a chain reaction which proceeds in the direction of growth. CS-A may be such a substance. Its effects on the genetic mechanism will be more closely investigated.

Summary. Evidence is presented which further indicates that under specific conditions chondroitin sulfate A will act as a growth stimulating agent. Effective in tissue cultures in concentrations as low as those ascertained for several hormones, production of RNA, DNA and phospholipid is increased simultaneously within a few hours after application (the total extractable phospholipid is reduced indicating an increased rate of turnover), but the stimulating effect is gone by 24-48 hours. The demonstrated action of small amounts of chondroitin sulfate A in several types of cells, together with the finding that this material is normally present in human plasma in such amounts suggests that it may be a metabolic agent in the regulation of cell processes.

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Hydroxyproline in Human Blood: Forms in Which It Is Present.* (30255)

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The amino acid hydroxyproline is present essentially only in collagen of all the body proteins, but it cannot enter into this protein except through proline. It has, therefore, merited considerable interest as a guide to the metabolism of collagen. Much is known of its excretion in urine, but until very recently little was known of its presence in blood. For some time we have been studying the forms in which hydroxyproline is excreted in urine and have found that about half is in the form of the dipeptide prolylhydroxyproline. We wanted, therefore, to determine this dipeptide in blood serum and to estimate how much of the hydroxyproline of serum is in this form. Keiser *et al*(1) had shown that the method of Prockop and Udenfriend(2) cannot be used to determine total serum hydroxyproline, but that much of the hydroxyproline of alcohol precipitates can be extracted with hot trichloroacetic acid. We attempted to estimate the protein hydroxyproline of serum by this means(3). Later, however, we found that a considerable portion of the material containing hydroxyproline in the hot TCA extracts is dialyzable. LeRoy *et al*(4) have recently produced data to indicate that the hydroxyproline of alcoholic precipitates is largely protein. Further study by us is consistent with this view since it indicates that dialysis of the hydroxyproline in hot TCA extracts of alcoholic precipitates is undoubtedly due to hydrolysis under the conditions of the hot acid extractions. In the present paper we report values of the

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different fractions of hydroxyproline which we determined in pooled human serum.

Materials and methods. Fresh pooled normal human serum purchased commercially was divided into separate containers and stored in the freezer at —20°C until used for analysis.

Free and total dialyzable hydroxyproline was determined after dialysis of 15 ml of serum with 700 ml of distilled water with stirring in the refrigerator for 24 hours. The dialyzate was concentrated *in vacuo* at 40°C to 7.5 ml, and total hydroxyproline was determined after hydrolysis, and free hydroxyproline without hydrolysis, by the method of Prockop and Udenfriend(2).

Alcohol-soluble hydroxyproline was determined by adding 320 ml of absolute alcohol to 80 ml of serum usually in the refrigerator, and then filtering and washing the precipitated protein with 80% alcohol. The combined filtrate and washings were evaporated to dryness *in vacuo* below 40°C, and the residue was dissolved in 40 ml water and analyzed by the Prockop-Udenfriend method.

The procedure of Keiser *et al*(1) was followed to dissolve precipitated protein. The alcohol precipitate was freed of most of the alcohol at the vacuum pump and then stirred with 4% trichloroacetic acid at 90°C for 30 minutes and filtered hot. The extracts from 3 such extractions were concentrated *in vacuo* to ⅓ their original volume and analyzed for total hydroxyproline by the Prockop-Udenfriend method before and after exhaustive dialysis against distilled water. It was found that only 23 to 40% of the solubilized hydroxyproline was undialyzable.

Other methods were tried to liberate the apparent nonprotein hydroxyproline that does not appear in alcohol extracts or in dialyzates of untreated serum. These consisted of heating serum for as long as 3 hours in boiling water at pH 2.0, 4.6, 5.5, and 7.9. One portion of the solutions was adjusted to pH 5.0 and heated a few minutes more if necessary for complete coagulation of protein before filtration. The other was dialyzed without adjusting pH, further heating, or filtering. The filtrates and dialyzates were then concentrated *in vacuo* and analyzed by the Prockop-Udenfriend method.

