Immunophoretic Evaluation of Blood Serum Poteins in Chickens. II. Patterns in Vitamin E Deficiency Encephalomalacia.* (31239)

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Alteration of serum protein patterns in chickens on vitamin E-deficient diets is characterized primarily by an elevation of the beta globulin fraction and a lowering of the albumin/globulin ratio(1-4). In addition, a decrease in the albumin level is found in the chronic syndromes of exudative diathesis and nutritional myopathy, but not in encephalomalacia. A more refined method, namely immunophoresis, has been used in attempting to identify the nature of the abnormality in the beta globulin fraction, to relate its possible significance to the production of the specific syndromes of antioxidant deficiency states and to examine its connection with the probable peroxidation of highly unsaturated fatty acids (*e.g.*, linoleic and arachidonic acid) *in vivo* under such conditions. The principle of identifying chicken serum protein fractions by analogy with comparable findings in human serum has been discussed in the first paper of this series(5). The findings in blood serum of chickens on encephalomalacia-producing diets are presented here. The findings related to myopathy-producing diets will be discussed later(6).

Methods and materials. Thirty-eight chicks† were divided into 2 groups. (1) *Nutritional encephalomalacia.* Twenty-five chicks were placed on purified diet(1) deficient in vit E or any synthetic antioxidant, and containing 4.8% linoleic acid. Diet was begun on day of hatching or at latest when the chicks were 4 days old. (2) *Normal chicks.* Thirteen chicks from the same stock were placed on a commercial chick feed, well balanced for growth factor and containing supplements of Santoquin (ethoxyquin 125 mg/kg).‡ The findings in these normal chickens and another group of normal chickens from one to 210 days of age are discussed elsewhere.

Chickens of each group were sacrificed under identical conditions serially so that experimental and normal chickens of identical ages were available for comparison. A series of chickens from 4 days to 26 days of age were used in this part of the study.

Generally when symptoms of ataxia became severe, the chick on the purified diet was sacrificed, since survival for 24 hours more usually would not occur. Methods of collection of blood and immunophoresis have been previously described(5).

Immunophoretic tests were always prepared with the sera of 2 chickens on the same slide reacting with the same antibodies. This permitted a comparison of the distribution pattern of the immunological precipitation curves produced under identical conditions in 2 samples of serum. On enlarging the stained slide by projection or photography, it became possible to demonstrate whether the fractions in the 2 samples were identical or dissimilar. The following series of tests were made.

1. Comparison of sera from experimental chickens after varying periods on the purified diet, with sera from normal chickens of the same age. A series of preparations demonstrating the effects of dietary stress on serum proteins were thus obtained (Fig. 1, 2).

