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Studies on Nutritional Muscular Dystrophy. I. Dietary Factors, II. Fibrosis and Lipomatosis of Tissues

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STUDIES on NUTRITIONAL MUSCULAR DYSTROPHY

I. DIETARY FACTORS
II. FIBROSIS AND LIPOMATOSIS OF TISSUES

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A THESIS

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FURTHER STUDIES ON DIETARY FACTORS ASSOCIATED WITH NUTRITIONAL MUSCLE DYSTROPHY

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FIVE FIGURES

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Morgulis and Spencer ('36) concluded that muscular dystrophy must be regarded as a multiple deficiency disease. Wheat germ being an excellent source of the essential dietary factors we fractionated this material and fed the separate fractions as an adjunct to the basal diet 13 or 313. The fractions were always administered in quantities equivalent to 20 to 30 gm. wheat germ.

FAT-SOLUBLE FRACTION

1. Ether extract of wheat germ. Both a commercial ether extracted oil and an oil which we prepared with specially purified ether was found to give greater protection than the cold pressed oil. The animals invariably become dystrophic, but the time required was occasionally as long as four months. The greater prophylactic action of this oil is attributed to the fact that continuous extraction with ethyl ether probably removes small amounts of the water-soluble fraction also.

2. Petroleum ether extract of wheat germ. On the dystrophy-producing diet supplemented with this fraction the rabbits become severely dystrophic but the time of onset is somewhat delayed, the effect being very similar to that of the cold pressed oil. The extracted residue of the wheat germ fed in conjunction with diet 13 does not prolong the

onset of dystrophy. On adding the petroleum extract to this combination, the animals have been cured and once more made dystrophic by omitting the residue.

3. Acetone extract of wheat germ. This extract has both preventive and curative properties. Rabbits receiving 2 to 3 cc. of this acetone preparation together with diet 13 were in good health and attained a body weight similar to that of control animals of the same age. The acetone extracted wheat germ, on the other hand, had no effect on the development of dystrophy showing that the acetone completely removes the essential dietary factors.

4. Hexane fraction of the acetone extract. In view of the fact that the acetone extract is complete, so far as muscle dystrophy preventing or curing factors are concerned, we fractionated this by means of hexane which removes only lipid-soluble material. This hexane fraction added to the diet 13 acted very much like the petroleum ether extract or the cold pressed oil of wheat germ. The animals invariably became dystrophic but more slowly than on diet 13 alone.

5. Non-saponifiable fraction of wheat germ oil. The non saponifiable fraction of wheat germ oil is a source of vitamin E. Our studies with this fraction as a supplement to diet 13 show that the animals become dystrophic just as they do on cold pressed oil, the hexane fraction or the petroleum ether extract.

WATER-SOLUBLE FRACTION

The petroleum ether extracted wheat germ has no protective or curative action if fed as an adjunct to diet 13. This residue was used as the source for obtaining the water-soluble fraction by means of exhaustive extraction with 70% alcohol. The alcohol extracts were concentrated under reduced pressure and filtered, the residual aqueous solution being preserved with benzoic acid. Feeding this extract alone as a supplement to diet 13 had no effect and the rabbits quickly became dystrophic.

COMBINATION OF THE FAT-SOLUBLE AND WATER-SOLUBLE FRACTION

Neither the fat-soluble nor water-soluble fraction obtained rom wheat germ can prevent or cure muscle dystrophy in abbits fed diet 13. We studied therefore their combined ffect. In figure 1 we reproduce a few representative growth urves to illustrate the effect of such combinations.

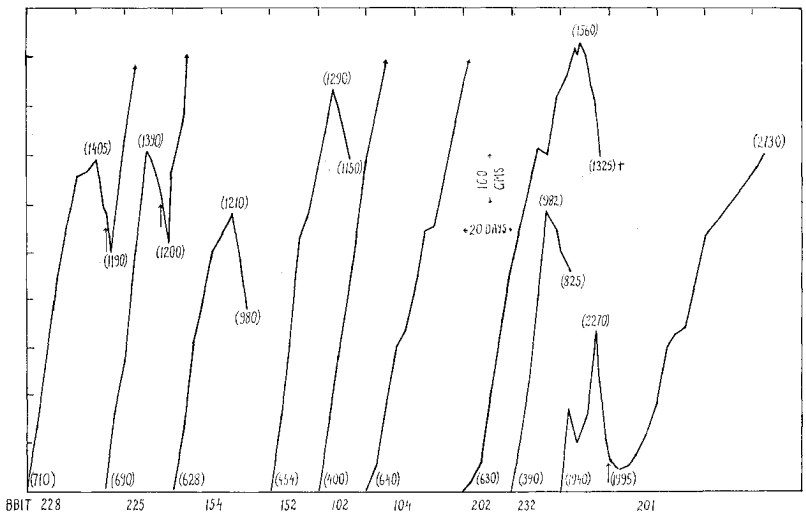

Fig. 1 Rabbit no. 154, dystrophic on diet 13. Rabbit no. 152, dystrophic on et 313. Rabbit no. 102, normal growth on diet 313 + whole wheat germ. Rabbit . 104, normal growth on diet 313 + acetone extract of wheat germ. Rabbit no. 2, dystrophic on diet 13 + petroleum ether extract of wheat germ (fat-soluble action). Rabbit no. 232, dystrophic on diet 13 + water-soluble fraction of ieat germ. Rabbit no. 201, dystrophic on diet 13 + petroleum ether extract d cured by the addition of the water-soluble fraction from wheat germ. The owth curve is plotted beginning with the twenty-third day of the experiment. ıbbit no. 228, dystrophic on diet 13 and cured by adding petroleum ether tract +5% yeast. Rabbit no. 225, dystrophic on diet 13 and cured by adding e non-saponifiable fraction of wheat germ oil and the water soluble fraction of ieat germ.

1. Petroleum ether and alcohol extracts. Animals made ystrophic on diet 13 supplemented with the petroleum ether ctract from wheat germ recover from their paralysis uickly when the water-soluble fraction is added to their

ration. It has been found necessary at times to administe this extract by pipette for a few days because the sick animal were unable to eat or drink. Nevertheless, they were able t stand up after even a few administrations of the wate soluble extract.

Rabbits supplied with both petroleum ether and alcoh extracts make quick and really spectacular recovery fro dystrophy, and we have kept them in good condition for few months afterward. Most of the animals, however, hav ultimately suffered a relapse. Since there was invariably pulmonary involvement, we are not certain at this tim whether the relapse which occurred 1 to 3 months subs quent to the cure was not due to an intercurrent infection.

Fig. 2 Rabbit 250. Dystrophic after 24 days on diet 13.

Fig. 3 Rabbit 250 after 9 days on curative diet of diet 13 + non-saponifiat fraction of wheat germ oil + water-soluble fraction of wheat germ.

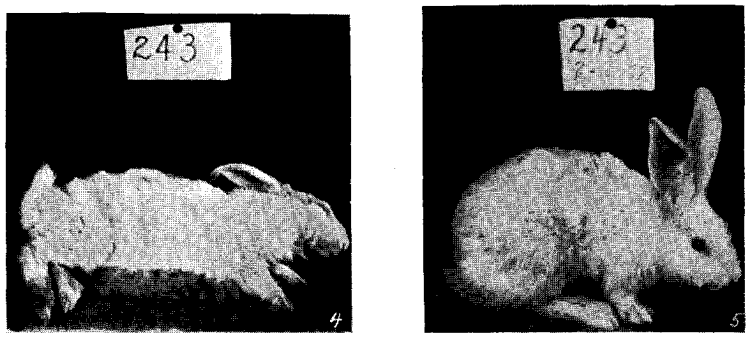

Fig. 4 Rabbit 243. Dystrophic after 22 days on diet 13.

