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The Relative Metabolic Inertia of Tendon Collagen in the Rat

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The protein collagen, various polysaccharides and other substances have been known for many years to be present in the extracellular spaces of the body. Changes in these tissue components in disease have been demonstrated by pathologists, and recent clinical and anatomical studies on rheumatism and allied diseases have greatly stimulated further work on the 'ground substances' of the extracellular spaces. Very little is known, however, about the metabolism of these substances even in the normal subject. Information, therefore, on the metabolism of collagen would seem to be of interest. This protein is widely distributed throughout body tissues and is found in large amounts in bone, cartilage, tendons and skin. At an estimate, about one-third of the total mass of the protein of the mammalian body consists of collagen and it is thus important to establish whether this extracellular protein is, like most intracellular proteins, constantly broken down and resynthesized, even in the adult animal. Collagen is particularly rich in glycine, and glycine labelled with ^{14}C in the methylene group was therefore used to determine the turnover of collagen in three series of rats. For preliminary communication, see Perrone & Slack (1951).

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EXPERIMENTAL

General

Treatment of animals and administration of labelled glycine. Male albino rats of the Institute stock were used throughout. Series *A* consisted of four rats with body weight ranging from 358 to 390 g.; their ages were not known exactly, but all animals of this group were more than 14 months old. Series *B* consisted of five young adults (initial body wt. 203–207 g.), whilst series *C* consisted of five young rats with initial body weight ranging from 50 to 80 g. All animals were apparently in normal health and had not been used for other experiments. Throughout the experiment they were given the Institute stock diet. The labelled glycine, which was kindly supplied by Dr H. R. V. Arnstein, had an activity of 1 $\mu\text{C}/\text{mg.}$; the required dose was mixed with about 30–50 mg. non-isotopic glycine and fed in the diet over a period of 3 days at the beginning of each experiment. The rats of groups *A* and *B* each received 10 $\mu\text{C.}/100$ g. initial body weight. Three of the rats of group *C* (nos. 99, 100 and 104) received 40 $\mu\text{C.}/100$ g. initial body weight, whilst the other two (nos. 115 and 116) received 6.25 $\mu\text{C.}/100$ g. initial body weight. The animals were killed by stunning at varying times during the experiment.

Growth of animals. The old rats of group *A* showed no marked or consistent change in body weight (Table 1). The young adults of series *B* showed a steady increase of weight during the experiment, the rate varying somewhat with different animals (Table 2). The young rats of series *C* increased their body weight very markedly during the experi-

ment (Table 3). Since this matter is of considerable importance for the interpretation of the isotope results the actual growth curves of the last group are shown in Fig. 1.

Fig. 1. Growth curves of the young rats of series C (Table 3).

Preparation of samples

Liver and muscle. The livers and representative muscles from the four limbs were removed and finely minced. The samples were spread around the inner walls of flasks of 250 ml. capacity, with the aid of a little distilled water and freeze-dried. Drying was begun within 3 hr. of killing the animal. Free glycine in tissues was removed by repeated extraction of the dry samples with boiling 75% (v/v) ethanol; the samples were then defatted by ether extraction. Hydrolysis was carried out by boiling, under reflux, suspensions of the dry material (2 g. approx.) in 6N-HCl (approx. 100 ml.) for 48 hr. The solutions were taken to dryness and the residue dissolved in water. Treatment with 1:2:4-fluorodinitrobenzene was carried out by a slight modification (Perrone, 1951) of the method of Sanger (1945).

Collagen. The collagen studied in this investigation was all obtained from white tendons. These were dissected free of muscle fibre and fascia, and consisted of a mixed sample of about 1 g. (fresh weight) of cleaned tendon obtained from all four limbs, the back and tail of the rat. The method of purification was similar to that of Bowes & Kenten (1948) with the following additional procedure. After thorough cleaning by alternate and repeated extractions with distilled water and 10% (w/v) NaCl the collagen was refluxed with approx. 0.1% acetic acid (50 ml.) overnight. The residue of elastin was filtered off. For determinations of dry weight, the gelatin was precipitated by careful neutralization of the filtered acetic acid solution with ammonia, followed by addition of acetone (950 ml.). The solid material was centrifuged off and dried *in vacuo* over P_2O_5 . The purification of the collagen from some tissues presents difficulties (Lowry, Gilligan & Katersky, 1941), but rat tendon appears to yield pure collagen readily. The collagen obtained by the above

procedure gave negative reactions for aromatic amino-acids and showed no selective absorption between 257.5 and 287.5 m μ . (Loofbouroow, Gould & Sizer, 1949). Further evidence of purity is the very low specific activity found in the glycine obtained by hydrolysis of the collagen of the tendons of the old rats.

