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THE EFFECT OF A PROTEIN-FREE DIET ON THE COLLAGEN CONTENT OF MICE

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Experiments with isotopically labelled amino acids (e.g. Neuberger, Perrone & Slack, 1951; Neuberger & Slack, 1953) have shown that collagen is metabolically generally inert compared with other body proteins. In certain circumstances nevertheless collagen may be metabolized rapidly, as for example in the involuting uterus (Harkness & Harkness, 1954; Harkness & Moralee, 1956). These observations suggested that the reported inertness of collagen is in part dependent on stable metabolic conditions, and it appeared of interest to investigate whether this inertia was maintained during general loss of nitrogen by the body.

We therefore gave mice a diet free from protein. Over the experimental period of about 20 days they lost approximately half their initial body weight. This treatment led to a significant diminution in total body collagen, although the loss was proportionately less than that of protein as a whole. Examination of individual tissues showed that collagen was lost mainly from the skin.

Our experiments have also established the total amount of collagen in the mouse and its distribution between various tissues.

METHODS

Animals and diet. Adult male albino mice were used throughout. The protein-free diet contained 83% sucrose, 12% arachis oil, and 5% of the salt mixture described by Hubbell, Mendel & Wakeman (1937) with vitamin supplementation (Perez-Tamayo & Ihnen, 1953). The animals were allowed to eat and drink *ad lib.*

Experimental method. Two experiments were done, differing slightly in details. In each the animals were divided at the start into two equal groups by reference to a table of random numbers. Both groups were weighed and one (controls) killed with ethyl ether and stored at $-10°$ C in sealed vessels. Experimental animals were given the protein-free diet and weighed on alternate days. They were killed and stored at $-10°$ C for a few days before both groups were thawed and dissected for analysis.

In Expt. 1, twenty mice of a rather wide range of body weight were used. The experimental group was given the protein-free diet for 17 days. In all animals the abdominal viscera, excluding

generative organs and kidneys, were weighed and analysed separately from the remainder of the body.

In Expt. 2, twenty mice were selected from stock in ten pairs, each pair having less than 1 g difference in weight between its two members. One member of each pair was then chosen at random to form the experimental group; the remainder were controls. The protein-free diet was given for 20 days. The body of each animal was analysed in five parts, each of which was weighed in a sealed bottle before analysis. First the two quadriceps muscles were removed. They were transected just above their patellar insertions and dissected free from their proximal attachments. Next the two femora were removed and scraped as far as possible free from ligamentous and muscular attachments. The skin was then removed, and last the abdominal viscera, excluding the reproductive organs and kidneys. The remainder, which contained the greater part of the muscle and bone of the animal, is referred to as the carcass.

Chemical methods. Collagen was brought into solution with hot trichloroacetic acid (TCA) (Fitch, Harkness & Harkness, 1955). In the first experiment the tissues were extracted twice with about ten times their volume of hot 5% (w/v) TCA and three times with cold TCA, soluble and insoluble material being separated by centrifugation. The tissues were homogenized in a small blender of the Waring type after the first heating with TCA. The combined extracts were made to a known volume. A sample was heated to dryness on a boiling water-bath and hydrolysed in 6 N-HCl for estimation of hydroxyproline by the method of Neuman & Logan (1950). In the second experiment, after the tissues had been heated in TCA the soluble and insoluble materials were not separated but an equal volume of 10% TCA was added to them. This brought the TCA approximately back to the original concentration since it was found that most of the original TCA had decomposed by this time. The material was heated for a further half hour and then made to a known volume. The suspension was mixed and a sample removed for estimation of total nitrogen by Ma & Zuzaga's (1942) modification of the Kjeldahl method. The remaining suspension was centrifuged at 2500 rev/min for 30 min, and portions of the supernatant were removed for estimation of hydroxyproline by Neuman & Logan's (1950) method, after evaporation to dryness on a boiling water-bath, and hydrolysis with 6 N-HCl in a sealed tube for 4 hr at 40 Lb./sq.in. (2·8 kg/cm^2) pressure in an autoclave. The collagen content of the tissue was calculated from the hydroxyproline content by multiplying by a factor of 7·46. Non-collagenous nitrogen was calculated by deducting collagenous nitrogen from the total.

