish due to its probably being polluted from the dead eggs. From then on the eggs were removed as they died and the water changed from time to time. Examinations were made approximately every two hours.

The first attempt due to a certain degree of technique ignorance was not very successful. Subsequent attempts with vastly improved technique were far more satisfactory.

Of the 167 eggs pricked the first time only about a half dozen illustrated cleavage and these died almost immediately thereafter. It would practically an impossibility to cite all the factors entering into the experiment which would determine whether or not it would be successful. In work of this nature the number of variables is unlimited and satisfactory results are obtained only after repeated attempts and development of faultless technique.

The following three attempts were far more successful and quite a few eggs reached the many celled stage. Below are records of the history of the eggs pricked in all four attempts.

Records of Eggs Pricked

	First Set	Second Set
Species of frog ---	Rana pipiens	Rana pipiens
Type of water -----	Tap water	Pond water
pH of water -------	7.3	7.7
Water temperature -	23°C.	23°C.
Number of eggs ----	125	304
Time pricked ------	4/8/41 3 P. M.	4/15/41 3 P. M.
% of eggs reaching cleavage stage ----	8%	11%

	Third Set	Fourth Set
Species of frog ---	Rana pipiens	Rana pipiens
Type of water -----	Tap water	Pond water
pH of water -------	7.3	7.7
Water temperature -	23°C.	23°C.
Number of eggs ----	460	619
Time pricked ------	4/16/41 3 P. M.	4/24/41 3 P. M.
% of eggs reaching cleavage stage ----	15%	21%
% of eggs reaching many celled stage -	7%	11%

In so far as I was able to determine eggs placed in sperm-sterile tap water and eggs placed in sperm-sterile pond water showed no difference in development. About the same number of eggs in each medium underwent cleavage, at about the same time, and to the same degree. Eggs placed in Ringer's solution remained dormant. I assumed that the Ringer's solution was toxic to the eggs but it 's possible that such was not the case and that imhibition of development might have been brought about by any one of the many conditions of which I spoke of before as for example; (1) the damaging effect of handling the eggs which includes abnormal pressure in stripping, (2) desiccation of the eggs while on the slide waiting to be pricked, (3) injury caused during the smearing with blood, (4) injury incurred during the actual pricking which is no doubt the most difficult step in which to become proficient. It should be realized that the eggs are extremely delicate and quite liable to injury. The point of puncture, the depth of the puncture and the diameter of the needle are all important factors and should be the object of careful consideration.

Cleavage and the Origin of the Germ Layers

Promptly following the union of the male and female sex cells, the fertilized ovum enters on a series of cell divisions which give the first external sign of development in the ordinary sense. This initial period in the development of a new, many-celled individual is called cleavage, or less appropriately segmentation. By it the egg is split up, or fractionated, into a number of smaller cells, termed blastomeres.

The object of this paper is of course to prove that the exact same development of a frog's egg such as occurs after the fusion of the female and male pro-nuclei may be initiated by a mechanical force alone (by pricking with a fine needle), that this development is materially aided by a chemical agent (blood), and most important, to suggest the possible significance of each step in a light that will aid and stimulate further investiga-

tion. Below follows a discussion of cleavage or segmentation and the origin of the germ layers which phenomena are identically alike whether induced artifically or occurring naturally.

Cleavage proceeds by an orderly succession of mitoses which typically tends to follow the doubling sequence 2, 4, 8, 16, etc., although in practice the regularity of this series is sooner or later disturbed and becomes irregular. A further characteristic of cleavage is that the rate of cell division is too rapid to permit the customery intervening growth of the daughter cells as a whole, although the nuclei do grow and maintain their size. Consequently, at each mitosis throughout cleavage the blastomeres are progressively halved in size, until finally the size-relations between the originally over-large cell bodies and their nuclei are reduced to normal. In a strict sense, therefore, cleavage is a fractionating process which provides building units, rather than a process of truly constructive development. Such cleavage divisions are always mitotic and each daughter cell receives the full assortment of chromosomes, half from each parent. Naturally in so far as the eggs in this experiment are concerned there is only one parent, the female, and opinions regarding the chromosome number of artifically parthogenized eggs differ. This particular phase of egg development will be taken up separately near the end of this paper. Returning to the subject of cleavage divisions once again - the resulting cluster of cohering blastomeres is sometimes called a morula from its general resemblance to a mulberry. By this time the blastomeres have so arranged themselves as to surround a central, free space. Their continued subdivision produces a hollow shpere, the blastula, whose central cavity is the blastocoele, or cleavage cavity. This state of development marks the end of the cleavage period.