Fig. 5 Rabbit 243 after 12 days on curative diet of diet 13 + petroleum etl extract of wheat germ + 5 gm. yeast per day.

2. Hexane fraction and alcohol extract. Three rabbits ıade dystrophic on the basal diet were definitely cured by dding both the hexane fraction of an acetone extract from /heat germ and the alcohol extract.

3. Non-saponifiable fraction and alcohol extract. The on-saponifiable material from wheat germ oil was fed in apsules and the water-soluble fraction of the wheat germ alcohol extract) was given at first by pipette but later it was ncorporated in the diet. Definite cures were effected by this ombination as can be seen from figures 2 and 3. However, here was a recurrence after about 4 weeks. We have reason o believe that possibly mixing with the basal diet results ı destruction of some of the factors, but this question will eed to be studied further.

4. Petroleum ether extract of wheat germ and yeast. The /ater-soluble fraction has such obvious relation to the vita- ıin B complex that it seemed advisable to study the effect f combining our fat-soluble fraction of wheat germ with a ich vitamin B source. We chose yeast for this purpose. 'igure 4 shows a rabbit which became severely dystrophic; gure 5 shows this animal 12 days later after the basal diet /as supplemented with a petroleum ether extract and with gm. yeast per day. Experiments now in progress with arious other sources of the vitamin B complex are also roving very successful.

STUDIES ON LETTUCE

Morgulis and Spencer ('36) found that the addition of 0 gm. lettuce per day to diet 13 practically doubled the ime required for the onset of dystrophy but if wheat germ il was also added dystrophy could be prevented or cured. lince the accumulating evidence favors the view that the at-soluble factor is closely associated, or is perhaps even lentical with vitamin E, and lettuce is a good source of itamin E, we experimented with feeding fresh lettuce ad bitum. Growing rabbits consume 300 to 500 gm. per day reely, and we found that such a supplement to diet 13 is

highly satisfactory for normal growth and maintenance o good muscle condition. We have used this supplement bot as a preventive and curative measure with perfect success.

MISCELLANEOUS STUDIES

Limitations of space make it impossible to more than men tion dietary studies with other foodstuffs. Studies o cottonseed meal, acetone extracts of cottonseed and the non saponifiable fraction of the cottonseed oil show that thes behave like similar preparations from whole wheat germ but the cottonseed contains the necessary factors in smalle amounts.

Cornmeal, on the other hand, even in as high an amoun as 50% of the ration, only delayed the onset of disease, th rabbits becoming severely dystrophic in about 2 months An acetone extract as well as different commercial corn oil gave similar results. Since corn oil is a good source o vitamin E, corn either lacks or is very low in the water soluble factor present in wheat germ, cottonseed or lettuce

Experiments with egg yolk show that it behaves like whea germ oil. Egg yolk as the only supplement to diet 13 canno prevent the onset of dystrophy, but dystrophy so develope disappears rapidly upon the further addition of 50 gm lettuce per day to the ration.

DISCUSSION AND SUMMARY

Dietary studies on the prevention or cure of nutritiona muscular dystrophy in rabbits demonstrate clearly th multiple factor nature of this deficiency disease. All the es sential factors are present in acetone extracts of wheat gern or cottonseed, though not in adequate amounts in the latte Fresh green leaves of lettuce fed in large amounts with th basal diet can cure dystrophic rabbits and maintain them i good health for a long time.

Whole wheat germ was fractionated into its fat-solubl and water-soluble components. Neither fraction by itself ca either prevent or cure dystrophy. The fat-soluble materia

: the non-saponifiable portion of it, incorporated into the ısal diet delays the onset of dystrophy while the water-ıluble fraction, either as the extract or as the residue left ver from the extraction with petroleum ether, does not even ffect the rapidity with which dystrophy develops on diet 13. owever, when the two extracts are fed together to severely ystrophic rabbits they recover very quickly and grow gorously. Many animals after they made good progress ı these curative diets for long periods of time have had remission of the disease. Since in every case there has ɔen some pulmonary complication, the recurrence of dys-ophy after the animals have been gaining steadily and be-ıved entirely normally even for months will need further vestigation.

Cures have been effected not only by combining wheat germ l and the water-soluble fraction of wheat germ but also by ɔmbining the latter fraction with a petroleum ether extract ' wheat germ or with its non-saponifiable fraction, as well as r combining the fat-soluble fraction with yeast or with dif-ırent vitamin B concentrates.

It may, therefore, be regarded as proved that one of the ısential dietary factors is present in the non-saponifiable action of the wheat germ oil. The fact that this factor is ɔsent in linseed oil but is found also in lettuce, cottonseed : corn oil, showing that its distribution is similar to that ' vitamin E, and the fact that it is destroyed by the same eatment which destroys vitamin E indicate that both are ther very closely associated or possibly even identical.

Pappenheimer and Goettsch ('31) and Morgulis and pencer ('36) found that muscle dystrophy in rabbits cannot ɔ cured by vitamin E concentrates alone but, on the other ınd, foods which prevent or cure this condition contain tamin E. We have shown that a petroleum ether extract ' wheat germ or the non-saponifiable fraction of wheat germ l as supplements to diet 13 merely delay the onset of ystrophy but do not prevent it or cure it. We tested these eparations for the vitamin E content by studying their

effect in restoring fertility in rats which have ceased to r produce on a vitamin E-free diet, and found them to l effective sources of this vitamin. But they are effective curing or preventing muscle dystrophy only when they a administered in combination with the water soluble fracti of wheat germ, or various concentrates of the vitamin complex.

Judging by the distribution of the water-soluble fact (or factors?) this obviously belongs in the vitamin B cor plex since yeast, wheat germ and lettuce are all good source and experiments now in progress further substantiate th assumption. The dystrophy-producing diet itself must co tain enough of the recognized growth factors since Pappe heimer and Goettsch ('31) maintained rats for three gener tions and we reared two generations of rats on diet 13 313 without any ill effects. The antineuritic vitamin B_1 excluded automatically and B_2 (riboflavin) can also be rule out safely because Guha ('31) has shown that this fact is not destroyed by Perhydrol (Merck) while our diet 31 which is treated with Superoxol, is as effective in producir dystrophy as is diet 13 treated with $FeCl_3$. Vitamin B_6 ma likewise be disregarded because it is not extracted by ac tone, while our acetone extract of wheat germ is very effe tive in preventing or curing nutritional dystrophy. We mus therefore, consider vitamin B_4 which Elvehjem ('35) four to be very labile, especially in the presence of oxygen. It present in wheat, yeast and green foods (lettuce, alfalfa) ju as the water-soluble dystrophy-preventive factor which w have been studying. The fact that Pappenheimer ar Goettsch ('34) produced a purely myopathic condition i ducklings on the same synthetic diet which in chicks causes myelopathic disease thought to be similar to a B_4 deficienc further suggests a possible connection between this vitami and our water-soluble factor. However, there is one ver serious objection to identifying these two dietary factor which must not be overlooked, namely, that rats do well eve for several generations on the diets which produce musc dystrophy in rabbits.

