STUDIES ON THE FORMATION OF COLLAGEN

II. The Influence of Growth Rate on Neutral Salt Extracts of Guinea Pig Dermis*

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The collagen fraction extracted from fresh connective tissues with cold neutral salt solutions appears to represent a stage in the development of this protein which precedes the laying down of fibrils. Isotope incorporation studies indicate a relatively high turnover rate for this fraction as compared to acid-soluble and insoluble collagen (1, 2). The collagen extracted with neutral salt solutions may be readily polymerized *in vitro* to typical cross-striated fibrils simply by warming (3–5).

Preliminary investigations on the influence of vitamin C deficiency on the neutral salt-extractable components of guinea pig skin were complicated by the observation that the growth rate of the animal profoundly influenced the amount of collagen extracted (6). This paper deals with a study of the effect of different growth patterns of guinea pigs on the compositions and properties of cold neutral salt extracts of fresh skin. Growth rate is measured as weight gain.

The rate of growth was manipulated by caloric intake alone. Fig. 1 provides examples of the four types of growth patterns studied. One group of animals was forced to lose weight for varying periods by fasting after a control period of active growth. Another group was maintained at constant weight by dietary restriction after an active growing period. A third group was allowed to gain weight rapidly for varying times after a period of static weight and a fourth group of actively growing animals served as controls.

Experimental Methods

Guinea pigs of both sexes were fed a standard diet of Purina rabbit chow supplemented with 50 gm. of lettuce daily with free access to water. All animals received 25 mg. of ascorbic acid by mouth 3 times weekly except when otherwise stated.

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Weight regulation experiments were performed as follows:—

Group A (symbol ⁄). Normally growing controls fed *ad lib.* There was some daily irregular fluctuation in weight gain in all but a few animals; however a general increase was maintained over a period of 14 days for all prior to sacrifice.

Group B (symbol ⁄^). Normal weight gain on *ad lib.* diet for 14 days followed by weight loss induced by diet restricted to water and 50 gm. of lettuce daily for 2, 4, and 6 days.

Group C (symbol ⁄—). Normal weight gain for 14 days followed by static weight for 14 days maintained by reducing caloric intake to 12 to 17 gm. plus 50 gm. of lettuce.

Group D (symbol ⁄—⁄). Growth was manipulated as for Group C, then at end of 14 day static weight period groups of animals were fed *ad lib.* for 3, 5, 7, 9, and 12 day periods before sacrifice.

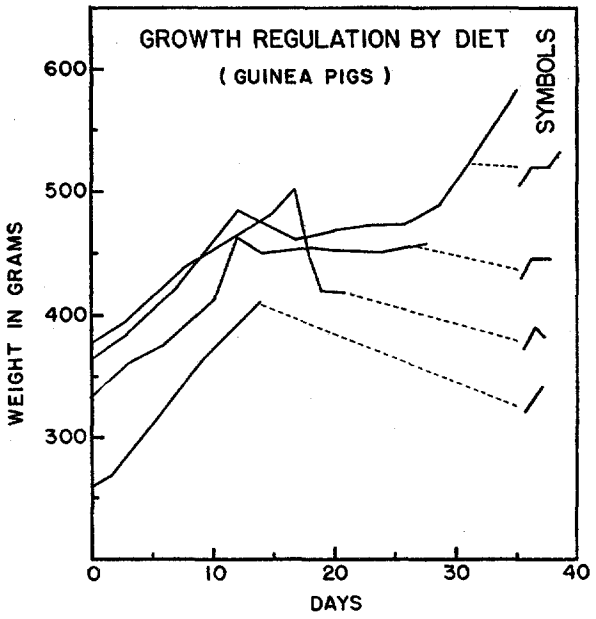

FIG. 1. Individual protocols of typical growth curves for each type of experiment

Group E (symbol $_0{}^8$⁄). Suckling animals growing steadily from birth, sacrificed on 8th day. Weights measured for last 6 days.

Group F (symbol $_0{}^8$⁄^$_2$). Suckling animals were separated from mothers after 8 days of continuous growth from birth and fed water and lettuce plus 10 mg. ascorbic acid daily per os.

