17. Snedecor, G. W., *Statistical Methods,* Iowa College Press, Ames, 1956.

18. Harper, P. V., Jr., Neal, W. B., Jr., Hlavacek, G. R., *Metabolism,* 1953, v2, 69.

19. Lofland, H. B., Moury, D. N., Clarkson, T. B., Middleton, C. C., *Circulation,* 1964, v30, No. 4, Suppl. III, 20.

20. Swell, L., Field, H., Jr., Schools, P. E., Jr., Treadwell, C. R., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 651.

21. Zilversmit, D. B., Sweeley, C. C., Newman, H. A. I., *Circ. Res.,* 1961, v9, 235.

22. Goldman, D. S., Chaikoff, J. L., Reinhardt, W. O., Enterman, C., Dauben, W. G., *J. Biol. Chem.,* 1950, v184, 727.

23. Zilversmit, D. B., McCandless, E. L., Jordan, P. H., Jr., Henly, W. S., Ackerman, R. F., *Circulation,* 1961, v23, 370.

24. Zilversmit, D. B., Shore, M. L., Ackerman, R. F., *ibid.,* 1954, v9, 581.

25. Newman, H. A., Zilversmit, D. B., *ibid.,* 1959, v20, 967.

26. Swell, L., Field, H., Jr., Schools, P. E., Jr., Treadwell, C. R., PROC. SOC. EXP. BIOL. AND MED., 1960, v105, 662.

27. Hoffman, C. W., Master's Thesis, Bowman Gray School of Med., 1963.

28. Weinhouse, S., Hirsch, E. F., *Arch. Path.,* 1940, v30, 856.

Received July 17, 1964. P.S.E.B.M., 1964, v117.

Collagen Synthesis and Turnover in the Growing Rat Under the Influence of Methyl Prednisolone.* (29653)

M. E. NIMNI† AND L. A. BAVETTA

Department of Biochemistry and Nutrition, School of Dentistry, University of Southern California, Los Angeles

The physical as well as the chemical properties of collagen have been shown to change with age. The content of soluble collagen of skin decreases rapidly with age whereas its tensile strength becomes progressively greater. The literature in this connection has been reviewed by Harkness(1).

Following injection of labeled glycine, the specific activity of skin collagen was greater in young animals than in older ones(2,3,4), reflecting the increased synthesis during the period of rapid growth. Growth retardation induced by dietary as well as hormonal imbalance were significant factors in reducing the rate of skin collagen synthesis as judged by a decreased radioactive uptake(3,5).

Experiments following administration of C^{14}-proline indicate a turnover time of more than 150 days for the insoluble fraction of skin collagen and 25 days for the citrate soluble fraction(6). Values reported by Harkness(1) reflect a turnover of the order of hundreds of days. This relative inertness of collagen is further supported by the findings of Popenoe and Van Slyke using C^{14}-lysine as a tracer(7). On the other hand experiments also using C^{14}-lysine rendered half life values of 28 days for the insoluble collagen and 17 days for the soluble fraction (8).

In most of these experiments only a few animals were used, and observations were made over relatively short periods of time, thus making extrapolation of values corresponding to long half lives inaccurate. Because of this lack of quantitative information, the effects of added variables, such as age, hormones, diet and others become hard to interpret. The following experiments were attempted to try to overcome some of these problems. The incidental variation was minimized by performing small skin biopsies from the same animals at the different time intervals considered.

Experimental procedure. At initiation of the experiment 21 male rats of the Holtzman strain weighing in the range of 142 to

* Supported by Public Health Research Grants DE-01021 and DE-00626, Nat. Inst. Dental Research.

† Post Doctoral trainee, U. .S. Public Health #5-T1-DE-94-02.

150 g were injected intraperitoneally with a solution of glycine-1-C^{14} in normal saline (10 μc/ml); (Spec. Act. 3.06 mc/mM). Each animal received 0.2 ml of solution (2 μc) per 100 g of body weight. On the third day following the injection, all animals were implanted subcutaneously with a polyvinyl plastic sponge weighing 100 mg ($\pm$ 2 mg) and a skin biopsy was performed. The skin sample was weighed immediately (300-400 mg) and kept frozen until time of analysis. At this point in the experiment the rats were divided into 3 groups; one served as control and continued consuming the standard laboratory diet (ground Wayne); the other 2 groups were fed the same diet supplemented with 5 mg and 20 mg per kg respectively of Methyl prednisolone. At subsequent intervals (20, 43, 70 and 115 days) similar biopsies of dorsal skin were performed in different areas and after weighing they were saved for final analysis. At the end of experiment rats were sacrificed and the subcutaneously implanted plastic sponges removed.