RESULTS

The results of the first experiment are given in Table 1. The animals of the experimental group lost about 45% of their initial body weight during 17 days on the protein-free diet and the total collagen content of their bodies was

TABLE 1. Collagen content of control mice and mice fed on protein-free diet (Expt. 1)

	Body wt. at death (g)	Total collagen (mg)	Skin and carcass collagen (mg)	Visceral* collagen (mg)
Control	35·0±1·2	1000±40	980±70	23·6±1·6
Protein-free diet	19·7±2·2	870±30	850±20	20·1±0·8

* Abdominal viscera excluding kidneys and genital organs.
The estimate of variation is the standard error of the mean.

87% of that of the control animals. This difference was significant when tested by analysis of co-variance to eliminate the effect of difference in initial body weight between the two groups ($0{\cdot}01 > P > 0{\cdot}001$). The abdominal viscera of the deficient animals also contained less collagen than the controls, but the difference was not significant ($P < 0{\cdot}05$).

The results of the second experiment are given in Tables 2–4. The experimental diet produced a significant loss of total body collagen ($0{\cdot}01 > P > 0{\cdot}001$). The loss (11%) was less than the loss in body weight (44%) or non-collagenous N (33%). Collagen formed $2{\cdot}6 \pm 0{\cdot}1$% of the body weight and $17{\cdot}7 \pm 0{\cdot}7$% of total N in the control animals.

TABLE 2. Weight, collagen and non-collagenous nitrogen (NCN) in different parts of the body in normal mice and mice fed on a protein-free diet (Expt. 2)

	Controls					Protein-free diet				
	Wet wt. (g)	Total collagen (mg)	Collagen as % of wet wt.	NCN (mg)	NCN as % of wet wt.	Wet wt. (g)	Total collagen (mg)	Collagen as % of wet wt.	Total NCN (mg.)	NCN as % of wet wt.
Skin	6·67 ± 0·17	442* ± 26	6·7 ± 0·4	216* ± 8	3·23 ± 0·08	3·96 ± 0·11	369 ± 26	9·3 ± 0·6	171 ± 20	4·35 ± 0·13
Carcass	24·11 ± 0·37	539 ± 12	2·20 ± 0·06	503* ± 22	2·09 ± 0·06	14·03 ± 0·48	496 ± 51	2·90 ± 0·08	327 ± 14	2·34 ± 0·08
Viscera	7·53 ± 0·38	32 ± 1	0·50 ± 0·03	159 ± 18	2·15 ± 0·12	3·67 ± 0·12	29 ± 1	0·80 ± 0·03	91 ± 8	2·53 ± 0·15
Femora	0·160 ± 0·005	15·1† ± 0·5	9·40 ± 0·35	2·3† ± 0·4	1·45 ± 0·09	0·170 ± 0·006	15·6 ± 0·8	8·9 ± 0·5	2·8 ± 0·6	1·66 ± 0·16
Quadriceps	0·53 ± 0·02	4·3 ± 0·1	0·81 ± 0·02	18·0 ± 0·6	3·38 ± 0·07	0·29 ± 0·02	4·1 ± 0·1	1·45 ± 0·05	10 ± 0·7	3·57 ± 0·21
Total	39·00 ± 0·54	1028* ± 25	2·6 ± 0·11	893* ± 36	2·26 ± 0·08	22·12 ± 0·64	913 ± 33	4·2 ± 0·3	602 ± 21	2·49 ± 0·22

The estimate of variation is the standard error of the mean.

* Nine animals only, one analysis of skin being lost accidentally.

† Seven animals only, three analyses being lost accidentally. For computation of total body collagen figures were estimated for femora from their wet weight and percentage collagen in other animals. The femora formed such a small proportion of the total (1·5%) that no appreciable error is introduced by this procedure.