The embryo next makes an important advance by becoming two layered; the stage itself is designated the gastrula, and the process is gastrulation. The two germ layers, thus formed, are the outer ectoderm and the inner entoderm. Directly following, or even overlapping, gastrulation comes the addition of

a third germ layer, the mesoderm, inserted between the other two. This last step is somewhat complicated by the simultaneous development of the notochord (primitive backbone). Since the gut and neural tube soon appear also, the fundamental ground-plan of the vertebrate body is laid down early.

Most eggs have a principal axis connecting the two opposite poles (animal and vegetal), and are radially symmetrical with respect to this axis. Some, like the frog's egg, become bilaterally symmetrical during fertilization, and perhaps even earlier. Moreover, different animal groups vary as to the existence of a relation between the plane of the first cleavage and the future median plane of the body. In some the first cleavage separates the egg into prospective right and left halves of the body; in others it cuts across the future median plane, while in still others a constant relation is lacking. The degree of potency of individual blastomeres is another variable feature. In certain animals (e.g., sea urchin) these can be separated and made to develop into perfect embryos; that is, the early blastomeres are totipotent and development is 'regulative.' Other cleavage stages (e.g., tunicates) are unalterable and from the beginning have their various formative substances sorted out among the component blastomeres; in such forms the early morula is like a mosaic, and isolated blastomeres develop into partial larvae. Cleavage stages of frogs' eggs must likewise be unalterable because all efforts on my part to derive embryos from component blastomeres of those frog eggs which reached the many celled stage in my experiment resulted in failure. In all animals, however, this segregation into a fixed mosaic-whole enters eventually, even though tardily. Since it is known that two or more embryos sometimes develop from a single mammalian ovum, including that of man, it follows that in these instances the egg is regulative, much like that of the sea urchin.

It is the active protoplasm of the egg that accomplishes division. The inert, stored yolk-substance is not involved beyond acting as an impediment that retards the process of mitosis and even prevents it from extending into

overdense regions. In this way the relative amount of yolk and its even or uneven distribution throughout the egg have a profound influence on cleavage and the distribution of the germ layers. Yet, in spite of the hindering yolk, which causes proportionate modifications in the physical appearence of these stages, the processes at work and the results accomplished are fundamentally comparable in all vertebrate types. The simplest explanation of this basic uniformity is the directing influence of a common inheritance which labors as best it can with eggs variously endowed with yolk.

On the basis of the abundance and distribution of yolk, cleavage is classified as follows:

(A) Total. Entire ovum divides; holoblastic ova.

1. Equal. In isolecithal ova; blastomeres are of equal size; e.g., Amphioxus and true mammals.
2. Unequal. In moderately telolecithal ova; yolk accumulated at vegetal pole retards mitosis, and fewer but larger blastomeres form there; e.g., lower fishes and amphibia.

(B) Partial. Protoplasmic regions alone cleave; meroblastic ova.

1. Discoidal. In highly telolecithal ova; mitosis restricted to animal pole; e.g., higher fishes, reptiles and birds.
2. Superficial. In centrolecithal ova; mitosis restricted to the peripheral cytoplasmic investment; limited to arthropods.

Observations on cleavage bring to light certain general priciples which can be formulated as rules. Nevertheless, these must not be regarded as invariable laws because they are occasionally disturbed by other, incidental influences.

1. A mitotic spindle occupies the 'center of density' of its protoplasmic mass. (In an isolecithal ovum the spindle is located centrally; in a telolecithal ovum it is nearer the animal pole.)

