Lysosomal enzymes and vitamin E deficiency

1. Muscular dystrophy, encephalomalacia and exudative diathesis in the chick

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1. The activities of several lysosomal hydrolases were measured in the tissues of chicks suffering from nutritional muscular dystrophy, encephalomalacia or exudative diathesis.

2. In dystrophic breast muscle, β-glucuronidase was raised five- to six-fold, cathepsin fourfold and acid phosphatase 1·5-fold. No change was found in the subcellular distribution of β-glucuronidase.

3. Chicks with encephalomalacia showed no changes in the β-glucuronidase, β-galactosidase, acid phosphatase or β-acetylglucosaminase activities of cerebellum or brain. Subcellular distribution of β-glucuronidase and β-galactosidase in these tissues was also unchanged.

4. In exudative diathesis, hydrolases were found in the exudate, and there was increased activity in the subcutaneous tissue first showing haemorrhages. Increased hydrolytic activity was found in liver, spleen and kidney. Breast muscle was not always affected by the exudative condition, but, when it too degenerated, its hydrolase activity increased.

5. β-Glucuronidase activity was measured in the serums of chicks suffering from each of the three deficiency diseases. None of the diseases caused a rise in activity.

Vitamin E deficiency in several species has been shown to be accompanied by striking changes in the activities of lysosomal hydrolases. Skeletal muscle of vitamin E-deficient rabbits displayed increased proteolytic and dipeptidase activity, but liver, spleen and kidney were not affected (Weinstock, Goldrich & Milhorat, 1955, 1956). Increased activities of several other hydrolases in dystrophic rabbit muscle were found by Zalkin, Tappel, Caldwell, Shibko, Desai & Holliday (1962) and there was also marked liberation of enzyme activity from the particles into the cell sap. Breast muscle of chicks with nutritional muscular dystrophy was found to have raised activities of five acid hydrolases (Desai, Calvert, Scott & Tappel, 1964), and similar changes in activity were found by Desai (1966) in lambs with white muscle disease. Brain tissue of chicks with encephalomalacia also had increased hydrolase activity and a greater proportion of the activity was in the supernatant fraction (Jibril & McCay, 1965; Tappel, Sawant & Shibko, 1963).

Our studies were designed to extend this survey of changes in lysosomal hydrolases to exudative diathesis in the chick. We also studied muscular dystrophy and encephalomalacia in order to compare our results with those of other workers.

EXPERIMENTAL

Animals, diets and enzyme substrates

Dietary ingredients. Casein, 'low vitamin content', was purchased from Genatosan Ltd, α-protein from the Chemurgy Division of Central Soya Inc., London, and torula yeast from Lake States Yeast and Chemical Division of St Regis Paper Co., Rhinelander, Wisconsin, USA. The salt–vitamin mixture used in all the chick diets supplied salt mixture (Bunyan, Diplock, Edwin & Green, 1962) 6·75%, vitamin mixture (Bunyan *et al.* 1962) 0·2%, choline dihydrogen tartrate 0·4%, chlortetracycline 0·2 ppm and, in the form of a stabilized powder, vitamin A, 25 i.u. and vitamin D_3, 3·3 i.u./g.

Enzyme substrates. β-Glycerophosphoric acid disodium salt obtained from Kodak Ltd (maximum α-content = 0·1%) was recrystallized twice from hot water and ethanol. *p*-Nitrophenyl phosphate disodium salt and *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside were obtained from Koch–Light Laboratories Ltd. 4-Methylumbelliferone glucuronide was prepared by the method of Mead, Smith & Williams (1955). 4-Methylumbelliferone galactoside was prepared by the method of Robinson (1964). Substrate solutions were prepared freshly each day.

Chicks. One-day-old cockerels were purchased from a commercial breeder. They were reared in electrically heated wire-floored cages and provided with food and water *ad lib.* These chicks were either of the Rhode Island Red × Light Sussex type or Warren sex-linked Rhode Island hybrids, as indicated in the tables.

