# PHYSICAL PROPERTIES OF THE ALLANTOIC AND AMNIOTIC FLUIDS OF THE CHICK

## II. Hydrogen Ion Concentration

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The importance of the hydrogen ion concentration of the external medium of cells, tissues, and organisms has long been recognized. In the case of the developing chick, the external medium is represented by the amniotic fluid, and since the allantois soon surrounds the other components of the egg, its contained fluid must also be considered as a part of the embryo's external environment. This question of the hydrogen ion concentration of these extra-embryonic fluids has already received some attention, notably in the work of Aggazzotti (1913), Buytendijk and Woerdeman (1927), and Yamada (1933), but the results of these investigations do not agree, in part perhaps because the accuracy of the various techniques employed is open to question.

Accordingly, it was considered desirable to reexamine this problem, using the glass electrode technique, which is admirably suited for use with small quantities of fluid. Haugaard (1934) further points out that the glass electrode can also be used in work with solutions containing carbon dioxide (as do both fluids under investigation) which can only be measured with the hydrogen electrode by the use of special devices.

The type of glass electrode recommended by MacInnes and Belcher (1931) was employed, together with a silver-silver chloride electrode and calomel half-cell, following MacInnes and Dole (1929), designed for use with very small volumes of fluid. The silver-silver chloride electrode was constructed according to the specifications of Jones and Hartmann (1915). In practice, the procedure given by MacInnes and Dole (1929) for making pH determinations with this apparatus was followed. The entire electrode assembly (silver-silver chloride electrode, glass electrode, calomel half-cell) was kept in an incubator set to operate at 25°C. and maintained at this temperature throughout the course of the experiments.

As recording device, a galvanometer and single tube direct-current amplifier were used. To minimize vibrational effects, the galvanometer was mounted in a special suspension. The direct current amplifier was essentially the one now manufactured and sold commercially as the Beckman pH meter. A series of five standard buffer solutions was made up for calibration purposes, and pH values of unknown solutions determined graphically from the pH *vs.* deflection curves obtained with these buffers. An accuracy of about 0.01 pH unit was thus secured.

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The eggs used all came from the same flock of Barred Plymouth Rock hens. Samples of the two fluids were taken as previously described (Walker (1943)), and these were stored in stoppered glass vials within the incubator (at 25°C.) for at least
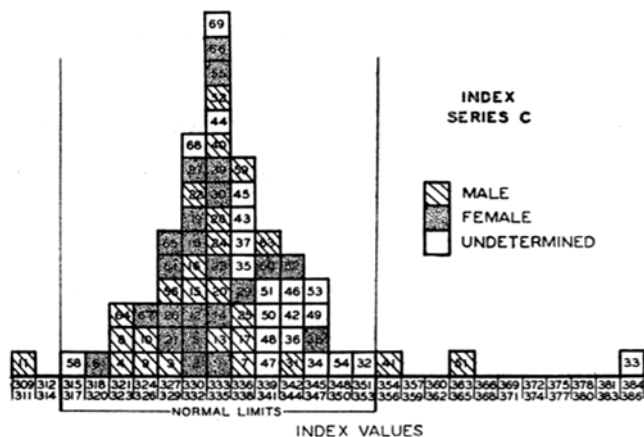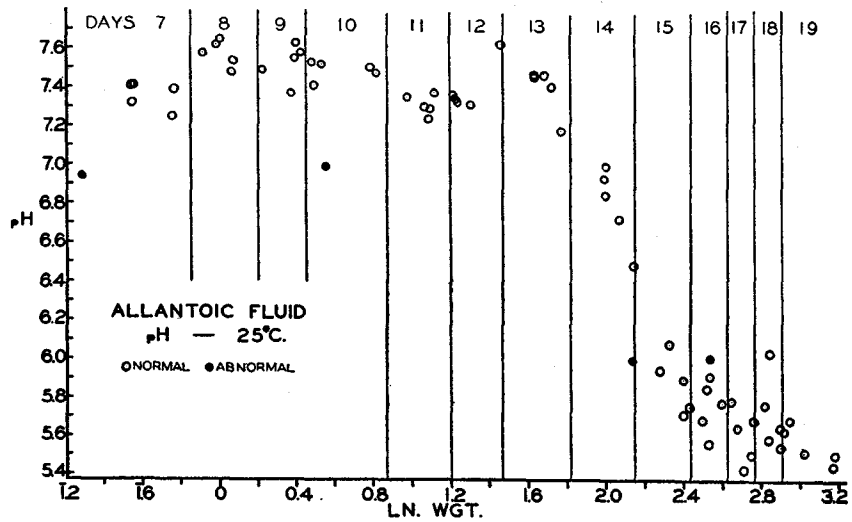