The purified gelatin from 1 g. fresh white tendons was hydrolysed by refluxing with 6N-HCl (50 ml.) for 24 hr. The solution was reduced to dryness, the residue taken up in water and the 2:4-dinitrophenyl (DNP) amino-acids prepared as described above.

Isolation of DNP-glycine. Glycine from the hydrolysates of liver, muscle and collagen was separated as the DNP derivative on Celite columns containing m-NaH₂PO₄ as the stationary phase and 10% (v/v) ether in CHCl₃ as the mobile phase. Rapid and effective separation of glycine is achieved by this method (Perrone, 1951). From the hydrolysate of 1 g. fresh tendon, amounts of crystalline DNP-glycine greater than 50 mg. were always obtained using a column containing approx. 50 g. Celite 545. Crystallization was effected by dissolving material obtained from the column in dioxan and adding hot cyclohexane. DNP-glycine so obtained appeared to be pure as shown by chromatographic behaviour, melting point which was 205° (uncorr.) and mixed melting point.

Radioactivity measurements

The specific radioactivities of the DNP-glycine samples were determined with a bell-shaped helium-filled Geiger-Müller counter having a thin mica window. The counter has an unusually low background count of 11–13 counts/min. The measurements were carried out on solid samples placed on disks of identical geometry and 2 sq.cm. area. The material was spread evenly by the pellet technique (Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949; Popják and Beekmans, 1950). Each sample contained at least 25 mg./sq.cm.; under such conditions of infinite thickness, the number of counts is linearly proportional to activity. No correction for self-absorption was made. Results are expressed as counts/min./2 sq.cm.

RESULTS

Changes in the specific activities of glycine obtained from the mixed proteins of liver and muscle

The glycine obtained from liver protein showed a very high activity 24 hr. after the feeding of the labelled amino-acid had been completed. This activity decreased very rapidly over the following 35–50 days (Tables 1–3). In the young adults (Table 2) the isotope content decreased over the first 3 weeks by a factor of approx. 2.5/week, but it appears from the rather scanty data that the activity-time curve of the liver glycine flattened out later. In the young rats (Table 3) the activity fell more sharply with time than in the older animals. This is presumably associated with the rapid rate of growth which was found with these animals. This point will be discussed later.

The radioactivity of the glycine obtained by hydrolysis of the mixed proteins of muscle showed,

Table 1. *Specific radioactivities of 2:4-dinitrophenylglycine samples obtained from liver, muscle and tendon collagen of old rats fed $\text{NH}_2^{14}\text{CH}_2\text{COOH}$*

Rat no.	Body weight		Dose of ^{14}C -labelled glycine/100 g. body wt. (μC .)	Duration of experiment (weeks)	Specific activities (counts/min./2 sq.cm. disk, infinite thickness) of DNP-glycine samples of		
	Initial (g.)	Final (g.)			Liver	Muscle	Collagen
101	373	355	10	7	184*	348*	15
102	385	380	10	7			—
105	358	371	10	3			24
106	390	370	10	3	505	493	12

* Pooled samples.

Table 2. *Specific radioactivities of 2:4-dinitrophenylglycine samples obtained from liver, muscle and tendon collagen of young adult rats fed $\text{NH}_2^{14}\text{CH}_2\text{COOH}$*

Rat no.	Body weight		Dose of ^{14}C -labelled glycine/100 g. body wt. (μC .)	Duration of experiment (days)	Specific activities (counts/min./2 sq.cm. disk, infinite thickness) of DNP-glycine samples of		
	Initial (g.)	Final (g.)			Liver	Muscle	Collagen
1	234	237	10	1	3700	698	46
2	233	242	10	7	1414	603	68
3	203	229	10	14	550	422	50
4	206	252	10	21	286	454	127
5	205	236	10	35	148	383	151

Table 3. *Specific radioactivities of 2:4-dinitrophenylglycine samples obtained from liver, muscle and tendon collagen of young rats fed $\text{NH}_2^{14}\text{CH}_2\text{COOH}$*

