Changes Induced by E-Avitaminosis on the Proteins of Rabbit-Muscle Extracts

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A number of biochemical studies have been carried out in recent years on muscle affected by E-avitaminotic dystrophy in order to discover the primary biochemical changes responsible for the onset and the development of the dystrophic condition.

Since 1951 Aloisi and co-workers have shown that in this condition quantitative and qualitative changes take place in the contractile proteins of rabbit muscle (see Aloisi, 1951; Aloisi, Ascenzi & Bonetti, 1953). The myosin content of muscle-fibre extracts decreases rapidly and actin loses its power of polymerization (Aloisi, Ascenzi & Bonetti, 1952*a, b, c*; Bonetti, Aloisi & Merucci, 1952).

There is also other circumstantial evidence that in the dystrophic condition actin or actomyosin can be found in a depolymerized form: observations with the polarizing microscope and electron-microscopic investigations on isolated dystrophic myofibrils (Bompiani, 1954) and on solutions of muscle proteins (Aloisi *et al.* 1952*a*) are consistent with this assumption. Also Feuer & Frigyes (1952) have found a loss of myosin in nutritional dystrophy, and Crepax (1952) has obtained the same results by electrophoretic investigations on muscle extracts. This author has also shown two gradients which are supposed to correspond to the *G*-actin and to the *Y*-protein of Dubuisson (1950). In late-lactation paralysis of rats Rumery, Maurer & Mason (1955) have found a reversible decrease of a fraction of muscle proteins which seems to correspond with actomyosin.

Changes in myosin and in actomyosin have also been found by Fischer (1950) in denervation atrophy. In this condition actomyosin has been reported to have a diminished capacity for contraction, and myosin is less uniform in dispersity and has decreased adenosine triphosphatase activity.

The purpose of our work has been to analyse in some detail the changes of contractile proteins during the development of the dystrophy. A preliminary note on the results here presented has already been published (Azzone & Aloisi, 1955). As will be shown below, we have divided muscle

extracts made by extraction at high ionic strength into three parts which have been called fractions I, II and III.

Fraction I in normal muscles corresponds to the part which is discarded as actomyosin during the preparation of myosin (Szent-Györgyi, 1945; Mommaerts & Parrish, 1951); fraction II corresponds to myosin; fraction III is obtained from the muscle extract by precipitation with ammonium sulphate at 48% saturation, after separation of myosin and actomyosin. We have chosen this nomenclature since our data show that the usual procedures for separating myosin and actomyosin cannot be relied upon in advanced dystrophy and the fractions obtained from dystrophic muscles are differently constituted, when compared with those obtained from normal rabbits.

METHODS

Young rabbits (0.7–1 kg.) were divided into two groups: the first group was kept on the diet of Houchin & Mattill (1942) slightly modified by Aloisi *et al.* (1952*b*); the control animals were fed on the same diet supplemented with 50 mg. of α -tocopherol given by mouth twice weekly. Animals in the first group were killed after variable periods in order to follow the development of changes in contractile proteins. The animals were kept on the diet for different lengths of time and were classified as subdystrophic or fully dystrophic according to the absence or the clear appearance respectively of clinical and histopathological signs of dystrophy. Muscle sections were examined histologically and submicroscopically by polarized light. Animals in the second group were killed after 1 month and muscles were examined in the same way as those from the first group. Forty-eight rabbits were employed in the experiments here described.

Protein extraction. The muscles, obtained from the killed animals (previously anaesthetized with MgSO_4), were chilled in ice, minced and extracted with Guba & Straub (1943) solution (pH 6.5; *I* 0.55) for 15 min. at 0°. The suspension was pressed through cloth and centrifuged. The separation of the contractile proteins was carried out either according to Mommaerts & Parrish (1951) by dialysis or according to Szent-Györgyi (1945) by dilution. In the first case, the extract was dialysed against distilled water (10 vol.) and centrifuged; the precipitate was dissolved in 0.5M-KCl, then diluted to 0.25M and centrifuged. The centrifuged precipitate is called fraction I.