2. Comparison of immunophoretic patterns

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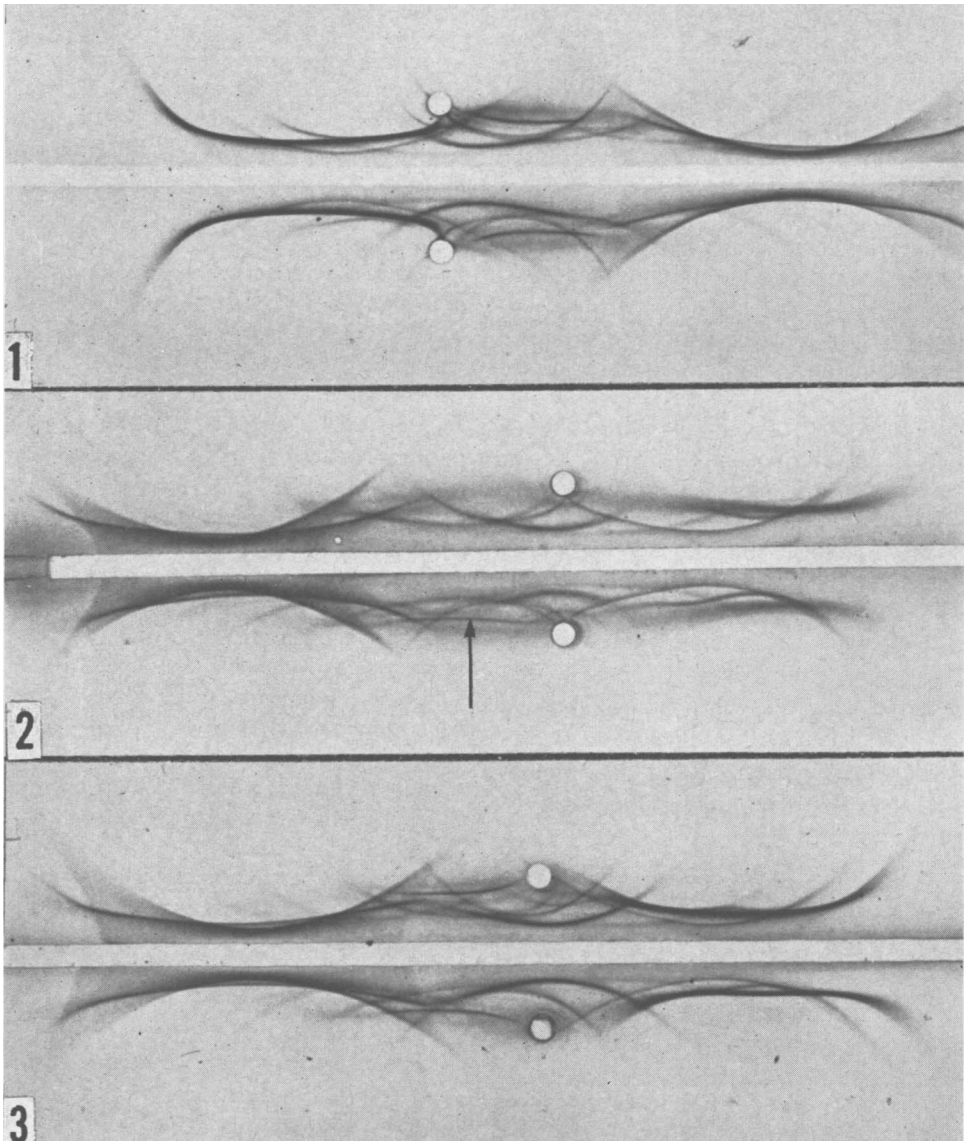
† Chicks were received from Nicolas Lohman Co., Cuxhaven, Germany one day after hatching. These were of unselected breed, but rather consisted of Leghorns and red chickens, resembling Rhode Island Reds. Eggs provided by the same firm were of the same mixture of strain. Sex of chickens was not determined since all were sacrificed long before they reached maturity.

‡ Vollkraftkorn K. Kuken Alleinfutter. Bergische Kraftfutterwerk, H. Schmidt, K. G. 4 Dusseldorf 1, Postfach 1709.

from normal and experimental chickens of differing ages. In some instances the experimental chicken was older, in others the normal chicken was older (Fig. 3, 4).

3. Comparison of immunophoretic pattern of sets of chickens on the experimental diet, with severe degrees of encephalomalacia to demonstrate the constancy of the patterns (Fig. 5).

Exact quantitative measures of the frac-



Immunophoretic patterns in chicken blood serum.

FIG. 1. Comparison of precipitation curves in 4-day-old chickens on normal and purified vit E-deficient diets. Upper trace, normal chick; lower trace, symptomless chick on purified diet. Note similarity of traces at this early age.

FIG. 2. Twenty-day-old chicks. Upper trace, normal chick. Lower trace, chick on purified diet, with mild symptoms of encephalomalacia. Note abnormal curve at arrow in lower trace.

FIG. 3. Comparison of traces of 5-day-old normal chick (upper trace) with 25-day-old chick on purified diet. Symptoms of encephalomalacia severe. Note persistence of precipitation line in beta lipoprotein position in both traces.