Corollary: Blastomeres divide into two equal parts unless the yolk is unevely stored.

2. The axis of a spindle occupies the longest axis of its protoplasmic mass. (Evident in ovoid blastomeres.)

Corollary: The ensuing division plane cuts across this long axis, and the daughter cells revert to a more spheroidal shape.

3. Each new division plane tends to intersect the preceding plane at right angles. (Acts to maintain spheroidal shape of blastomeres.)

4. The speed of cleavage is inversely proportional to the amount of yolk encountered. (In telolecithal ova, animal cells divide faster than vegetal cells.)

The simplest approach to cleavage and the formation of the germ layers is to follow through these stages in type animals. In this manner the increasing influence of yolk in modifying the primitive developmental plane can be traced and appreciated. Since this paper is concerned primarily with the development of frogs' eggs the following description will apply particularly to amphibia.

Cleavage. - The moderately telolecithal ova of these vertebrates are commonly several millimeters in diameter and contain sufficient yolk to crowd the nucleus and most of the cytoplasm near the upper, or animal pole. The first cleavage spindle appears in this 'denser' cytoplasm, above the center of the egg. The earliest visible sign of cell division is a furrow that starts at the animal pole and passes down opposite meridians to meet at the vegetal pole. Extension of this circular constriction inward bisects the egg into two blastomeres. The second cleavage division is also meridional, but the plane of separation is at right angles to the first; the four resulting cells are equal. The spindles for the third cleavage are again located nearer the animal pole, but the division takes place in a horizontal plane. Consequently the upper four cells, thus cut off, are distinctly smaller than the lower four. In the further cleavages that follow, the larger, yolk-laden cells divide more slowly than the smaller, more purely protoplasmic ones of the animal pole. After about 32 cells have been formed, tangential divisions

(i.e., parallel with the surface) begin to occur along with the other types already described. Since in the meantime a central blastocoele makes an appearence, it follows that the wall of the hollow blastula, which results, is several to many cells thick. The cleavage cavity is relatively smaller than that of Amphioxus and it is eccentric in position; the thick floor of this cavity is made up of the larger and less numerous vegetal cells. Cleavage in this group is thus total but unequal.

Gastrulation. - Simple invagination of the vegetal hemisphere of the frog's blastula is not mechanically possible, and accordingly gastrulation is accomplished in a modified matter. Three different processes operate: (1) A certain amount of true invagination occurs along a transverse, crescentic groove located just below the equator of the blastula; here cells move inward and the groove itself deepens into a shallow, cleft-like pocket of entoderm. The cavity thus created is the beginning of the archenteron whose entrance is the blastopore. Although other factors now begin to operate in extending the archenteron, the yolk-laden vegetal cells do become lifted up and are displaced toward the circumference. This re-arrangement of the yolk entoderm obliterates the blastocoele, whereas the capacious space left vacant by the shifted cells is added to the original, narrow archenteron. (2) A zone of actively dividing animal cells occupies the equator of the blastula, bordering on the vegetal hemisphere. This is the germ ring. The segment of it that overlaps the early, shallow archenteron constitutes a fold of tissue known as the dorsal lip of the blastopore. Cells not only proliferate in it but they also move past the margin and continus inward, where they are added progressively to the entoderm. This inturning of marginal cells is involution.
(3) The archenteron further increases in extent greatly by active growth and bodily movement on the part of the dorsal blastoporal lip. This results in a spreading downgrowth over the vegetal hemisphere. Such overgrowth (epiboly) is at first confined to the original crescentic lip, but as other regions of the ring join in the downspread, the crescent grows into a semicircle whose

ends finally meet in a complete circle on the ventral side of the embryo. At the end of this period of overgrowth and confluence, the blastopore is a narrow, circular aperture surrounding an uncovered part of the vegetal hemisphere; the latter bulges as the so-called yolk plug.