Rat no.	Body weight		Dose of ^{14}C -labelled glycine/100 g. body wt. (μC .)	Duration of experiment (weeks)	Specific activities (counts/min./2 sq.cm. disk, infinite thickness) of DNP-glycine samples of			Activities (counts/min./2 sq.cm. disk, infinite thickness) corrected for dose ^{14}C of 10 μC /100 g. body weight		
	Initial (g.)	Final (g.)			Liver	Muscle	Collagen	Liver	Muscle	Collagen
115	79	115	6.25	1	722*	594*	428	1155*	950*	685
116	80	97	6.25	1			376			601
104	50	119	40	3	592	799	947	148	200	237
99	50	246	40	7½	88	308	526	22	77	131
100	50	160	40	8½	—	—	773	—	—	193

* Pooled samples.

— Means not determined.

like that of liver, a steady fall with time in all three groups of rats (Tables 1–3). In group *B* the initial value was only about 20% of that of the glycine of the liver, but the rate of decrease was very much slower so that after 2–3 weeks the activity of the glycine of muscle equalled that of the glycine of liver, and with animals killed 35 days or more after the beginning of the experiment the activity of the former always exceeded that of the latter by a large amount. The specific activity of the glycine from muscle protein like that from liver protein, decreased more sharply in rapidly growing animals than in adults.

Changes of specific activity of the glycine obtained from the collagen of the tendons

The glycine isolated from the collagen of old rats after 3 and 7 weeks showed a very low radioactivity (Table 1). In young adults the activity was slightly

higher, but still very low, compared with that of the glycine of muscle or liver protein. Unlike the latter, the activity of the glycine of the collagen increased slowly during the experiment (Table 2). With the very young rats, on the other hand, initial activity was very high, only slightly lower than the corresponding value for muscle and about half that for liver (Table 3). In this experiment the activity decreased with time in marked contrast to the young adults.

Effect of growth on the changes of specific activity with time

The young rats used in the third series grew rapidly during the experiment, whilst those of the second series were older and grew only at a low rate. The changes in body weight of the old rats were small and probably due entirely to changes in fat or water content of the body. The considerable differences

found between the three groups of rats especially with respect to collagen, can be entirely explained in terms of growth. During the period while the labelled glycine is being fed and for a short time afterwards, glycine with a high isotope content is incorporated into body proteins. Later, however, the radioactivity of the glycine available for protein synthesis decreases rapidly and, at least in the rat, becomes negligible within a few days compared with the initial radioactivity. If the newly formed protein is metabolically inert like the haemoglobin of the red cell, the radioactivity of the protein will then become constant, provided no growth takes place. If, however, the net amount of the protein increases throughout the experiment, the initial radioactivity will fall, owing to dilution by deposition of new protein of rapidly decreasing isotope content. If the protein under investigation is not metabolically inert, this fall of activity, which is specifically associated with growth, will be superimposed on the decrease of isotope content which is caused by the workings of normal catabolic and anabolic processes. For a correct evaluation of this 'growth dilution' effect, it would be necessary to know the exact increase in the particular protein and also the changes of specific radioactivities of the glycine used for protein synthesis throughout the experimental period. Such data do not exist, and we have therefore assumed that the net increases in muscle protein, liver protein and collagen are linearly proportional to increase in body weight. We have further assumed that the isotopic material is introduced into the body over an infinitesimally short period at the beginning of the experiment and that the isotope introduced into the protein later can be neglected. The specific activities found were therefore multiplied by the ratio of final to initial body weight and the corrected values thus calculated are shown in Table 4. This very simplified treatment is unlikely to give accurate results, but may yield an indication of the order of magnitude of

this effect of 'growth dilution' on activity-time curves.

Table 4 shows that the rate of decrease of activity of the glycine of liver and muscle protein is more or less the same in all three groups of rats, i.e. the apparently greater decrease in young rats as compared with older animals, can be ascribed to a net increase of protein involving dilution with material of low isotope content. The activity of the glycine of collagen, on the other hand, decreases either not at all or only slightly in these young rats and the decrease observed in the uncorrected values is almost entirely due to 'growth dilution'.