In the second procedure the extract was diluted with 4 vol. of water (i.e. to 1/0.1) and then allowed to super-precipitate; this precipitate is also called fraction I. After separation of fraction I in both cases the solutions were diluted to 1/0.05. The precipitate so obtained (which in normal animals should correspond to myosin) is called fraction II. Fractions I and II were washed with 0.02M-KCl and redissolved in 0.5M-KCl. Any material remaining undissolved was separated by centrifuging at 15 000 rev./min.

To the supernatant of the Guba & Straub extract, after separation of fraction II at 1/0.05, 30 g. of $(\text{NH}_4)_2\text{SO}_4$ (48% saturation) was added for every 100 ml. of solution. After 3 hr. the precipitate was spun down, then dissolved in a little water and dialysed against 0.02M-KCl to eliminate any free ammonium salt. The solution was then adjusted to 0.2M with respect to KCl and centrifuged to remove any insoluble material; the clear supernatant solution is called fraction III.

Usually fraction I was prepared from Guba & Straub extract treated by the dilution method, and fractions II and III were prepared from extract treated by the dialysis method.

The protein concentration of every fraction was estimated as nitrogen after digestion, by the micro-Kjeldahl technique. The nitrogen values are multiplied by 6.25.

Salting-out curves. On the above-mentioned protein solutions salting-out experiments were performed according to a slightly modified form of the method described by Tenow & Snellmann (1954). To every tube 3 ml. of water and $(\text{NH}_4)_2\text{SO}_4$ in varied quantity were added, in order to reach the desired degree of saturation in a final volume of 5 ml. A protein solution was prepared containing 3 mg. of protein/ml. of 0.1M-phosphate buffer, pH 7, which was also 0.65M with respect to KCl. Of this solution 2 ml. was then added, with shaking, to each tube. The solutions were placed in a cold room for 18–20 hr. to establish equilibrium between the phases. The suspensions were then filtered through Whatman no. 1 filter paper in the cold room and the absorption of the filtrates was measured whilst still cold, at 278 and 250 m μ . Readings at the latter wavelength were made in order to estimate the nucleotide content of all filtrates. The complete absorption curves of those filtrates corresponding to 12, 26–27 and 50% saturation of $(\text{NH}_4)_2\text{SO}_4$ were then plotted by subtracting the corresponding readings of each filtrate from those of the preceding one, for the purpose of obtaining the u.v. spectra of the main components demonstrable by the salting-out curves. The latter were obtained, by the method of Snellman & Tenow (1954), by plotting $-dS/dC$ against C and taking equal to 100% the amount of protein in the solutions in which precipitation had not taken place [S , absorption; C , percentage of saturation with $(\text{NH}_4)_2\text{SO}_4$].

Electrophoresis. The protein solutions were dialysed for 36–48 hr. against the medium used for the electrophoresis; this contained: 0.25M-KCl; 0.004M- KH_2PO_4 ; 0.032M- Na_2HPO_4 ; pH 7.4, I 0.35 (Crepax, 1951). The electrophoresis was performed in a Perkin-Elmer apparatus (model 38A) for 10–12 hr. The conductivity of the solutions and the mobility of the gradients were estimated.

Precipitation curves at different concentrations of potassium chloride. The solubility curves of protein solutions in KCl were obtained as follows: a batch of tubes was prepared containing equal amounts of protein solution

with phosphate buffer, pH 6.8, and to each of them KCl and water were added in order to have the desired molarity in a final volume of 5 ml. The tubes were shaken, centrifuged, or the contents filtered on Whatman no. 1 paper, and the absorption was measured at 278 m μ . In the precipitation experiments in the presence of adenosine triphosphate (ATP) the protein concentration in the precipitate and in the supernatant was determined by the biuret reaction, according to Gornall, Bardawill & David (1949).

Viscosity. Viscosity measurements were carried out in Ostwald viscometers, at 0° in 0.5M-KCl, without and with added ATP. The viscosity number and the ATP sensitivity were calculated according to Weber & Portzehl (1952).

Determination of nucleic acids. The extraction of nucleic acids was carried out according to Ogur & Rosen (1950). Protein solutions were extracted successively with ethanol, hot ethanol-ether and ether. The dried material was weighed, extracted with N-HClO_4 and the resulting solution was centrifuged after 20 hr. The absorption of solutions was then measured at 260 m μ and analysis for phosphorus and ribose content was carried out.