Diets for experiments on muscular dystrophy. The basal deficient diets had the following percentage composition. Diet 1: casein 15, gelatin 10, lard 4, salt–vitamin mixture 7·35, DL-tryptophan 0·14, L-leucine 0·44 and glucose 63·07. Diet 2: casein 20, lard 4, salt–vitamin mixture 7·35, L-arginine 0·45 and glucose 68·2. Diet 3: casein 5, α-protein 5, torula yeast 10, lard 4, salt–vitamin mixture 7·35, glycine 1, L-leucine 0·2, L-glutamic acid 1·15, DL-phenylalanine 0·5, DL-tryptophan 0·1 and glucose 65·35. This diet is similar to one described by Scott & Calvert (1962), except for the use of α-protein as the isolated soya-bean protein, a decrease of glycine from 2·5 to 1% and the omission of their supplements of selenium and DPPD (*N*,*N*′-diphenyl-*p*-phenylenediamine). Diet 4: α-protein 24, salt–vitamin mixture 7·35, glycine 0·5 and glucose 68·15.

All these diets were designed so that the sulphur amino acids limited growth. Diets for control chicks were made by the addition of suitable amounts of DL-methionine.

Diets for experiments on encephalomalacia. The basal diet had the percentage composition: casein 20, gelatin 8, lard 20, salt–vitamin mixture 7·35, glucose 44·65 and Se, 0·15 ppm, as sodium selenite. A control diet was prepared by adding D-α-tocopheryl acetate, 140 ppm, to the basal diet.

Diets for experiments on exudative diathesis. The basal diet had the percentage composition: torula yeast 60, lard 4, salt–vitamin mixture 7·35, DL-methionine 0·4, glycine 0·5 and glucose 27·75. Control diets were prepared by adding to the basal diet supplements of either Se, 0·15 ppm, as sodium selenite, or D-α-tocopheryl acetate, 100 ppm.

Signs of deficiency and selection of chicks for testing

Deficient chicks, together with the appropriate controls, were killed and tested at times described below. Individual chicks were tested in experiments on muscular dystrophy, but, in those on encephalomalacia and exudative diathesis, it was often necessary to combine the tissues of two or three chicks for testing.

Muscular dystrophy. White striations in the breast muscle first became visible through the skin at about $4\frac{1}{2}$ weeks of age when the deficient diet was given throughout, or about 5 days after withdrawing methionine from the supplemented diet at 4 weeks old. Dystrophic chicks were killed and tested at 36–54 days old, and the degree of degeneration of their muscles was graded approximately as slight, moderate or severe.

Encephalomalacia. Deficient chicks were examined daily from 2 weeks old for signs of encephalomalacia: at first a staggering gait, followed by limb spasms and head twisting and, finally, prostration, paralysis and death. When examined, many brains were found to be oedematous and many cerebella had petechial haemorrhages.

Exudative diathesis. This condition was recognized externally by a green discoloration of the abdomen. Haemorrhages were seen on the surface of the breast and leg muscles and on the viscera of many chicks with exudates. At a later stage, some chicks developed haemorrhagic degeneration of the breast muscle itself with some white striations, although these did not exceed the 'moderate' grading used in the other tests on muscular dystrophy. In addition to these lesions, changes in the abdominal viscera were sometimes found: livers were pale, kidneys often enlarged and spongy, and spleens pale and small.

Tissue preparations and enzyme methods

Preparation of tissue homogenates. Chicks were killed by breaking their necks and the tissues required were immediately excised, washed, blotted and weighed. Coagulated exudate was removed from the skin and breast muscle and weighed. Breast muscle was chopped finely and homogenized in a top-drive steel-bladed macerator (MSE homogenizer) for about 2 min at 1000 rev/min. Soft tissues (liver, spleen, kidney, exudate, cerebellum and the remainder of brain) were cut into small pieces if necessary and homogenized in a glass homogenizer tube, using a nylon pestle with 0·76 mm total diameter clearance. The homogenizer was precooled to 0° and thereafter kept in ice-water. The tissue was homogenized for about 1 min by passing the tube up and down several times while the pestle was rotated at about 1000 rev/min. Homogenates of muscle and the soft tissues were filtered through two layers of surgical gauze. This suspension was called the 'whole homogenate'.

When only the total enzyme activity was required, tissues were homogenized in ice-cold de-ionized water. However, when a measure of free activity was also required and when subcellular fractions were prepared, homogenates were made in 0·25 M-sucrose solution (containing 0·001 M-diamino-ethanetetraacetic acid disodium salt). Total enzyme activity was usually released from the homogenate in sucrose solution by including Triton X-100 in the incubation mixture at a final concentration of 0·1 % except for the phosphatase assay (see p. 130). Blood was collected immediately after

death from the heart or from the vessels of the neck after the head had been severed, and the clot was centrifuged to separate the serum.