Table I provides detailed protocols for each experiment.

Preparation of tissues and extracts and the analytical procedures are described in the preceding paper (5). The extracting medium used was 0.45 M NaCl, 2 volumes per gm. of wet tissue. The ground dermis of each of 8 animals of group A (⁄) and 4 of group C (⁄—) was divided in half and one portion extracted with 0.45 M NaCl and the other with 0.14 M NaCl.

At least one extract from each group was examined in the Spinco model L analytical ultracentrifuge (two samples were examined simultaneously by using a wedge cell window) and in the Perkin-Elmer electrophoretic apparatus at 3°C. after dialysis against veronal citrate-NaCl, pH 8.6, $\mu = 0.2$. Viscosities were measured in an Ostwald viscometer (flow time 60 seconds at 5 ± 0.1°).

Water and collagen content were determined in samples of fresh dermis from each animal by drying in an Aberhalden chamber *in vacuo* at 108°C. for 24 hours and analyzing the dried tissue for hydroxyproline.

RESULTS

The rate of increase in wet weight of the dermis is about 5 per cent of that of the body weight over a range of 60 to 700 gm. (Fig. 2). The dry weight

TABLE I

Details of Regulated Growth Experiments

Group and symbol	Duration of growth stages	Numbers of animals	Range of weight change in final stage	Range of final weight
	days		*gm./day*	*gm.*
A	14	9	(+)5.7-8.8	319-531
B	14, 2	5	(−)21-54	216-485
	14, 4	2	(−)6.5-17.2	418-504
	14, 6	2	(−)17.0	323-397
C	14, 14	6	$< \pm 1.0$	229-458
D	14, 14, 3	4	(+)6.5-12.5	215-420
	14, 14, 5	5	(+)8.5-13.6	349-420
	14, 14, 7	4	(+)7.0-10.3	286-466
	14, 14, 9	6	(+)8.0-13.0	514-650
	14, 14, 12	3	(+)8.1-13.9	514-650
E	8	11	(+)4.5-11.5	78-172
F	8, 2	8	(−)7.0-13.5	76-144

is relatively constant over the same range of body weight. The data include animals of all the growth categories.

The mean collagen concentration and standard deviation for 32 animal skins representing all types of experiments was 50.8 per cent ± 17.2. The differences between experimental groups were not statistically significant. This relatively broad spread can probably be accounted for by the difficulty in uniformly removing epidermis, fat, subcutaneous tissue, etc.

A. Correlation of Growth Rate with Extractable Collagen.

A summary of the data in terms of viscosity and hydroxyproline concentration for the 0.45 M NaCl extracts in each experimental series is presented

in Fig. 3. Simple inspection reveals that viscosity closely parallels hydroxyproline concentration independently of the growth pattern, a relationship clearly seen in Fig. 4 in which all the hydroxyproline-viscosity data obtained in these experiments are plotted. The amounts of collagen are markedly reduced in the extracts of all animals not gaining weight. There does appear to be a minimum extractible hydroxyproline value; 24 μg./ml. (344 μg. collagen/gram wet tissue) was the lowest value, observed in an animal fasted for 7 days with loss of more than ⅓ of the body weight. This small amount of collagen is represented by the low, slow moving, poorly diffusing boundary present in the electrophoretic and ultracentrifuge patterns (see Figs. 6 and 7).