Phosphorus analyses were performed according to Berenblum & Chain (1938). Ribose was estimated by the orcinol reaction (Ogur & Rosen, 1950). The orcinol was twice recrystallized from benzene. Purines and pyrimidines were determined chromatographically by the method of Smith & Markham (1950).

Enzymic activity of muscle proteins. Adenosine triphosphatase activity was determined at 25° in glycine buffer, pH 9, with CaCl_2 (total volume 2 ml.). The reaction was stopped by adding 1 ml. of 10% (w/v) trichloroacetic acid and free phosphorus was determined by the Berenblum & Chain (1938) method.

RESULTS

Analysis of fraction I. In normal rabbits a dilution (1:5) of a Guba & Straub (1943) extract produces, after a lag time due to splitting of ATP, the precipitation of the fraction I which only in part is constituted by true *F*-actomyosin and which constitutes a variable proportion (10–30%) of the myofibrillar proteins present in the extract. On the other hand by diluting (1:5) a Guba & Straub (1943) extract prepared either from subdystrophic rabbits or from rabbits in advanced dystrophy a large precipitate of proteins formed rapidly; this contained 60–100% of the myofibrillar proteins contained in the extract. In normal muscle the extractability of fractions I + II is in the range of 15–18 mg./g. of muscle; these values are reduced in subdystrophic animals to 8–12 and in advanced dystrophy to 4–5 mg./g. of muscle (averages from six to eight experiments).

The salting-out curves and the electrophoretic patterns reveal that the composition of fraction I and therefore its physicochemical properties change according to the development of the dystrophic process. Fraction I always does in fact contain a mixture of actomyosin and myosin but,

in subdystrophic animals, the salting-out curves (Fig. 1) show a small first component which precipitates at 14–16% (a small amount of which is present also in the normal), a small precipitate at 30% saturation (actomyosin) and a main component which precipitates at 38–42% (myosin). In the electrophoretic patterns obtained from more advanced dystrophic animals (Fig. 2) there is a considerable increase of the first component which precipitates at 12–16%; there is always a large precipitation between 20 and 30%, but the amount of the component which is precipitated at 38–42% (myosin) is clearly decreased.

It seems therefore evident that whereas in subdystrophic animals larger amounts of the myosin precipitate in fraction I, together with the *F*-actomyosin of this fraction, in advanced dystrophy, owing to the loss of myosin, fraction I appears to consist mainly of actomyosin and the unknown component. The predominance of myosin in fraction I of subdystrophic animals is likely to explain the physicochemical properties of this fraction, as reported below.

The electrophoretic patterns confirm the salting-out curves. Electrophoresis of fraction I from sub-

dystrophic animals (Fig. 3) shows a main component which has the mobility of myosin

$$(2.8 \times 10^{-5} \text{ cm.}^2 \text{ v}^{-1} \text{ sec.}^{-1});$$

this gradient after electrophoresis for 10–12 hr. may show a very slight tendency to split (confirming therefore the presence of scanty amounts of actomyosin); behind myosin there is a very small peak with a mobility intermediate between that of myosin and that of the group of myogens (about $2.2 \times 10^{-5} \text{ cm.}^2 \text{ v}^{-1} \text{ sec.}^{-1}$). Fraction I from rabbits in advanced dystrophy shows, on the contrary, well-distinguished actomyosin and myosin peaks (Fig. 4); behind them there is another peak, often very abundant and sometimes splitting into two or three components, with a mobility between 2.1 and $1.6 \times 10^{-5} \text{ cm.}^2 \text{ v}^{-1} \text{ sec.}^{-1}$.

Potassium chloride-precipitation curves of fraction I from subdystrophic animals are close to those of myosin (that is, complete solubility is found in 0.1 M-KCl); if, however, the KCl-precipitation curve is carried out in the presence of ATP, a large precipitate with 0.1 M-KCl is formed, involving 50–90% of the total protein (Fig. 5). KCl-precipitation curves of fraction I from animals in

Fig. 1. Salting-out curve of fraction I from subdystrophic animals. For details see the text.

Fig. 2. Salting-out curve of fraction I from dystrophic rabbits. For details see the text.

