Comparison of Plasma Protein Changes in Antioxidant Deficiency Muscular Dystrophy and Genetic Muscular Dystrophy in the Chicken.* (30089)

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Alteration of the blood serum protein profiles in chickens manifesting the vitamin E deficiency syndrome of encephalomalacia(1) and of exudative diathesis(2,3) is characterized by an elevation of the B globulin fraction and a lowering of the A/G ratio. Similar changes have been reported by Oppenheimer *et al*(4) for the rabbit with muscular dystrophy of antioxidant deficiency. On the other hand, in man and in mouse, genetic forms of muscular dystrophy do not show such serum protein modifications(5,6). While investigating certain aspects of antioxidant deficient states in chickens, it has been possible to make comparisons between chickens of the New Hampshire breed(7), subject to hereditary (or genetic) muscular dystrophy, with a healthy strain of the same breed of chickens which developed nutritional myopathy following a diet deficient in antioxidants and sulfuramino acids(8,9). The present study is concerned with an analysis of the plasma proteins in these 2 sets of chickens. Comparison of the plasma protein profiles in these experimental states will be made with those of chickens affected by the other 2 syndromes of antioxidant deficiency.

Methods and materials. Twenty-eight chickens, one-day-old, were received from Asmundson,† and divided into 2 sets. One set was fed a diet deficient in vit E and sulfuramino acid (Table I). The other set was given the same diet, to which supplements of a synthetic antioxidant (Santoquin) and methionine hydroxy analogue were added (Table II). Each set of chickens contained both the genetic dystrophic and the normal strains. Thus 4 groups of chickens were used in this experiment as follows:—

Group I: N.C.D. Nine chicks of normal strain on the diet containing supplements of the antioxidant and sulfuramino acid. (Control chicks).

Group II: N.D.D. Seven chicks of the same strain on the experimental diet deficient in antioxidant and sulfuramino acid.

Group III: D.C.D. Five chicks of the genetic dystrophic strain on the supplemented diet provided for Group I.

Group IV: D.D.D. Seven chicks of the genetic dystrophic strain on the deficient diet, as provided for Group II.

Chicks were weighed weekly and examined for evidence of clinical paralysis of the breast muscles, chiefly by the ability of the chicken to right itself when laid on its back. Chickens of Group II, when manifesting such paralysis were sacrificed concomitantly with chickens of Group I which served as a control. Sacrifice of chickens in Groups III and IV depended upon their clinical states. Those of Group IV were more sick than any of the other 3 groups and were sacrificed at an earlier date; those of Group III were allowed to survive until the experiment was terminated. During the experiment, several chickens of each group were bled bi-weekly to trace the development of any alterations in the plasma proteins, and to correlate such findings with the clinical state of the chickens.

Sodium pentobarbital anesthesia, (100 mg/kilo intraperitoneally) was used to permit ventricular puncture or sacrifice by bleeding. In older birds, venipuncture of wing vein was used for procurement of repeated samples of blood. Anticoagulants of 2 functionally similar types were used on different occasions: a potassium ammonium oxalate mixture, and ethylenediamine tetraacetic acid (EDTA). The non-coagulated blood was centrifuged

* This project was partially supported by a grant from the Muscular Dystrophy Assn. of America and NIH, Nat. Inst. of Child Health and Human Development.

† Chicks were supplied by V. S. Asmundson, Univ. of California Dept. of Poultry Husbandry, Davis.

TABLE I. Dystrophy-Producing Diets.

Assay protein C-1	5.0
Gelatin	10.0
Casein (purified)	10.0
Chick Salts A*	6.0
"Stripped" Lard	4.0
Choline chloride	.20
Vitamin mix S-35*	.50
" A (10000 I.U./g)	.05
" D (7500 I.U./g)	.03
Alphacel	1.00
Procaine penicillin G (50%)	.004
NF 180	.05
Cerelose	q.s. 100

* Poultry Sci., 1958, v37, 1460.

TABLE II. Control Diet Supplements.

L-cystine	.40
Methionine hydroxy analogue (MHA)*	.30
dl-alpha-tocopherol acetate	.01
Santoquin†	.02

* Registered trademark of Monsanto Chemical Co. for calcium dl-2-hydroxy,4-methylthio butyrate, the hydroxy analogue of methionine.