FIG. 1. Histogram resulting from application of index to experimental material.

TABLE I

*Average Measurements of All Embryos Used*

| Age | Crown-rump length | Weight | ln W. | pH | |
|---|---|---|---|---|---|
| | | | | AL.F. | AM.F. |
| *days* | *cm.* | *gm.* | | | |
| 7 | 2.6 | 0.68 | $\bar{1}.63$ | 7.36 | 7.15 |
| 8 | 2.95 | 1.00 | 0.00 | 7.57 | 7.28 |
| 9 | 3.3 | 1.44 | 0.36 | 7.53 | 7.11 |
| 10 | 3.6 | 1.88 | 0.62 | 7.49 | 6.90 |
| 11 | 4.2 | 2.89 | 1.06 | 7.31 | 6.83 |
| 12 | 4.7 | 3.65 | 1.29 | 7.39 | 6.65 |
| 13 | 5.25 | 5.41 | 1.69 | 7.39 | 6.58 |
| 14 | 5.9 | 7.72 | 2.04 | 6.79 | 6.54 |
| 15 | 6.7 | 10.69 | 2.37 | 5.87 | 6.40 |
| 16 | 7.1 | 12.67 | 2.54 | 5.75 | 6.92 |
| 17 | 7.4 | 15.22 | 2.72 | 5.60 | 7.12 |
| 18 | 7.85 | 17.58 | 2.86 | 5.71 | 7.48 |
| 19 | 8.4 | 21.13 | 3.05 | 5.55 | 7.87 |

20 minutes before use. As before, a great deal of variation was found in embryos of the same incubation age, and accordingly the material was classed primarily by wet weight. Chicks of a certain weight class have been grouped together more or less arbitrarily, to approximate the familiar "days incubation." Five individuals of each

of these "age" groups were tested.   The index of functional normality described by
Walker (1938) was again employed, and Fig. 1 is the histogram obtained for this



FIG. 2. pH of the allantoic fluid—measurements upon individual chicks (approxi-
mate incubation age indicated on top scale).



FIG. 3. pH of the amniotic fluid—measurements upon individual chicks (approxi-
mate incubation age indicated on top scale).

series of embryos.   As before, the median class is that represented by embryos with
index values of 333 to 335, with limits of functional normality from 315 to 353.