Fig. 3. Electrophoretic patterns of fraction I from subdystrophic rabbits: Electrophoresis for 668 min. at 2.66 v/cm., *I* 0.35, pH 7.4; concn., 7.6 mg./ml. The ascending front is shown on the top.

advanced dystrophy seem to be more similar to that of actomyosin, since a gradual increase of solubility is observed from 0.1 to 0.3 M-KCl. The viscosity measurements confirm also the different composition of fraction I from subdystrophic and dystrophic rabbits. In the former the viscosity values are almost identical with those of myosin and the sensitivity to ATP is very low (Table 1); in the latter, on the contrary, there is a high viscosity and a high sensitivity to ATP.

Analysis of fraction II. This fraction (which is precipitated by diluting to I 0.05) in normal muscle extracts is almost exclusively myosin.

Fig. 4. Electrophoretic patterns of fraction I from dystrophic rabbits. Electrophoresis for 610 min. at 2.32 v/cm., I 0.35, pH 7.4; concn., 6.7 mg./ml. The upper pattern corresponds to the ascending front.

Some preliminary observations (Azzone & Aloisi, 1955) on this fraction seemed to suggest that solubility and viscosity changes in myosin were appearing during dystrophy. Later experiments have demonstrated that this hypothesis was incorrect, since fraction II in dystrophic muscles is not composed solely of myosin, and therefore the solubility and viscosity changes of fraction II cannot be attributed to myosin itself.

In advanced dystrophy a dramatic decrease of this fraction takes place up to a point where only scanty amounts of it can be obtained [average about 0.7 mg./g. of muscle instead of 15 mg./g. in normal muscle (six experiments)].

Ammonium sulphate salting-out curves show that, in fraction II from dystrophic muscles, the component which is precipitated at 12–16 % saturation is clearly predominant; only a small precipitate is found at 30 % and an even smaller one at 38–42 % (Fig. 6). The electrophoretic patterns show that in this fraction from normal or sub-

Fig. 5. Precipitation curves in potassium chloride solution of fraction I from subdystrophic rabbits: O, with ATP added; Δ, without ATP added.

Table 1. *Viscosity measurements*

Viscosities without ATP were calculated as: $Z\eta = \frac{2.3 \times \log \eta_{rel}}{c}$. Sensitivity was calculated, according to Weber & Portzehl (1952), from the equation: $\text{sensitivity} = \frac{(Z\eta \text{ without ATP}) - (Z\eta \text{ with ATP})}{Z\eta \text{ with ATP}} \times 100$; c = concn. in mg./ml. Figures are averages from the number of experiments indicated.

	No. of experiments	Without ATP	With ATP added	Sensitivity
Fraction I				
Subdystrophic rabbits	9	0.2	0.19	5
Dystrophic rabbits	5	0.25	0.19	28
Fraction II				
Dystrophic rabbits	6	0.13	0.13	0

Fig. 6. Salting-out of fraction II from dystrophic rabbits. For details see the text.

Fig. 7. Electrophoretic patterns of fraction II from sub-dystrophic rabbits. Electrophoresis for 570 min. at 2.32 v/cm., I 0.35, pH 7.4; concn., 9.45 mg./ml. The ascending front is shown on the top.

Fig. 8. Electrophoretic patterns of fraction II from dystrophic rabbits. Electrophoresis for 570 min. at 2.32 v/cm., I 0.35, pH 7.4; concn., 9.4 mg./ml. The upper figure shows the ascending front.

Fig. 9. Potassium chloride-precipitation curve of fraction II. O, Fraction II from dystrophic rabbits; Δ , fraction II from normal animals.

dystrophic animals (Fig. 7), the main component has a myosin mobility; on the contrary, in dystrophic rabbits (Fig. 8) the peak showing myosin mobility is much reduced or is present only in traces, and a component predominates which has a low mobility ($1.4 \times 10^{-5} \text{ cm.}^2 \text{ v}^{-1} \text{ sec.}^{-1}$) and which seems to have a slow diffusibility. The latter corresponds to the protein material which precipitates at 12–16% saturation with $(\text{NH}_4)_2\text{SO}_4$. Moreover, we have noted the presence of a very small peak with very high mobility ($12 \times 10^{-5} \text{ cm.}^2 \text{ v}^{-1} \text{ sec.}^{-1}$) which is running very quickly out of both fronts. In these experiments there has always been found a considerable anode-cathode asymmetry.

