that stimulation of the medial thalamic structures may result in a "spike and wave" activity of large areas of the cortex. This activity was dependent on a stimulus frequency of about 3 per second. It was also found(3) that with metrazol sensitization and photic stimulation, repeated stimuli applied with a frequency of about 3 per second produce "spike and wave" formations in the cortex. However, the "spike and wave" discharges lasted only as long as the stimulation and have not been produced as a consistent self-maintained seizure activity after the withdrawal of the stimulus. If a self-sustained discharge occurred it consisted of "fast" spikes of the type seen during the "tonic" phase of a "grand mal" seizure.

The principles of development of the electrocorticogram of the cat indicate a tendency of the immature cortex to produce "slow" frequencies. This physiological electrical characteristic of immaturity is also manifested during the pathological state of an epileptic after-discharge. On this basis it is possible that the electrical picture of focal and non-focal "spike and wave" discharges so frequently found in the EEG of epileptic children may be explained by the readiness of the immature cortex to produce this type of discharge.

Summary. Metrazol and electrically induced seizure activity in the cortex of kittens at various stages of maturation reveals a relation of form and frequency of discharges to age. Focal and non-focal "spike and wave" discharges of frequencies seen in the EEG of the epileptic human infant and child may be reproduced in young animals. With increasing age spikes of faster frequencies replace this slower "spike and waves". The findings indicate the importance of electro-ontogenetic studies in investigations of the electrical patterns of epileptic seizures.

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Electrophoretic Mobility—Ionic Strength Studies of Proteins. I. Heterogeneity of Human Serum Albumin.* (21008)

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The moving-boundary method of electrophoresis as developed by Tiselius(1) offers a powerful tool for testing one aspect of the purity of proteins, *i.e.,* their homogeneity with respect to mobility(2). Such crystalline proteins as serum albumin(3) conalbumin, chymotrypsin, ribonuclease, etc.(4), B-lactoglobulin(5) and many other proteins were found to give a single moving boundary over a certain range of pH but *2 or more* boundaries under other conditions. By the technics of electrophoretic mobility, v.s. pH (3,6-10), prolonged electrophoresis(11,12), or electrophoresis in the presence of certain anions (13,14), human serum albumin showed *2* or more moving boundaries. Leutscher(3,6) carried out his original studies at pH 4.0 and with low ionic strength (.02) acetate buffer and reported separation of normal human serum albumin into 2 "components". Miller, *et al.*(9) also used acetate buffer at pH 3.9 but of high ionic strength (0.2, 90% NaCl) and reported separation of human serum albumin into *4* "components". It was our belief that this discrepancy in the number of boun-

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daries formed, as well as in their sharpness of resolution for quantitative analysis, could best be resolved by a careful, systematic study of the effect of variation of the buffer ionic strength on the electrophoretic mobility of purified human serum albumin. The importance of the ionic strength factor, *especially at low salt concentrations,* on the electrophoretic mobility of proteins has been stated by Svensson(15) as being of the same order of magnitude as that of pH. The experimental studies presented in this paper show that it is possible to separate human serum albumin, which shows a single boundary at pH 8.6, 0.1 ionic strength barbiturate buffer, into at least four distinct peaks at pH 4.0, ionic strengths 0.06 to 0.03, acetate-NaCl buffer, for the ascending boundaries.

A decrease in the serum albumin component at pH 8.6, 0.1 ionic strength is a characteristic of many chronic diseases, *e.g.,* multiple sclerosis(16), as well as of most acute diseases (17,18). Leutscher(6) has shown that for such diseases as nephrosis, or cirrhosis of the liver, a reversal of the normal ratio of the areas under the two peaks formed occurs at pH 4.0 and .02 ionic strength, acetate buffer. It is believed that the quantitative fractionation of human serum albumin into four or more components, as described here, by moving boundary electrophoresis may prove to be useful in the clinical study and evaluation of many diseases.