In Figs. 2 and 3 will be found the results of the pH determinations on the individual chicks used in these experiments. Average pH values for this series are shown in Table I and in Fig. 4. In this last figure, the data of Aggazzotti (1913), of Buytendijk and Woerdeman (1927), and of Yamada (1933) have been included for purposes of comparison.
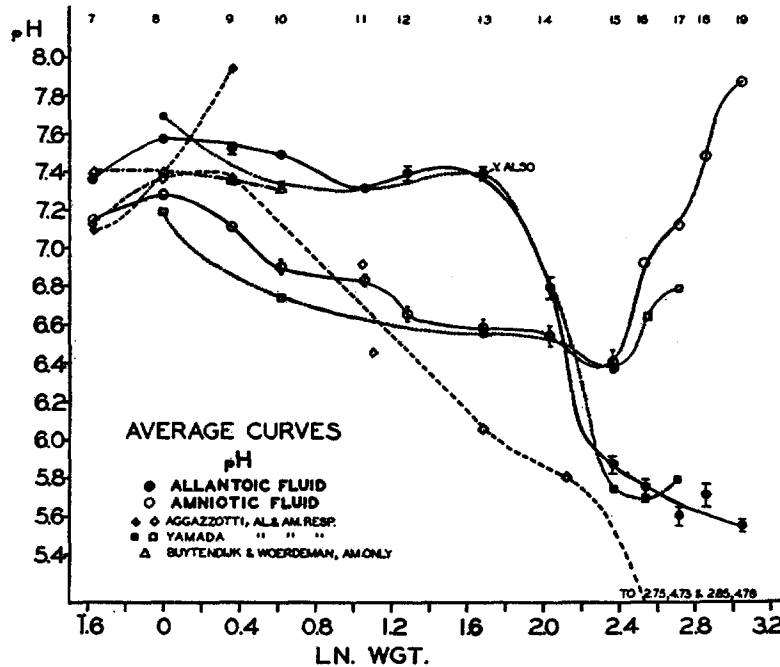


FIG. 4. Average curves, hydrogen ion concentration of the allantoic and amniotic fluids—each point on the above curves represents the average values of both weight and pH of the five individuals of each "age" studied.

DISCUSSION

In the case of the allantoic fluid, we find the hydrogen ion concentration maintained at an approximately constant level during the first half of the period of development under investigation, with pH's slightly on the alkaline side of neutrality (range: 7.31 to 7.57). The hydrogen ion concentration of this fluid should in large measure be determined at these early stages by the character of the renal secretions which are being discharged into it. In this connection, Needham (1925; 1926a; 1926b) has shown that there is a succession of end-products of nitrogen metabolism in the chick: first, ammonia is produced, reaching a maximum concentration on the 4th day; secondly, urea, with a maximum on about the 9th day; and finally, uric acid. Needham

(1931 a) has shown that uric acid is first excreted in the form of soluble urates, which on dissociation yield free basic ions, and these ions, together with the small amount of ammonia which is presumably still present and other un-identified basic ions, evidently counterbalance the urea[1] to such an extent that an equilibrium is reached which is slightly on the basic side of neutrality.

Another factor which must receive consideration here is the probable presence of carbon dioxide in this fluid (and in the amniotic fluid also). Unfortunately, the carbon dioxide content of these extra-embryonic fluids has not yet been determined, but in view of the respiratory function of the allantois, the allantoic liquid must contain a significant amount of this substance. From the results of chemical analyses, it is impossible to estimate the buffering capacities of this fluid, although the possible existence of a phosphate system is indicated. However, unless the buffering powers were rather high, carbon dioxide present in this fluid by diffusion from the allantoic blood vessels would exert a considerable effect upon its pH. This effect is not seen during the early portion of the period under investigation even though Pott and Preyer (1882) have shown that the amount of carbon dioxide produced by the embryo increases markedly with development (approximately tenfold in the period between the 7th and 19th days). These early observations of Pott and Preyer have since received abundant confirmation, as for example in the work of Murray (1927).

During the last week of incubation, the picture changes. A definite shift to the acid side becomes evident with an abrupt fall in pH from 7.39 on the 13th day to 5.87 on the 15th day; this is followed by a more gradual decrease to 5.55 on the 19th day. This marked increase in acidity foreshadows the conditions seen in adult avian urine (pH 5.0, Takamatsu (1935)), and is presumably due to a combination of the following factors. In the first place, since carbon dioxide production is continually increasing with development, there may be such an increase in the concentration of carbonic acid present in the fluid that its buffering capacity is overbalanced. Secondly, there occurs at this time a reduction in the basic constituents of the allantoic fluid because the excretory products are now being formed as free uric acid rather than its salts. Thirdly, as Fell and Robison (1934) have shown, calcification of avian cartilage and osteoid tissue begins at about the 15th day of incubation, a phenomenon which would result in a mobilization and withdrawal of calcium and phosphorus from other regions of the egg. That this would affect the extra-embryonic fluids is clearly suggested by the analyses of Iseki (1930). This loss of calcium and phosphorus from the allantoic fluid would doubtless greatly reduce its buffering powers.