Collagen fractionation. Both skin and sponge samples were fractionated by a micromodification of the technique suggested by Houck and Jacob(9) followed by an extraction with 0.3 M TCA(10). Each fraction was then dialyzed 48 hours against water. The first fraction was obtained by homogenizing with 10 ml of 0.15 M NaCl during 15 minutes using a Virtis 45 homogenizer, and maintaining the temperature at 4°C. After allowing to stand overnight in the cold the samples were centrifuged also in the cold at 25,000 $\times$ *g* for 1 hour. The residue was reextracted with 0.5 M NaCl and 0.5 M citrate pH = 3.6. The remaining portion was extracted twice with 0.3 M trichloroacetic acid in a 90°C water bath during 30 minutes. All fractions were dialyzed; aliquots were removed and analyzed for radioactivity and collagen content. Collagen was calculated based on the amount of hydroxyproline present in the acid hydrolysate, determined by a modification of the Neuman and Logan method(10). Radioactivity was measured using a gas-flow thin window Geiger-Muller tube, after plating the extracts on aluminum planchets.

In calculating the turnover data, corrections for isotopic dilution due to growth were made taking into account changes in surface area of the animal, in skin thickness as well as variations in content. Values plotted in the abscissa are average specific activity values for each group calculated in the following manner: Corrected specific activity = cpm/mg collagen $\times$ surface area (cm^2) $\times$ skin g/cm^2 $\times$ % collagen in wet skin. It is felt that this method of correcting specific activity takes into account all the variables which can be quantified in such a system, and is more valid than using total body weight for the purpose. Isotope reutilization through exchange between tissues has to occur via its transport by the blood stream, and it is known that the specific activity of circulating glycine decreases rapidly compared to that of the collagen fraction with more rapid turnover. Smith(4) has shown that in young growing rats injected with radioglycine, the specific activity of this amino acid was greater by the 4th day of experiment in the soluble collagen fraction than in the plasma. Therefore it would be reasonable to expect that all collagen molecules formed after this time would possess lower specific activity and would not appreciably alter the subsequent curves of radioactive collagen decay corrected for isotopic dilution.

Results. Table I summarizes changes in body weight and food consumption of the different groups in response to oral administration of 2 different levels of methyl prednisolone. Growth was considerably retarded by the corticoid, especially at the higher dosage, in spite of a satisfactory food intake, thus reflecting the catabolic nature of this steroid. The average daily dose was calculated based on those for food intake. The specific activity of the soluble collagen fractions (0.15 M NaCl, 0.5 M NaCl and 0.5 M citrate) and of the insoluble fraction (0.3 M TCA extractable) at different intervals following isotope administration is shown in Table II. Shortly after the injection of

TABLE I. Changes in Body Weight, Food Consumption, and Average Methyl Prednisolone Dosage of the Different Experimental Groups Observed During a 115 Day Period.*

Treatment	Body wt increment, g/115 days	Food intake, g/day	Avg methyl prednisolone dose, mg/day
Control	+323	22.9 (± .7)	—
5 mg M.P./kg diet	+238	21.2 (± .6)	.11
20 mg M.P./kg diet	+102	18.2 (± .7)	.36

* 7 Rats per group.

TABLE II. Specific Activity of Skin Collagen Fractions Isolated at Different Time Intervals Following Injection of 1-C^{14}-glycine.

Treatment	Fraction	Skin specific activity*				
		Day 3	Day 20	Day 43	Day 70	Day 115
Control	.15M NaCl	561	257	83	41	9
	.50M NaCl	350	154	59	25	12
	.5 M citrate	250	140	92	38	27
	.3 M TCA	261	146	68	45	32
.11 mg/M.P./day	.15M NaCl	691	318	110	56	30
	.50M NaCl	433	161	61	39	34
	.5 M citrate	240	180	103	78	65
	.3 M TCA	245	164	100	66	60
.36 mg/M.P./day	.15M NaCl	678	501	174	70	64
	.50M NaCl	401	281	106	60	39
	.5 M citrate	220	200	141	91	95
	.3 M TCA	214	175	124	69	71

* Each value represents an average of 7 animals.