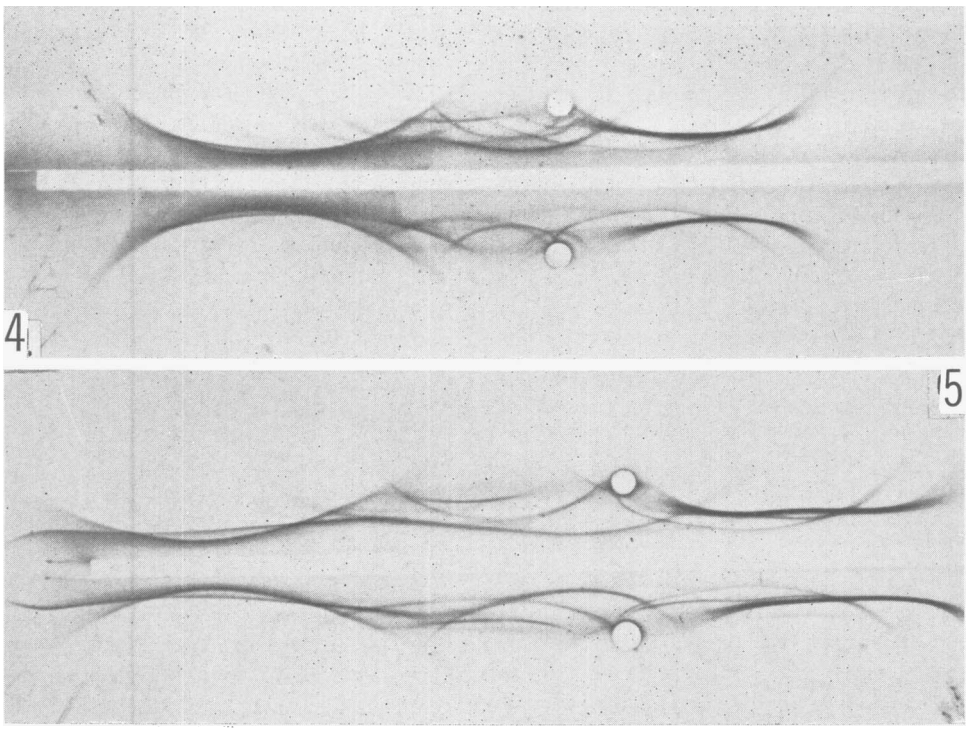


FIG. 4. Comparison of traces from 16-day-old chick (upper trace) on purified diet (severe encephalomalacia) with trace from normal chicken age 56 days (lower trace).
FIG. 5. Traces from two 25-day-old chickens with severe degree of encephalomalacia. Both chicks on vit E-deficient purified diets.

tions could not be made. The presence or absence of a precipitation curve could be noted; when the precipitate appeared dense it was designated as increased, where faint as decreased, always in terms of the pattern of healthy sera.

Results. Clinical symptoms. For these studies chicks on the purified diets generally developed symptoms at ages younger than those cited by Pappenheimer(7); however, a variability in the susceptibility of individual chickens was noted. Most chicks developed symptoms by the 10th day, although 4 in our series showed no ataxia after 21 days on the purified diet. Chicks with most severe ataxia showed punctate hemorrhages in the cerebellar folia; others showed no gross brain changes.

Growth was definitely retarded in the chicks on the purified diet; chicks weighed from 20 to 60 g less than the normal chicks of the same age, depending on age at sacrifice.

In classifying the severity of symptoms, the same criteria were used as reported previously(1,8) as follows:

Category I. Preclinical stage. Usually up to 6 days after the beginning of the purified diet.

Category II. Moderate degree of ataxia. Between 6 to 12 days of dietary deficiency.

Category III. Severe ataxia or *in extremis*. Twelve to 21 days on the diet. Several chickens which showed severe symptoms at an early date were found to have pericardial effusion. No other signs of exudative diathesis were evident. These chickens were included in the group classified in *Category III*. In previous papers(1,2) determinations of blood serum total protein had been made in chickens in each symptomatic category and were not repeated in this study. Hematocrits were determined in some of the experimental chickens and were found to correspond to the values found in control chickens.

Serum immunophoresis. Immunophoretic

preparations showed the following: 1. *Normal chickens.* As already reported(5), the significant findings in normal chickens show that of the more readily identifiable fractions, age and maturity of hens do not significantly modify the pattern of protein fraction distribution. On the other hand, the precipitation curve in the position of beta lipoprotein is not seen in sera from chicks 10 days or older; similarly, prealbumin curves, very prominent in young chicks, become faint after the 12th day, and are not observed in mature chickens. The gamma globulin fractions, present as 4 separate precipitation curves in neonatal chicks, appear generally as 3 fractions, until after the 12th day, when a fourth fraction frequently can be identified.