Furthermore, according to Sula (1935), uric acid is less soluble in acid media

[1] Urea dissociates with the formation of free hydrogen ions, according to Cristol, Fourcade, and Seigneurin (1935).

than it is in alkaline; thus in the existence of this high concentration of hydrogen ions during the last few days of incubation, a mechanism is provided whereby this uric acid is left behind, encrusted on the remnants of the allantois, when the chick emerges from his shell.

In the case of the amniotic fluid, the average pH is slightly above neutrality, but slightly below that of the allantoic fluid, on the 7th day. A maximum pH value is indicated on the 8th day, but this rise may not be significant. Generally speaking, during the period from the 7th to the 15th day of incubation, there occurs a gradual decrease in pH; by the 10th day, it has become slightly acid in its reaction. This change has probably been brought about by the increasing carbon dioxide production of the embryo with the attendant diffusion of this gas into the amniotic cavity; possibly this takes place by way of the allantoic circulation, but even more probably by direct diffusion from the embryo's tissues into the enveloping amniotic fluid. If this is so, the buffering capacity of the amniotic liquid is less than that found in the case of the allantoic.

After the 15th day of incubation, the amniotic fluid becomes rapidly more alkaline, reaching a pH of 7.9 on the 19th day. These high values are interesting in that the allantoic fluid exhibits a decidedly acid reaction at this time. This demonstrates that the amnio-allantoic membrane cannot be permeable even to so small and readily diffusible an ion as hydrogen. Unquestionably, therefore, carbon dioxide could not be reaching the amniotic cavity by diffusion from the allantois during this period.

This change in the hydrogen ion concentration may possibly be linked with the absorption of certain materials from the amniotic fluid by the embryo, which is known to occur during this time. Protein materials from the albumen sac have been injected into this fluid (Hirota (1894)), only to disappear very shortly, according to the findings of Schenck (1932). This obviously results in a predominance of basic constituents, but again, the results of chemical analyses offer no satisfactory explanation as to their nature.

Comparisons between the specific conductance of these fluids (Walker (1943)) and their hydrogen ion concentration reveal certain interesting general relationships. In the case of the allantoic liquid, both specific conductance and pH are decreasing during the last few days of the period investigated. This decrease in the ionic strength of this fluid apparently does not involve a decrease in the number of hydrogen ions present in solution. The same correlation may be observed in the minima occurring for both properties on the 15th day in the case of the amniotic fluid. The high pH values for this latter fluid occurring on the 19th day correspond to fairly high conductance values, but the highest conductance values occur during a period in which pH is quite close to neutrality.

The investigation of changes in the pH of these two fluids has been studied previously by other workers. The present study, made with more advanced

technique, should be compared critically with these earlier results. In Fig. 4, certain of these data have been plotted together with the results of the present series of experiments.

Of these earlier data, the two most important sets are those of Aggazzotti (1913) and of Yamada (1933). The former has for a long time been credited with reliability, as for example by Needham (1931 b). The data of Buytendijk and Woerdeman (1927) are incomplete, and as seen in Fig. 4 run slightly higher than, but close to, those of Yamada and the present data. Gueylard and Portier (1925), although credited by Needham (1931 b)[2] with observations on the allantoic fluid, actually measured the pH of various mixtures of both amniotic and allantoic fluids; their results have little bearing on the present situation. Abe's observation (1927) that the pH of the amniotic fluid is constant at 7.1 throughout the course of incubation indicates the use of an inadequate method of measurement. The values obtained by Rubinstein (1932) for the amniotic fluid show too great a variation to be reliable.