Experimental. In order to determine the electrophoretic mobility and homogeneity of the albumin† solution used in these studies, 2 ml of 25% solution are diluted to 10 ml with barbiturate buffer(20) (pH 8.6, 0.1 ionic strength) and then dialyzed in Visking tubing for a minimum of 5 hours in a mechanical dialyzer(21) against 400 ml of the buffer at 25°. The analysis was performed with the portable Aminco-Stern electrophoresis apparatus employing a previously published technic(16). Electrophoresis was carried out at 2 degrees and the pattern obtained was photographed and traced (Fig. 1a). In the experiments shown in Fig. 1b and 1c, the 5% serum albumin solutions were run electrophoretically in barbiturate buffer at pH 8.6, ionic strengths .02 and .005 respectively. A series of electrophoretic determinations were performed on an albumin sample diluted in the same manner but dialyzed against acetate-NaCl buffer(22) and pH 4.0 and at the ionic strengths shown in Fig. 2. The final pH of each buffer solution after dilution with distilled water was checked with a glass electrode and adjusted to pH 4.0 ± 0.02 pH units. The electrophoretic analyses were run exactly as described above except for changes in the field strength and duration of run as shown in Fig. 2a to 2h. The mobility of the leading component for each ionic strength given in Fig. 2 was calculated from the ascending pattern and the results obtained in these runs are plotted in Fig. 3. A number of other samples fractionated with Cohn's technic were obtained and showed the same splitting into 4 distinct peaks under our experimental conditions. In addition, an albumin sample was

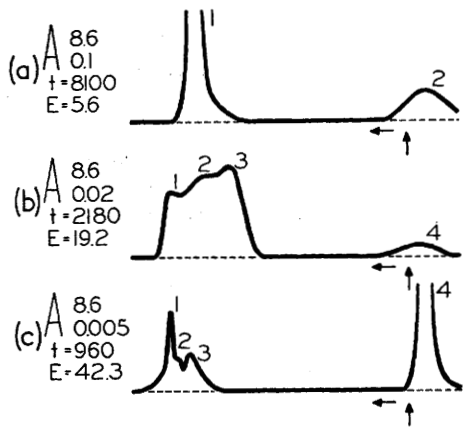

FIG. 1. Fractionation of 5% human serum albumin (A) solution; pH 8.6 barbiturate buffer, ionic strengths (a) 0.1, (b) 0.02, (c) 0.005. All conditions were kept constant except potential gradient (E = volts/cm) and duration (seconds) as noted for each diagram. Vertical arrows indicate starting positions. All photographs and diagrams in this paper are of the ascending boundaries. Peak 2 in Fig. 1a represents the classical protein-buffer salt (or delta) boundary.

† Albumin in these studies obtained from American National Red Cross through the courtesy of Dr. J. N. Ashworth. It is prepared by the ethanol-low temperature fractionation procedure of Cohn and his associates(19). It consists of 25% albumin solution and contains 0.02 M sodium caprylate and 0.02 M sodium acetyl tryptophanate as preservatives.

prepared from normal human serum by salt fractionation with 28% sodium sulfite by the procedure of Wolfson, *et al.*(23), the excess salt was removed by dialysis and the sample concentrated to about 2% albumin by evaporation in a cellophane bag. The results obtained for human serum albumin prepared in this manner and run at pH 4.0 and 0.03 ionic strength are shown in Fig. 4a. In order to make certain that the splitting of the albumin into a number of boundaries under our experimental conditions does not represent denaturation products or artefacts obtained at pH 4.0, the following series of experiments were performed: a) A 5% serum albumin sample was run electrophoretically in the manner described above at pH 4.0 and 0.03 ionic strength and the usual 4 peaks were obtained (Fig. 4b). The protein sample was withdrawn and the acetate buffer removed by overnight dialysis in cold running water. The solution was then dialyzed mechanically at 25° against repeated changes of barbiturate buffer (pH 8.6, 0.1 ionic strength) and rerun electrophoretically, giving a *single* peak as is shown in Fig. 4c. Satisfactory results were also obtained with the reverse experiment, *i.e.*, running the sample first at pH 8.6, 0.1 ionic strength and then at pH 4.0, 0.03 ionic strength to obtain the 4 boundaries. b) In an experiment performed as described in (a) above, the run was continued at pH 4.0, 0.03 ionic strength for 1 hour until 4 distinct boundaries were obtained. The polarity of the electrodes was then reversed and the same potential applied for the same time, until the pattern reformed into a single but less-sharp boundary. c) In a run performed as described under (a) above, photographs were taken every 15 minutes for a total of 4 exposures and the mobility of the leading component calculated (Fig. 4d).