TABLE 3. Distribution of weight, collagen and non-collagenous nitrogen (NCN) in bodies of control normal mice and mice fed on a protein-free diet (Expt. 2)

	Controls			Protein free diet		
	Wet wt. as % of total wet wt.	Collagen as % of total collagen	NCN as % % of total NCN	Wet wt. as % of total wet wt.	Collagen as % of total collagen	NCN as % of total NCN
Skin	17·11 ±0·46	42·71 ±1·76	24·65 ±0·81	17·92 ±0·37	40·06 ±1·76	28·46 ±0·79
Carcass	61·84 ±0·63	52·31 ±1·72	55·25 ±1·33	63·37 ±0·46	54·54 ±1·61	54·54 ±1·06
Viscera	19·27 ±0·86	3·11 ±0·13	17·79 ±0·94	16·59 ±0·43	3·24 ±0·21	15·26 ±0·62
Femora	0·42 ±0·01	1·49 ±0·06	0·27 ±0·02	0·79 ±0·02	1·71 ±0·08	0·47 ±0·05
Quadriceps	1·43 ±0·05	0·41 ±0·01	1·99 ±0·07	1·31 ±0·03	0·45 ±0·02	1·70 ±0·05

The estimate of variation is the standard error of the mean.

The results of analyses of different tissues showed that some lost a greater proportion of their collagen than others. No loss of collagen was found in bone, as judged from the femur. The collagen content of the quadriceps muscles was only 5% lower in the experimental mice, a difference which was not

significant. The collagen content of the carcass was however significantly lower ($0{\cdot}05 > P > 0{\cdot}02$) in the experimental animals. There is some apparent anomaly in the fact that while the carcass showed a significant reduction in deficient animals two of its major constituents, bone and muscle, did not. It is obvious that the carcass is in effect a collection of units similar in type to quadriceps muscle and femur. Part of the explanation may lie in experimental error in measuring comparatively small amounts of collagen.

The difference between the mean collagen content of the viscera of the control and experimental groups was not significant. However, the fact that a similar but again just not significant difference was found in the first experiment suggests that the viscera do in fact participate in the general loss of collagen. The largest significant difference (16%) in the collagen content between the control and experimental animals was found in the skin ($0{\cdot}05 > P > 0{\cdot}02$).

TABLE 4. Losses of wet weight, collagen and non-collagenous nitrogen calculated from difference between means for experimental and control groups (Expt. 2)

	Losses, absolute and as % contribution to total loss			Percentage difference, control mice minus experimental mice		
	Weight (g)	Collagen (mg)	NCN (mg)	Weight	Collagen	NCN
Skin	2·71 (16·0%)	72·6 (61·4%)	45·0 (15·3%)	40·6	16·4	20·8
Carcass	10·07 (59·6%)	43·0 (36·3%)	176·1 (59·9%)	41·8	7·9	34·9
Viscera	3·87 (23·0%)	3·0 (2·5%)	65·1 (22·1%)	51·4	9·3	41·6
Femora*	−0·0099 (−0·05%)	−0·0005 (−0·4%)	−0·41 (−0·1%)	−6·0	−3·0	−2·7
Quadriceps	0·24 (1·4%)	0·2 (0·1%)	8·1 (2·7%)	45·1	3·7	18·9

* Negative values indicate apparent gains by experimental group.

The relative importance of losses from the different parts of the body may be demonstrated by expressing the loss from each part as a percentage of the loss for the whole body (Table 4). Whereas the greatest part of the loss of weight and non-collagenous nitrogen (59·7 and 59·9% respectively) was from the carcass, the greater part (60%) of the loss of collagen was from the skin. Losses of non-collagenous nitrogen and wet weight from muscle, carcass and viscera were similar and comparable to those in the whole body; from the skin they were small, possibly owing to the fact that hair is in a stable condition and can be lost only by falling out or friction. There was no loss of weight of non-collagenous N from the femora.