One of the most striking features of the present data, however, is the confirmation afforded Yamada's results. The curve for the allantoic fluid is practically identical with that given by the Japanese worker, the fluctuations which do appear being of a very minor nature. As for the amniotic fluid, although the data are slightly higher than those obtained by Yamada, the agreement is nevertheless quite good. His determinations were carried only as far as the 17th day, but the upward trend shown by the present data is certainly indicated.

In contrast to these two sets of data stand the results obtained by Aggazzotti, who claims to have found the existence of extremely acid conditions within the amniotic fluid during the latter portion of the incubation period. pH values as low as 4.7 are reported on the 18th to 19th days. In fact, Aggazzotti's results are consistently lower than the present data and those of Yamada over practically the entire range of development. Aggazzotti does not give details of his method of pH determinations but merely states that the values were determined electrometrically. The fact that he confirmed his electrometric pH determinations by means of determinations of the titrable acidity gives more weight to his figures and therefore only accentuates this discrepancy.

However, although his physical measurements seem to have been carried out with reasonable exactitude, there is reason to question his methods of obtaining samples of the fluids. For example, in connection with the allantoic fluid, he states that by the 8th or 9th day, he was no longer able to obtain pure samples of this fluid because of contamination by yolk. No such difficulties were encountered in the course of the present investigations, nor have they been reported by others who have worked with this material. Moreover, in

[2] Needham, J., Chemical embryology, London, Cambridge University Press, 1931 b, 857.

connection with the amniotic liquid, he describes it as quite clear and colorless, a description which does not agree with that of Yamada nor with observations made in the course of the present investigation. Yamada describes the amniotic fluid of 12 day embryos as slightly turbid, and at the end of the 2nd week of incubation it becomes, in addition, pale yellow in color. He further notes that it becomes very viscous at about this time. His observations have been afforded complete confirmation throughout the present investigation. Aggazzotti, strangely enough, fails to note any of these changes in the appearance of this fluid, a fact which would seem to indicate that he was not dealing with samples of the amniotic fluid at all.

As the result of what is apparently *a priori* reasoning, Aggazzotti claimed that the embryonic urine was acid in reaction. Since his data show that the allantoic fluid is alkaline in all stages studied and that the amniotic first becomes acid on the 11th day, he has drawn the following conclusions: (1) the mesonephros is not functional; and (2) the metanephric secretions are deposited in the amnion. Fiske and Boyden (1926), however, have pointed out that it is impossible to predict, *a priori*, what the reaction of the embryonic excreta would be.

Aggazzotti then proceeded to attempt to explain how this embryonic urine could become deposited in the amniotic cavity. Contrary to the earlier work of Gasser (1880) on the development of the cloacal opening, he holds that, from about the 11th day on, the urinary secretions of the embryo pass out through some "physiological connection" between the cloaca and the amniotic cavity at the region of the Bursa of Fabricius. According to Lillie (1930),[3] this is without morphological basis. The experiments of Hanan (1925), (1927) on the excretion of trypan blue by the mesonephros of the developing chick show that physiological evidence is likewise lacking. Thirdly, chemical analyses of the amniotic fluid fail to reveal any appreciable amounts of uric acid at any time during incubation.

In view of these criticisms of Aggazzotti's interpretations, and the possibility that he was not dealing with pure samples of amniotic fluid, and in view of the excellent agreement between the present data and those of Yamada in spite of differences in technique employed, the latter sets of values should be considered as representing the hydrogen ion concentration of these extra-embryonic fluids, and the data of Aggazzotti should be discarded.

### SUMMARY

1. The hydrogen ion concentration of the allantoic and amniotic fluids of the developing chick has been determined over the period of incubation between the 7th and 19th days using the glass electrode technique.

[3] Lillie, F. R., The development of the chick, New York, Henry Holt, 2nd edition, revised, 1930, pp. 314 *ff*.

2. Changes in this property have been related to changes in the chemical composition of these two fluids.

3. The results of this investigation have been compared with those obtained by other workers. Excellent confirmation has been afforded the work of Yamada, whereas the work of Aggazzotti, which has long been accepted, is shown to be in error.

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