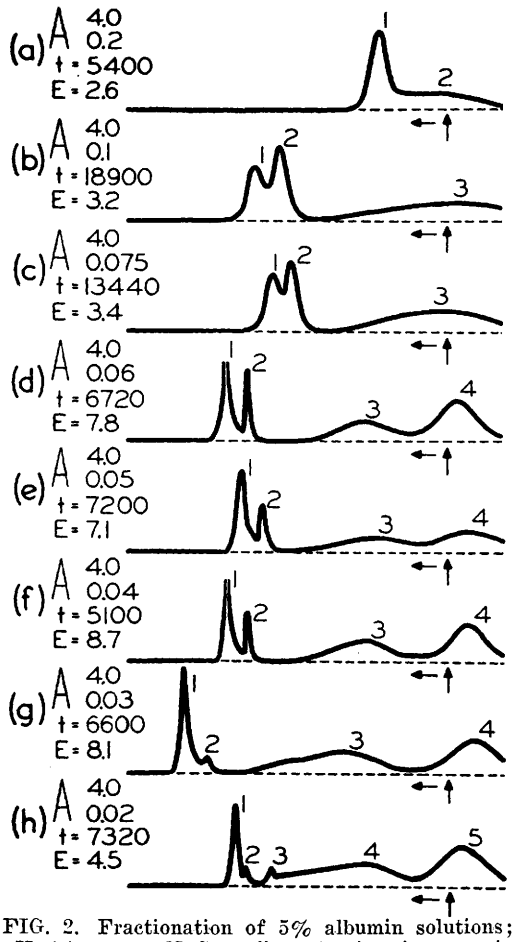

FIG. 2. Fractionation of 5% albumin solutions; pH 4.0 acetate-NaCl buffer, showing increase in number of boundaries with decrease in ionic strength of the buffer. Note change in relative areas in peaks labeled 1 and 2 with decreasing ionic strength.

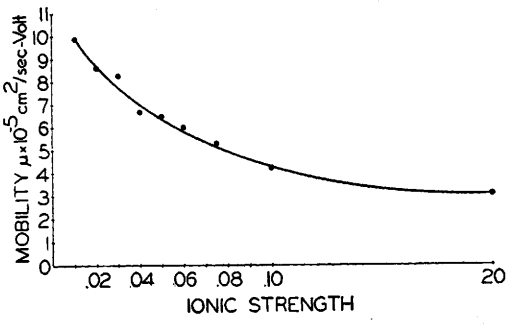

FIG. 3. Change in electrophoretic mobility of fastest moving boundary (peak 1) with increase in ionic strength of acetate-NaCl buffer.

Results. A) *Effect of ionic strength variation with barbiturate.* The ascending electrophoretic patterns obtained for 5% human serum albumin solutions at pH 8.6, barbiturate buffer, and at ionic strength of 0.1, 0.02 and 0.005, respectively, are shown in Fig. 1. These patterns provide experimental proof that sufficiently large variations of the ionic strength factor can be just as important in affecting the mobility relationships of a protein mixture as are the pH changes previously