TABLE III. Collagen Content of Different Skin Fractions Isolated from Rats Receiving Methyl Prednisolone in Their Diets.*

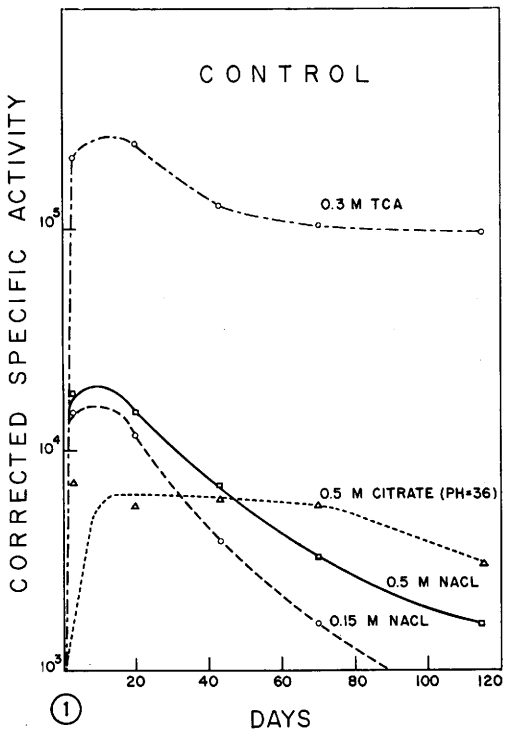
Treatment	Collagen, g/100 g wet skin			
	.15M NaCl	.5M NaCl	.5M citrate	.3M TCA
Control	.28 (±.01)	.72 (±.04)	.47 (±.07)	14.1 (±.5)
.11 mg M.P./day	.22 (±.02)	.42 (±.03)	.87 (±.13)	16.0 (±.7)
.36 mg M.P./day	.20 (±.03)	.34 (±.01)	1.10 (±.09)	17.2 (±.7)

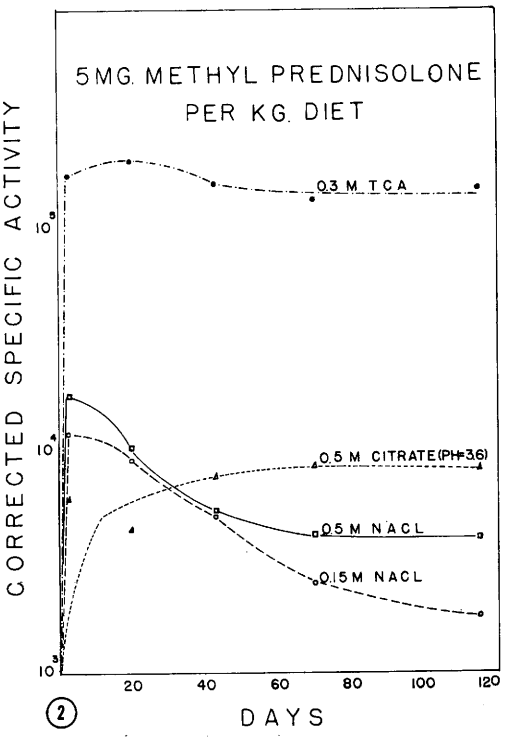
* Values obtained at end of experiment (7 animals per group).

glycine-1-C^{14} the soluble collagens showed a greater degree of labelling than the insoluble fraction, but these differences gradually decreased. These relative changes in specific activity reflect the more rapid turnover of the soluble collagen compared to the insoluble collagen.

Table III shows the distribution of the different collagen fractions in the skin at the end of experiment. Per unit weight of skin, the insoluble collagen was greater in the methyl prednisolone treated rats. Among those animals receiving corticoids, the soluble fractions seem to show a change in distribution leading to an accumulation of citrate soluble collagen with a decrease in the saline soluble fractions. The radioactive decay curves of the different skin collagen fractions corrected for the isotopic dilution caused by growth are shown in Fig. 1, 2 and 3. These values were corrected to take into account changes in pool size of each one of the fractions, surface area of the animal and skin thickness.