Subcellular fractions. These were prepared by differential centrifugation at about 4°. The size of the smallest particle completely sedimented (S_{min}) was calculated by the method of de Duve & Berthet (1953). We used the following expression for S_{min} (in Svedberg units of 10^{-13} sec):

$$S_{min} = 3{\cdot}9 \times 10^{8} \left(\frac{R_{av} \log_{10} (R_{max}/R_{min})}{g \times min} \right),$$

where R_{av}, R_{max} and R_{min} are, respectively, the average, maximum and minimum radii of the column of liquid while centrifuging. Average centrifugal force (g) in the middle of the liquid was calculated as $1{\cdot}1118 \times 10^{-5} \times (\text{rev/min})^2 \times R_{av}$. The time–force integral, $g \times$ min, was calculated from the area under the graph of average centrifugal force against time.

The whole homogenate was freed of cell debris and nuclear material by centrifuging in cooled buckets in an MSE Minor Mk II centrifuge. The supernatant fraction was called the 'homogenate' and had $S_{min} = 2 \times 10^{5}$. A 'lysosomal' fraction ($S_{min} = 2 \times 10^{5}$–2300) was also prepared, by centrifuging in an MSE 'Superspeed 25' refrigerated centrifuge and washed once with sucrose solution. This fraction corresponds approximately to the sum of the heavy and light mitochondrial fraction of rat liver ($S_{min} = 1{\cdot}5 \times 10^{5}$–2300) described by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955), if we assume that particles from other tissues have similar characteristics. The first supernatant solution over the lysosomal fraction was combined with the washings and called the 'supernatant' fraction ($S_{min} < 2300$).

Enzyme assays. Enzyme assays and blanks were carried out in triplicate, except for *p*-nitrophenyl phosphatase for which tests were duplicated. For all enzymes apart from cathepsin, blank estimations were made by leaving the addition of homogenate until last.

Enzyme units. With the exception of cathepsin, each enzyme unit was defined as the amount of enzyme that catalysed the hydrolysis of 1 μmole of substrate per min under the conditions stated. The unit of cathepsin activity was defined as the amount that liberated 1 μg of tyrosine (or substances of equivalent optical density) per min. For convenience, some results were expressed as milli-units (m-units).

Acid phosphatase (*EC* 3.1.3.2, *orthophosphoric monoester phosphohydrolase*). (*a*) β-glycerophosphatase. Homogenate in water (1 ml) was added to 1 ml of buffered substrate (0·02 M-β-glycerophosphate and 0·25 M-sucrose in 0·1 M-acetate buffer, pH 5·0) at 37°. Enzyme action was stopped after 10 min by adding 3 ml trichloroacetic acid solution (4·17%, w/v) mixing by inversion. The solutions were cooled in ice-water and centrifuged. Inorganic phosphate was determined in a portion (3 ml) by adding 3 ml sodium molybdate solution in 2 N-H_2SO_4 (2·5%, w/v). Colour was developed by adding 1 ml reducing solution (0·3 g $SnCl_2.2H_2O$ and 3·0 g hydrazine sulphate per l. of N-H_2SO_4 (Hurst, 1964)) and measured at 700 nm after about 20 min in a Unicam SP 600 spectrophotometer, with appropriate blanks. (*b*) *p*-Nitrophenyl phosphatase. Homogenate in sucrose solution (1 ml) was added to 1 ml of buffered substrate (0·02 M-*p*-nitrophenyl phosphate and 0·25 M-sucrose in 0·1 M-acetate buffer,

pH 5·0) at 37°. For measuring total activity, the mixture also contained Triton X-100 (0·1 %, finally). Enzyme action was stopped after 10 min by adding 8 ml N-NaOH. Liberated *p*-nitrophenol was determined by its optical density at 400 nm.