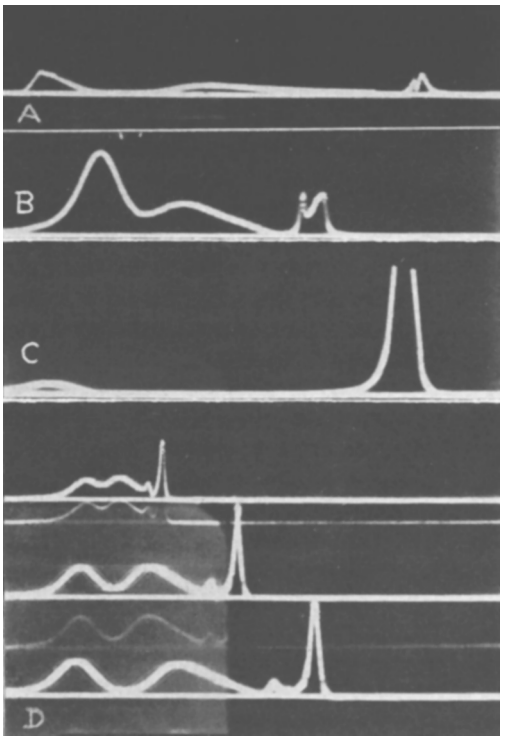

FIG. 4. (a) Electrophoretic pattern of normal human serum albumin prepared by sodium sulfite (28%) fractionation of serum. Acetate-NaCl buffer pH 4.0, ionic strength 0.03. (b) American Red Cross serum albumin prepared by ethanol-low temperature fractionation. Electrophoretic subfractionation in acetate-NaCl buffer, pH 4.0, ionic strength 0.03. (c) Recovered solution from (b), redialyzed and rerun electrophoretically in barbiturate buffer pH 8.6, ionic strength 0.1. (d) Sequence photographs (every 15 min.) of the subfractionation of 5% albumin solution in acetate-NaCl buffer, pH 4.0, ionic strength 0.03. Solution was recovered from a previous run in barbiturate buffer, pH 8.6, ionic strength 0.1.

investigated (2-10). It can be noted that while at an ionic strength of 0.1 (Fig. 1a), the albumin appears as the usual sharp, single boundary with a calculated descending mobility of $-6.8 \times 10^{-5} cm^2$ per sec. per volt, a 5-fold decrease in the ionic strength of the buffer to 0.02 (Fig. 1b) gives the "shoulder" type of boundary found by Miller, *et al.* (9) at pH 3.9, 0.2 (90% NaCl) ionic strength, acetate buffer. When the ionic strength of the barbiturate buffer at pH 8.6 is decreased 20-fold to 0.005, the further separation of the serum albumin into a number of separate boundaries is clearly evident as is seen in Fig. 1c. In these runs all other variables were kept constant except those noted in Fig. 1.

B) *Effect of ionic strength variation with acetate buffer.* The acetate buffer, pH 4.0, used in these runs is that proposed by Miller and Golder (22) in which 90% of the ionic strength is furnished by the NaCl content of the solution. The buffering capacity of this acetate buffer is less and its conductivity greater than the acetate buffer of the same pH and ionic strength employed by Leutscher (3,6) in his studies of albumin fractionation. This difference in the acetate buffers used by these investigators could serve to explain the fact that with Miller's buffer at pH 4.0, *0.20* ionic strength, the two boundaries obtained by us and the relative areas under them, correspond closely to those found by Leutscher (3) for his acetate buffer at pH 4.0, *0.02* ionic strength, although the boundaries are somewhat sharper in the latter case. As the ionic strength of the acetate-NaCl buffer at pH 4.0 is decreased from 0.20 to 0.02, the number of distinct boundaries formed increases from 2 to 5 as seen in Fig. 2. Only ascending boundaries are shown in these tracings. In general we have tended to designate with numbers only those boundaries which are both distinct and capable of quantitative measurement of their areas. A decrease in the ionic strength under our experimental conditions, as is clearly evident in Fig. 2, also results in a change in the relative areas under the two fastest moving ascending boundaries, *i.e.*, peaks 1 and 2. As the ionic strength decreases from 0.075 to 0.02, there is a continuous increase of the area under peak 1 and a corresponding decrease of the area under peak 2. According to the Debye-Huckel extension of the diffuse "double layer" theory of Gouy (24), as the ionic strength is increased, the layer becomes thinner and the mobility decreases. That this is indeed the experimental findings for the fastest moving ascending boundary is shown graphically in Fig. 3 where the mobility decreases continuously toward an asymptotic level as the ionic strength is varied from 0.01 to 0.2. All other experimental conditions were kept constant in these runs except for those noted in Fig. 2a to 2h.

