PHYSIOLOGICAL ONTOGENY.

A. Chicken Embryos.

XI. The pH, Chloride, Carbonic Acid, and Protein Concentrations in the Tissues as Functions of Age.

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(Accepted for publication, April 21, 1926.)

A previous paper (1) reported preliminary analyses of the changes with age in the chief organic constituents (carbohydrate, protein, and fat) of the chicken embryo. The present study concerns itself rather with the physicochemical conditions of the tissues, at least in so far as these can be defined by a knowledge of the concentration of protein, chloride, bicarbonate, and hydrogen ions. The notion was entertained that their simultaneous representation with time variable would provide a simple description of the changing internal environment. It is assumed that the reacting substances and their milieu are mutually interrelated, so that a knowledge of the latter will bear upon the vital activity of the former. It is understood that the present study is statistical in so far as the analyses give artificial values which are to be taken quite arbitrarily to represent some average, the significance of which is not rationally to be deduced. Moreover, in the present state of our ignorance in respect to the physicochemical organization within the functioning cell there is considerable doubt as to the value of these analytic results for the representation of physiological conditions. Particularly is it necessary to examine with suspension of judgment such questions as the normal average range of hydrogen ion concentration in the tissues. Experimental findings and theoretical considerations show that the pH as well as the concentration of other ions varies in different parts of the body, of an organ, and of any one cell. In a metazoan, there-

fore, any figure obtained for the whole organism would be an average value, not necessarily corresponding to the actual conditions at any one locus. It is just these average values, however, which we at present seek to find so that correlations may be made between various chemical constituents in terms of the age of the organism.

Methods.

pH.

There is uncertainty about any described technical procedure, and, but for the relevancy of the H ion concentration in defining important relationships, we should not have undertaken to determine it. No procedure has as yet been developed which does not require an assumption of the constancy of a number of theoretically variable factors. In so much as we seek the changes in pH with age rather than the actual value at any one time, an unknown error, if relatively constant, would not vitiate a conclusion as to the direction of change which the results might seem to indicate.

For multicellular organisms four chief methods for the determination of average pH have been described, two of which are: (*a*) injection of vital dyes (2) and (*b*) microscopic observation of the color of an indicator within cells after small fragments of tissue bathed in dye solution have been squeezed sufficiently to rupture cell membranes and then released so as to allow the indicator to flow back into the interior with some of the expressed cytoplasm (3). These methods have given results of interest for other studies, but as the values obtained are purely local and vary from apparent values of pH 3.0 for intracellular granules in some cells to values higher than in blood for bone and cartilage, one is not able to arrive at an average pH figure for the whole organism. Moreover, one is unable to predicate (1) whether the dye itself affects the equilibrium which it is supposed to measure, (2) what corrections are necessary for protein and other factors within the cell—for these cannot be minimized by dilution,—and (3) to what degree exposure of the surface of the section, organ, or body to oil or air affects the color *in vivo*.

The two other important methods depend upon: (*c*) potentiometer readings at the moment of the thawing of tissues which have been frozen, triturated, and introduced in the form of solid masses into the hydrogen electrode cell (3), and (*d*) gross compression of tissues with colorimetric determination of the expressed fluid (3, 4).

When frozen tissue is placed within an electrode cell a progressive change in pH is recorded as the temperature is gradually raised. It seems that in certain parts of the temperature curve obtained the pH changes are rather sudden, and

Vlès, who has worked with this method, is inclined to attribute these sudden shifts to dissociations involving different radicles of the cell proteins. Vlès' results showed great variations under fixed conditions. The technique is somewhat elaborate, and as we do not know what changes occur upon freezing and thawing and at what point one may consider the reading obtained to represent the true pH of the tissue fluids, we turned to method (*d*) or some modification of it as more suitable for our purposes. Michaelis, and Vlès who have used this method, made no satisfactory provision for the immediate escape of CO_2 from tissue surfaces exposed to air. In our experiments we covered the material with oil to minimize this error. Moreover, there is something to be said in favor of our method from the standpoint of simplicity. The only special piece of apparatus