In none of the groups did the decay curves for the insoluble collagen indicate the presence of a single component, but rather seemed to reflect a progressive insolubilization of the





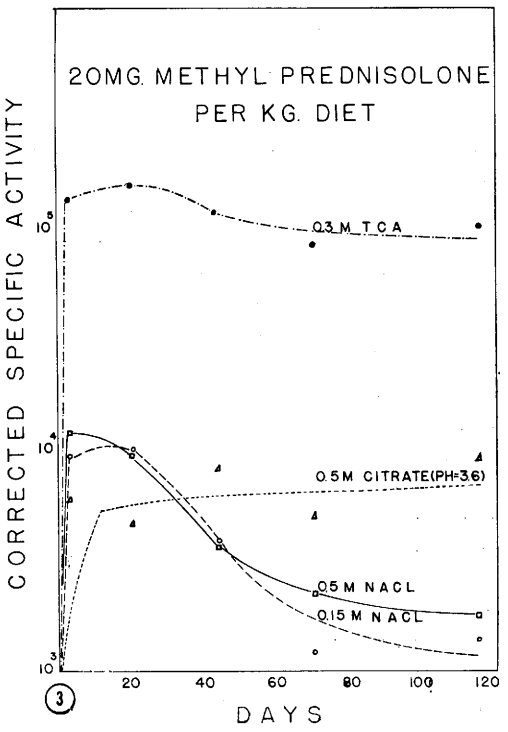


FIG. 1. Specific activity of different skin collagen fractions isolated from normal growing rats at different time intervals following injection of glycine-1-C^{14}. These radioactivity values have been corrected for changes in surface area, skin thickness and collagen content of skin.

FIG. 2. Specific activity of different skin collagen fractions isolated from rats receiving the lower level of methyl prednisolone (0.11 mg/day).

FIG. 3. Specific activity of different skin collagen fractions isolated from rats receiving the higher level of methyl prednisolone (0.36 mg/day).

collagen conducive practically to inertness. In the normal growing rat (Fig. 1) the extrapolated turnover time ($T\frac{1}{2}$) was 28 days if measured before the 43rd day of the test, 88 days if measured between the 43rd day and the 70th day, and 300 days if measured after the 70th day.

For animals receiving 5 mg of methyl prednisolone per kg of diet the turnover calculated at similar time intervals was 35 days, 100 days and $>$ 300 days (Fig. 2). Those animals receiving higher doses of methyl

TABLE IV. Collagen Fractions Present in Sponges Removed from Experimental Animals 112 Days Following Implantation. The only fraction which exhibited significant amounts of radioactivity was the insoluble collagen.

Treatment	mg Collagen/sponge .15M NaCl	.5M NaCl	.5M citrate	.3M TCA	Specific activity .3M TCA fraction*
Control	.83	.61	.95	31.3	1.9
.11 mg M.P./day	.75	.57	1.03	28.3	3.5
.36 mg M.P./day	.65	.51	1.03	32.5	6.6

* cpm/mg collagen.

prednisolone (20 mg/kg of diet) revealed turnover times of the order of 40, 80 and $>>$ 300 days respectively (Fig. 3). The 0.3 M citrate soluble fraction seemed to be in equilibrium with the insoluble collagen judging by the shape of the curves.

The salt soluble fractions showed a rapid turnover time with values ranging between 15 and 26 days from the 0.15 M NaCl fraction and between 18 and 24 days for the 0.5 M NaCl fractions. In the corticoid treated animals these soluble fractions began to slow down their decay rate after approximately 2 half lives.

The values for the different fractions of collagen synthesized around the polyvinyl sponges removed 112 days following their subcutaneous implantation are shown in Table IV. The total amount of collagen synthesized at this site did not differ significantly among groups. As in the case of the skin, the more soluble fractions tended to decrease in size following corticoid administration. The radioactivity of the soluble fractions was undetectable, whereas the insoluble collagens showed significant differences in specific activity, becoming progressively more radioactive as the dose of methyl prednisolone increased.