In conclusion it may be said that of the essential nutri-
onal factors necessary for the integrity of the skeletal
uscles the fat-soluble one is very closely associated or per-
aps even identical with vitamin E and is present in the oil
wheat germ, cottonseed, corn, also in lettuce and alfalfa.
is also present in the non-saponifiable portion of the oil
id in our ether, petroleum ether or acetone extracts of wheat
rm. It can be separated from the acetone extract by means
hexane. The water-soluble fraction can be obtained by
traction of wheat germ with 70% alcohol or with acetone,
id is also present in the wheat germ residue after extrac-
on with petroleum ether. This fraction belongs to the
tamin B complex because it can be furnished by lettuce,
ast and a variety of vitamin B concentrates.

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A MICROMETHOD FOR THE DETERMINATION OF GELATIN AND A STUDY OF THE COLLAGEN CONTENT OF MUSCLES FROM NORMAL AND DYSTROPHIC RABBITS

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In the course of our investigation of nutritional muscle dystrophy the need for some chemical method for determining the degree of dystrophy was soon recognized. Since in the dystrophying muscle there is a progressive accumulation of fibrous tissue, it seemed to us that a microdetermination of the collagen content, in the form of gelatin nitrogen, would furnish the necessary clue for such an estimation.

Method

In recent years two procedures have been suggested for the determination of collagen as gelatin N. Mitchell, Zimmerman, and Hamilton (4) worked out a method requiring a 25 to 100 gm. sample of meat. The meat is macerated in a ball mill, filtered through a 40 mesh sieve, and the residue is thoroughly washed with water. The washed residue, suspended in water, is then autoclaved for 2 hours at 16 to 18 pounds pressure. The filtrate from the autoclaved residue, together with the washings, is decanted into a volumetric flask, and the N determination is made on an aliquot portion of this solution containing all the gelatin.

Farkas (1) proposed a method for the analysis of gelatin-protein mixtures based upon the fact that gelatin-picrate is soluble at 40°, and could thus be separated from other precipitated proteins at that temperature. By making the precipitation with picric acid at 40° and subsequent extraction of the precipitate with warm

water all of the gelatin in the original material could be removed. When the solution has cooled, the gelatin is precipitated and is washed free from picric acid with Brücke's reagent (10 per cent KI saturated with HgI_2) on the filter. The washed residue is then digested and analyzed for N. Than (9) utilized this procedure in determining the collagen content of 0.5 gm. of dried meat.

Obviously, the Mitchell method cannot be used for small amounts of tissue, while the Farkas-Than method is very tedious and time-consuming, and not well suited for routine work.

Stoke's method (8), accepted by the Association of Agricultural Chemists for the determination of gelatin in milk products, employs $Hg(NO_3)_2$, which precipitates all proteins except gelatin. To the filtrate, containing the gelatin in solution, picric acid is added to precipitate the gelatin. Our objection to the Farkas-Than procedure holds equally well for this method. Jacobs and Jaffe (2) modified this official method by employing basic lead nitrate as the reagent to precipitate various proteins and by subjecting the gelatin-containing filtrate to a preliminary treatment with activated charcoal, which adsorbs pseudogelatins and proteans. The gelatin contained in the final filtrate was then precipitated with tannic acid.

In developing our procedure which is quick, simple, and applicable to very small quantities of material we availed ourselves of the best features of these various methods: minced muscle is dried and powdered, the collagen is converted to gelatin by autoclaving, the gelatin is extracted with hot water and is, finally, precipitated with tannic acid.

Procedure

The muscle to be analyzed is freed from adhering tissue, minced, and a weighed amount is dehydrated with acetone. For this purpose the minced tissue in a weighing bottle is completely covered with acetone which is allowed to evaporate spontaneously at room temperature. The material is then dried to constant weight in a vacuum desiccator and ground to a fine powder. A 100 to 200 mg. sample of the powder (corresponding to about 0.5 to 1 gm. of fresh tissue) is weighed into a small centrifuge tube, suspended in about 4 cc. of water, and autoclaved for 2 hours at 15 to 20 pounds pressure. After cooling, the tube is centrifuged

and the liquid is poured off into a large centrifuge tube. The residue in the original tube is extracted three times with small amounts of hot water, centrifuged, and the washings also transferred to the large tube, which now contains all the gelatin. The solution is slightly acidified, 5 cc. of a 5 per cent tannic acid reagent added, and the tube set in the ice box for at least 1 hour, whereupon the gelatin is quantitatively precipitated. The precipitated gelatin is centrifuged off and is dissolved by heating gently with 1 cc. of 2 N NaOH and 5 to 10 cc. of water. The gelatin solution is transferred to a small Kjeldahl flask, to which are also added the washings; the contents are neutralized with 1 cc. of 2 N H_2SO_4 and digested, with 2 cc. of a 1:1 H_2SO_4 solution and H_2O_2. The digested material after cooling is transferred to a 100 cc. volumetric flask and diluted to volume. The N determination is carried out on aliquot portions of this solution, containing 0.5 to 1.0 mg. of N, by direct nesslerization.

The total N of the muscle powder was determined by a procedure which we described elsewhere (7).

Analyses of Isoelectric Gelatin

To test the accuracy of this micromethod we analyzed isoelectric gelatin which we prepared according to Loeb's direction (3). Our preparation contained 17.2 per cent of N. Using a 0.21 per cent solution of this isoelectric gelatin, we determined the N of the tannic acid precipitate made either directly or after a preliminary autoclaving of the gelatin solution. The procedure was exactly as previously described. In both instances the N content was found to be 17.2 per cent as of the original preparation.

Experiments with the isoelectric gelatin solution were made to determine the time necessary to allow the tannic acid precipitation to become quantitative, and we found that 1 hour in the ice box secures quantitative precipitation of the gelatin.

Isoelectric gelatin was added in known amounts to muscle powder, the gelatin content of which had been determined. Analyses have proved that added gelatin was quantitatively recovered.

Finally, we studied the effect of various non-protein nitrogenous substances on the gelatin determination. We added creatinine, uric acid, or urea to samples of the isoelectric gelatin solution, and analyzed for gelatin following all the steps of the determination as

previously described. In every instance the gelatin determination was quantitative.

The experiments with the isoelectric gelatin of known N content show, therefore, that the tannic acid is capable of precipitating small amounts of gelatin quantitatively, that the gelatin itself is not affected by autoclaving either alone or together with muscle powder, and that the presence of various non-protein nitrogenous substances does not interfere with the quantitative gelatin determination by tannic acid precipitation.

Collagen Content of Normal and Dystrophic Muscles

In this study of the collagen content of muscles from normal rabbits and from rabbits made dystrophic by special dietary treatment our chief aim was to trace the development of the disease. The animals used for this experiment were all practically of the same age (between 30 and 40 days old) but owing to the large number required they belonged to six different litters. As was pointed out in a previous paper (5) rabbits on the Goettsch-Pappenheimer Diet 13 become dystrophic in about 3 to 6 weeks and usually die shortly after the onset of the disease, unless furnished with foodstuffs which secure recovery. In these experiments we employed our modified Diet 313, in which treatment with superoxol is substituted for the ethereal $FeCl_3$ treatment and which is just as effective. The body weight curve of rabbits on this dystrophy-producing diet is, for a time, the same as of the control animals. After a while, however, the daily gains become irregular and alternate with losses. We designate this as the *period of wavering weight.* Finally, an abrupt drop in the body weight occurs, and the condition of the animal progresses rapidly to death. We designate the sharp break in the weight curve as the *critical point.* Usually, a few days beyond this point, the condition of the animals is so far advanced that it is no longer possible to save them by dietary treatment. A number of interesting and critical changes occur at this time in the metabolism (6).