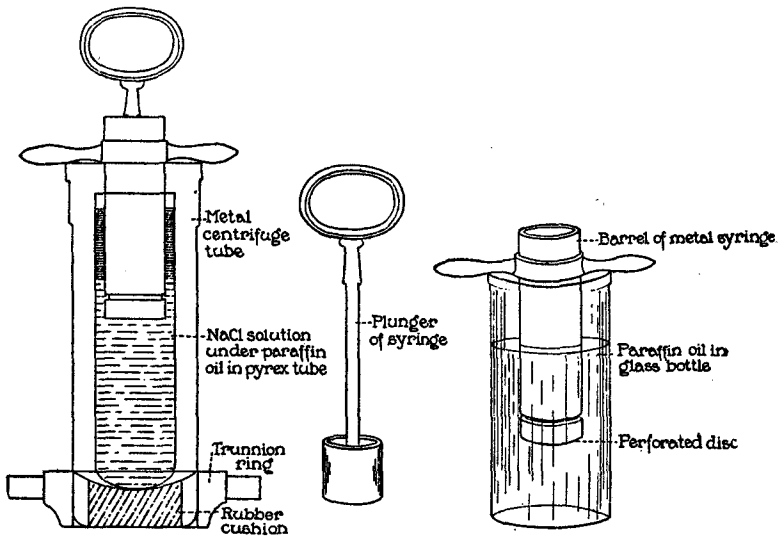

FIG. 1. The apparatus employed for determining the average pH of the tissues of chicken embryos.

required is a metal syringe for the compression of embryonic tissue, with a perforated bottom to allow for the filtration of the expressed fluids. The syringe has a diameter of 15 mm., which is a convenient size for embryos up to 15 days of age. The barrel, capped with a perforated disc, is half submerged in paraffin oil in a small glass bottle (Fig. 1). The egg is opened and as expeditiously as possible the membranes are cut, the embryo lifted out with forceps, and dropped into the oil-containing syringe. The piston is then inserted and the syringe inverted to expel the air. It is immediately introduced into the pyrex tube *B* which contains the indicator dissolved in sodium chloride under oil at such a level that the perforated surface is well below the oil in the tube. The arms of the syringe engage

the edge of the metal centrifuge tube so that all possible force can be applied downward upon the handle of the piston. Juice, with disintegrated formed elements, passes through the sieve into the NaCl solution. 2 minutes of centrifuging throws down the red blood cells and formed elements, leaving the clear solution of tissue juice and indicator in the supernatant saline. The pH of this fluid is read by comparison with colorimetric standards. The method is not unlike Hawkins' procedure for blood (5). For making the standards the bicolor principle was used. Each pH standard value is obtained by placing 2 tubes in a comparator block in line with a blank tube containing dissolved tissue juice of appropriate concentration without dye. One tube containing a certain concen-

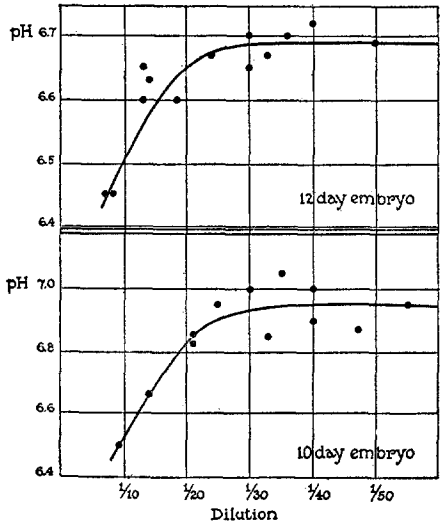

FIG. 2. The effect of dilution (as measured by the amount of formed elements thrown down in centrifuging) upon the value of the pH of tissues.

tration of the dye in its alkaline form and the other a certain concentration of the same dye in its acid form. The tubes were selected from a graded series of each form so as to provide by their combination the color shade corresponding to the desired pH. For the preparation of the standards the technique recently perfected by Hastings and Sendroy has been followed in detail (6).

As has previously been shown for blood, considerable dilution is necessary before constant readings practically independent of further dilution could be made. In the case of tissues it was found that dilutions of 1/30 to 1/70 (as measured by the amount of formed elements thrown down in centrifuging, which is equivalent to about

1/25 to 1/60 in terms of the total volume of extract) gave satisfactory results; or in other words that with 20 cc. of saline the total amount of tissue extract, *i.e.* formed elements + fluid used must lie between 0.33 and 0.80 gm. approximately (Fig. 2).