† Registered trademark of Monsanto Chemical Co. for 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline.

immediately and the plasma samples were analyzed by paper electrophoresis employing the Beckman "Spinco" apparatus model R, and the separations were traced on the Beckman Analytrol model RB. Plasma was examined instead of serum because difficulty was encountered in obtaining a uniform clotting from the blood samples, making uncertain the comparison of the several samples of serum obtained. Total plasma protein concentration was determined by the Biuret method as modified by Ferro and Ham(10). For convenience, the gamma globulin and fibrinogen peaks(11) were combined as globulin in the calculation of the A.G. ratio. All results were evaluated statistically by the paired T test.

Results. Group I. The chickens comprising Group I gained weight well and appeared extremely healthy and normal. Gross examination of the pectoral muscles at time of sacrifice revealed that there were no white striations in the muscle fibers. Histologically the muscle was normal.

Group II. With this group, growth by weight was retarded as much as 50% probably due to a lack of an optimal amount of sulfuramino-acids in the diet. Myopathic symptoms were manifested between the 8th and 14th weeks of life. The "righting ability" was either completely lost or considerably slowed as the wings became progressively less functional. Concomitantly with the onset of the disease, a generalized muscular weakness was noted, especially in the legs of those affected at an early age. A variable amount of localized or diffuse white striations of the pectoral muscle was noted. Histological sections through grossly diseased parts of the pectoral muscle showed the characteristic changes seen in nutritional myopathy, *viz.*, a coagulation necrosis of whole or parts of sarcolemmal fibers, splitting and fragmentation of fibers, phagocytosis of damaged fibers, interstitial infiltration by round cells, aggregation of sarcolemmal nuclei within and at the surface of damaged or empty sarcolemmal tubes and increased connective tissue in the interstices replacing necrotic or lost muscle fibers.

Group III. The weight growth curve of the genetically dystrophic chickens on the supplemented diet, resembles that of Group I until the onset of the dystrophic symptoms at about the eighth week of life. All birds exhibited the clinical signs of the disorder by the twelfth week, as was expected. Upon gross and histological examination, all pectoral muscles revealed the general appearance of dystrophic muscles as described elsewhere (12). However, activity and tonus of the muscles, especially in the legs, was much better than in the nutritionally deprived birds.

Group IV. The genetically dystrophic, nutritionally deprived group showed 70% growth retardation, extreme muscular weakness, and a generally sickly appearance. These chickens were unable to stand for more than one minute at a time, walked with extreme difficulty, and seemed to be approaching the terminal stage of the disease. The last of the group were sacrificed at the eleventh week of life since death seemed imminent. In addition to manifesting the characteristic fatty striations, the pectoralis and other muscles were extremely atrophic. Histologically a greater degree of derangement of, and damage to muscle fibers than occurred in Group III was evident.

TABLE III. Relative Amounts.

Group	No.	Days age	Albumin	Alpha I	Alpha II	Alpha III	Beta	Gamma + fibrinogen
I NCD	9	86.6	38.71 ± 1.11	10.29 ± .85	9.64 ± .71	5.51 ± .98	16.84 ± 1.11	18.97 ± 2.63
II NDD	7	88.4	30.92 ± 2.16*	10.37 ± 1.36	8.74 ± .74	8.48 ± .70	24.47 ± 1.35†	18.00 ± 3.06
III DCD	5	99.6	38.40 ± 1.66	13.55 ± .73	8.73 ± .53	6.64 ± .88	18.30 ± 1.40	14.37 ± 1.52
IV DDD	7	68.3	37.54 ± 2.26	11.25 ± 1.45	10.42 ± 1.06	5.14 ± .58	20.77 ± 2.26	14.86 ± .90

* As compared with Group I, P value is less than .01.
† As compared with Group I, P value is less than .001.
‡ As compared with Group I, P value is less than .025.
§ As compared with Group III, P value is less than .001.
Table includes standard error of mean.

TABLE IV. Absolute Amounts.

Group	A/G	Albumin	Alpha I	Alpha II	Alpha III	Beta	Gamma + fibrinogen	Total proteins
I NCD	.764 ± .046	1.49 ± .04	.39 ± .03	.38 ± .04	.22 ± .04	.66 ± .05	.76 ± .10	3.9 ± .2
II NDD	.487 ± .048†	.97 ± .06†	.34 ± .04	.31 ± .03	.26 ± .03	.81 ± .08	.69 ± .13	3.2 ± .2‡
III DCD	.725 ± .036	1.27 ± .04	.47 ± .04	.29 ± .02	.26 ± .03	.57 ± .04	.48 ± .04	3.4 ± .1
IV DDD	.640 ± .047	.90 ± .06§	.27 ± .04	.24 ± .03	.12 ± .01	.49 ± .04	.29 ± .02	2.3 ± .1§

See Table III footnotes.

