was only about 4 times that of the controls. Their results, however, clearly show that additional work with tagged fatty acids in severly deficient animals is indicated. It also seems important to determine what change, if any, occurs in the protein and phospholipid moiety of the low-density lipoproteins during choline deficiency. Recent experiments have shown a decided drop in phospholipid phosphorus content of the low-density lipoproteins of rats after 3 weeks on the choline deficient diet, and its return to normal following choline administration.

Summary. The cholesterol and triglyceride content of the high- and low-density lipoproteins of rats on a choline deficient diet for at least 3 weeks has been compared with that of controls on the same diet supplemented with choline. The deficient animals showed only about one-half the concentration of these lipids in the low-density lipoprotein fraction compared with the controls. Supplementation of the diet of the deficient animals with choline raised the concentration of these lipids to normal after a few days. The cholesterol content of the high-density lipoproteins seemed not to be affected by the deficiency state. The triglyceride content, however, was depressed.

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Diketopiperazine of Prolylhydroxyproline in Normal Human Urine.*† (29756)

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The dipeptide prolylhydroxyproline represents a major fraction of the total hydroxyproline of urine from normal subjects and from patients with collagen diseases both on a regular diet and on a collagen-free diet(1). We have recently shown that this dipeptide cyclizes quite readily to the diketopiperazine even at room temperature at pH 8 and higher, and that negligible amounts cyclize at lower pH(2,3). Since physiological pH is not far removed from 8, it was desirable to know whether any of the diketopiperazine would be formed normally in man. Our study describes a quantitative analysis for this substance by the principle of isotope dilution in urine excreted by a healthy man, 20 years old, normally on a regular diet and by the same individual after ingestion of bicarbonate to produce urine having a pH of 8.

Methods. Determination of the dipeptide. A preliminary description of our method for determining dipeptide prolylhydroxyproline in urine(3) will illustrate the method we have used for determination of the diketopiperazine. The principle of isotope dilution permits quantitative results in spite of the major losses which are difficult to avoid in a long and difficult determination. At first we

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detected the C^{14} areas after electrophoresis and chromatography with autoradiographs, but we later developed a scanning device for this purpose(4). It is essential only that the final substance that is isolated and that will be used for calculation from specific activity be pure. This is accomplished by obtaining the dipeptide by chromatography, converting it to the diketopiperazine by heating overnight in 0.2 N ammonia, and then isolating the diketopiperazine in the same system of chromatography. Since the Rf values of dipeptide and diketopiperazine in butanol-acetic acid-water, 4:1:5, are 0.24 and 0.60, respectively, it is evident that any contaminants containing hydroxyproline picked up at the Rf of dipeptide would be eliminated in the region of Rf of the diketopiperazine. A simple determination of hydroxyproline and a measurement of the radioactivity of the isolated diketopiperazine would be all that are required to calculate the content of the dipeptide, prolylhydroxyproline, in urine.

Determination of diketopiperazine. With this background we devised a method for quantitative determination of the diketopiperazine (D.K.) of prolylhydroxyproline in urine. C^{14} diketopiperazine was prepared from chromatographically pure C^{14} dipeptide by heating for 16 hours at 50° in 0.2 N ammonia. After removal of ammonia the compound was isolated by chromatography on paper in butanol-acetic acid-water, 4:1:5. It contained only one component on chromatography in 3 different systems and yielded theoretical amounts of proline and hydroxyproline on hydrolysis. In addition, there is other evidence for the purity of synthetic C^{12} diketopiperazine(2) prepared in the same way. Preliminary experiments indicated that urine can be treated with 250 mg of carbon per 100 ml to remove most of the pigment but without removing a significant amount of diketopiperazine, as measured with the C^{14} compound, in much the same way we did for dipeptide. Less carbon must be used, however, than was possible with the dipeptide. With enough carbon, all of the diketopiperazine can be removed; in fact, this procedure is actually utilized to obtain a control urine free of the diketopiperazine.