Total hydroxyproline of alcohol precipitated serum was determined in essentially the same way as that described by LeRoy *et al*(4), by hydrolysis followed by separation of the liberated amino acid on a column of ion exchange resin.

After every determination a complete absorption curve was obtained with a Bausch and Lomb Spectronic 20 spectrophotometer between 440 and 580 mμ in order to detect the presence of the false chromophore(1) with a peak at 450. If present, the determination was discarded.

Prolylhydroxyproline was determined by a modification of our method for urine on the principle of isotope dilution after addition of C^{14} prolylhydroxyproline(5). The first such experiment with 350 ml of fresh human serum, however, proved a failure. After addition of the C^{14} dipeptide, the proteins were precipitated with trichloroacetic acid, and the mixture was left over the weekend before filtration. Under these conditions the dipeptide apparently remained adsorbed on the protein. Experiments were, therefore, performed with C^{14} prolylhydroxyproline to determine how to remove the serum proteins while leaving the dipeptide in solution.

The dipeptide was added to fresh serum, the proteins were precipitated, and the precipitates were then filtered after 30 minutes. It was found that 72% of the C^{14} was recovered in the TCA filtrate after removal of most of the trichloroacetic acid with ether. With picric acid, 86.7% of the C^{14} was recovered in the filtrate, and with 90% alcohol, 66.5% of the C^{14} was in the filtrate. In the latter, an additional 9.4% was removed from the precipitate by washing the filter with 90% alcohol, making a total recovery of 75.9%.

An outline of the complete method for serum is as follows:

350 ml serum + C^{14} Pro HyPro (DP)
|
HCl to pH 5 + alcohol to 90%
|
Filter and wash with 90% alcohol
|

Concentrate-acidify to pH 2 with TCA
|
Filter-electrophoresis at 2500 volts—2 hr
|
Detect C^{14}—elute
|
Chromatography in BuOH-Acetic—16 hr
|
Detect C^{14}—elute DP
|
Convert DP to Diketopiperazine (DK)
| (0.2 N NH_3 at 50°)
|
Chromatography in BuOH-Acetic—7 hr
|
Detect C^{14}—elute DK
|
Determine HyPro by the Stegemann method and c.p.m.

Then, $DP = m\left(\frac{S}{s} - 1\right)$ by the usual equation for isotope dilution. The device described in our improved method(6) is used to detect counts from C^{14}.

Results and discussion. Table I shows the results of experiments to detect the presence of nonprotein hydroxyproline in serum that does not appear initially in dialyzates or in alcohol extracts. It is apparent that the additional hydroxyproline solubilized after protein coagulation is entirely undialyzable. It is interesting that heating at pH 2, which would be comparable to extraction with hot TCA, is most effective in solubilizing the protein.

Values of the different fractions of hydroxyproline determined in serum appear in Table II. Fig. 1 shows absorption curves corresponding to the values obtained with the Prockop-Udenfriend method. The curves for the various fractions of serum compare quite well with the curve for the hydroxyproline standard (curve a). Although the hot TCA extracts that were used for analysis and dialysis did not absorb in the region of 450 mμ, others obtained by further extraction of the alcohol precipitates did. This is consistent with the findings of the previous workers(1). Similarly, the chromophores corresponding to the results of Table I showed no absorption in the region of 450 mμ. However, more drastic conditions produce both a peak at 450 mμ and additional nonprotein hydroxyproline.

TABLE I. Effect of pH and Heat on Hydroxyproline of Serum.

pH	Time of heating (hr)	Hydroxyproline: In alcohol extract (μg/ml)	Hydroxyproline: After heating, Undialyzed (μg/ml)	Hydroxyproline: After heating, Dialyzate (μg/ml)
2.0	.5	2.4	4.5	2.5
2.0	1	2.6	5.5	2.5
2.0	2	2.3	4.6	2.5
2.0	3	2.5	7.1	2.5
4.6	.75	2.5	3.0	2.5
5.5	.25	1.4	2.1	1.8
7.9	1	2.2	2.8	2.2
7.9	2	2.4	3.8	2.4
7.9	3	2.2	3.7	2.4

TABLE II. Hydroxyproline Fractions of Blood Serum.