Bicarbonate and Chloride.

As Van Slyke and others have shown for blood, it would seem that the Cl ion and the HCO_3 ion were perhaps quantitatively and physiologically the most important anions in the body. The method devised by Van Slyke for the estimation of total carbon dioxide in solid carbonates (7), with a few modifications kindly suggested by Dr. Van Slyke himself was used.

Embryos were rapidly separated from their membranes and dropped into 3 cc. of 1.0 N NaOH solution in a weighing bottle. Exposed to the air they were expeditiously and thoroughly cut up with fine scissors, weighed, and then transferred with 10 cc. of water to a 250 cc. suction flask. In the flask was then inserted a tube containing 10 cc. of approximately 0.05 N $Ba(OH)_2$ solution. In the tube was put a small glass rod, which, when the flask was gently rotated, served to stir effectively the contents of the tube. The flask was then evacuated and after standing more than an hour, 5 cc. of 4.0 N lactic acid was allowed to flow into the embryonic extract, so that the NaOH was more than neutralized and because of the evacuated condition of the flask there was a ready evolution of carbon dioxide which combined with the barium hydroxide and was precipitated as barium carbonate. After a 24 hour interval in which the bottle was shaken frequently the barium hydroxide solution in the tube was filtered through a Gooch crucible, washed with 40 cc. of water, and titrated with HCl. Two controls were done with each set of eight determinations and the difference between the titration results for each embryo and the average value for the two controls indicated the amount of carbonic acid given off by the embryonic extract.

The chlorides were estimated by Van Slyke's recently simplified method for tissues, whereby the entire analysis is carried out in one flask (8).

Protein.

The nitrogen estimations reported in a previous paper (1) were used for our calculations. The results multiplied by 6.25 were assumed to be equal to the concentration of protein. A complicating factor is the growth of feathers during the last week of incubation. The feathers, like bone, may be said to have no real part in the dynamic activity of the body, and thus their inclusion in the analysis of

the whole embryo would tend to distort the composite picture by which one might seek to represent the physicochemical pattern of the functioning tissues. The weight of the feathers was determined from the time that they attain measurable mass, *i.e.* from the 13th day of incubation onwards, and then corrections in the total protein

TABLE I.

The Concentrations of Certain Chemical Constituents of the Tissues of Chicken Embryos as Functions of Age.

Age.	pH		Chloride.				Total carbonic acid.				Feathers, per cent of total weight.	Protein, gm. per 100 gm. H_2O.*	Fat, gm. per 100 gm. H_2O.*
	pH	Standard error. ±	Per cent of total solid.	Standard error. ±	Gm. per 100 gm. H_2O.	Millimols.	Per cent of total weight.	Standard error. ±	Gm. per 100 gm. H_2O.	Millimols.			
days													
5												3.95	0.81
6			4.82	0.30	0.291	82.1						4.17	0.87
7			4.76	0.08	0.289	81.5	0.062	0.004	0.066	15.0		4.38	0 95
8	7.00	0	4.48	0.12	0.281	79.3	0.066	0.007	0.070	15.9		4.67	1.03
9	6.96	0.03	4.27	0.10	0.285	80.4	0.074	0.004	0.079	18.0		4.88	1.11
10	6.95	0.03	3.73	0.06	0.272	76.7	0.064	0.002	0.069	15.7		5.31	1.24
11	6.91	0.03	3.10	0	0.265	74.8	0.083	0.005	0.090	20.5		5.88	1.42
12	6.69	0.02	2.58	0.04	0.250	70.5	0.075	0.004	0.082	18.6		6.81	1.69
13	6.71	0.03	2.27	0.13	0.243	68.6	0.092	0.005	0.102	23.2	0.30	7.62	2.05
14	6.69	0.02	1.88	0.09	0.244	68.8	0.095	0.002	0.108	24.6	1.10	8.61	2.61
15	6.64	0.04	1.53	0.04	0.239	67.4	0.089	0.006	0.104	23.6	1.77	10.19	3.40
16			1.27	0.03	0.235	66.2	0.118	0.003	0.141	32.0	2.74	10.88	4.14
17			1.14	0.05	0.225	63.4					2.81	11.25	4.73
18			1.00	0.09	0.225	63.4					2.23	11.76	5.42
19											2.02	11.24	6.08

* Figures in this column were derived from a previous paper (1).

made on the basis that feathers are composed of approximately 90 per cent protein (keratin). This factor may be neglected in estimating the concentration of the electrolytes.