C) *Effect of time of dialysis on electro-*

phoretic patterns obtained. Few studies(14, 25,26) have been made of the ionic strength-mobility relationships of proteins over a wide range of values for a particular buffer system although pH-mobility studies of proteins appear quite frequently in the literature. Except for Luetscher's(3) work with serum albumin little work has been done in the low ionic strength range, *i.e.*, below 0.05. One of the reasons for the lack of such studies has been pointed out by Armstrong and his coworkers (14). They state that at the extremes of the ionic strengths studied, *i.e.*, above 0.3 or below 0.05, variability in resolution of the individual diagrams proved too great to permit the evaluation of the influence of the ionic strength factor. Dialysis of protein solutions is usually carried out against the buffer to be employed for periods of 12 to 48 hours at 4-5° sometimes with several changes of the buffer solution used, (9,27). While this may be adequate for ionic strengths from 0.05 to 0.2, albumin solutions in low ionic strength buffers require a dialysis period of 72 hours or longer at 4° for reproducible results. For the sake of convenience it is preferable to use a mechanical dialysis set-up as recommended by Reiner and Fenichel(21) for a minimum of 5 hours at room temperature. Duplicate patterns thus obtained with a 5% protein solution in 0.03 ionic strength, acetate-NaCl buffer, run under identical conditions but several days apart, are practically superimposable and give almost identical measurements of the areas under the various peaks.

D) *Effect of variation of albumin concentration.* It would be advantageous for clinical studies in protein metabolism to be able to work at as low an albumin concentration as would be consistent with good reproducible results for the areas under each of the 4 peaks. A series of determinations were then run as above, at pH 4.0, 0.05 ionic strength, in which the albumin concentration was varied from 0.5 to 5.0%. The results obtained in such runs, which are not shown here, indicate that clear-cut separations into 4 distinct peaks are obtained at protein concentrations between 1.0-5.0%.

E) *Effect of method employed for albumin separation.* Experimental evidence that the observed boundaries for human serum albumin are protein in nature, and not artefacts, is provided by the work of Hoch and his coworkers(12,28) and by Reinhold and Gilman (10). Further evidence in this direction is provided by running 5% albumin in acetate-NaCl buffer ionic strength 0.1, pH 4.0; withdrawing the sample after its fractionation, as in Fig. 4b, and rerunning after suitable dialysis in barbiturate buffer pH 8.6, ionic strength 0.1. Under the latter conditions the albumin migrates as a single boundary as seen in Fig. 4c. Opposite results were obtained by the reverse procedure, that is, running the albumin sample in barbiturate buffer at pH 8.6 first. Similarly the reversible boundary-spreading test(2) while indicating the heterogenity of serum albumin also shows the separation of the albumin into 4 or more components to be reversible since the original boundary can be reformed by changing the polarity of the electrodes. This would indicate that the forming of multiple boundaries with serum albumin solution is not due to either denaturation or irreversible dissociation into smaller molecular weight units.

The constancy of the mobility of the leading boundary with time would also provide evidence against any change in the surface charge density of the protein molecules such as would occur with either denaturation or dissociation of the molecule. In addition the photographs shown in Fig. 4d, illustrate the changes of the electrophoretic pattern with time, until maximum development into separate boundaries occurred, under the given experimental conditions.

Discussion. The use of the word "component", with its implied meaning of a distinct molecular species, has been avoided. Instead the terms "boundaries" or "peaks" have been employed interchangeably. This usage more accurately describes the phenomena which occur during the electrophoresis of proteins and which is observed and recorded photographically by the schlieren lens system(27). Provided no false boundaries are present, the number formed with the electrophoretic method must be considered to be a *minimum* estimate of the number of distinct molecular species (or components) present in a mixture.