β-Glucuronidase (*EC* 3.2.1.31 *β-D-glucuronide glucuronohydrolase*). The tissue preparation (0·5 ml of a suitable dilution in water or sucrose solution) was added to 1 ml of buffered substrate (5×10^{-4} M-4-methylumbelliferone glucuronide and 0·25 M-sucrose in 0·1 M-acetate buffer, pH 5·0) at 37°. Enzyme action was stopped after 10 min by adding 8·5 ml 0·2 M-NaOH–glycine buffer, pH 10·4. (The buffered substrate contained Triton X-100, 0·26 % (w/v) when the total activity of a sucrose homogenate was required). The amount of 4-methylumbelliferone liberated was determined by measuring the fluorescence of the solution in a Specker fluorimeter (Hilger & Watts, Ltd) or in a Locarte fluorimeter. The primary filter in each instrument transmitted maximally at 365 nm, whereas their secondary filters transmitted maximally at 440 and 410 nm, respectively. Quinine sulphate solution (in 0·1 N-H_2SO_4) was used as reference standard.

β-Galactosidase (*EC* 3.2.1.23, *β-D-galactoside galactohydrolase*). This enzyme activity was determined in the same way as *β*-glucuronidase but 4-methylumbelliferone galactoside ($1{\cdot}24 \times 10^{-3}$ M) was used. The use of this substrate has been discussed before (Robinson, 1957).

Cathepsin. Proteolytic activity was measured at pH 5 with haemoglobin as substrate. This enzyme activity in rat liver is optimal at about 3·3. However, we intended to design an assay system that would allow measurement of free activity of lysosomal fractions as well as total activity of various fractions, and so account had to be taken of the tendency of lysosomes to rupture at acid pH. Therefore, following Gianetto & de Duve (1955), pH 5·0 was selected for all the assays.

The substrate solution was prepared by suspending 2·6 g finely ground ox haemoglobin in 100 ml 0·255 M-acetate buffer, pH 5·0, containing 0·25 M-sucrose and 0·15 % (w/v) Triton X-100. The suspension was kept at 37° for 1 h with occasional shaking, filtered through glass-wool and then centrifuged at 10000 rev/min for 5 min in the refrigerated centrifuge. The supernatant solution was stored at 4° until required. For the enzyme assay, 1 ml substrate solution was warmed to 37° for 5 min before adding the tissue homogenate (0·5 ml). Enzyme action was stopped after a further 62 min at 37° (total time 67 min) by adding 4 ml 10 % (w/v) trichloroacetic acid solution. Blank estimations were made by delaying addition of the homogenate until the substrate had been at 37° for 65 min and then stopping the reaction at 67 min, thus ensuring that the amount of hydrolysis of haemoglobin by incubation at 37° alone was the same as in the test reaction. The test and blank solutions were cooled in ice-water for 30 min and then filtered through Whatman no. 42 filter paper, without suction. Soluble protein, in terms of tyrosine equivalents, was determined by measuring optical density of the filtrates at 280 nm in a Uvispek spectrophotometer (Hilger & Watts, Ltd) calibrated with a solution of L-tyrosine (Kunitz, 1947). Enzyme activity during 60 min was given by the difference between the test and blank results for 62 and 2 min respectively.

β-Acetylglucosaminase (*EC* 3.2.1.30, *β-2-acetamido-2-deoxy-D-glucoside acet-*

amidodeoxyglucohydrolase). Enzyme activity in brain was measured by the liberation of *p*-nitrophenol from the substrate, *p*-nitrophenyl 2-acetamido-2-deoxy-*β*-D-glucopyranoside (Borooah, Leaback & Walker, 1957; Findlay, Levvy & Marsh, 1958). Homogenate (0·5 ml) was added to 0·5 ml buffered substrate (0·005 M in 0·1 M-acetate buffer, pH 5·0 containing 0·25 M-sucrose) at 37°. Enzyme action was stopped after 20 min by adding 8·5 ml N-NaOH. The cloudy solution was filtered through Whatman no. 42 filter paper without suction, and *p*-nitrophenol was determined by the optical density at 400 nm.

RESULTS

Muscular dystrophy. Enzyme changes in dystrophic muscle are shown in Table 1. These results are completely in agreement with those of Desai, Calvert, Scott & Tappel (1964) in showing great rises in enzyme activity. On average, *β*-glucuronidase rose five- to six-fold, cathepsin by fourfold and acid phosphatase by only 1·5-fold. Some increases were as much as eight- to twelve-fold for *β*-glucuronidase and sixfold for cathepsin. Tests on subcellular fractions showed no decrease in the proportion of *β*-glucuronidase activity bound within lysosomes of dystrophic muscle (52 (SD ± 10) % compared to the control result of 41 (SD ± 13) %); nor was serum *β*-glucuronidase affected, the results for dystrophic birds being a little lower than those for normal chicks. Desai, Calvert, Scott & Tappel (1964) did not comment on the subcellular distribution of enzymes in the muscles they studied.