Plasma protein characteristics. Data describing the blood plasma protein profile of each of the 4 groups of chickens are tabulated in Table III showing the relative proportions of each protein fraction. The absolute amounts of each fraction, tabulated in Table IV, were calculated in terms of the total protein determinations. Using Group I chickens as the control data for comparison, it is evident that Group II chickens, affected by nutritional myopathy, shows a significant alteration of the plasma protein patterns. In terms of the relative percentages of the fractions albumin is decreased by 20% ($P<.01$), B globulin is increased by 45% ($P<.001$), and the A/G ratio is decreased from .764 ± .046 to .487 ± .048 ($P<.001$). Other globulin fractions appear to be unaltered. Total plasma protein concentration was decreased ($P<.025$). Reflecting this change the total amount of albumin shows a striking decrease ($P<.001$). The increase in the absolute amount of B globulin was not statistically significant when compared with the control; however, when the decrease in albumin is considered in computing the absolute value of B globulin, then a significant increase in B globulin is established, particularly since other globulin fractions in the abnormal plasma parallel both the relative and absolute values of these fractions in the control area. Visual scanning of the electrophoretic trace readily demonstrates the fall in albumin, rise in B globulin and constant level of other globulin fractions. The total plasma protein concentration and electrophoretic profiles showed a progressive alteration in Group II birds as symptoms of myopathy developed and progressed.

Although the chickens of Group III were severely paralyzed by genetic muscular dystrophy at the time plasma samples were examined, the total plasma protein concentration and the electrophoretic protein profiles did not differ in any significant way from those found in normal chicks on the control diet (Group I). The chickens of Group IV, with the additional stress of a dietary deficiency added to the genetic muscular disease, while showing an alteration in plasma protein concentration, nevertheless maintained a normal electrophoretic protein profile. There was a uniform decrease in all proteins, albumin as well as globulins, with maintenance of a normal A/G ratio, and without manifestation of elevation of the B globulin fraction.

Discussion. The findings presented here provide evidence that plasma (or serum) proteins in all antioxidant deficiency syndromes in the chicken, are altered by an elevation of the B globulin fraction and a lowering of the A/G ratio. Transudation into

subcutaneous tissues is apparently not the consequence of the lowered A/G ratio, since other antioxidant deficiency syndromes with low A/G ratios can be produced without the complication of exudative diathesis. Additionally, the albumin fraction is markedly decreased in the myopathies due to vit E deficiency in the chicken and rabbit(5), and in exudative diathesis in the chicken(4,5). However, it remains unchanged in encephalomalacia of the young chick, where the altered A/G ratio is an expression of the increased B globulin(1). The alterations in plasma (and serum) protein observed in antioxidant deficient states must be regarded as an expression of the metabolic disorder itself, rather than a cause of the symptoms. The variations in character of the purified diets used to produce these symptoms probably account for the difference.

Chickens of the genetic dystrophic strain, on a properly balanced purified diet, develop paralysis of pectoral muscles within 8 to 12 weeks. Total plasma protein concentration, and the electrophoretic profile of the protein fraction are comparable to that found in the chicken of the normal strain on the same diet. When the dystrophy strain chicken is stressed with the myopathy producing diet, however, the severity of the paralysis is increased, and longevity of the chicken lessened. Under these conditions, despite a pronounced drop in total plasma proteins, and in concentration of the albumin fraction, globulin concentration dropped in a parallel manner, although the electrophoretic profile of the globulin fraction remained normal. Thus the A/G ratio maintained a normal value. Although these chickens were sacrificed at an earlier age than those of the other group, age alone could not account for the alterations in total protein concentration. They did not compare with Vanstone's(13) values for these ages in healthy chickens. Whether the protein alterations found in this group are an expression of their inanition cannot be stated.