For a time the archenteron remains a narrow cleft between the blastoporal lip and the massive entoderm, but the movement of the yolk-entoderm cells, as already described, expands the primitive gut cavity and produces a fairly typical gastrula, except as it is distorted in one region by the presence of larger, yolk-laden cells. Ectoderm and entoderm meet at the rim of the circular blastopore. The entodermal lining of the archenteron is composed partly of the large cells that originally occupied the vegetal pole, and partly of cells that were added by invagination, involution and epiboly.

Mesoderm Formation. - Both mesoderm and involuted entoderm originate at the same time from out of the blastoporal lip. The edge of the ever-advancing lip proliferates and leaves behind a trail of tissue destined to become these layers. But entoderm and mesoderm are not distinguishable as such when first laid down; rather, the new tissue is an undifferentiated, cellular mass. Soon, however, this mass splits into two layers which are the definite entoderm and mesoderm. The mesodermal sheet spreads both through its own growth activity and as the result of continued additions from the blastoporal growth zone. Extending foward and downward it finally becomes a complete, solid middle layer

The thickened plate of mesoderm in the dorsal midplane is the presumptive notochord. It separates away from the rest of the mesoderm and differentiates into an elongate, cellular rod. The adjoining mesoderm, on each side of the notochord, thickens and becomes cut up into a series of block-like somites, segmentally paired. The mesodermal sheet lateral to each set of somites splits into somatic and splanchnic layers; the intervening cleft (coelom), so formed, eventually meets the corresponding cavity of the opposite side at the midventral line of the embryo. While these events have been going on, the ectoderm of the mid-dorsal line thickens into a neural plate, which rolls up into the

neural tube and then detaches. In tailed amphibia the roof of the archenteron consists, for a brief period, of the notochordal plate and part of the general mesoderm, somewhat as in Amphioxus. However, the free edges of the entoderm soon elevate, meet and complete the gut tube. On the other hand, the archenteron of the frog possesses a practically complete entodermal roof from the beginning. In the end the structural relations of both types are conformable with the body plan of a typical vertebrate.

Method of Inducing Ovulation in Rana Pipiens

In order to insure the eggs leaving the ovaries, entering the oviducts and residing in the uterus as mature ova, the following technique, based on the method of Rugh, was followed.

A female frog was incapacitated by vigorously bumping its head on a table. It was then decapitated and the lower jaw cut off. The skin was removed from the dorsal surface of the cranium and then this dorsal surface removed by first making a longitudinal cut with scissors and then picking off the cartilage and bone with forceps thus exposing the dorsal surface of the brain. Next the brain was picked up with forceps by the medulla oblongata and deflected back upon itself exposing the pituitary body cradled in the bone of the ventral surface of the cranium just posterior to the optic chiasm and quite easily identified by its vasculation.

The pituitary gland is a small body of double origin, attached by a stalk to the base of the brain. In making the dissection as above it is necessary to examine the ventral surface of the brain to determine whether or not the pituitary is adhering to this surface which it does occasionally, or is situated as described above, just posterior to the chiasma. The infundibulum, a slightly bilobed outgrowth from the posterior ventral wall of the diencephalon behind the chiasma, and hypophysis, just posterior and attached to the infundibulum, combined are termed the pituitary body.

The different parts of the pituitary have different functions. The

secretion of the anterior lobe is best known through its effects on growth and on the sex plands (gonads). It is for this latter effect that the pituitary is injected into the frog from which the eggs for pricking are to be obtained. Repeated transplants of anterior lobe substance induce precocious sexual maturity and ovarian growth.

After its excision the pituitary was placed at once in approximately one c.c. of a .6 N NaCl solution (a .6 N NaCl solution having the same osmotic pressure as frog blood), teased, thoroughly and completely macerated, and injected by means of a syringe into the lower right quadrant of the coelom of the frog in which ovulation was to be induced.

If the pituitary is not to be used immediately it may be kept indefinitely in absolute alcohol but must be dissolved in .6 N NaCl solution for injection. During the injecting great care must be exercised to make certain that the coelomic wall only is penetrated and not the intestines lying directly beneath. In this experiment two glands were injected the afternoon of the first day and the eggs were in the uterus and ready for stripping the next afternoon. The frog was kept at room temperature throughout the entire course of the experiment and the water was changed daily.