The dystrophy was associated with a striking decrease in growth rate and lesser changes in the protein and water content of the muscle. Dystrophic chicks weighed only about 70 % of controls, but the weight of breast muscle remained a fairly constant proportion of body-weight. Muscle protein, however, was decreased by about 16 % and the moisture content increased by about 3·5 %. The rises in enzyme activity noted above would be still greater if expressed on a protein basis.

Encephalomalacia. The enzymes *β*-glucuronidase, *β*-galactosidase, acid phosphatase and *β*-acetylglucosaminase were studied in cerebellum and the remainder of brain (Table 2). In cerebellum, mean values for the deficient chicks were from 5 to 25 % lower than for normal controls, whereas they were from 5 to 15 % higher in the rest of the brain. None of these differences was statistically significant. The lysosomal fractions of cerebellum and brain were found to contain 78–85 % of the *β*-glucuronidase activity and 72–80 % of *β*-galactosidase (Table 3). These results are at variance with those of Jibril & McCay (1965) who reported a fivefold rise in *β*-glucuronidase and a twofold rise in acid phosphatase; their control values for these two enzymes were higher than ours by two and four times, respectively. These authors also reported an increase in the acid phosphatase and neuraminidase activity of the supernatant fraction; we found no change in the subcellular distribution of *β*-glucuronidase, which might be expected to be similarly affected. In our tests, deficient chicks were smaller than controls, but there was little difference in the weights and protein contents of cerebellum or brain.

Exudative diathesis. Results are shown in Table 4. The green exudate always showed some enzyme activity even when tested on the day that it first appeared. There was

also a significant rise in β-glucuronidase activity of the skin in the region where the first subcutaneous haemorrhage occurred. There were large rises in β-glucuronidase and catheptic activity of breast muscle. However, these rises only occurred when the muscle had visibly degenerated, and low values were found in three chicks that were otherwise

Table 1. *Muscular dystrophy and lysosomal enzyme activity in chicks*

(Muscular dystrophy was induced either (A) by giving the chicks a deficient diet or (B) by giving them that diet supplemented with methionine until about 28 days old and then withdrawing the supplement. The severity of the dystrophy was scored as severe (3), moderate (2) and slight (1). Enzyme activities are given as means with standard deviations and the no. of tests is shown in parentheses)

Deficient diets	Mean dystrophy score	Method for producing dystrophy	Tissue	Enzyme	Enzyme activity: Dystrophic chicks*	Enzyme activity: Normal chicks*
1, 2, 3, 4	2·0	A	Breast muscle	β-Glucuronidase (m-units/g)	30 ± 14 (4)	10 ± 6 (10)
1, 2, 4	2·1	B	Breast muscle	β-Glucuronidase (m-units/g)	56† ± 35 (8)	
4	2·1	B	Breast muscle	Cathepsin (units/g)	43‡ ± 22 (4)	11 ± 7 (4)
4	1·7	B	Breast muscle	Acid phosphatase (units/g)	0·38 ± 0·27 (3)	0·25 ± 0·11 (3)
4	1·7	B	Serum	β-Glucuronidase (m-units/100 ml)	66 ± 26 (5)	85 ± 9 (5)

* One chick only was used in each test. Rhode Island Red × Light Sussex cockerels were used in early experiments and Warren cockerels in later experiments.
† Significantly higher than corresponding value for normal chicks, $P < 0.001$.
‡ Significantly higher than corresponding value for normal chicks, $P < 0.05$.