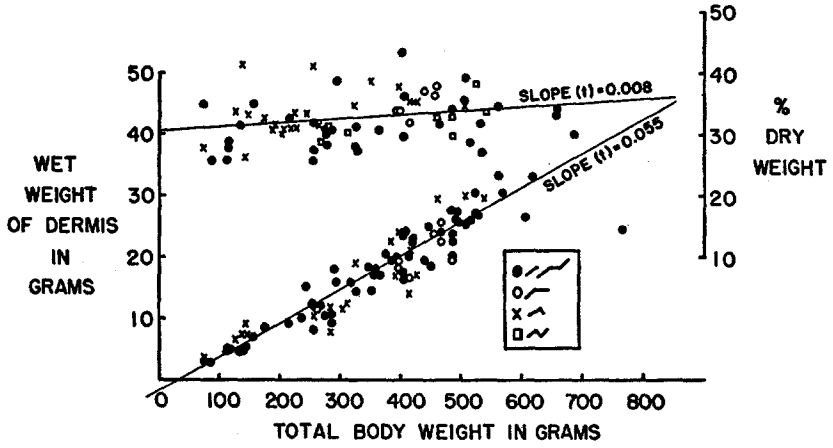

FIG. 2. Total wet weight of dermis and percentage dry weight are recorded as functions of body weight of animals used in all experiments. The lower line of data refers to the wet weight ordinate. †, line drawn by method of least squares.

Two days of weight loss reduced the collagen content of the extracts considerably as indicated in Figs. 3 and 6. That age is not an active factor, at least through young adulthood, is illustrated by comparing the last two columns of Fig. 3 (groups E and F) with the first two columns (groups A and B). Two days fasting of suckling animals 8 days old (about 120 gm. in weight) induced a reduction of salt-extractible collagen in the extract to the same levels as that similarly induced in 12 week old animals (about 600 gm.).

The amount of extractible collagen obtained from animals of group D (^{3}) approximated the low levels for the restricted animals of group C ( ). Five to seven days of renewed *ad lib.* feeding was required for the reappearance of appreciable amounts of salt-extractible collagen (Fig. 3).

There was considerable variation in the amount of collagen extracted from the actively growing normal controls, young adults as well as 8 day old ani-

mals. However, the lowest values never approached those induced by short periods of fasting in either group. No correlation was found ($P > 0.1$) between weight gain and extracted hydroxyproline for animals above 300 gm., whereas there was significant direct correlation ($P < 0.01$) for the 8 day old suckling animals (Fig. 5). The dotted portion of the curve in Fig. 5 is derived from the fact that a small amount of collagen is always extractible even in animals losing much weight, suggesting that the roughly linear relationship

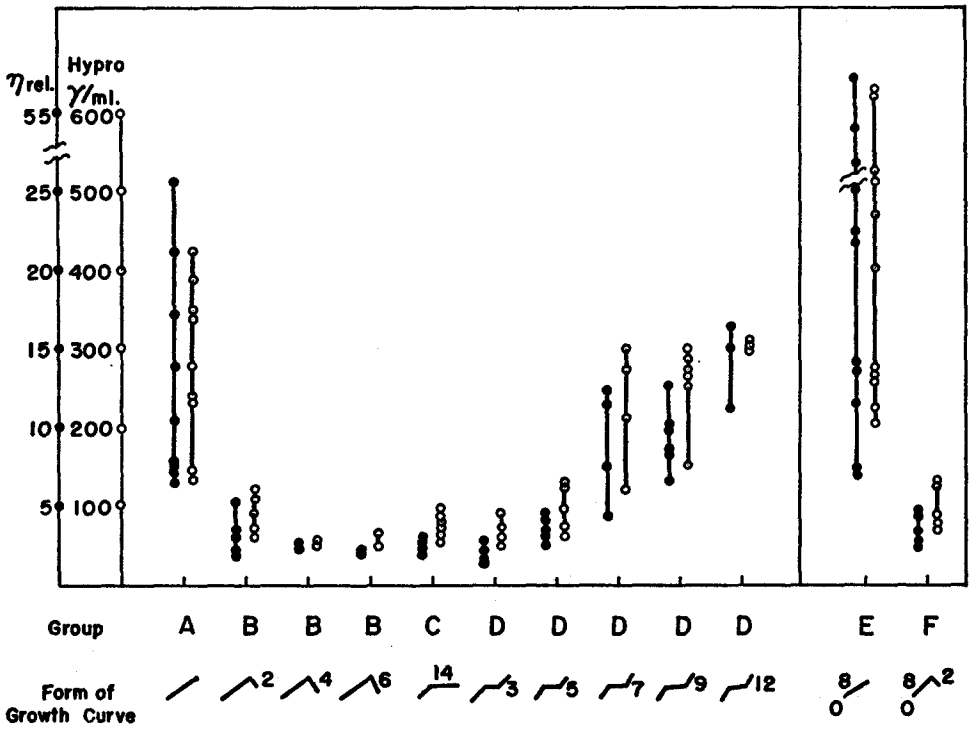

FIG. 3. Relative viscosity and hydroxyproline (Hypro) content of all the extracts in the different growth manipulation experiments represented symbolically along the baseline.