Our object was to follow the changes in collagen content of the muscles from the time the rabbits have been placed on this Diet 313 until they develop definite signs of dystrophy. Since no two animals behave exactly alike, the disease developing at a variable and totally unpredictable rate in each, it was not possible to so

select animals for the study of their muscles as to obtain a continuous series of stages. About 50 young rabbits were separated into a control and experimental group, care being taken that they represented approximately the same distribution as regard litters and initial weights. The experimental rabbits were given our Diet 313, while the control animals received this diet supplemented with wheat germ, which we have shown to be an excellent preventive of the disease. Every animal was weighed daily and individual weight charts were kept. At different times rabbits were selected from both groups, and usually three muscles (the left gastrocnemius, left biceps femoris, and left triceps brachii) were taken for the collagen determinations. The material was analyzed in accordance with the procedure previously described. The results of these analyses are presented in Table I.

The control rabbits were killed at different times between the age of 36 and 79 days. An examination of the total and gelatin N content of the three sets of muscles shows that this is apparently not affected by age. The total N of the gastrocnemius, biceps, and triceps was, on the average, 13.92, 13.93, and 13.64 per cent, respectively. These values are calculated on the basis of the dry weight of the muscles. The total gelatin N for the corresponding muscles was 1.91, 1.47, and 1.77 per cent. The proportion of total muscle N in the form of collagen N in the three muscles was 13.7, 10.5, and 13.0 per cent, respectively.

It is important to point out that the muscles with the highest collagen content show the smallest range of variation. Thus, the gastrocnemius has an average ratio of gelatin N to total N of 13.7 per cent, with a range between maximum and minimum values of 2 per cent. The triceps muscle, with an average of 13.0 per cent, has a range of variation of 4 per cent, and the biceps, with an average of 10.0 per cent, has a range of 5 per cent (if one exceptionally high value is not included in the calculation, otherwise the average is 10.5 per cent and the range is 7 per cent).

Before describing the condition found in their litter mates in the course of development of dystrophy, we shall make brief reference to analyses made on the same muscles from several animals, belonging to other experimental series, which were all in a more or less advanced stage of dystrophy. When the average values (13.34, 13.77, and 13.44 per cent) are examined, it will be

TABLE I

Collagen Content of Normal and Dystrophic Rabbit Muscles (on Basis of Dry Weight)

Rabbit No.	Age at death	Days on Diet 313	Gastrocnemius			Biceps femoris			Triceps brachii			Remarks
			Total N	Gelatin N	$\frac{\text{Gelatin N}}{\text{Total N}} \times 100$	Total N	Gelatin N	$\frac{\text{Gelatin N}}{\text{Total N}} \times 100$	Total N	Gelatin N	$\frac{\text{Gelatin N}}{\text{Total N}} \times 100$	
	days		*per cent*	*per cent*		*per cent*	*per cent*		*per cent*	*per cent*		
158	36					13.50	1.65	12.2	13.40	1.79	13.4	Control; Diet 313 + wheat germ
137	37					14.04	1.67	11.9	13.68	1.61	11.8	" "
122	41								13.53	1.81	13.4	" "
120	47		14.04	2.01	14.3	14.37	2.08	14.5	14.03	1.85	13.2	" "
131	54		13.90	2.02	14.5	13.65	1.44	10.5	13.28	2.00	15.1	" "
149	56		14.20	1.82	12.8	13.90	1.22	8.8	13.79	1.56	11.3	" "
136	69		13.68	2.00	14.6	13.42	0.98	7.4	14.04	1.77	12.6	" "
127	75		13.75	1.73	12.6	14.23	1.42	10.0	13.01	1.79	13.8	" "
147	79		13.96	1.89	13.5	14.33	1.28	8.9	13.96	1.79	12.8	" "
Average			13.92	1.91	13.7	13.93	1.47	10.5	13.64	1.77	13.0	
128	40	5	13.33	1.79	13.4	13.68	1.39	10.2	14.03	1.52	10.8	Apparently normal
123	51	10	13.40	2.22	16.6	13.54	1.31	9.7	13.90	1.47	10.6	"
129	52	17	14.04	2.27	16.2	13.50	1.48	11.0	13.47	2.22	16.5	" "
143	54	18	13.90	2.37	17.1	13.53	1.89	14.0	13.96	1.92	13.8	" "
124	59	18	13.90	1.79	12.9	13.33	1.86	14.0	13.60	2.17	16.0	" "
151	59	22				13.75	2.08	15.1	13.73	2.08	15.1	" "
126	63	22	13.60	1.87	13.7	13.64	1.20	8.8	13.13	1.77	13.5	" "

141	61	25	13.75	2.00	14.5	13.96	1.66	11.9	13.33	2.57	19.3		Apparently normal
148	62	25				13.88	1.67	12.0	13.90	2.08	15.0		Slight dystrophy
144	62	26				13.81	1.79	13.0	13.62	2.94	21.6		Apparently normal
132	63	28	13.60	1.79	13.2	14.70	1.75	11.9	13.33	2.03	15.2		Wavering in weight
152	70	32				14.33	2.35	16.4	13.61	2.78	20.4		Slight dystrophy
135	72	35				14.70	1.60	10.9	13.79	3.03	22.0		Wavering in weight
138	72	36				13.60	2.56	18.8	13.40	3.23	24.1		Slight dystrophy
134	73	36				13.83	2.73	19.7	13.33	3.33	25.0		Definite dystrophy
121	79	39				14.33	1.54	10.7	13.53	2.17	16.0		Wavering in weight
154	71	32				13.68	3.38	24.7	13.61	2.94	21.6		Definite dystrophy
142	76	40				12.96	2.32	17.9	13.54	2.62	19.3		Slight dystrophy
139	83	46				13.50	3.23	23.9	13.44	3.03	22.5		Definite dystrophy
130	90	46				13.47	1.91	14.2	13.44	3.17	23.6		9 days on curative diet; recovering
153	96	46				13.40	2.08	15.5	13.53	2.57	19.0		20 days on curative diet; recovering
119	79		13.20	4.31	32.7	13.37	3.28	24.5					Definite dystrophy
101	114		13.40	4.24	31.6	13.56	2.65	19.5	13.47	4.63	34.4		" "
114	95		13.33	3.91	29.3	13.40	2.85	21.3	13.33	4.43	33.2		" "
117	110		13.30	4.16	31.3	14.31	4.17	29.1	13.33	4.51	33.8		" "
107	116		13.47	4.95	36.7	14.19	2.78	19.6	13.50	3.32	24.6		" "
154	70								13.55	3.33	24.6		" "
Average.....			13.34	4.31	32.3	13.77	3.15	22.8	13.44	4.04	30.1		

noted that the total N content of the seven diseased rabbits was somewhat decreased, but the diminution, except in the case of the gastrocnemius, is not significant. The collagen content, however, is from 2 to 2.5 times as great, the per cent of total N as gelatin N increasing to 22.8 to 32.3 per cent. In other words, the collagen, which makes up about one-fiftieth to one-seventieth of the dry weight of normal muscles, is about one-twentieth to one-thirtieth of the dystrophic muscles. In the group of muscles studied the largest increase is found in the gastrocnemius and the smallest increase in the biceps femoris, and this also corresponds to the general collagen content of these three muscles.