Discussion. The turnover of collagen in the skin of the rat was found to be an age dependent process. As growth of the animal became slower, the turnover rate decreased, and this occurred most markedly with the insoluble fraction. The $T_{1/2}$ for this fraction in the young, rapidly growing animal was 28 days. This value increased progressively with time, until an extrapolated value of 300 days could be approximated during the latter part of the test. The span of intermediate values covers a range which encompasses most of those reported in the literature for this particular fraction of collagen. Corticoid treatment, known to inhibit the synthesis of collagen, was apparently able to retard the process of collagen breakdown. In general, administration of methyl prednisolone slowed down the turnover of collagen. The collagen content of the skin, as well as its distribution among the different fractions isolated, was also affected. The salt soluble fractions showed a relative decrease following hormone therapy, whereas the citrate and insoluble collagen fractions increased. This resembles the phenomenon which was observed in aging guinea pigs, where the reduction of collagen synthesis brought about a diminution of all components extractable by neutral salt solutions(11).

Subcutaneously implanted plastic sponges which were maintained in place throughout the experiment were removed and the collagen analyzed. The corticoid did not affect the net amount of collagen synthesized at that site, but here again, it seemed to induce a redistribution of the collagen, causing an increased accumulation of the less readily soluble fractions. Radioactivity was detectable only in the insoluble fractions, in which instance the specific activity was significantly greater in those animals receiving corticoid. This finding can also be interpreted as an indication of a decrease in turnover, since the net amount of collagen present in the sponges did not differ between groups. A decrease in synthesis, coupled with a decrease in breakdown could cause the sponges to retain a larger amount of radioactive molecules, and still reach similar levels of total collagen.

Our findings allow us to conclude that corticoids do not only inhibit the synthesis of collagen, but also retard its breakdown. The decrease in turnover time would seem to indicate that the inhibition of collagen catabolism is greater than the inhibition of the synthetic process. This would also be in agreement with the relative increase in skin collagen which accompanies treatment with a high dosage of corticoid. This same pattern of events may occur during aging(12) and during the feeding of amino acid deficient or imbalance diets(3), where the amount of collagen per unit of body weight or of fat-free dry skin increased significantly in spite of a decrease in rate of synthesis.

Summary. At different time intervals, over a 115 day period skin biopsies were performed in growing rats which had received an initial tracer dose of C^{14}-glycine. Specific activity values for the different fractions of skin collagen isolated (0.15 M NaCl, 0.5 M NaCl, 0.5 M citrate pH = 3.6 and insoluble) were corrected for growth taking into account changes in surface area, skin thickness and pool size of the particular fraction. The 0.15 M and 0.5 M NaCl fractions in the normally growing rats decayed almost exponentially with $T_{1/2}$ of 17 and 20 days. The curve for the insoluble collagen did not reflect the presence of a single component but seemed to indicate a progressive insolubilization. A $T_{1/2}$ of 28 days was evident during the period of rapid growth whereas a value close to 300 days could be extrapolated towards the end of the experiment. Corticoid treatment (0.11 and 0.36 mg/day) decreased the turnover rate of all fractions, and caused a decrease on the amount of collagen extractable by neutral salt.

1. Harkness, R. D., *Biol. Rev.,* 1961, v36, 399.

2. Nimni, M. E., Bavetta, L. A., PROC. SOC. EXP. BIOL. AND MED., 1961, v108, 38.

3. Nimni, M. E., Hom, D., Bavetta, L. A., *J. Nutrition,* 1962, v78, 133.

4. Smith, Q. T., *Am. J. Physiol.,* 1963, v205, 827.

5. Bavetta, L. A., Bekhor, I., Nimni, M. E., PROC. SOC. EXP. BIOL. AND MED., 1962, v110, 294.

6. Gerber, G., Gerber, G., Altman, K. I., *J. Biol. Chem.,* 1960, v235, 2653.

7. Popenoe, E. A., Van Slyke, D. D., *ibid.,* 1962, v11, 3491.

8. Kao, K. Y. T., Hilker, D. M., McGavack, T. H., PROC. SOC. EXP. BIOL. AND MED., 1961, v106, 121.

9. Houck, J. C., Jacob, R. A., *ibid.,* 1960, v105, 324.

10. Fitch, S. M., Harkness, M., Harkness, R. D., *Nature,* 1955, v176, 163.

11. Gross, J., *Am. Inst. Biol. Sci.,* 1960, Publ. 6, 192, c.a., 1963, v59, 4328.

12. Sobel, H., Zutraven, H. A., Marmorston, J., *Arch. Biochem. Biophys.,* 1953, v46, 221.

Received July 20, 1964. P.S.E.B.M., 1964, v117.