in growing animals becomes non-linear and levels out at zero or negative growth. Examination of the growth charts revealed a day to day fluctuation in weight gain for the more mature controls in most cases, whereas the daily gain in weight for the suckling animals was relatively constant for each individual. It seems likely that the effects of daily fluctuations in growth rate in the older animals caused the large variations in salt-extracted collagen, even though average growth rates may have been comparable.

Fig. 6 compares the electrophoretic patterns, relative viscosities, and hydroxyproline values of extracts of the skins of representative animals of

groups A (/) and B (/\). The major change in the electrophoretic pattern is a great diminution in the height of the slow moving collagen peak as a function of weight loss. The fall in viscosity and hydroxyproline content correlates well with this alteration. Ultracentrifuge diagrams also illustrate the same diminution in the slow moving, hypersharp boundary. This type of change is illustrated in Fig. 7, in which the sedimentation patterns of representative extracts of the pelts of an animal of group A (/) and group C (/⌒) are compared.[1] In this instance, comparison is also made between 0.14 M extracts obtained from separate portions of the same skins. The difference

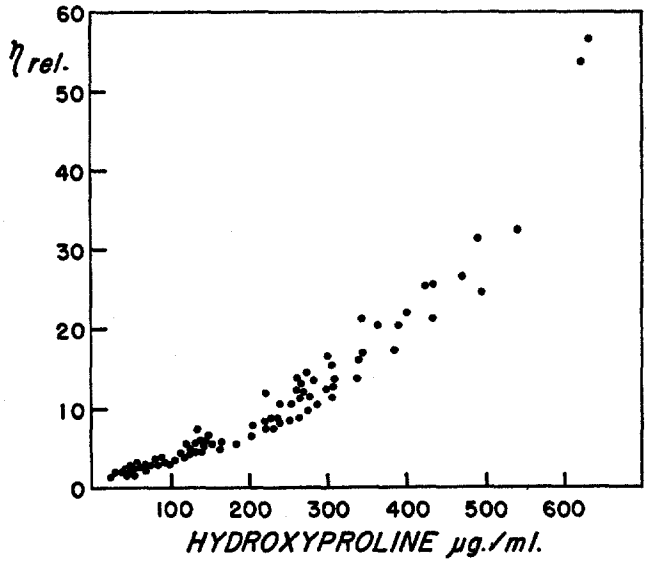

FIG. 4. Hydroxyproline concentration of extracts as a function of viscosity. Each point represents a single extract from one animal. All animals used in dietary manipulation experiments are represented here.

between the extracts from growing and non-growing animals are similar for the two different extracting media.

B. Total Amount of Neutral Salt-Extractible Collagen Obtained from the Dermis of Actively Growing Guinea Pigs and Those Starved for 2 Days.—

All the data reported thus far was obtained from single extracts. In order to determine how much of the skin collagen is removed in cold saline extracts, the fresh ground tissues of two actively growing guinea pigs each weighing 450 gm. were repeatedly extracted for 16

[1] The hypersharp, slow moving peak in group A (/) extracts (0.45 M) appears as a vertical bar running the full depth of the pattern. At lower concentrations, as in the 0.14 M extract in the upper half of the picture, the peak that remains still diffuses poorly.

hour periods with 2 volumes of 0.45 M NaCl at 3°C. and the extracts analyzed for hydroxyproline. The liquid was separated from the tissue by centrifugation and clarified by filtration as described earlier. Extraction was repeated until hydroxyproline was no longer measurable in the solution. Methiolate, 1:1000 was added to the media to suppress bacterial growth.