RESULTS.

The results of the pH determinations were more regular and consistent than we had anticipated (Table I). They showed that be-

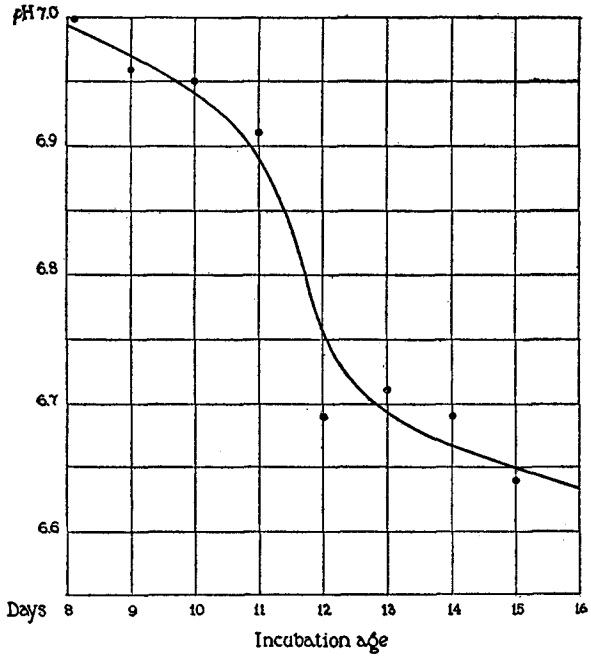

FIG. 3. The pH of the tissues as a function of age.

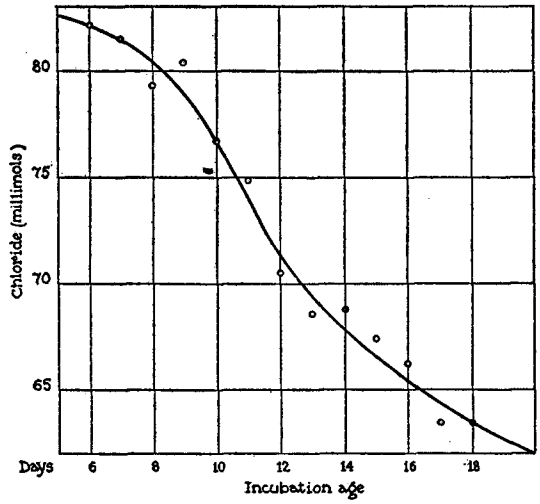

FIG. 4. The molar concentration of chlorides in the tissues as a function of age.

tween the 11th and 12th days of incubation a well marked change towards acidity occurred (Fig. 3). In form the curve resembles somewhat a simple gelatin titration curve, the steep portion in our diagram indicating a relatively unbuffered phase between two states of comparative stability.

About the same period (Table I) the molar concentration of chloride also falls (Fig. 4) whereas the bicarbonate increases (Fig. 5). At

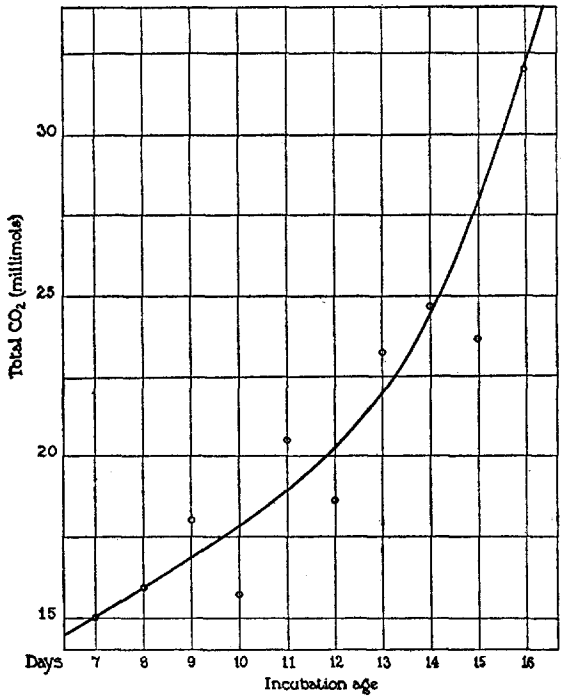

FIG. 5. The molar concentration of total carbonic acid in the tissues as a function of age.