At various intervals we have analyzed the muscles of the animals which were on the dystrophy-producing Diet 313 from 5 to 46 days (age 40 to 83 days). For about 25 days there are no outward signs by which one can detect the onset or progress of dystrophy, and such animals are designated as "apparently normal." The first symptoms of the onset of dystrophy, apart from the wavering weight curve, is the peculiar posture and inability of the animal to right itself, and, depending upon the severity of these outward signs, the condition is described as slight or definite dystrophy. Unfortunately, owing to the fact that the muscles were used also for other investigations, we omitted the gastrocnemius in animals in the more advanced stage of the disease. Of the eight rabbits whose gastrocnemii were analyzed within the first 25 days on Diet 313, five have collagen values entirely within the normal range but three rabbits, though outwardly they appear entirely normal, show a slight but definite increase in the collagen content between the 2nd and 3rd week.

The triceps brachii was analyzed in all of the nineteen experimental rabbits. Here, likewise, for about 4 weeks the collagen values are practically within the normal range. Soon after that, however, there is a marked rise in collagen content. It is rather interesting to point out that of the five animals examined within the 5th week two animals, with no outward signs of dystrophy, showed a large increase in collagen content, and one was within the normal range. But two animals, already affected, have a collagen content within the uppermost normal range. Of course, this may mean that the latter have started at the lowest normal range, while the former had started at the highest normal range

and that actually both groups of animals have a markedly increased collagen content. Another point in this connection which merits attention is the fact that a considerable dystrophic change in the muscle may take place before the outward signs of dystrophy become manifest. However, between the 5th and 6th week, when the outward signs are present and the condition increases in severity, there is a very abrupt and rapid development of collagen in this muscle.

The results with the biceps femoris are essentially the same. We have already mentioned that the normal range of variation of the collagen content of this muscle is large. We find, therefore, that four out of thirteen rabbits examined with outward manifestations still show a collagen which falls within the upper range of normal variation, but four animals, without any external signs, show a small but unmistakable increase in collagen. By the end of about 5 weeks not only does the disease develop rapidly, but the collagen content of this muscle, like that of other muscles examined, increases rather abruptly and quickly.

Two animals of this series, after they had developed definite dystrophy, were changed to a curative diet (Diet 313 + wheat germ). They were sacrificed at the end of 9 and of 20 days on this diet. They had both recovered from the outward signs of the disease. The collagen content of the biceps had almost returned to normal, while the recuperative changes in the triceps muscle are decidedly slower but nevertheless progressive.

SUMMARY

A method is described for the microdetermination of collagen as gelatin N, which depends upon the conversion of collagen to gelatin by autoclaving 100 to 200 mg. of substance dried by means of acetone. The gelatin is precipitated by means of tannic acid; the precipitate is dissolved in dilute alkali and digested with H_2SO_4 and H_2O_2. The digest is made up to a known volume and aliquot portions are directly nesslerized. The quantities are so adjusted that the aliquots contain 0.5 to 1.0 mg. of N.

The collagen content of the gastrocnemius, biceps femoris, and triceps brachii was determined in a number of growing rabbits. The collagen content, as well as the per cent of total N in the form of gelatin N, was found to be independent of the age of the animals.

The average per cent of total N in the form of gelatin N is greatest for the gastrocnemius and smallest for the biceps, while the range of variations is smallest for the gastrocnemius and greatest for the biceps. Rabbits in which nutritional muscle dystrophy has reached an advanced condition have 2 to 2.5 times as much collagen in the muscles as the control animals.

In the course of the development of the disease definite changes in the collagen content usually begin to appear when the animals have been on the dystrophy-producing Diet 313 about 3 weeks and become quite marked at the time the critical point is reached. The rather rapid development of the fibrous tissue in the muscles thus coincides with the general metabolic reaction at that period. When the animals are cured of the dystrophy, the collagen content of the museles regresses. The return to the normal condition was much more rapid in the biceps femoris than in the triceps brachii in these animals.

The collagen content may be markedly increased even before the outward signs of dystrophy become apparent. Different muscles of the same animal are apparently not affected to the same degree. However, the collagen determination may be used as a criterion of the early onset of the disease only in the case of a muscle like the gastrocnemius, whose range of normal variation in collagen content is very limited.

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STUDIES ON THE LIPID CONTENT OF NORMAL AND DYSTROPHIC RABBITS

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In a previous paper (6) it has been shown that hypercholesterolemia is an outstanding characteristic of rabbits afflicted with nutritional muscle dystrophy. Furthermore, a preliminary investigation of various tissues revealed that in different skeletal muscles the cholesterol increased considerably.

In view of the fact that the heart is not affected histologically (3), it is interesting to point out that chemically it is also apparently unaffected, at least so far as its lipid composition is concerned. Only the skeletal muscles of dystrophic animals have a very markedly increased cholesterol content, and we must, therefore, recognize that dystrophy is accompanied not only by a nonspecific hypercholesterolemia but also by a specific and very striking rise in the cholesterol content of the skeletal muscles.

The accumulation of cholesterol in the skeletal muscles naturally raises the question as to its origin. It has been proved quite conclusively that cholesterol is synthesized in the organism of man and higher animals (8), but neither the site of its formation nor its source is definitely known.

The first question which confronted us was whether we were dealing with an actual cholesterol synthesis or merely with a redistribution of cholesterol already present in the organism. To obtain a direct answer to this question is not so simple, but it seemed to us that a determination of the total cholesterol content of entire rabbits, both normal and dystrophic, would give a clue to its solution. The study of the cholesterol was supplemented also with analyses of the total lipid and of the lipoid P content.

Our second objective was to study the development of the lipomatosis which occurs in nutritional muscle dystrophy. The results of this study are given in the second portion of the paper.

Methods

The animals were killed by a blow over the head. They were depilated and freed from every trace of gastrointestinal contents. The carcass was weighed and autoclaved 2 hours at 35 pounds pressure. The material was then ground and mixed to a homogeneous mass. The finely ground mush was partially evaporated and material for analysis was taken from several localities with a large cork borer as a sampler. The material was repeatedly extracted with Bloor's alcohol-ether mixture (1). In the experiments on individual tissues described later the fresh tissue, carefully freed from adhering connective tissue or fat, was minced and mixed. One portion was used to determine the dry residue and another weighed portion was ground with fine sand and repeatedly extracted with Bloor's reagent. In every instance the alcohol-ether extract was so prepared that 100 cc. of the final volume corresponded approximately to 1 gm. of fresh tissue used.

The total lipid was determined gravimetrically after the alcohol-ether was driven off in a current of warm air, and the solution allowed to stand overnight at 80° and dried to constant weight in a vacuum desiccator. The lipoid P was determined on appropriate amounts of the extract to yield 0.015 to 0.030 mg. of P. The extract was evaporated to dryness and digested with H_2SO_4 and superoxol. The P determination was carried out by the Kuttner-Lichtenstein procedure (4). For the determination of free and combined cholesterol the micromethod of Schoenheimer and Sperry (9) was used. We checked our analytical results by determinations on known amounts of cholesterol and cholesterol acetate, and in our experience the method proved more accurate than the authors claim for it.