A total of 7 and 8 per cent of the total skin collagen was extracted. The first extracts contained 15 and 20 per cent of all the collagen removed.

To obtain a measure of the total amount of neutral salt-extractible collagen in normal growing animals as compared to the amount obtainable from

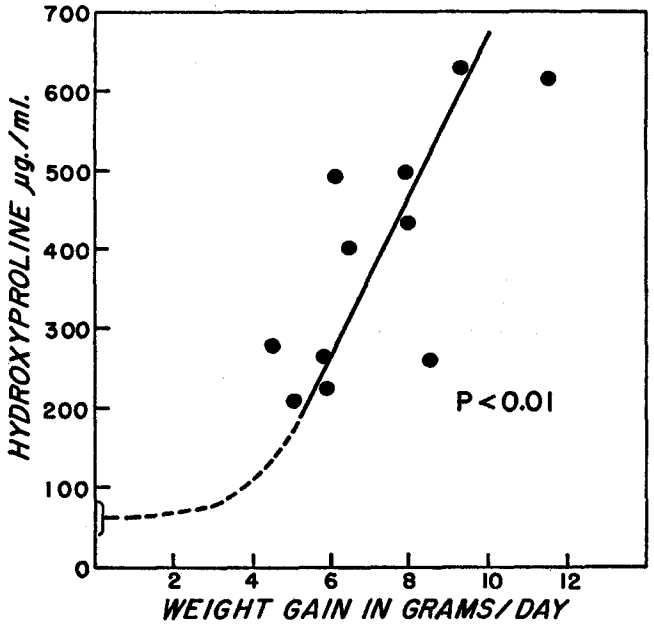

FIG. 5. Hydroxyproline concentration of extracts as a function of weight gain for eleven 8 day old animals. Dotted portion of curve is suggested extrapolation to minimum hydroxyproline values found in non-growing guinea pigs.

animals losing weight, repeated daily extractions were performed on the pelts of three suckling animals of group E (0%) and three of group F (0%↘) which had been fasted for the last 2 days and had lost an average of 12 gm. per day). All were given lettuce, water, and 10 mg. vitamin C daily.

Total collagen of the corium was determined by hydroxyproline analysis of dried samples using a conversion factor of 14 per cent.

Fig. 8 details the course of the series of extractions. The three periodically spaced peaks, in amount of extracted hydroxyproline, represent 48 hour extraction periods in contrast to the usual 24 hours.

The percentage of the total skin collagen removed from each of the three animals in each group is summarized in Table II. Twenty-one extractions

were required for the control animals and seventeen for the fasted guinea pigs. The data are broken down into values for the first extract, the first three, and for the sum of all.

An average of 46 per cent more collagen could be removed with 0.45 M NaCl from group E ($_0^s\diagup$) animals than from group F ($_0^s\diagup^2$).

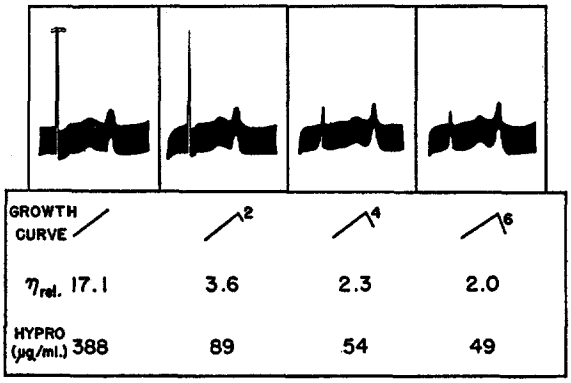

FIG. 6. Effect of different periods of fasting on the neutral extracts as evidenced by electrophoresis, viscosity, and hydroxyproline (Hypro) determination. Four individual and representative protocols.

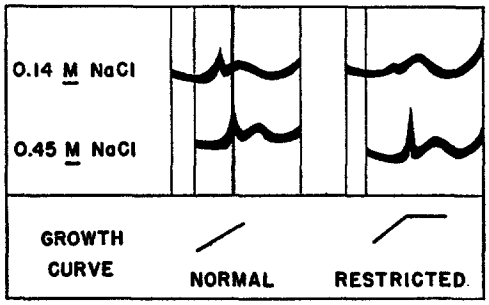

FIG. 7. Ultracentrifuge patterns of 0.45 M and 0.14 M NaCl extracts (simultaneous double run using a wedge cell window), comparing those from a normally growing animal with those from one whose weight was maintained constant for 14 days.