The complete method for determination of the diketopiperazine is as follows: Mix 80 ml of urine at pH 2 with C^{14} diketopiperazine; add carbon (2.5 mg/ml), filter and concentrate; paper electrophoresis for 2 hours at 3000 volts; then elute; chromatograph in butanol-acetic acid-water 7 hours; elute; chromatograph in isoamyl alcohol-pyridine-water 7 hours; elute and concentrate. Determine hydroxyproline and counts per minute. Then, $\text{D.K.} = m\left(\frac{S}{s} - 1\right)$ μg in 80 ml, where m is the amount of C^{14} diketopiperazine added and S and s are the specific activities of the added and isolated substances. Instead of depending on conversion of dipeptide to diketopiperazine to remove contamination, the method for the diketopiperazine depends largely on eliminating hydroxyproline contaminants by electrophoresis at pH 2. The diketopiperazine remains near the origin and does not migrate as a cation, as do peptides containing hydroxyproline. To be certain of removing all contamination, electrophoresis was prolonged for more than 2 hours at 3000 volts, and this was followed by chromatography, first in butanol-acetic acid-water, 4:1:5, and then in isoamyl alcohol-pyridine-water, 35:35:30. The C^{14} diketopiperazine, after scanning, was cut out in the region of the Rf of the diketopiperazine in each of these systems. As a final check of purity, proline (5), as well as hydroxyproline(6), was determined. This gave a one to one ratio of proline to hydroxyproline. Still further proof was found that hydroxyproline contaminants were not obtained under these conditions and that the amount of diketopiperazine that was determined actually existed in the urine analyzed. We did this by treating the same urine with 10 g of Norit per 100 ml to remove all existing diketopiperazine, and then analyzing the filtrate in the same way after addition of C^{14} diketopiperazine.

Results and discussion. Table I shows the results obtained in urine under normal conditions, after ingestion of bicarbonate, and after removal of diketopiperazine with carbon. The diketopiperazine that was isolated in the control urine had only 7% less specific activity than the C^{14} diketopiperazine that

TABLE I. Values of Diketopiperazine of Prolylhydroxyproline Determined in Urine.

				Diketopiperazine hydroxyproline (DK HyPro)		
	m*	S†	s‡	In 80 ml, μg	In 24 hr mg	% of total
Normal urine Total hydroxyproline = 72.4 mg/24 hr	29.24	1750	364	111.5	1.345	1.86
Alkaline urine§ Total hydroxyproline = 71.7 mg/24 hr	29.24	1750	701	43.8	1.31	1.83
Control urine‖ Total hydroxyproline = 72.4 mg/24 hr	87.6	2015	1885	6.13	.074	.10

* μg DK HyPro added to 80 ml urine. † S = specific activity of added DK $= \frac{\text{cpm}}{\mu\text{g DK HyPro}}$.
‡ s = specific activity of isolated DK $= \frac{\text{cpm}}{\mu\text{g DK HyPro}}$. § 24 hr volume calculated from value of creatinine. ‖ Same as normal urine in which DK has been removed with carbon.

had been added and yielded a negligible value of 6 μg hydroxyproline, as diketopiperazine, in 80 ml. It is interesting that urine of pH 8.3 voided after administration of bicarbonate had essentially the same amount of diketopiperazine in relation to its total hydroxyproline as that normally excreted: 1.86% in one and 1.83% in the other. Although this amount of diketopiperazine is small, our experience with determination of dipeptide in urine, and our failure to obtain any of the cyclized substance in urine free of diketopiperazine attest to the validity of this finding. It is probably representative of the amount excreted generally by normal individuals. Actually, since diketopiperazine originates only from the dipeptide, prolylhydroxyproline, the amount that was found is not so small. Since dipeptide varies generally between 20 and 60% of the total hydroxyproline in urine, the amount of diketopiperazine determined indicates that about 5% of the dipeptide was cyclized in our samples of urine. Moreover, it is plausible that a larger fraction of the dipeptide prolylhydroxyproline would be cyclized, either in the blood and tissues or in the bladder, in certain diseases that are accompanied by prolonged alkalinity.

Summary. Diketopiperazine of prolylhydroxyproline was determined in urine voided under normal conditions and after ingestion of bicarbonate, as well as in urine from a control which had been treated with carbon to remove diketopiperazine. The normal and alkaline urines had the same small amount of this substance, whereas the urine after carbon treatment had essentially none.

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