Table 2. *Encephalomalacia in chicks and lysosomal enzyme activity in brain and serum*

(All chicks receiving the basal diet showed ataxia and limb spasms. Some were also prostrate. Chicks receiving vitamin E were normal. Enzyme activities are given as means with standard deviations and the no. of tests is shown in parentheses)

Tissue	Age (days)	Enzyme	Enzyme activities*: Deficient chicks†	Enzyme activities*: Normal chicks
Cerebellum	14–31	β-Glucuronidase (m-units/g)	46 ± 11 (4)	58 ± 12 (4)
		β-Galactosidase (m-units/g)	68 ± 19 (3)	84 ± 14 (3)
		Acid phosphatase (units/g)	0·48 ± 0·16 (3)	0·63 ± 0·12 (3)
		β-Acetyl glucosaminase (units/g)	0·19 ± 0·03 (3)	0·20 ± 0·01 (3)
Brain without cerebellum	14–35	β-Glucuronidase (m-units/g)	37 ± 4 (3)	32 ± 5 (3)
		β-Galactosidase (m-units/g)	93 ± 2 (3)	88 ± 11 (2)
		Acid phosphatase (units/g)	0·48 ± 0·08 (3)	0·46 ± 0·08 (3)
		β-Acetyl glucosaminase (units/g)	0·20 ± 0·018 (3)	0·24 ± 0·022 (3)
Brain	41	β-Glucuronidase (m-units/g)	42 (1)	52 (1)
Serum	21–35	β-Glucuronidase (m-units/100 ml)	76 ± 18 (5)	107 ± 32 (5)

* In each test the tissues of two or three chicks were combined.
† About two-fifths of these chicks (Warren hybrids) were prostrate in the final stages of the disease.

severely affected by the exudative, haemorrhagic condition. Liver, kidney and spleen all had significantly raised values for cathepsin, but only in spleen was there significantly greater β-glucuronidase activity. Blood serum was tested for β-glucuronidase activity, but showed no change due to the deficiency disease.

Chicks with exudates were generally smaller than controls and had smaller livers.

Table 3. *Encephalomalacia and lysosomal enzymes in chicks: subcellular distribution of β-glucuronidase and β-galactosidase*

(All chicks receiving the basal diet showed ataxia and limb spasms. Some were also prostrate. Chicks receiving vitamin E were normal)

Tissue	Age (days)	Enzyme		Enzyme activity* Deficient chicks	Enzyme activity* Normal chicks
Cerebellum	25	β-Glucuronidase (m-units/g)	Whole homogenate	60·3 (2)	69·4 (2)
			Homogenate†	32·7	36·8
			Lysosomal fraction	27·0	27·9
			Supernatant fraction	4·8	5·6
Brain without cerebellum	14		Whole homogenate	36 (1‡)	38 (2)
			Homogenate†	23	25
			Lysosomal fraction	18	21
			Supernatant fraction	5	6
Cerebellum	18	β-Galactosidase (m-units/g)	Whole homogenate	49·4 (3)	67·9 (3)
			Homogenate†	28·6	49·3
			Lysosomal fraction	19·6	31·5
			Supernatant fraction	7·2	12·1
Brain without cerebellum	35		Whole homogenate	93·7 (1‡)	93·3 (1)
			Homogenate†	68·4	66·9
			Lysosomal fraction	41·4	42·4
			Supernatant fraction	13·2	10·8

* Figures in parentheses show number of chicks tested (all Warren hybrids).
† After removal of nuclei and cell debris (see p. 130).
‡ This chick was prostrate in the final stages of the disease.

Table 4. *Exudative diathesis and lysosomal enzymes in chicks*

(All the chicks receiving the basal diet showed the exudative, haemorrhagic condition between days 17 and 28; some also had haemorrhagic degeneration of the breast muscle (see footnote). Chicks receiving Se or vitamin E were normal. Results (for the tissues of two or three chicks, combined) are given as means with standard deviations and the no. of tests is shown in parentheses)

Tissue	Enzyme activities: Deficient chicks	Enzyme activities: Normal chicks
β-Glucuronidase		
Exudate (m-units/g)	17 ±9 (8)	—
Skin* (m-units/g)	64† ±26 (5)	35 ±8·1 (4)
Serum (m-units/100 ml)	113 ± 13 (6)	119 ±63 (5)
Breast muscle (m-units/g)	31‡ ±34 (3)	15 ±5·5 (3)
Liver (m-units/g)	300 ±73 (4)	330 ±32 (6)
Kidney (m-units/g)	270 ±35 (2)	300 ±20 (3)
Spleen (m-units/g)	430† ±95 (3)	280 ±54 (4)
Cathepsin (units/g)		
Exudate	8·5 ±6·4 (2)	—
Breast muscle	26‡ ±28 (4)	4·8 ±5·3 (4)
Liver	183† ±63·6 (3)	100 ±15·3 (3)
Kidney	132† ±52·2 (4)	70 ±13·8 (4)
Spleen	215† ±16·1 (3)	174 ±25·2 (4)

* The first tissue to be affected by the deficiency condition was a small area of skin just below the lower end of the sternum. This area developed a subcutaneous haemorrhage and the earliest exudates seemed to derive from it.