first glance it would seem that this indicated an inverse relationship as if the tendency for maintaining osmotic equilibrium were the controlling factor, but there are reasons for believing that this is not the case. We assume that the figures for Cl are reliable, and under the conditions of our analyses have significance as indices of tissue conditions. In the case of carbonic acid, however, certain other

phenomena must be taken into consideration, principally the absorption of carbonates from the shell and their reprecipitation out of solution during active bone formation. Hence the concentration of total acid including as it does the amount of salt in solid phase is no indication of the activity of the ions in functioning protoplasm.

One is inclined to correlate the increasing acidity of the tissues with the accumulation of the CO_2 of catabolism, and to attribute the differences in the rate of pH change to variations in (1) the prevailing ratio in the tissues, (2) the catabolic rate, *i.e.* the rate of CO_2 production, (3) the functional effectiveness of systems of the organism, such as the circulation, which concern themselves with the transport and disposal of carbonic acid, and (4) phenomena, such as bone formation, which affect the carbonate equilibrium.

Little is known about the acid-base ratio in the tissues. From theoretical considerations this balance would seem to be quantitatively the least significant of the factors enumerated; and besides, since the buffer salts of protoplasm are of diverse nature with different dissociation constants, the conditions of heterogeneity would be such as to yield graphically a straight rather than an S-shaped line on titration. Catabolism is also a factor of lesser importance, and since its rate decreases with age it cannot be held accountable for an increasing acidity. Variations in the circulation, on the other hand would greatly affect the pH, and as it has been shown by Cohn (9) that during the early days of incubation there is a marked increase in the rate and regularity of cardiac contraction, and thus presumably a heightened efficiency of the blood vascular system in disposing of the respiratory carbon dioxide, this factor might explain the relative stability of the pH up to the 11th day.

From then on, if one can judge from the heart rate, there being no improved effectiveness in the circulation to compensate for the accumulating CO_2 the hydrogen ion concentration would rise.

The fourth factor listed above, calcium metabolism, may be significantly related to succeeding events. Leaving other facts out of consideration the period of rapidly decreasing pH might be coincidental with the formation of calcium salt deposits at ossification centers which as Little reports (10), may be seen as early as the 9th or 10th day in the chick, whereas the absorption of calcium from the shell,

assuming that this process began about the 12th day, might account for the subsequent relative stability of the pH.

In this connection, Aron's collected analyses (11) of the ash of human embryos indicate that with development there is a relative increase of calcium and phosphorus as compared to potassium, sodium, and choride. The extent of these relative proportional changes may be accounted for by the ossification of the skeletal parts of the body. Aron had no data for carbonates, but on theoretical grounds one might expect a relative increase in their concentration roughly proportional to the phosphates. In the chick Plimmer and Scott (12) have shown that the inorganic phosphates increase markedly during the last days of incubation, being derived from phosphorus organically combined with the lipoids of the yolk. In bone the molar strength of calcium phosphate is to that of calcium carbonate as 3 is to 1. Since our analyses do show an increase in total CO_2, we are led to assume that it is the result of the absorption of carbonates from the shell and their precipitation in osseous structures. For instance:

$$\underset{\text{(Shell)}}{CaCO_3} + H_2CO_3 \rightarrow \underset{\text{(Blood)}}{Ca(HCO_3)_2} \rightarrow \underset{\text{(Bone)}}{CaCO_3} + H_2CO_3$$

and

$$\underset{\text{(Shell)}}{3\,CaCO_3} + 3\,NaH_2PO_4 \rightarrow 3\,\underset{\text{(Blood)}}{CaHPO_4} + 3\,NaHCO_3 \rightarrow \underset{\text{(Bone)}}{Ca_3(PO_4)_2} + 2\,NaH_2PO_4 + Na_2HPO_4$$