The Chromosomes of Parthenogenetic Frogs

Below follows observations made by Charles L. Parmenter on the important features of interest in connection with Parthenogenetic frogs, namely, the chromosome number and the sex determining mechamism, of which I made brief mention above. Parmenter, at the suggestion of Loeb, undertook the investigation of these problems by using some of the parthenogenetic frogs and tadpoles which the latter had thus far raised.

Previous to 1919, Loeb had succeeded in raising twenty frogs to the adult condition; 15 of these were males, 3 were females, and the sex of the remaining 2 was undetermined. In 1919 Loeb succeeded in raising sixty-five tadpoles

to metamorphosis. One of these has metamorphosed, 17 have been fixed for cytological purposes, 5 have died, and the rest are still tadpoles.

The chromosomes of the gonads of one of these adult males and of 13 of the tadpoles have been examined. In all these individuals the number is clearly diploid. The only two spermatogonial complexes of the adult male sufficiently clear for study, show about 20 chromosomes distinctly and others superimposed. Among the cells undergoing maturation are tetrads in the late prophase stage. These tetrads appear as rings, either completely closed or slighlty open at one point. They are apparently of the same form as tetrads of the normal material. Their number is clearly haploid, but an eact count has not yet been made. In the secretions of the gonads of the 13 tadpoles there are many complexes in which all but one or two chromosomes are entirely clear, and several mitoses in which all the chromosomes are well separated but cannot be counted with certainty because the cell has been cut in sectioning. However, the number of chromosomes in a limited number of complexes of two individuals is definitely twenty-six.

Since none of the individuals studied had the haploid number it is probable that the diploid number is characteristic of the majority, if not for all the parthenogenetic individuals. The diploid number as well as the similarity in form of the tetrads of the parthenogenetic and normal animals may have been brought about by the retention of the second polar body, or by a premature division of the chromosomes without the division of the cell body just before the first cleavage. It is hoped in the future to determine how this condition has arisen.

At the present time the mechanism producing the two sexes in both the normal and parthenogenetic frogs is undetermined. Levy in Rana esculenta, and Swingle in Rana pipiens, describe a sex chromosome in the normal male, but the evidence of neither of these authors is convincing. There are some interesting theoretical possibilities by which a predominance of parthenogenetic males over females as indicated by the numbers so far obtained, might be

produced, and it is hoped that further observations will reveal the exact mechanism.

Conclusion:

Experiments in artificial parthenogenesis are based on, and help to prove a fundamental postulate of physiology, namely, that all vital phenomena may be explained by the laws of physico-chemical science.

Previous to the first artificial parthenogenesis experiments fertilization was believed to be effected by the combination of the male and female pro-nuclei. This belief told us little if anything of the nature of the actual activating force and the function of the sperm was interpreted and viewed from a chemical, physical and more often than not, from a mystical standpoint.

When the development of eggs was initiated by a hypertonic solution of sea water such as was discussed in great detail at the beginning of this paper, the old theory of fertilization being caused by something inherent in the sperm which was carried over into the mature ovum, was of necessity discarded and the phenomena of fertilization was regarded in a new and different light - from the point of view of chemical stimulation. But, when frogs' eggs were caused to develop artificially by simply pricking them with a suitable needle, investigators in the field were obliged once again to alter their stand and acknowledge that development may be initiated by a purely mechanical force.

The purpose of this paper is not to advance any new explanation of the phenomena of fertilization, natural or artificial, but simply to gather together, present, and comment on existing theories advanced by authorities with the purpose of indicating new paths of experimentation which it is hoped will furnish us a satisfactory explanation of the old problem of the true nature of fertilization as a physico-chemical phenomenon.

The explanation probably will be forth coming when the exact relationship

between the physical stimulus and the chemical medium is determined. At present the function of the blood is unknown. Does it affect the tonicity of the egg; does a leucocyte enter the egg and act like a male pronucleus; what would be the affect of using blood from other animals, and what would be the effect of centrifuged blood - It is the answers to these questions which should be sought in order to throw light on what is now a mere observed fact.

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