2. *Experimental chickens.* (a) Albumin fraction remained constant while the prealbumin fraction decreased with age as in the normal chicken. (b) A definite increase in the slower moving of the beta globulin fractions was present, not apparently related to the age of the chicken or to the severity of the symptoms. (c) A pronounced increase in the precipitation curve in the position of the beta lipoprotein fraction was constantly observed and was present at all ages of the series studied. (d) Transferrin was increased over normal in younger chickens, but lessened in amount with increasing age. (e) Gamma globulin fractions did not increase in numbers to the extent of that found in healthy chickens of the same age. This may reflect a failure of development of the immunoglobulins. The gamma 7S globulin fraction was frequently short and thick and deficient in a large number of chicks. Fraction gamma IM globulin appeared unaffected by the dietary stress.

It seems that no direct correlation between the observed serum protein changes and the severity of clinical symptoms could be made. A more significant correlation could be established with the age of the chicken and the length of exposure to the deficient diet.

Discussion. Currently it can be reported that the primary difference between chicks fed the experimental diet and those fed a stock diet is characterized by a persistent heavy precipitate in the mobility range of beta lipoprotein, which does not appear in chickens older than 10 to 12 days when fed on the normal diet. This same precipitation curve has been found in older chickens fed a purified diet deficient in vit E and sulfur amino acid(6). Preliminary findings in a study currently in progress, however, do not confirm that this precipitate represents beta lipoprotein. In fact, ultracentrifuge flotation tests of chicken serum show that while Svedberg flotation units are equal for alpha lipoprotein in both normal chicks at 15 days and in chicks of the same age on vit E-deficient diet, the Sf units for beta lipoprotein in the vit E-deficient chick were approximately half that of the 15-day-old normal chick(9). This finding would give more specific evidence of a specific vit E deficiency, if the control diets were identical with the experimental diet, but supplemented with adequate quantities of vit E. This procedure is projected at this time.

The significance of the precipitation line in the position of beta lipoprotein remains unclear. One might conjecture that it may represent a denatured protein, possibly of beta lipoprotein. The precipitate undoubtedly explains the increased beta globulin content of chicken blood serum in vit E deficiency. The reported increase in cholesterol in serum and tissues of animals on similar diets(10,11) would have some bearing on the interpretation of our findings. Cholesterol is associated with a lipid fraction of proteins which in electrophoresis moves largely with beta lipoproteins(12). Tocopherols are also transported in the lipoproteins(13) where they may stabilize these lipid structures by preventing oxidation. The presence of an abnormal fraction in the beta globulins, together with a decrease of the beta lipoprotein fraction which has approximately the same mobility as the abnormal fraction, suggests that the beta lipoprotein breaks down in the absence of vit E. In *in vitro* experiments of Nishida and Kummerow(14) it appears that hydroperoxide of methyl lineolate and beta lipoprotein have an affinity for each other with the resultant denaturation of the latter. Precipitation of such denatured protein has been observed in blood vessels. We ourselves have observed aortic subintimal fatty crystal deposits in young chicks affected by nutri-

tional encephalomalacia(15). Whether such deposits in other blood vessels or on other tissues can account for altering cell membrane permeability will require further research. The requirement for linoleic acid in the diet to produce encephalomalacia and the augmenting effects of supplements of linoleic acid in producing nutritional myopathy support such a possibility(16,17).

The findings that prealbumin and albumin are relatively unaltered by the encephalomalacia-producing diet confirm previous observations by Tureen *et al*(1).

Gamma globulin fractions, relatively unchanged under the experimental conditions, nevertheless, in a few instances, more often than in control chickens of the same age show a precipitate curve of the gamma 7S globulin fraction very similar to that observed in immunophoretic preparations of tissue (muscle, brain) homogenates. No explanation for this observation is offered.

Summary and conclusions. 1. Twenty-five newly hatched or very young chickens were subjected to a purified diet, deficient in vit E or any synthetic antioxidant and supplemented with 4.8% linoleic acid. If permitted to survive, these chicks developed encephalomalacia between the 10th and 26th day. Forty-two chicks, fed on a conventional stock laboratory diet, developed normally and were well at the time blood samples were examined. 2. Immunophoresis patterns of blood sera from experimental and normal chickens were obtained. 3. In the experimental sera, an antigen-antibody precipitation curve with the mobility of beta lipoprotein persisted whereas this curve did not persist after the 10th day in normal chickens. This curve was interpreted as due to the presence of an abnormal protein, possibly a denatured beta lipoprotein. 4. The role of possible denaturation of beta lipoproteins in the blood serum in the presence of autoxidation products of linoleic acid in the serum as a possible mechanism for production of vit E deficiency syndromes is discussed.

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