Fraction	Hydroxyproline (μg/ml)
(a) Free dialyzable	2.3
(b) Total "	3.0
(c) Protein	7.1
(d) Alcohol soluble	2.9
(e) Prolylhydroxyproline	.12

In Table II the sum of the total dialyzable hydroxyproline (b) and the protein hydroxyproline (c) is 10.1 μg per ml for total hydroxyproline of serum. Total dialyzable (b) and alcohol-soluble (d) hydroxyproline values are essentially the same. This has been found repeatedly and agrees with the find-

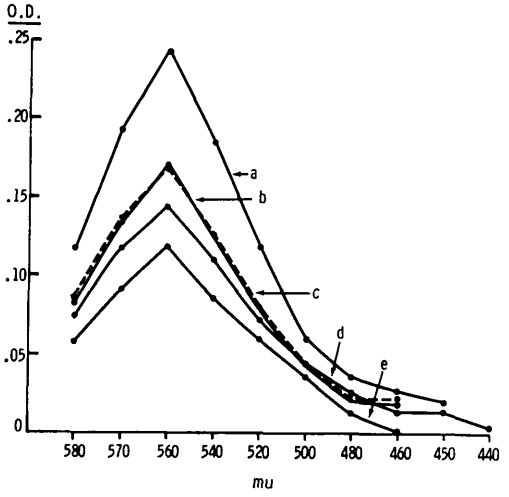

FIG. 1. Absorption curves of chromophores from the Prockop-Udenfriend method. a—Hydroxyproline Std., b—protein, c—alcohol-soluble, d—total dialyzable, e—free dialyzable.

ings of LeRoy *et al*(4). The content of prolylhydroxyproline in serum is 0.21 μg per ml as such, or 0.12 μg per ml as hydroxyproline (e). This represents 1.1% of the total hydroxyproline of serum (b + c), and 4.0% of the total dialyzable hydroxyproline (b), whereas it is 17% of the bound dialyzable hydroxyproline (b—a). Only the latter value is significant at present since free hydroxyproline comprises only a small fraction of the total excreted in urine and since we know nothing about the hydroxyproline of serum that is present in the form of protein. Prolylhydroxyproline therefore is 17% of the bound dialyzable hydroxyproline of serum, whereas it is 50% of the total hydroxyproline of urine(5).

Summary. Estimates of the different forms of hydroxyproline in blood serum were made by methods which now account for all of the hydroxyproline. In micrograms per milliliter of serum they are: total, 10.1; as protein, 7.1; dialyzable, 3.0; free, 2.3; peptide, 0.7; prolylhydroxyproline, 0.12. The latter represents only 1% of the total hydroxyproline of serum whereas it is about 50% of the total in urine.

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Ceruloplasmin: *in vitro* Inhibition in the Rabbit.* (30256)

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Despite numerous investigations [see (1) for review] little information is available on the role of blood ceruloplasmin in body function in general or its relationship to copper distribution and metabolism. It has been known since the discovery of Wilson's disease that the central nervous system is particularly sensitive to a disturbance in copper metabolism, although the significance of low ceruloplasmin to the condition is unknown. With the important finding that a copper deficiency state could be produced in animals maintained on a diet containing high molybdate and sulfate, an experimental technique became available for studying the relationship of copper to neurological function. Ewes maintained on a similar diet gave birth to lambs with swayback disease, a demyelinating condition presumably similar to Wilson's disease(2). It was the objective of the present study to determine the effects of a high molybdate and sulfate diet on blood ceruloplasmin in an effort to help elucidate the role of this copper protein.

Materials and methods. Twenty-four white albino rabbits, 14-15 weeks old, were maintained on an intake of 0.05% molybdenum, as sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$) and 1.8% sulfate, as sodium sulfate (Na_2SO_4) mixed with a Purina rabbit dry ration containing 2 mg % copper. Deionized distilled drinking water was provided, *ad libitum*.

The weight of the animals, the level of total copper in blood, serum oxidase activity, hemoglobin levels, and alkaline phosphatase levels were examined weekly. Tissue copper determinations were performed after 10 weeks on one-third of the animals, while the rest of the determinations were made after death.

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