Experiment 1—Four normal and five dystrophic rabbits were used in this experiment. The dystrophic rabbits were taken at 2 and 13 days after they had attained the "critical point" (7). The analytical data are summed up in Table I.

While the weights of the control animals vary as much as 100 per cent, it is to be noted that this has little if any effect upon

the values for the various lipid fractions. That the lipid increases are not of dietary origin is demonstrated by the following considerations. The first two control rabbits, on Purina Rabbit Chow, which has a low fat content, and the remaining two control animals on Diet 13 of Goettsch and Pappenheimer (3) plus 30 per cent wheat germ, a diet with a lipid content similar to the dystrophy-producing Diet 13, all have remarkably similar values for the lipid fractions studied, as contrasted to the values of the

TABLE I

Lipid Content of Whole Rabbits, Normal and Dystrophic

Rabbit No.	Live weight	Weight loss from maximum	Total lipid		Phospholipid		Cholesterol		Lipid / Phospholipid	Lipid / Cholesterol	Phospholipid / Cholesterol
	gm.	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*			
Normal											
A	2826		260.9	9.58	21.22	0.75	3.957	0.14	11.1	59.5	5.4
B	2790		445.3	15.96	23.99	0.86	4.533	0.16	17.4	92.0	5.3
174	1578		185.6	11.77	14.64	0.93	2.488	0.16	11.5	67.7	5.8
175	1364		154.8	11.35	13.31	0.98	2.233	0.16	10.5	62.5	6.0
Average...	....	.....	261.7	12.17	18.29	0.88	3.303	0.155	12.6	70.4	5.65
Dystrophic											
172	966	−17.2	142.2	14.74	11.05	1.15	3.132	0.32	11.6	40.9	3.5
177	856	−19.7	71.3	8.32	8.87	1.04	2.575	0.30	6.7	23.2	3.4
60	991	−21.4	95.5	9.63	9.67	0.98	2.993	0.30	8.5	27.6	3.2
59	692	−26.4	65.0	9.40	8.57	1.24	2.056	0.30	6.3	26.4	4.1
69	761	−37.0	80.5	10.58	10.39	1.37	4.207	0.55	6.4	15.7	2.5
Average...	....	.....	90.9	10.53	9.71	1.16	2.993	0.355	7.9	26.8	3.36

dystrophic animals on Diet 13 alone. This is further substantiated by the values for individual muscles given in Table II. The dystrophic and normal animals were on the same diet, while the controls were fed our Diet 313[1] plus 20 per cent wheat germ. The similarity of the control and normal values, as contrasted with the dystrophic values, is most remarkable.

It is noteworthy that even when the dystrophy has advanced

[1] Diet 13 treated with ethereal superoxol instead of $FeCl_3$.

so far that the weight of the rabbits decreased by one-fifth to one-third, the per cent of total lipid in the body was still quite high. The per cent of phospholipid and cholesterol is remarkably constant, and is much greater than in the control rabbits. What is particularly significant, however, is the fact that, whereas the absolute amount of lipid is decreased to less than one-third and of the phospholipid to one-half, the amount of cholesterol is only about one-tenth less in the dystrophic animals, although their live weight is only about 60 per cent (on the average) of that of the control rabbits. Furthermore, the relative lipid content is about one-seventh smaller in the dystrophic animals, while the phospholipid is one-fourth greater and the relative cholesterol content is even more than doubled.

A study of the lipid to phospholipid, lipid to cholesterol, and phospholipid to cholesterol ratios throws interesting light on the direction of the change in the chemistry of the dystrophic animal. These ratios, particularly the last one, reveal considerable constancy and show that the lipid of the dystrophic animals contains about 1.5 times as much phospholipid but 2.5 times as much cholesterol as the lipid from the control animals. If we calculate the pre-dystrophic cholesterol values (using the control average and the calculated live weights just before the onset of the dystrophy) and compare them with the values given in Table I, the cholesterol in these five dystrophic animals has increased by 1.32, 0.93, 1.04, 0.60, and 2.34 gm. respectively. This suggests as a very strong possibility that we are dealing with an actual synthesis and accumulation of cholesterol in animals afflicted by the dystrophic process.

Experiment 2—Lipomatosis and fibrosis are two most characteristic transformations in muscles undergoing dystrophy. Just as in the case of fibrosis, which has been described in a previous (10) paper, we set ourselves the problem of following the development of the lipomatosis. The same series of rabbits used in the study on the development of fibrosis has been used also for this purpose, except that in addition to the gastrocnemius, biceps femoris, and triceps brachii muscles we extended the analysis also to the abdominal and intercostal muscles, the gastrointestinal tract, heart, lungs, kidney, liver, spleen, and brain. In this series the determinations were carried out on twenty-one

rabbits which were on Diet 313. At certain intervals animals were killed and the condition of their muscles was checked by histological examination. Eight rabbits with entirely negative histological findings are grouped as normal; the remaining thirteen showed various degrees of dystrophy. In addition, two rabbits which had become dystrophic were placed on a curative diet and their tissues were analyzed after 9 and 18 days of recovery.

In addition to this we also analyzed the tissues from another group of nine control and seven definitely dystrophic animals. With the exception of one rabbit, only the gastrocnemius, biceps femoris, and triceps muscles were analyzed in this series of definitely dystrophic rabbits, and they all showed a very extensive development of lipomatosis. The total lipid content of various organs in both control series was very similar. Unfortunately it would be impossible to present the data in detail and in Table II only the average results for each group are reported. The data pertaining to such organs as the heart, gastrointestinal tract, liver, kidney, and lungs in which there has been no change in total lipid or in lipoid P have not been tabulated.

Surveying the results obtained in the first series made up of control and severely dystrophic rabbits, we note that the lipoid P is materially increased in all the muscles studied, but the rise in cholesterol is particularly great, the total cholesterol content of different muscles increasing from 100 to nearly 350 per cent. The abdominal muscles show the least change, while the gastrocnemius shows the greatest change. Furthermore, the cholesterol esters which in normal muscles make up about 4 to 8 per cent increase to about 12 to 27 per cent of the total cholesterol in the dystrophic muscles.

We have included in Table II the results on the spleen, one of the organs in which no change in the lipids occurs during dystrophy. Neither the total lipid, lipoid P, nor total and free cholesterol is materially different in the spleens from the normal and dystrophic groups. Unfortunately only one brain was analyzed in the severely dystrophic group, but this had the highest total lipid we found in the large number of determinations. The lipoid P content was increased proportionately, so that the lipid to phospholipid ratio remained unaffected. The cholesterol of