As there is a gradient from left to right, *i.e.* from shell to bone, it seems that the phenomenon considered by itself would tend to have two significant effects for the present argument: (1) an increase in the alkaline reserve and thus a decrease in the negative acceleration of the pH and (2) the precipitation of calcium carbonate and phosphate as bone, thus increasing the total concentration of these salts in the organism. It is actually the case that soon after the onset of ossification, there is a temporary cessation in the fall of the pH, and an increase in the concentration of carbonate, so that we are inclined at present to account for it on the basis of the initiation of the absorption of calcium carbonate from the shell. The lowered pH would further the absorption of carbonates, a phenomenon, which would

in turn tend to buffer the pH change. If the amount of osseous disposition may be considered as a local affair independent of the general physicochemical equilibrium, how may we ascertain what is the concentration of carbonate in functioning tissue?

For adult blood it has been found that HCO_3 is approximately 0.23 Cl (13) when expressed in mols. This relationship may be fairly universal in protoplasm and body fluids since our figures show that it is approximately true in the case of the tissues of the young embryos before any bone formation occurs. The average values of the concentration of chlorides and carbonates from the 7th to the 10th day inclusive yield the following ratio: HCO_3/Cl equals 0.20. If a constant value for the HCO_3 ratio is assumed, the HCO_3 may be known by a determination of the chlorides as Haldane has suggested (14); furthermore, with a knowledge of the pH one might obtain H_2CO_3 since $pH = 6.1 + \log \frac{BHCO_3}{H_2CO_3}$; and, finally, having estimated the total CO_2 by the method described above, the amount of carbonate in bone might be calculated, since:

$$\text{total } CO_2 = H_2CO_3 + BHCO_3 + \text{Bone } CO_3 \text{ (approximate)}$$

In this way theoretical values for the distribution of carbonate in the body, and its concentration in functioning protoplasm might be calculated. The present suggestion that the ratio Cl/HCO_3 has a more or less constant value (*i.e.*, about 0.20–0.23, as found in young chicken embryos and human adult blood) would lead to the conclusion that the concentration of total CO_2 in the tissues decreased with age with the chlorides.

Aron's figures show a decrease in the total Na/Ca ratio no doubt due in large part to bone formation. The concentration of active calcium ions in tissue fluids, assuming a constant supply of calcium, would be functional to the existing physicochemical equilibrium and other factors being equal would tend to increase with the acidity.

This hypothesis might be confirmed or dismissed if it was found possible to make estimations of the amount of bone laid down, or determinations of the CO_2 tension in the tissue fluids or both.

It may be seen (Table I) that the concentration of protein increases markedly with age (Fig. 6), the greatest change occurring around the

15th day—3 or 4 days later than the changes in the concentration of electrolytes.

With the exception of hemoglobin it would seem that the isoelectric points of animal proteins were below pH 6.6, so that with the fall in pH between the 11th and the 12th days the proteins would give up basic ions. Later, with the increase in the concentration of protein, more cations would come to be associated in organic combination; in other words, there would be more protein molecules per gm. of water, though possibly each molecule might be less effective as a

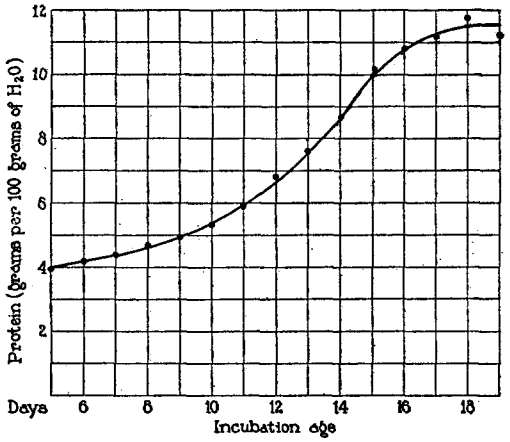

FIG. 6. The concentration of protein (gm. per 100 gm. of water) as a function of age.

buffer. There are no data, however, to show what is the total effect on the protein buffer value made by the changes taking place between the 10th and the 19th days of incubation.