C. Comparison of Amounts of Collagen Extracted from Skins of Normal ($_0^s\diagup$) and Fasted ($_0^s\diagup^2$) Guinea Pigs in Neutral and Acidic Media.—

In an attempt to further assess relationships between neutral salt-extracted collagen and that extracted by citrate buffers at low pH and by dilute acetic acid, those tissues remaining after the exhaustive extractions described in the preceding section were treated as follows:

The six residues were dialyzed free of NaCl and each reextracted three times with 5 volumes (*v/w*) of citrate buffer 0.1 M, pH 3.5 as described by Orekhovitch *et al.* (7). The cloudy non-

viscous extracts were clarified in the Spinco model L preparative ultracentrifuge at 50,000 *g* for 3 hours and analyzed for hydroxyproline. Relative viscosity was never above 3.0. After three extractions each residue was dialyzed to remove citrate and reextracted 2 times with 15 volumes (*v/w*) of 0.5 M HAc, pH 3.5 (five volumes of medium was completely absorbed by the tissue). The extracts were clarified and analyzed as described above.

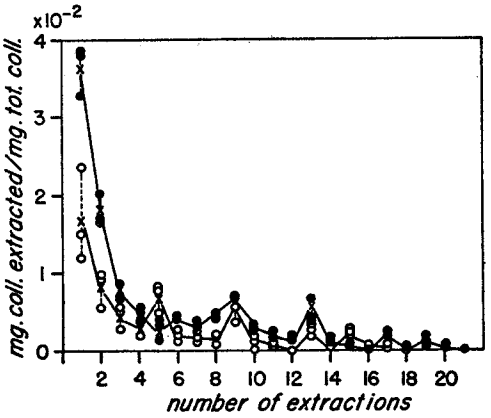

FIG. 8. Fraction of total skin collagen removed in each of a series of successive extractions from the skins of three normally growing animals as compared to three animals of about the same age which had been fasted for 2 days. ●, normal ($_0^8\!/$), ○, fasted 2 days ($_0^8\!/\!\!\wedge_2$)

TABLE II

Per Cent of Total Skin Collagen Extracted

Type of extraction	Normal growth ($_0^8\!/$)				2 day weight loss ($_0^8\!/\!\!\wedge_2$)			
	1	2	3	Average	4	5	6	Average
NaCl 1 ×	3.8	3.5	3.7	3.7	2.3	1.2	1.5	2.4
3 ×	6.2	5.6	6.3	6.0	3.8	2.0	2.9	2.9
17–21 ×	11.0	9.3	10.5	10.3	6.8	4.0	6.1	5.6
Citrate 3 ×	2.3	3.3	1.2	2.4	1.8	0.9	1.8	1.5
Acetic acid 2 ×	4.0	3.6	3.0	3.5	6.0	2.9	4.4	4.4

The per cent of total dermis collagen removed in this series of extractions are also summarized in Table II.

The amount of collagen removed by citrate in three extractions was less than half that removed in three NaCl extracts for both groups. However, this acidic medium removed an average of 40 per cent more collagen from the actively growing animal tissue than from that of the fasted group. The amounts of collagen removed in each of the three extracts was about the same. Two extractions with acetic acid removed more collagen than did three citrate extractions but less than the first NaCl extract. Twenty per cent

more collagen was removed from the group F ($_0^{5}/_{2}$) animals than from controls ($_0^{5}/$). The second acetic acid extracts contained much less collagen than did the first. It is interesting to note, however, that the sum of the fractions of collagen removed in both acidic media is the same for both groups of animals.

D. Relationship of Collagen and Non-Collagenous Components to Viscosity.— It is evident that the viscosity of the extracts is directly related to the collagen content and this in turn is dependent upon growth of the animal.