Table 2. *Nucleic acid content*

Ribonucleic acid (RNA) content has been calculated by assuming a P content in RNA of 8.4%, as found by Smith & Markham (1950) for yeast ribonucleic acid.

	Preparation	P content (%)	RNA (%)	ϵ_P	'Purine-bound' P/ribose	
					Ribose (%)	Ratio
Fraction I	A	0.59	7.0	9.400	1.53	0.39
Fraction II	A	0.43	5.1	9.960	1.45	0.3
	B	0.73	8.7	9.390	2	0.36

Fig. 10. Electrophoretic patterns of fraction III from normal rabbits. Electrophoresis for 663 min. at 2.32 v/cm., I 0.35, pH 7.4; concn. 11 mg./ml. The ascending front is shown on the top.

Fig. 11. Salting-out curves of fraction III from normal animals. In the upper curve the solutions were filtered at 0°; in the lower the solutions were refiltered at room temperature.

Fraction II from dystrophic muscles has a KCl-solubility curve completely different from normal myosin and seems clearly to be less soluble (Fig. 9). The drop in viscosity of fraction II parallels the changes in the salting-out and electrophoretic patterns (Table 2); this fraction has no sensitivity to ATP. On the other hand, enzymic

determinations show that in advanced dystrophy fraction II has retained only traces of adenosine triphosphatase activity (Q_P between 0 and 229, measured on six preparations), whereas a certain adenylic-deaminase activity is still present.

Analysis of fraction III. Whereas fractions I and II correspond in normal muscle to well-defined and -studied protein entities (respectively actomyosin and myosin), fraction III is, even under normal conditions, made up of a mixture of proteins not yet studied and defined. As explained in the Methods section, this fraction is collected by adding 30 g. of $(\text{NH}_4)_2\text{SO}_4$ to 100 ml. of the supernatant solution, after separation of fraction II. The analysis of this fraction has been carried out on the assumption that a part of the myosin could still be soluble, in dystrophic-muscle extracts, at I 0.05, as found by Fischer (1950) in denervation atrophy. Moreover, this fraction is rather interesting since it probably concerns also the so-called 'extra material' of Szent-Györgyi, Mazia & Szent-Györgyi (1955), which is composed of proteins extractable from myofibrils at high ionic strength, but which remain in solution during separation of myosin and actomyosin.

The salting-out and the electrophoretic diagrams of this fraction from normal muscles show that it is made up of at least three components (Figs. 10 and 11); the first of these, which moves faster on electrophoresis, is very likely to be tropomyosin; in fact, pure rabbit tropomyosin added to the solution does not separate from this faster peak during electrophoresis. In our Laboratory, Corsi (1957*a, b*) has demonstrated that one of the components of the 'extra material' of Szent-Györgyi *et al.* (1955) is tropomyosin.

Behind tropomyosin there is a main component which has a mobility like that of myosin

$$(2.7-2.8 \times 10^{-5} \text{ cm.}^2 \text{ v}^{-1} \text{ sec.}^{-1})$$

and which sometimes, after prolonged electrophoresis, appears to be splitting (as in Fig. 10); behind this, there is another small gradient with a slower mobility. The main component might be partly related to the *Y*-protein of Dubuisson (1950) since his extraction procedure is very similar to ours.

The salting-out curves carried out at 0° reveal, however, a peculiar phenomenon (Fig. 11). After

an initial precipitation at 26% saturation a repeated redissolution and reprecipitation of protein material up to 50% saturation is observed. This phenomenon disappears when the tubes are filtered at room temperature. This is probably connected with the formation of unstable complexes in the presence of tropomyosin; another example of a similar phenomenon has been shown, by Snellmann & Tenow (1954), to occur in the presence of uterine actotropomyosin.