The criteria for the purity of a protein has been reviewed by Li(29) who states that the more satisfactory evidences are the physicochemical properties such as solubility, electrophoresis, etc. As previously mentioned few purified proteins, if any, meet even these tests of homogeneity. These experimental findings had led Haurowitz(30) to assert that it is possible that completely pure proteins do not exist, and that each of the so-called "pure" proteins is in reality a mixture of very similar protein molecules. The experimental work reported here for human serum albumin would tend to support this viewpoint.

At ionic strengths of 0.01, or less, the patterns obtained do not give the clearly resolved, measurable peaks formed at the higher ionic strengths. Since we are more concerned with the *quantitative* rather than the qualitative aspects of albumin fractionation, we arbitrarily chose an ionic strength of 0.03 (pH 4.0 acetate-NaCl buffer) which gives four clearly resolved and measurable boundaries, as the most convenient experimental conditions for most of our studies.

As stated by Longsworth(28) and Alberty (2) "ideal" electrophoresis of protein mixtures should be carried out at low protein concentrations and with high ionic strength buffers so that the protein itself would carry a negligible charge. In actual practice it is not possible to carry out electrophoresis under "ideal" conditions and as a result there are always differences between the ascending and descending patterns. While the conditions under which our experiments would run would appear to be "non-ideal", a number of mitigating factors exist. For example, most of our experimental work was performed at pH 4.0 at which the albumin (isoelectric point, pH 4.5) carries only a slight positive charge. In addition, reduction of the albumin concentration to 1%, or less, still gives similar patterns as were obtained at a 5% protein concentration. However, it is an experimental fact that human serum albumin can be fractionated electrophoretically into a number of peaks in low ionic strength buffers whether the protein carries a large negative charge (pH 8.6, .005 ionic strength, barbiturate buffer) or a small positive charge (pH 4.0, .03 ionic strength, acetate-NaCl buffer). We have preferred to use the latter conditions for the same reasons that most investigators working with plasma proteins prefer to use barbiturate buffer at pH 8.6 rather than phosphate buffer at pH 7.7, namely, that additional ascending boundaries are obtained and there is better resolution of these boundaries for quantitative purposes.

It should perhaps again be emphasized that Miller's(22) acetate-NaCl buffer is *not* the same as the acetate buffer employed by Leutscher(3,6) in his studies, although both can be made up to an equal ionic strength, *i.e.*, 0.02. The fact that the addition of NaCl to a buffer to increase the ionic strength causes changes in the electrophoretic patterns has been previously cited by other investigators (13,31). In fact, Armstrong, *et al.*(14), found that a synthetic mixture of albumin and beta-globulin which gave one boundary with sodium diethylbarbiturate buffer, 0.1 ionic strength, was separated readily by electrophoresis in sodium diethylbarbiturate-sodium caprylate buffer, 0.1 ionic strength. By combining the experimental conditions of Luetscher(3) with the acetate-NaCl buffer of Miller and Golder(22) we have been able to verify Miller's(9) findings that serum albumin contains at least 4 "components" and that the results obtained are highly reproducible.

Electrophoretic phenomena, similar to our observations on serum albumin, at pH values just below the isoelectric point have been reported by Longsworth and Jacobsen(32) for bovine serum albumin and B-lactoglobulin. These authors suggest that under these conditions the equilibria prevailing in the body of the protein solution are being continually adjusted in the boundary layers as electrophoretic separation takes place. It is also a well known fact that serum albumin binds readily with large molecules, *e.g.*, lipoproteins (14) and bilirubin(11), and with small ions (33). The authors prefer to withhold their own interpretation of these phenomena pending further experimental work. The electrophoretic separation and the quantitative analysis of the substances present under each peak seen in the electrophoretic patterns is now in progress in this laboratory. Some pre-

liminary results already obtained for other proteins, *e.g.*, human gamma globulin, indicate that we are dealing with a generalized phenomena in which additional boundaries can be obtained by means of electrophoretic separation just below the isoelectric point of the protein in the presence of suitable low ionic strength buffers. The extension of the technic of sub-fractionation described here to clinical studies of isolated protein fractions from sera of diseased cases such as nephrosis(6), cirrhosis(6), multiple sclerosis, cancer, etc., may provide useful information about their protein metabolism.