DISCUSSION

The turnover rates of liver and muscle proteins

Schoenheimer, Rittenberg and their colleagues, using amino-acids labelled with ^{15}N , have carried out extensive investigations on the turnover rates of the mixed proteins of various organs of the rat (see, for example, Ratner, Rittenberg, Keston & Schoenheimer, 1940; Shemin & Rittenberg, 1944). Our data on liver and muscle extend somewhat the results of the American workers. The data on the specific activities of the glycine obtained from the mixed liver proteins of the young rats fit, apart from the last figure, reasonably well the equation

$$\ln i_t = A - kt,$$

where i_t is the activity (in counts/min.), t the time (in days) and A and k constants. The best values for A and k are 8.36 and 0.145, respectively. From this the half-life $t_{20.5}$ can be calculated to be about 4-5 days. Values obtained later than 21 days do not fit a logarithmic curve and the activities are higher than expected. This is probably partly caused by the heterogeneous character of the liver proteins; collagen in particular with its high glycine content may retain the labelled amino-acid for long periods

Table 4. *Results of experiments (Tables 1, 2 and 3) corrected for increase in body weight and reduced to a dose of 10 μc /100 g. body weight, expressed as counts/min./2 sq.cm. disk, infinite thickness*

(For details of the correction, see text.)

Time (days)	Old rats			Young adults			Young rats		
	Liver	Muscle	Collagen	Liver	Muscle	Collagen	Liver	Muscle	Collagen
1	—	—	—	3700	698	46	—	—	—
7	—	—	—	1470	626	70	1530	1188	852
14	—	—	—	623	478	58	—	—	—
21	{ 505 466	{ 493 454	{ 12 24	350	555	159	391	480	564
35	—	—	—	170	441	174	—	—	—
49	184	348	{ 15 17	—	—	—	—	—	—
53	—	—	—	—	—	—	109	379	645
60	—	—	—	—	—	—	—	—	618

— Means not determined.

and cause a tailing of the activity-time curve. Another complicating factor is the reversible transfer of amino-acids between tissues of high turnover rate such as liver and those of low turnover rate, like muscle, a point already emphasized by Shemin & Rittenberg (1944). Since muscle has a higher activity than liver in the later stages of the experiment, such a transfer of labelled glycine would reduce the rate of decrease of isotope in muscle in the early stages of the experiment and in liver in the later stages.

The data for muscle fit a logarithmic curve rather badly, but very approximate values for k can be calculated. These are between 0.023 and 0.029, corresponding to half-life periods of 24–30 days. It is probable, and this is also suggested by unpublished observations of Sprinson & Rittenberg (1949), that muscle proteins are metabolically very heterogeneous and average half-life figures have therefore not much theoretical significance in this case. It is of interest that the activity-time curves of muscle and liver glycine cross at about 20 days. The fact that the activity of the sample isolated after 5 weeks was only 40 % lower than that found after 1 day (Table 2) shows that the radioactive glycine is retained in the muscular tissue for a relatively long time. Shemin & Rittenberg (1944) analysed glycine obtained from the combined skin, muscle, tendons, etc., and found a steadily increasing isotope content. Since their 'carcass' fraction contained considerable amounts of collagen and since their rats were still growing and therefore deposited new collagen, the observed increase in isotope content was almost certainly due to the glycine derived from collagen.

The turnover of tendon collagen

All the results reported in this paper are compatible with the assumption that collagen, once it is deposited in the intercellular spaces, becomes metabolically inert and is, if at all, only very slowly degraded in the normal animal. That in the young rat dietary glycine is utilized by the fibroblasts for collagen formation is shown by the high initial activities found in the rapidly growing rats and the moderate activities observed in the slowly growing young adults. The reasons for the decrease in specific activity of the collagen glycine in the young rats have already been discussed, but it remains to consider the causes for the steady increase found with the young adults. In growing rats there are two processes which oppose each other in their effects on activity changes in the collagen. On the one hand, the glycine circulating in the body and available for protein synthesis after the feeding of the labelled compound has been discontinued, has a significant, but rapidly diminishing radioactivity and the total isotope content of the glycine of the collagen is bound to increase, provided the deposited collagen is metabolically inert. But, if

the animal grows rapidly, the rate of increase of total collagen may be greater than the rate of increase of total radioactivity. In such a case the specific activity of the glycine will decrease and so explain the fall found with the uncorrected values of young rats (Table 3). If, on the other hand, growth is slow, the specific activity of the newly formed collagen may be higher than that of the collagen already deposited and thus specific activity will increase until the activity of the 'free glycine' of the body has decreased to that of the glycine residue in collagen. This would explain the rise of activity in the slowly growing young adults. The very slight activities found in the old rats (Table 1) cannot be explained by general growth, but it is likely that the collagen in certain organs increases in these old animals. Thus the collagen content of rat muscle (Lowry, Hastings, Hull & Brown, 1942) and also that of the aorta (Myers & Lang, 1946) increases appreciably on ageing.