Fraction III prepared from dystrophic rabbits has the following properties: (a) the amount of the fraction as a whole is clearly increased in comparison with normal values: from 2.2 to 4.4 mg./g. of muscle (five experiments); (b) the salting-out diagram at 0° no longer shows the phenomenon of the redissolution of the precipitate (Fig. 12) but the precipitation is regular between 26 and 50% saturation; (c) in the electrophoretic patterns the

faster gradient of tropomyosin is not distinguishable any more but it seems to be confused with the main intermediate component, giving a peak (Fig. 13) which runs with a mobility higher than that of the intermediate component in the normal ($3.4 \times 10^{-5} \text{ cm.}^2 \text{ v}^{-1} \text{ sec.}^{-1}$); (d) contrary to the normal, in fraction III there are always present traces of adenosine triphosphatase activity which is enhanced by Ca^{2+} and not by Mg^{2+} ions.

Fig. 12. Salting-out curves of fraction III from dystrophic animals. As in Fig. 10, the two curves show the difference between filtration in the cold (upper) or at room temperature (lower).

Fig. 13. Electrophoretic patterns of fraction III from dystrophic rabbits. Electrophoresis for 512 min. at 2.32 v/cm., I 0.35, pH 7.4; concn., 10.7 mg./ml. The ascending front is shown on the top.

Fig. 14. Total organic P content in different preparations of fraction I. The ordinate gives the extinction at 278 mμ/mg. of protein.

Fig. 15. Ultraviolet-light absorption spectra of the different components present in the salting-out curve of fraction I from dystrophic rabbits: A, B, C and D indicate the whole fraction, the components between 12 and 26%, the components between 26 and 50% and the final supernatant respectively.

Ultraviolet spectra. In fractions I and II of normal animals the extinction at $250\text{ m}\mu$ ($E_{0.1\%}$) is respectively 0.6 and 0.5 (four experiments). In dystrophic animals these values are always markedly increased: in five experiments we had 1.3 for fraction I and 3.3 for fraction II. Also the $278/250\text{ m}\mu$ absorption ratio is changed in dystrophy: the normal values of 1.4 and 1.5 (for fractions I and II respectively) are decreased to 1.0 and 0.8.

In fraction I the increase in the extinction coefficient has been found to be related to the total organic phosphorus content (Fig. 14). From the u.v. spectra of the different components as revealed by the salting-out curves of fractions I and II (Figs. 15, 16) we realize that the shift in the absorption maximum from 278 to $260\text{ m}\mu$ concerns particularly the first component which precipitates at 12–16% saturation.

Above 50% saturation the absorption ($278\text{ m}\mu$) of filtrates of salted-out fractions I and II from dystrophic muscles is markedly increased: 20% of the initial absorption is here still present, instead of 5–6% as in normal cases.

Nucleic acid content. The assumption that the u.v.-light shift should mainly be due to an increased nucleic acid content was found to be true

by extraction and analysis of the three fractions studied. Apart from fraction III, which has a low nucleic acid content, our results (Table 2) show that considerable amounts of ribonucleic acids (5–8%) are present in both fraction I and II. The presence of ribonucleic acid was confirmed by chromatographic analysis where, after hydrolysis with N-HCl , four spots were recognizable, namely, those of guanine, adenine, cytidylic and uridylic acids. Dische's (1955) reaction with diphenylamine, carried out on hot 0.5N-perchloric acid extract (Ogur & Rosen, 1950), has failed to reveal any trace of deoxyribonucleic acid.

DISCUSSION

From the results here reported it seems evident that during the dystrophic changes in muscle fibres in E-avitaminosis the proteins extractable at high ionic strength undergo different biochemical changes. Besides a considerable quantitative decrease of protein present in the extract (particularly of myosin) (see also Bonetti *et al.* 1952; Feuer & Frigyes, 1952; Crepax, 1952) there is also a sharp change in the composition of the different fractions obtained.