† Significantly higher than corresponding value for normal chicks, $P < 0.05$.

‡ Individual results for degenerate muscle (with striations and haemorrhages) were much higher than for other chicks in the same group: 20 and 69 m-units β-glucuronidase/g, compared to 4 m-units/g. Similarly, for cathepsin; degenerate muscle had 38 and 60 units/g compared to 2 and 4 units/g.

Other features of the disease were an apparent enlargement of the kidney and a shrinkage of the spleen. The exudative condition did not alter the protein content of liver, kidney, spleen or breast muscle.

DISCUSSION

Our finding of increased lysosomal enzyme activity in nutritional muscular dystrophy is in agreement with the results of Desai, Calvert, Scott and Tappel (1964); some of the changes found in our tests were even greater. These authors found that dietary linoleic acid was essential for the production of muscular dystrophy and that pathological changes were correlated with increased 'peroxidizability' in vitro of the tissues, as measured by the thiobarbituric acid reaction. They concluded that 'nutritional muscular dystrophy in the chick is initiated by peroxidative tissue damage'. Zalkin *et al.* (1962) reached a similar conclusion in their work on vitamin E deficiency in the rabbit. The earliest pathological changes in the muscle coincided with the first increases in creatine and amino acids in urine, and lysosomal hydrolase activity of muscle; from this the authors concluded that the first consequence of vitamin E deficiency was free-radical damage to lipoprotein membranes of the cell and its subcellular organelles. The injured tissue would then be invaded by macrophages and phagocytic leucocytes, both rich sources of hydrolases. Desai, Calvert & Scott (1964) carried out a more searching time-sequence study of muscular dystrophy in the chick. They found that methionine would reverse the symptoms even when, in the absence of vitamin E, peroxidizability of the tissues remained high. Their conclusions were that 'peroxidizability of the tissues was not correlated with the recovery from dystrophy as effected by methionine' and, further, from their studies on β-glucuronidase in muscle, that 'lysosomal enzymes are not directly implicated in the cause of nutritional muscular dystrophy'.

We did not find any alteration in hydrolase activity in the cerebella or brains of chicks with encephalomalacia, as was found by Jibril & McCay (1965) and Tappel *et al.* (1963). Some of the chicks we studied showed only early signs of deficiency whereas others were almost moribund, and, to judge by the fate of other chicks of the same group, just a few hours from death. However, at no time was there a rise in β-glucuronidase, the enzyme which Jibril & McCay (1965) found to increase most in brain and which we found to increase greatly in dystrophic muscle. Similarly, we did not find the raised acid phosphatase and β-galactosidase activities reported respectively by Jibril & McCay (1965) and Tappel *et al.* (1963). There is no obvious explanation for these discrepancies, unless it lies in a difference between the rates at which encephalomalacia developed in their chicks and ours. Possibly a slower development of the brain lesion might have been accompanied by enzyme changes.

Exudative diathesis seems to be intermediate in its rate of progress between encephalomalacia, which often kills chicks within 24 h of the first signs of ataxia, and muscular dystrophy, which can last several weeks. Similarly, it is intermediate in the extent to which lysosomal hydrolase activity is altered. It must be admitted that the presence of hydrolytic activity in the exudate, and in the subcutaneous skin most

immediately concerned in the appearance of the exudate, suggests that the lysosomal hydrolases are implicated in the causation of the disease. However, this evidence alone is not sufficient to prove that the rise in hydrolase activity is not due to some other underlying pathological change caused by the deficiency of Se and vitamin E. The increased hydrolase activity of tissues such as liver, spleen and kidney was not associated with any complete breakdown of organ function as occurs in liver necrosis in rats but may have been responsible for the general decline in growth and health and for the eventual death of the chicks in this condition.

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