The absence of plasma protein changes in paralyzed genetically dystrophic chickens points again to the differences between the metabolic features of this disorder and that of nutritional myopathy. In the latter condition, a generalized metabolic disorder is present affecting many organs and systems. In the former, only one system, *viz.*, the muscular structure is affected. The same is true of human and mouse muscular dystrophy, though variable and inconsistent patterns of lipids in the blood serum have been shown which nevertheless differ sharply from serum lipid changes in vit E deficiency states.

Summary and conclusions. 1. Chickens on an antioxidant and sulfuramino acid deficient diet, develop nutritional myopathy. Plasma protein changes, progressively developing during the course of the disease, are characterized by a significant reduction in the albumin fraction, a significant rise in the B globulin fraction and a significant fall in the A/G ratio. 2. Paralyzed chickens of a genetic dystrophic strain fail to show such changes. The plasma protein profile is comparable to that of healthy chickens on a control diet. 3. Genetic dystrophic chickens on an antioxidant and sulfuramino-acid deficient diet grow poorly, develop the most severe degree of paralysis, and have a shorter survival period. The plasma protein profile is characterized by a significant decrease in total protein concentration, although a rather normal plasma protein electrophoretic pattern is maintained. 4. It is concluded that the metabolic defect in genetic dystrophic chickens is one localized to the muscular system, while that in antioxidant and sulfuramino acid deficiency is a generalized one. 5. The significance of the protein changes is briefly discussed.

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Received November 23, 1964. P.S.E.B.M., 1965, v119.

Monocytin, a Protecting Substance Produced by Murine Monocytes. (30090)

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Bactericidal substances were isolated from white blood cells by several authors. The results of investigations performed from the very beginning of the bacteriological era(1) and those of later investigations were reviewed(2). A substance named "phagocytin" was extracted from rabbit granulocytes with a salt solution buffered at pH 5.6(3) and an arginine-rich fraction (histone B) was isolated from calf thymus(4). Phagocytin and histone B were identified as separate substances(5). A bactericidal substance had been accumulated in Eagle's medium incubated with murine granulocytes and living Gram-negative bacteria(6). Three lysozomal fractions rich in arginine and acting on *Escherichia coli in vitro* were isolated from guinea pig granulocytes(7).

The results presented here demonstrate that several bactericidal substances were freed by monocytes in the course of the phagocytic process. One of them was able to prevent infection, when administered at least 4 hours before intraperitoneal inoculation of a lethal dose of *Salmonella typhi* (strain Ty_2).

Materials and methods. Eighteen-hour cultures of *E. coli, S. paratyphi A, S. typhi* (strain Ty_2), *Shigella dysenteriae* and *Staphylococcus aureus* on nutrient agar were employed. Murine peritoneal exudate monocytes were used for production of bactericidal substances. Mice were pretreated by intraabdominal injections of 3 ml of 1% sodium caseinate 3 days before the harvest of the monocytes. The cells were collected in phosphate buffer saline (pH 7.4) and washed with the same buffer 3 times. Monocytes were incubated in Eagle's medium (pH 7.4) in the presence or absence of *S. typhi* (strain Ty_2). The phagocytosis experiments *in vitro* were carried out as previously described(6). Supernatant fluids were separated by centrifugation and discarded. The monocytes were washed with buffer and destroyed by 3-fold freezing and thawing. Debris was discarded by suitable centrifugation. The resulting cell extracts were dialyzed against 0.05 M buffer phosphate (pH 7.0), or against distilled water. Bactericidal substances were separated from infected dialyzed extracts by preparative electrophoresis. The bactericidal activity of their graded dilutions was examined by incubation with 10^5 bacteria in a total volume of 1.0 ml 0.05 M buffer phosphate (pH 7.0). The number of surviving bacteria was counted after 2 hours of incubation at 37°C. Mouse protective activities were examined by intraperitoneal or intramuscular administrations of various samples prior to or simultaneous with an intraperitoneal challenge of 2×10^8 microorganisms of *S. typhi* (strain Ty_2).

Preparative electrophoresis was carried out according to Osborn(8) with some modifications. The eluates were dialysed against 0.05 M buffer phosphate (pH 7.0) and concentrated by pervaporation. Analytical electrophoresis was carried out on 30/5 cm cellulose acetate strips (Oxoid-Sigma). The electrophoresis was carried out in TRIS-EDTA-

* Part of Ph.D. thesis to be submitted to Senate of Hebrew Univ., Jerusalem, Israel.