TABLE II

Average Lipid Content of Various Organs in Per Cent of Dry Substance

Condition and No. of rabbits	Organ	Total lipid	Lipoid P	Cholesterol			Lipid / Phospholipid	Lipid / Cholesterol	Phospholipid / Cholesterol
				Total	Free	Per cent free			
Control (9)	Gastrocnemius	14.1	0.185	0.404	0.380	94.1	2.0	22.5	11.4
	Biceps femoris	16.9	0.171	0.380	0.363	95.5	2.9	32.2	11.3
	Triceps	15.7	0.197	0.354	0.336	94.9	2.1	29.5	13.9
	Intercostal	25.8	0.156	0.494	0.454	91.9	5.5	43.4	7.9
	Abdominal	15.7	0.123	0.426	0.403	94.6	4.0	28.7	7.2
	Spleen	27.0	0.351	2.590	2.500	96.7	1.8	6.0	3.4
	Brain	49.7	0.840	9.170	9.100	99.2	0.9	2.1	2.3
Advanced dystrophy (7)	Gastrocnemius	31.0	0.210	1.405	1.059	75.4	4.6	17.3	3.7
	Biceps femoris	27.2	0.197	1.137	0.826	72.7	4.3	18.5	4.2
	Triceps	25.6	0.226	1.177	0.985	83.7	3.3	15.9	4.8
	Intercostal*	32.6	0.194	1.418	1.082	76.3	5.4	18.5	3.4
	Abdominal*	25.6	0.168	0.861	0.754	87.6	4.9	23.9	4.9
	Spleen†	26.2	0.366	2.700	2.500	92.7	1.6	5.3	3.4
	Brain*	66.1	1.109	14.750	14.750	100.0	0.9	1.6	1.9
Normal (8)	Gastrocnemius	14.1	0.178	0.419	0.391	93.3	1.9	22.0	10.6
	Biceps femoris	15.3	0.170	0.370	0.360	97.0	2.5	27.7	11.0
	Triceps	14.2	0.186	0.354	0.330	93.2	2.0	25.2	13.1
	Intercostal	24.5	0.165	0.505	0.488	96.6	4.8	39.3	8.2
	Abdominal	15.8	0.122	0.363	0.355	97.8	4.7	34.1	8.4
	Spleen	24.7	0.324	2.550	2.500	98.0	1.7	5.5	3.2
	Brain	49.0	0.851	9.300	9.240	99.4	0.9	2.0	2.3
Various stages of dystrophy (13)	Gastrocnemius	15.6	0.219	1.084	0.888	82.0	1.9	8.7	4.7
	Biceps femoris	19.1	0.184	0.592	0.524	88.5	1.9	16.7	7.8
	Triceps	17.1	0.230	0.851	0.724	85.1	1.8	12.3	6.8
	Intercostal	28.8	0.190	0.878	0.734	83.6	4.9	26.4	5.4
	Abdominal	21.2	0.143	0 533	0.505	95.0	4.9	34.1	6.9
	Spleen	24.1	0.375	2.700	2.620	97.1	1.3	4.5	3.5
	Brain	52.6	0.905	10.660	10.600	99.5	1.3	2.7	2.1

TABLE II—*Concluded*

Condition and No. of rabbits	Organ	Total lipid	Lipoid P	Cholesterol			Lipid / Phospholipid	Lipid / Cholesterol	Phospholipid / Cholesterol
				Total	Free	Per cent free			
Recovery, 9 days (1)	Gastrocnemius	17.2	0.245	0.835	0.793	95.0	1.7	12.2	7.3
	Biceps femoris	20.8	0.217	0.533	0.518	97.2	2.7	25.5	10.2
	Triceps	17.3	0.241	0.805	0.798	99.1	1.7	13.0	7.5
	Intercostal	26.8	0.157	0.663	0.612	92.3	5.7	33.5	5.9
	Abdominal	20.4	0.126	0.464	0.445	96.0	5.3	36.2	6.8
	Spleen	23.1	0.341	2.270	2.170	95.7	1.4	5.4	3.8
	Brain	54.6	0.909	10.800	10.800	100.0	0.9	2.0	2.1
Recovery, 18 days (1)	Gastrocnemius	23.7	0.208	0.635	0.615	96.9	3.4	27.9	8.1
	Biceps femoris	21.3	0.203	0.534	0.460	86.2	3.1	29.6	9.6
	Triceps	19.0	0.203	0.437	0.433	99.1	2.7	30.8	11.6
	Intercostal	27.4	0.177	0.775	0.614	79.2	5.1	28.5	5.6
	Abdominal	22.2	0.158	0.475	0.460	96.8	4.5	37.1	8.2
	Spleen	21.9	0.314	1.940	1.910	98.5	1.5	6.2	4.0
	Brain	50.7	0.881	10.050	10.050	100.0	0.9	1.9	2.2

* Single determination.
† Three determinations.

the brain is practically all in the free state, and this is also true for the brain from the severely dystrophic rabbit. However, the cholesterol content increased somewhat more (about 25 per cent) so that the lipid to cholesterol and the phospholipid to cholesterol ratios are lowered.

In the next series in which the development of lipomatosis was investigated, the briefly summarized results need to be elaborated in order to make the trends of change in the different stages of dystrophy manifest. While space limitations prevent the inclusion of the complete data, and since an average of values taken from animals varying from slightly to severely dystrophic does not present the correct picture, Fig. 1 is included as a typical example of the lipid findings for the gastrocnemius muscle from animals at various stages of dystrophy.

An examination of the data reveals that the total lipid content

of only certain organs, namely the skeletal muscles and the brain, increases. So far as smooth muscle (gastrointestinal tract), heart, lung, kidney, liver, and spleen are concerned, their total lipid is unaffected by dystrophy. The five types of skeletal muscles studied show somewhat variable behavior but in all of them

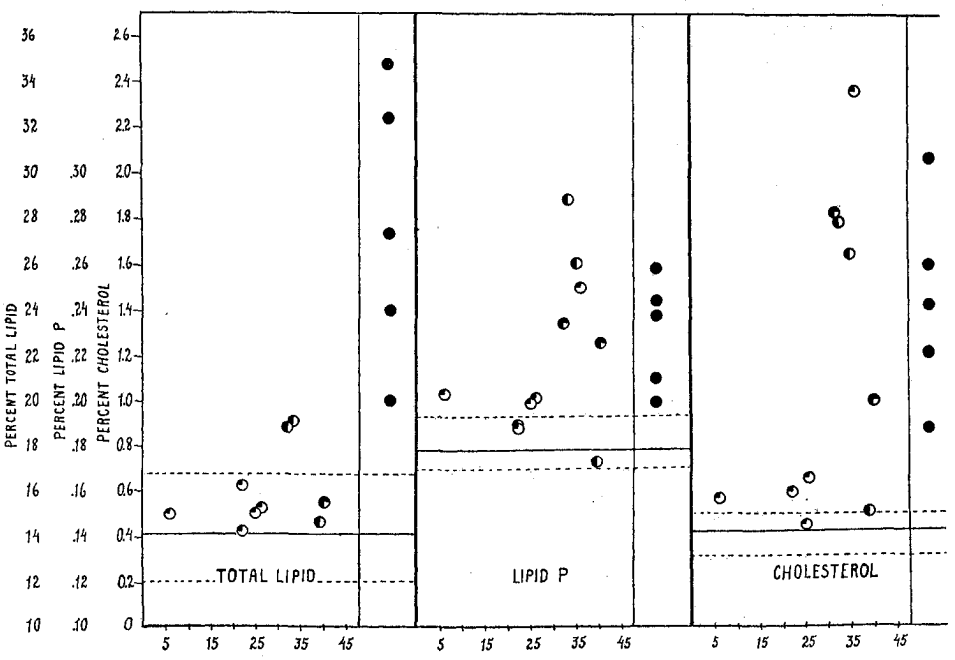

FIG. 1. Lipid distribution in gastrocnemius muscle of rabbits which have been on a dystrophy-producing diet for various lengths of time. The solid dots represent the values found in rabbits definitely dystrophic. The partially shaded circles represent the values found in rabbits killed at definite stages (days on Diet 313) and the one-, two-, or three-quarter shading corresponds to 1 +, 2 +, and 3 + reports of the histological examination of the dystrophic condition of the muscles. The broken lines mark off the extremes of variation, and the continuous line the mean value, found in the control animals.