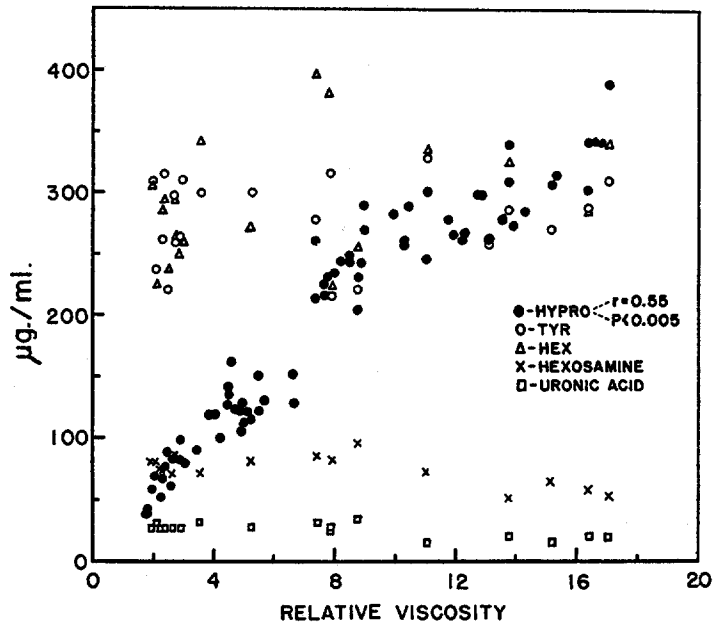

FIG. 9. Scatter diagram of concentrations of hydroxyproline tyrosine, hexose, hexosamine, and uronic acid as a function of extract viscosity.

However, examination of the changes with growth of the non-collagenous protein and carbohydrate seemed in order. To this end, tyrosine, hexose, hexosamine, and uronic acid were measured in dialyzed extracts of a number of representative animal skins in each group as indices of concentrations of these substances. Fig. 9 is a scatter diagram of the data obtained from skin extracts of some of the animals from all groups, plotted in μg./ml. as a function of relative viscosity of the extracts. It is evident on simple inspection that no correlation with viscosity exists for any of the indices other than hydroxyproline. There is nearly linear correlation between hydroxyproline concentration and relative viscosity. Values above ηrel. = 20 are not recorded. Similar correlation was observed for glycine and proline.

DISCUSSION

The diminution of neutral salt extractible collagen following restriction of total caloric intake may be explained either by polymerization in the tissue to insoluble fibrils or removal by metabolic degradation. The fact that the extracted collagen may be polymerized *in vitro* to typical banded collagen fibrils by warming to body temperature (3, 5) favors the FORMER possibility. Caloric restriction, or the resulting cessation of growth, may interfere with synthesis, thereby causing depletion of this collagen fraction. This hypothesis presumes that the precipitated collagen rapidly becomes insoluble in cold neutral salt solution. The presence of extractible collagen during active growth may mean that the rate of synthesis is greater than the rate of insolubilization.

It can be calculated that the amount of extractible collagen in the skin of an actively growing young guinea pig, about 10 per cent of the total, is almost equal to the daily increase in the dermal collagen. This estimation is based on the data obtained from a representative animal (No. 1, Table II) which weighed 148 gm. and gained 7 gm. per day. The estimated daily increment of skin collagen was 60 mg. and the total amount of extracted collagen was 66 mg. However, the observation that about 50 per cent of the extractible collagen is lost in 2 days of fasting may mean that perhaps 25 per cent of the daily increment of 60 mg. is unavailable as a result of continuous insolubilization. If this were actually the case, the value for total extractible collagen would include about 30 per cent more than the single day's increment.

The amount of neutral salt-extractible collagen in the skins of guinea pigs more than a year old is negligible, relative to that of young animals. However, this may not entirely reflect an age dependence but perhaps growth rate dependence, at least in part. Year old guinea pigs show no net growth. The fact that periods of fasting and weight loss reduced the amount of extractible collagen obtained from 1 week old (100 gm.) animals to the same low level as that found in partially fasted 12 week old animals (600 gm.) militates against age being the only factor.