To fraction I belong the proteins which precipitate suddenly by diluting the extract to $I\ 0.1$; according to Szent-Györgyi (1945) they are superprecipitated by the splitting of ATP. In normal muscles only actomyosin is precipitated at this ionic strength and it can carry down during precipitation a part of the myosin; in dystrophic muscles we find that a variable amount of actomyosin is mixed in the precipitate at $I\ 0.1$ with most of the myosin present in the extract. It follows that in subdystrophic rabbits fraction I will therefore show nearly all the physicochemical properties of myosin (but it can be superprecipitated with ATP at $I\ 0.1$), whereas in fully dystrophic rabbits, owing to the loss of myosin and to the increase of *F*-actomyosin, fraction I will change some of its properties (solubility in potassium chloride, viscosity number and ATP sensitivity; see Weber & Portzehl, 1952; Bailey, 1954) to those of actomyosin. It is possible that the increased amount of fraction I and consequently the higher content of myosin in this fraction in subdystrophic animals is to be related to the formation of a labile coupling of myosin with depolymerized actin (*G*-actomyosin). In fact, *G*-actin is present in the extracts of dystrophic muscles, as was shown by Crepax (1952).

On the other hand, the increased *F*-actomyosin content of fraction I in rabbits in advanced dystrophy may be related to the general metabolic disorder of degenerated muscles, which are supposed to contain a decreased amount of ATP (see

Fig. 16. Ultraviolet-light absorption spectra of the different components present in the salting-out curve of fraction II from dystrophic rabbits: A, B, C, D indicate the whole fraction, the components between 12 and 26%, the components between 26 and 60% and the final supernatant respectively.

Feuer & Frigyes, 1952), a condition which favours the formation of actomyosin during extraction.

Our results confirm the experiments of Dinning and others (Young & Dinning, 1951; Dinning, 1955), who have demonstrated an increased turnover of liver and muscle nucleic acids in E-avitaminosis and a high content of nucleic acids in dystrophic muscles. The presence of these nucleic acids (or of nucleoproteins, as may be suggested by the presence of a very fast component in the electrophoretic patterns) may probably explain the anomalous behaviour of fraction III in the salting-out and in the electrophoretic patterns. In fact Snellmann & Tenow (1954) have observed that actotropomyosin from the gravid uterus can easily be dissociated (showing thereby a different behaviour from this protein in the non-gravid uterus) because of a decreased nucleic acid content, and have suggested that ribonucleic acid may determine the formation of more stable heterogeneous protein complexes. We believe, however, that a more characteristic and specific phenomenon during dystrophy is the appearance in quite large amounts of a component which precipitates at 12–16 % saturation of ammonium sulphate in the salting-out curves. This component is present both in fractions I and II; in the latter it may constitute the major part of the fraction. When this component is found, as it is always in advanced dystrophy, it has a homogeneous electrophoretic behaviour in fraction II, whereas it is heterogeneous in fraction I and has a very slow mobility. The solubility properties of this component suggest that it is related to the myofibrillar proteins (its solubility in potassium chloride is rather similar to those of myosin and actomyosin) but its electrophoretic mobility is not comparable with any of the normal myofibrillar protein so far studied. Further work is in progress in order to identify and to study this new protein component which is particularly evident in the dystrophic condition and which may therefore be considered as 'abnormal' and is probably related to the processes which lead to the degeneration of muscle fibres.

SUMMARY

1. A muscle extract made at high ionic strength obtained from E-avitaminotic rabbits has been divided into three fractions corresponding (a) to actomyosin, (b) to myosin, and (c) to the proteins salted-out from the supernatant at 48 % ammonium sulphate saturation. Total extractable proteins are decreased in dystrophy and the proportions of the three fractions are also changed.

2. The first fraction, which is normally precipitated as actomyosin by the splitting of adenosine triphosphate, has mainly the physicochemical prop-

erties of myosin in subdystrophic animals, whereas in fully dystrophic rabbits the viscosity and potassium chloride-solubility properties are those of actomyosin. Salting-out and electrophoretic patterns of this fraction in advanced dystrophy also show a marked decrease of myosin content, and an unknown component appears which has a slower electrophoretic mobility and precipitates at lower ammonium sulphate saturation.

3. The second fraction still retains its myosin properties in subdystrophic rabbits, but in advanced dystrophy several changes take place: a decrease in viscosity, in potassium chloride-solubility and in adenosine triphosphatase activity. These effects are to be correlated with the disappearance of myosin as shown by salting-out and electrophoretic experiments; the latter indicate also that in this fraction the unknown component which is precipitated at 12–14 % saturation with ammonium sulphate predominates and has a very low electrophoretic mobility.