Summary and conclusions. 1. The ionic strength-electrophoretic mobility relationship of human serum albumin was studied at pH 4.0 in acetate-NaCl buffer over a wide range of ionic strength values. 2. A 5% albumin solution which gave a single boundary at pH 8.6 in barbiturate buffer, 0.1 ionic strength, showed separation into a number of ascending boundaries at an ionic strength of 0.005. 3. Similarly at pH 4.0 in acetate-NaCl buffer, the number of ascending boundaries increases from 2 to 5 as the ionic strength is decreased from 0.2 to 0.02. Under these conditions, the area under the fastest moving peak increases continuously at the expense of the slower-moving peak adjacent to it. 4. Using an ionic strength of 0.03 (pH 4.0) as a standard condition, since it gives 4 clearly resolved boundaries, various other factors which may influence the electrophoretic pattern were studied, *e.g.*, protein concentration, method of preparation of albumin fraction, time of run, and technic of dialysis used. 5. Reproducible values for the areas under each of the 4 peaks were obtained at protein concentrations between 1 to 5% and with mechanical dialysis for 5 hours or longer at 25° against the acetate-NaCl buffer, 0.03 ionic strength. 6. Preliminary work would indicate that the electrophoretic sub-fractionation of other proteins or protein fractions, *e.g.*, gamma globulin, is also possible and that this behavior may be part of a more generalized phenomena. 7. The possible explanation of these phenomena await quantitative analysis of the components present under each observed peak. However, evidence is presented that boundaries probably represent protein components and not artefacts.

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Alteration in Pentose Content of Chick Embryos Infected with Japanese Encephalitis Virus. (21009)

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The developing chick embryo has been used as a medium for the cultivation of a wide variety of obligate intracellular parasites, but the host-parasite relationship involved has received comparatively little attention. Since the embryo undergoes marked changes in size and composition during the growth period of the infectious agent, it necessarily constitutes a complex and dynamic medium.

The nucleic acids of the embryo are constituents of particular interest because of the direct correlation between ribonucleic acid concentration and the rate of protein synthesis (1-3). The invasion of unicellular hosts (*E. coli*) with bacteriophage has been shown by Cohen (4,5) to involve an inhibition of ribonucleic acid synthesis, whereas the synthesis of desoxyribonucleic acid proceeds at an essentially normal rate. Our observations have indicated that infection of chick embryos with the virus of Japanese encephalitis induces changes in tissue composition of comparable magnitude and in the same direction as those found in bacterial cells invaded by phage.

Materials and methods. Embryonating eggs of the White Leghorn were selected at random from several large and representative batches. All of the eggs used contained actively motile embryos with pronounced peripheral blood vessels visible upon candling. The eggs were incubated at 38°C and a relative humidity of 40 to 60%. Age of the embryo was designated as the number of days of incubator life. The embryos subsequently dying in the course of the experimental work were assumed to be dead when examination by candling disclosed that spontaneous motion of the embryo had ceased and the peripheral blood vessels had retracted. Infection with Japanese encephalitis virus (Nakayama strain, egg-adapted) was accomplished by inoculation of the chorio-allantoic membranes of 10-day-old embryos with 0.1 ml doses of a 1% suspension of infected chick-embryo tissue in phosphate buffered saline solution, pH 7.8. Twenty to 100 inoculated eggs were used in each experiment, with an equal number of control eggs from the same group. The eggs were maintained at 35°C and examined by candling at 4-hour intervals. All embryos which died less than 48 hours after inoculation were discarded. When the mortality ratio exceeded 20% (generally 60 to 72 hours after inoculation) both the infected and the normal