At least 5 days of active weight gain, induced by refeeding after a period of static weight, is required before there is an increase of salt-extractible collagen. This lag period probably represents the time necessary for building a reserve of protein by increased synthesis if it is being simultaneously removed by polymerization to fibrils. However, it is of interest that it takes 5 to 7 days before significant amounts of collagen appear in healing wounds in guinea pigs (8, 9, 10) and in the carrageenin granuloma (2) and in implanted Ivalon sponge (11). The collagen-synthesizing mechanism may require this lag period to get started.

Orekhovitch and colleagues contend that the collagen extracted by citrate buffer at pH 3–4 is a precursor of the fibrils (7, 12) and is in fact identical with that extracted in neutral media (13). The physical-chemical characteristics of the molecules may be identical but their respective positions in the scheme of fibrogenesis may be quite different. Incorporation studies with labelled glycine indicates that the neutral and alkali extractible fractions represent an earlier stage in collagen formation (1, 2). The observation that citrate extraction destroys the architecture of the collagen fibrils whereas neutral salt solutions do not (3), suggests that the former dissolves collagen from the fibrils in contrast to the latter. Orekhovitch (14) has stated "In starving animals the amount of procollagen in the skin is a little above typical average values for normally fed animals." This stands in sharp contrast with the effect of fasting on neutral salt-extractible collagen. In this study, however, the amounts of citrate-extractible collagen obtained from the skin (after all the neutral salt-soluble collagen had been removed) of normal ($_0^8\diagup$) guinea pigs was about 40 per cent more than that found in the 2 day fasted animals ($_0^8\diagup\!\!\diagdown_2$). Unfortunately only three citrate extractions were done. However, there is a suggestion here that citrate-extracted collagen may follow the pattern of the neutral salt-extracted fraction. When one adds the acetic acid–extracted collagen to the citrate-extracted fraction, the difference between acidic extracts from normal and fasted animals vanishes. As proposed earlier by others (1, 2) it is possible that the collagen removed by citrate or other acid buffers may have been more recently deposited on the fibril than the acetic acid–extractible and insoluble portion of the fibrils. The neutral salt-extractible collagen may or may not be associated with the periphery of the fibril. There may not be any physical chemical or composition difference in the molecules of any of these fractions except that imposed by their states of aggregation. An acid extraction may remove all three fractions together except for the amount that would complex at acid pH with acidic high polymers free in the ground substance (5).

The amounts of extracted non-collagenous components containing hexosamine, hexose, uronic acid, and tyrosine are essentially uninfluenced by the changes effected by dietary manipulation to which the collagen component is so sensitive.

The invaluable technical assistance of Miss Betty Dumsha, Mr. Joseph Fitzpatrick, and Mr. John McKay are gratefully acknowledged.

SUMMARY

The total amount of neutral salt-extractible collagen in the skin of growing, suckling guinea pigs amounted to about 10 per cent of the total collagen of the dermis. This is roughly equivalent to a 1 to 2 day increment in dermal collagen incident to growth.

Fourteen days of static weight maintained by limited caloric intake reduced the neutral salt-extractible collagen to very low levels. Following this period, 5 to 7 days of steady weight gain induced by *ad lib.* feeding was required to produce significant increases in this collagen fraction. Return to control levels occurred within 12 days of continuous growth.

The amount of collagen extracted from the dermis of young guinea pigs with cold neutral salt solutions varied directly with growth rate (weight gain) and was greatly diminished after short periods of restricted caloric intake. Two days of fasting diminished the total extracted collagen by one-half.

Three consecutive extractions with citrate buffer pH, 3.5, of the residues remaining after exhaustive saline extraction removed 40 per cent more collagen from the skins of actively growing animals than from those of animals fasted for 2 days. However, subsequent extraction of residues with dilute acetic acid equalized the total amount of collagen extracted at acid pH from the two groups.

The viscosity of cold neutral extracts was unrelated to the concentrations of non-collagenous proteins and carbohydrates but varied directly with the collagen content.

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