DISCUSSION

Although the quantitative distribution of collagen within the bodies of some animals can be approximately predicted from the literature, our results for mice appear to be the first direct measurements, except for a single guinea-pig analysed by Lightfoot & Coolidge (1948), who used a method of doubtful specificity (Harkness, 1952). Our results clearly indicate the importance of collagen among the body proteins, of which it constitutes about one-sixth in control animals. Of total body collagen, no less than 40% is in the skin, which forms only some 17% of the weight of the body. The latter figure agrees with that of 17·6% given by Welcher & Brandt (1903) for the mouse. The mouse, however, is a small animal and one might expect the proportion of the total collagen content of the body in the skin to be less in larger animals, which have a smaller relative skin surface area. It appears in fact that the thickness of the skin increases in proportion to other linear dimensions, since it forms about the same proportion of body weight in large as in small animals. Some figures from the literature of the percentage of the body weight contributed by the skin are 17% in the rat (Jackson & Lowry, 1912) 14·7% in the cat (Seldmair, 1899), 7·1–12·5% in sheep (Hennenberg, 1881), 20% including subcutaneous fatty tissue which forms half to three quarters or more of the total in the human new born and adult (Vierordt, 1906), 7–10% in cattle (Lawes & Gilbert, 1859; Moulton, Trowbridge & Haigh, 1922; Edinger, 1925), 15·5% in an elephant (Quiring, 1939).

Nitrogen constituted 3·6% of the wet weight in skin from control animals, and of this 28% was collagenous nitrogen. Eisele & Eichelberger (1945) found 3·37% nitrogen in wet, fat-free, human skin, of which no less than 74% was collagenous. The smaller representation of collagenous nitrogen in our animals was presumably due in part to the fact that hair had not been removed.

The rate of loss of collagen over the whole experimental period on the protein-free diet was slow compared with some of the other rates of loss recorded in the literature (see Harkness & Moralee, 1956). Our experiments agree with those of Neuberger *et al.* (1951) and Neuberger & Slack (1953) in showing collagen to have greater metabolic stability than other proteins taken as a whole. Neuberger & Slack (1953), however, found higher rates of incorporation of ^{14}C glycine into the collagen of bone and liver of adult rats than into skin, whereas in our experiments skin showed greater change than bone. Our experiments provided no evidence of change in bone, although this tissue is generally regarded as having relatively high metabolic activity. The control of the collagen content of bone is, however, peculiar and this may well explain our results. Reabsorption of calcium salts from bone appears to involve dissolution of whole bone tissue including the organic matter and it seems generally agreed that in the reabsorption of bone which accompanies normal growth

the organic component, which is principally collagen and the inorganic components are removed almost simultaneously (e.g. Kölliker, 1873; McLean & Bloom, 1940). When calcium salts are stored in or released from the skeleton in response to variation in intake of calcium in the diet, both components appear to change together (Bauer, Aub & Allbright, 1929; Bell, Cuthbertson & Orr, 1941; Walker & Arvidsson, 1954). Both components are also involved in the variations in calcium storage which occur in connexion with pregnancy and lactation (Ellinger, Duckworth, Dalgarno & Quenouille, 1952), and egg-laying (Bloom, Bloom & McLean, 1941). Bone reabsorption resulting from the administration of parathyroid extracts also seems to involve both components (Bauer *et al.* 1929; McLean & Bloom, 1941). The most potent cause of loss of material from bone appears to be immobilization and disuse. In this case also salts and organic matrix are lost together (Allison & Brooks, 1921).

There is no evidence to show why skin collagen should respond to protein depletion more readily than collagen elsewhere in the body. Little is known of the mechanisms controlling skin size, but it has been suggested (Billingham & Medawar, 1955; Abercrombie & James, 1957) that skin may grow in response to tension. The converse may be true: that a reduction in tension, as when weight is rapidly lost, may lead to regression in skin area and weight.

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SUMMARY

1. The effect of a protein-free diet on the collagen content of the body has been investigated in adult mice.

2. Collagen was found to form 2–3% of the wet weight of normal mice. Collagenous nitrogen formed 15–20% of the total nitrogen.

3. The experimental diet was given for 17–20 days and was found to produce a significant loss of collagen from the body, though this (10%) was smaller than the loss of body weight (45%) or non-collagenous nitrogen (35%).

4. Investigation of individual parts of the body (quadriceps femoris muscle, femur, viscera, skin and carcass) showed that the greater part of the loss of collagen (60%) was from the skin, which also lost a higher proportion (16%) of its original collagen than any other part.

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