The increase in the proteins, which act as anions at the pH under consideration, serves to replace the deficiency due to the loss of chloride ions. The interesting point is, however, that the two processes are not coincident, but that the chloride concentration has become established at a low level before the concentration of proteins has even reached the point of maximum rate of increase.

In the process of establishing an equilibrium between a mixed salt solution within a collodion sac and distilled water in which it is immersed it is usual to find fluctuations in the relative concentrations of

ions on each side of the membrane respectively due to differences in permeability or in the rates of migration of ions and molecules, before stability is attained. And thus at no moment in the movement towards equilibrium could the concentrations within and without

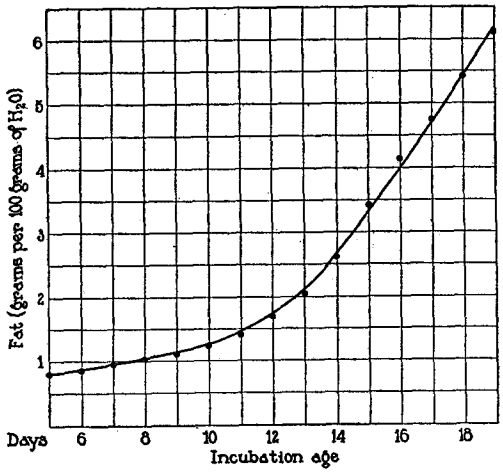

FIG. 7. The concentration of fat (gm. per 100 gm. of water) as a function of age.

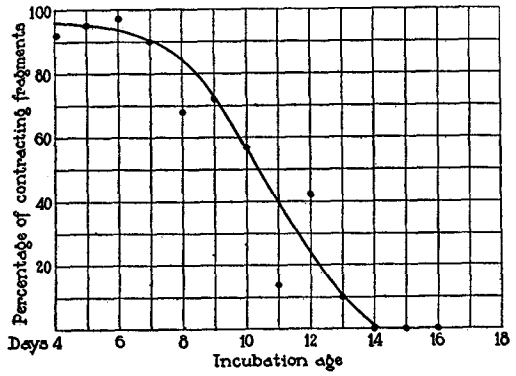

FIG. 8. The percentage of embryonic ventricular muscle fragments automatically contracting after implantation in plasma as a function of age.

the membrane be described by any of the usual physicochemical equations.

Similarly, results of our observations, which show that the electrolytes probably change several days before the protein, and the

latter several days before the fat (Fig. 7), lead to the conclusion that the processes of chemical differentiation are not to be described by a concept of dynamic equilibrium but rather by a notion of "follow the leader." The leader in this case is presumably the most rapidly permeating, reactive, and mobile molecule and tentatively we ascribe this rôle to the CO_2 of metabolism. This subject is to be discussed in a subsequent publication.

It would not be surprising to find that the changes in the chemical constitution of the tissues had considerable physiological significance. Already, in a few cases, we have had occasion to correlate chemical data with functional findings. For instance, in experiments upon the contraction of ventricular muscle fragments from chick hearts it was found that, whereas in the young embryos almost 100 per cent of the pieces planted in clotted plasma displayed autonomic, rhythmic contractions, in embryos of more than 13 days of incubation age, none contracted spontaneously (Fig. 8). Combining Cohn's results (15) with our own a graph to represent this phenomenon was obtained. It may be seen to bear some resemblance to the pH and chloride curves.

CONCLUSIONS.

Investigations of the chicken embryo during its incubation period show that:

1. The pH and the chloride concentration of the tissues decrease with age; the fall is most rapid between the 10th and the 13th days of incubation.

2. The concentration of total CO_2 increases with age. This fact is not considered inconsistent with a possible decrease in the concentration of active bicarbonate ions, since the increased CO_2 might well be the result of absorption of calcium carbonate from the shell and its precipitation as bone in the embryo.

3. The concentration of protein increases with age, especially between the 12th and the 16th days of incubation.

The fact that the electrolytes change with the greatest rapidity at about $11\frac{1}{2}$ days, the protein at 14 days, and the fat at $16\frac{1}{2}$ days might be taken as a demonstration of the phenomenon of unequal development in the realm of biochemical differentiation and consequently

that some notion of order, depending upon molecular reactivity and mobility would describe the process better than any concept of dynamic equilibrium.

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