But the present results by no means exclude the possibility that the collagen of the tendon participates to some extent in the dynamic processes of the body. In old rats the turnover rate must be extremely small, corresponding to a half-life period of several hundred days and of a similar order of magnitude as the normal life span of the rat. With young rats, such a turnover may be somewhat faster and the fact that the 'corrected' isotope contents of the young rats (Table 4) do not increase with time, like those of the young adults, indeed suggests that the collagen of the tendon is not completely inert in very young rats. The possibility that collagen changes in its metabolic behaviour with the age of the animal is suggested by the physical differences which have been observed (Lloyd & Marriott, 1935; Leplat, 1935) in collagen samples obtained from the tendons of young and old animals respectively.

It is impossible with the data available to decide as to which of the alternative interpretations is correct. But in any case the present results clearly indicate that the collagen of the rat tendon is metabolically inert relative to the mixed proteins of liver, other internal organs and even of muscle. It would be unjustified to assume that the collagens of the skin and bone behave in a manner identical with that of the tendon, but preliminary experiments indicate that the turnover rate of skin collagen is at least quite low. It is therefore probable that all extracellular proteins are very much less active metabolically than the intracellular proteins which predominate in liver, kidney, etc., and also in muscle. Sprinson & Rittenberg (1949) have emphasized recently the limitations of the concept of the dynamic state of body constituents as applied to some of the proteins of man and to a lesser extent to those of the rat. If the generalization is correct, that all extracellular proteins resemble collagen of the tendon, it would

follow that about 40 % of the total protein of the rat does not participate to an appreciable extent in the reversible and balanced processes which characterize the dynamic state of the body. It would also imply that about 75 % of the total glycine, a large part of the proline and almost all the hydroxyproline in the normal adult rat are in a static state.

The present results have some bearing on the question of health hazards involved in handling radioactive compounds which may become incorporated into the body proteins. Ingestion of radioactive glycine, for example, will result in high initial radioactivities in the proteins of the liver, intestine and organs of similar metabolic type. However, owing to the rapid metabolism of the proteins of these organs, such high activities will only be maintained for short periods, unless exposure is prolonged. The labelled compound is likely to be incorporated into muscle proteins to a smaller extent than into the liver proteins, but will be retained for relatively long periods. No appreciable entry into the collagen of the adult is likely to occur, but in the young growing subject the radioactive compound may become fixed in the extracellular proteins for a very long time. This would constitute a serious potential hazard peculiar to the child and adolescent.

SUMMARY

1. Glycine labelled with ^{14}C in the methylene position was fed to three series of rats, old rats, young slowly growing adults and rapidly growing young ones. The animals were killed at varying intervals of time and samples of liver, muscle and of

collagen from white tendons were obtained. 2:4-Dinitrophenylglycine from these three sources was crystallized after chromatographic separation and the radioactivity determined.

2. The specific activity of glycine from liver protein showed a very high initial activity which decreased rapidly during the subsequent 35–50 days. The results indicate that the half-life of the mixed liver proteins is 4–5 days. With muscle the initial activity was lower than in liver and there was a steady but slow fall with time. After 2–3 weeks the activity of muscle glycine equalled that of liver and later exceeded it.

3. The glycine from the collagen of old rats showed a very low radioactivity. In young adults the activity was slightly higher, but still very low compared with that of the glycine of muscle or liver. There was a slow increase of activity with time. With the very young rats initial collagen activity was very high, only slightly lower than the corresponding value for muscle and about half that of liver. The activities found in the young rats decreased with time in marked contrast to the young adults.

4. The results indicate that tendon collagen in the old rats is metabolically almost completely inert. In young rats interpretation is complicated by growth, but when correction is made for this factor it appears that collagen is relatively inert even in young actively growing animals, although the possibility cannot be excluded that the turnover rate of collagen is somewhat faster than in older animals.

5. It is pointed out that radioactive compounds may be deposited in the extracellular spaces of young subjects where they may remain for long periods.

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