4. Under normal conditions the third fraction contains, amongst other components, tropomyosin and Y-protein, but in dystrophic rabbits these proteins have a strong tendency to unite in rather stable complexes which probably include tropomyosin.

5. Fractions I and II show a high content of ribonucleic acid, in advanced dystrophy.

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The Hydroxylation of Nicotinic Acid by *Pseudomonas fluorescens*

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It has been shown that cell-free extracts of *Pseudomonas fluorescens* strain KB1 convert nicotinic acid into 6-hydroxynicotinic acid and that these extracts do not further oxidize this compound (Hughes, 1955). This paper reports studies in which ^{18}O -labelled oxygen has been used as a tracer to determine the origin of the hydroxyl group. It is concluded that the oxygen appearing in the hydroxyl group of 6-hydroxynicotinic acid is derived from water. Preliminary findings of this work have already been communicated (Hunt, Hughes & Lowenstein, 1957).

EXPERIMENTAL AND RESULTS

Growth of organisms. *Pseudomonas fluorescens* strain KB1 (Kogut & Podoski, 1953) was grown in 8 l. batches of medium containing inorganic salts (Hughes, 1955), yeast extract (Oxo Ltd.) 0.05% and nicotinic acid 0.1%, aerated at the rate of 5–6 l. of air/min. After growth for 16–24 hr. at 20–25° the cells were collected by centrifuging, washed well in 0.9% NaCl and stored at –15° until needed.

Preparation and handling of cell-free extracts. The washed cells were crushed in a Hughes (1951) press at –25° to –20° without the addition of abrasive. After crushing, the very viscous material was homogenized in a stainless-steel Potter-type homogenizer in an equal volume of ice-cold 0.5M-KH₂PO₄-Na₂HPO₄ buffer, pH 7.0, and centrifuged in 50 ml. cups for 20 min. at 1200 g. The viscous and cloudy supernatant was decanted from the more solidly packed intact cells and cell debris. It was stored at –20° until wanted and then thawed in ice-water, and added to Warburg cups containing the various ice-cooled substrates,

together with several 0.5 cm. glass beads. Oxygen uptake was followed manometrically. After completion of the reaction the liquid contents of the cups were transferred to conical centrifuge tubes.

Isolation of 6-hydroxynicotinic acid. In a previous paper (Hughes, 1955) 6-hydroxynicotinic acid was identified by paper chromatography, and isolated for chemical characterization by solvent-extraction procedures from relatively large-scale incubation mixtures. For the purposes of ^{18}O analysis it was essential to have a method yielding micro-mole quantities of 6-hydroxynicotinic acid in high purity from Warburg flasks. Preliminary experiments indicated that both nicotinic acid and 6-hydroxynicotinic acid could be absorbed and separated quantitatively by stepwise elution on Dowex-2 formate resin. The following procedure was used. After the completion of oxidation, reaction mixtures were transferred to 10 ml. conical centrifuge tubes and deproteinized by heating in a boiling-water bath for 2 min. The precipitate was removed by centrifuging. The supernatant was transferred quantitatively to a column of the formate salt of Dowex-2 ion-exchange resin (10% cross-linked, 200–400 mesh) with a resin bed 1 cm. in diameter and 5 cm. high, and a flow rate of approx. 1 ml./10 min. The column was washed with 5–6 vol. of water until the optical density of the eluate had fallen below 0.100 at 260 m μ . Small amounts of nicotinic acid, which remained after the reaction, were eluted with 0.04N-formic acid until the optical density at 260 m μ had again fallen to less than 0.100. The 6-hydroxynicotinic acid was then eluted with 0.2N-formic acid. Up to 100 μ moles of the compound could be eluted quantitatively in three or four 10 ml. fractions and the compound crystallized out from fractions containing the highest concentrations. The fractions containing 6-hydroxynicotinic acid were pooled and were evaporated slowly with gentle warming, and the 6-hydroxynicotinic acid was recrystallized once from a minimum of water and dried *in vacuo* over P₂O₅. The ratio $E_{260\text{ m}\mu}/E_{280\text{ m}\mu}$ of the isolated material agreed with that

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