the total lipid content increases as the dystrophy (on Diet 313) develops. Using the histological findings as a rough guide to the degree of muscle involvement, we find that the lipid content is entirely normal in those animals showing no dystrophy, even though on the diet for as long as 22 days (dystrophy develops in

30 days on the average). Moreover, most of the animals marked as 1 + still show a lipid content which falls within the normal range of variation. But as the dystrophic process develops and the condition histologically is already marked as 2 + or 3 + the total lipid content rises considerably beyond the normal range. When one takes into consideration the time element and the rapidity with which the muscular dystrophy proceeds, the accumulation of lipids, as well as of fibrous tissue, as was shown before (10), is almost cataclysmic. Considering only the tabulated average values, the increases range from about 5 to 25 per cent, though as the disease evolves into a more advanced condition the total lipid may be even doubled, as in the case of the gastrocnemius muscle. In the rabbits which were recovering from dystrophy for 9 and 18 days, respectively, there has been apparently no such marked recession of the lipomatosis as was already noticeable in the case of the fibrosis (10), except for the brain of one of these animals whose lipid composition has been completely restored to normal. The fact that neither smooth nor cardiac muscle nor any of the important internal organs is involved in this lipomatosis further emphasizes the fact that we are dealing with an almost purely skeletal muscle deficiency disease.

Studies on the lipoid P show a similar behavior. Like the total lipid content, the phospholipid content is definitely increased only in the skeletal muscles and in the brain. In the smooth muscles, in the heart, lung, kidney, and liver there is no change or, if anything, a tendency to decrease.

The situation is, however, much more striking from the point of view of the changes in cholesterol. Here definite, though not very large increases are observed even in the earliest stage of the dystrophic process and the increase in cholesterol content may go as high as about 50 per cent. But in the stages of dystrophy designated histologically as 2 + or 3 + the cholesterol rapidly attains increases of 100 to 350 per cent. Another striking fact is that the enormous rise in cholesterol esters does not really take place until the dystrophic process in the muscles is well under way. In rabbits still in the prodromal stage of the deficiency disease the cholesterol, though already considerably increased, may still be largely in the form of free cholesterol just as in the normal muscles, and only as the disease enters a more pronounced and

advanced stage do the cholesterol esters increase very greatly. In individual instances the cholesterol esters may even attain as high a proportion as one-third of the total. The highest values for the cholesterol esters are found in the gastrocnemius and biceps femoris muscles and the lowest in the abdominal muscles, which may be associated with the extent to which these different muscles are involved in the dystrophic process. Page (8) points out that the deposition of unusual amounts of esterified cholesterol is generally associated with disease, and it has come to be recognized as a sign of slow death of cells. In this series we also find that in the spleen and brain the cholesterol is almost entirely in the free state, as is the case in normal animals. During recovery there is not only a recession of the cholesterol so that in some muscles at least the content is again normal but the cholesterol esters also disappear, as can be seen from the figures for free cholesterol in Table II. This recession is apparently associated with the regeneration of muscle fibers.

In the brain of a severely dystrophic rabbit there was evidence of an appreciable (25 per cent) rise in cholesterol and it is, therefore, of interest to note that even in the earlier stages of dystrophy there is also evidence of such an increase, though of a much smaller degree.

A clearer picture of the changes in the lipid composition may be obtained from a study of the various ratios (last three columns in Table II). From this standpoint the spleen and brain show a remarkable stability, and among the skeletal muscles investigated the abdominal muscles undergo the least radical alteration in the dystrophic animals.

In organs whose total lipid content has not been affected there is no alteration in the relation between the different lipid components, a radically altered lipid pattern being a characteristic and specific change only of the skeletal muscles undergoing dystrophy.

A study of individual muscles shows that the total lipid may remain normal during the early stages of dystrophy but since the lipoid P content increases this tends to lower the lipid to phospholipid ratios. But in the advanced phases of dystrophy we note a decided increase in the ratios in spite of the fact that the phospholipid content of the muscles is appreciably greater. We must conclude, therefore, that initially it is the phospholipid of

the affected muscles which increases, the increase in other fatty material being a later phenomenon.

The study of the lipid to cholesterol ratio also reflects this time factor in the accumulation of fat in the dystrophic muscle. In the early stages the ratios for the gastrocnemius, biceps femoris, triceps, and intercostal muscles (the abdominal muscles show no effect as yet) are to a greater or less extent lower than at the advanced stage of dystrophy because the increase in cholesterol precedes the increase in fat content. In the later stages, although the cholesterol content still continues to rise very much, the deposition of fat in the muscles becomes so much more rapid that the ratios actually increase. In the rabbits on a recovery diet these ratios tend very quickly to return to normal values, thus showing that the regenerative process is associated not only with a disappearance of lipomatosis but also with a reestablishment of a normal lipid composition of the muscles.

The phospholipid to cholesterol ratios, which decrease during the development of dystrophy and become considerably smaller in the advanced condition, suggest that the initial change in the lipid pattern of the dystrophic muscles is due to an increase in the cholesterol which is followed later by the phospholipid and, finally, in the very advanced stage by a great increase in fat. This is also substantiated by observations on individual rabbits in which the rise in cholesterol was the first marked change observed in the lipid of their muscles at the time the histological picture was indicated as 1 +.

Lawaczeck (5) described a large increase in the cholesterol of the skeletal and cardiac muscles in pigeons suffering from beriberi. This condition, however, differs from that of dystrophic rabbits both qualitatively and quantitatively, and, furthermore, similar changes occur in fasting pigeons. Since animals suffering from beriberi, or avitaminosis B, are known to be in a state of chronic starvation, it is not entirely excluded that the condition described by Lawaczeck is purely an inanition effect, or at any rate is very seriously affected by it. In dystrophic rabbits, of course, inanition plays no part except in the last 3 or 4 days before death, and most of our animals have been killed prior to this. Furthermore, Ciaccio (2) found that the phospholipid content of muscles from pigeons with beriberi is diminished, which is also in contrast to our finding of a considerable rise in lipoid P in the muscles from dystrophic rabbits.

SUMMARY

Evidence is presented to show that the great increase in cholesterol found in the muscles of rabbits afflicted with nutritional muscle dystrophy results from synthesis and not from redistribution of preexisting cholesterol. Of the various tissues studied only skeletal muscles show a great increase in fat, lipoid P, and especially in cholesterol. No changes occur in the heart and various internal organs. In the brain there is also an increase in total lipid with a proportional increase in lipoid P but the cholesterol, especially in the very advanced stage, is increased in a somewhat larger proportion. Not all the skeletal muscles are affected in the same degree, the gastrocnemius being most and the abdominal muscles the least affected in the group of five types of skeletal muscle examined. There is evidence that the cholesterol is the first to increase in muscles affected by dystrophy, the lipoid P increasing next, and, finally, in the very advanced stage, the fat content rises very high. In the advanced condition of dystrophy the total lipid of the muscles may be doubled, and the cholesterol may even increase 100 to 350 per cent over the control. As dystrophy progresses not only the free cholesterol but also the cholesterol esters increase so that they may constitute 12 to 27 per cent of the total cholesterol instead of 4 to 8 per cent as in the normal muscles. The increase in cholesterol content may be regarded as a specific characteristic of dystrophied muscles. The changes which take place in the muscle lipids when the animal is recovering from dystrophy are discussed.

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