These chromatograms are shown in Fig. 5.

Discussion. The results described confirm the report of Bond(1,2) on the presence of a sex-associated protein component in liver extracts. The presence and absence of the protein fraction in response to varied hormone treatment of rats would indicate that it is a sex-hormone-dependent protein component.

The physiological significance of this liver component is not known. Reports indicate quantitative sex differences in liver-enzyme activities such as arginase, phenylalanine hydroxylase, and glucose-6-phosphatase(4,5,6, 7). Quantitative and qualitative differences due to sex have also been described for liver enzymes involved in metabolism of steroid hormones(8,9,10,11). The sex-associated protein was not found in extracts of male rat kidney, brain, or testis(2). It would be of interest if sex-hormone-dependent components exist in circulation in the blood. Attempts to isolate sex-associated proteins and to regulate the concentration of the protein components may be of aid in understanding the known problem of histoincompatibility between male and female rats(12,13,14,15).

Summary. The presence of a sex-associated protein component in extracts of livers from male rats has been confirmed. The protein fraction is not present in extracts of livers from females but appears on treatment of the female with testosterone or progesterone. The protein component is not present in livers of immature males and is decreased after treatment of males with estradiol.

We wish to express sincere thanks to Dr. Ailene Herranen for helpful guidance and discussion in this work.

1. Bond, H. E., Biochem. Biophys. Research Comm., 1960, v3, 53.
2. ———, Nature, 1962, v196, 242.
3. Anderson, N. G., Exp. Cell Research, 1956, v11, 186.
4. Campbell, R. M., Kosterlitz, H. W., J. Endocrinol., 1949, v6, 171.
5. Glock, G. E., McLean, P. S., Biochem. J., 1954, v56, 171.
6. Knox, W. E., Averbach, V. H., Lin. E. C. C., Physiol. Rev., 1956, v36, 164.
7. Freedland, R. A., Krakowski, M. C., Waisman, H. A., Am. J. Physiol., 1962, v202, 145.
8. Hubener, H. J., Amelung, G., Z. Physiol. Chem., 1953, v293, 137.
9. Forchielli, E., Brom-Grant, K., Dorfman, R. I., Proc. Soc. Exp. Biol. and Med., 1958, v99, 594.
10. Yates, F. E., Herbst, A. L., Urquhart, J., Endocrinology, 1958, v63, 887.
11. Rubin, B. L., Strecker, H. J., ibid., 1961, v69, 257.
12. Billingham, R. E., Koprowski, H., Nature, 1959, v184, BA6.
13. Billingham, R. E., Silvers, W. K., Science, 1958, v128, 780.
14. ———, J. Immunol., 1959, v83, 667.
15. Eichwald, E. J., Silmer, C. R., Wheeler, N., Ann. N. Y. Acad. Sci., 1957, v64, 737.

Received September 1, 1964. P.S.E.B.M., 1965, v118.

Effect of a Diet Deficient in Lipotropic Factors on the Lipoproteins of Rat Serum.* (29755)

J. C. FORBES, O. M. PETTERSON AND R. A. RUDOLPH
Department of Biochemistry, Medical College of Virginia, Richmond

Anionic polymeric polysaccharides have been extensively used as reagents for the precipitation of low density lipoproteins(1). Sakagami and Zilversmit(2) showed that under appropriate conditions dextran sulfate and calcium chloride precipitated the lipoproteins of densities less than 1.063 from hypercholesterolemic rabbit serum. Since these low-density lipoproteins are generally considered to be the chief vehicle for transporting triglycerides from the liver, it was decided to investigate the changes in lipid content of this fraction when animals were put on a choline-deficient diet. This report deals primarily with the changes in serum cholesterol and triglyceride content of this low-density

* Supported in part by NIH grant AM-07696-01.

fraction. Since chylomicra are also precipitated by the dextran sulfate procedure they were removed first by centrifuging the serum at 20,000 × *g* for 2 hours in a refrigerated centrifuge and the infranate used for the analytical procedures. The term low-density lipoprotein is used here to mean all the material precipitated by the dextran sulfate under the conditions employed. Although it may not exactly represent the low-density fraction as measured by ultracentrifugation technic it is assumed that changes in it as a result of the deficiency in lipotropic factors probably represent a change in the lipid transporting mechanism.

Procedure. Male rats weighing approximately 140 g were divided into 2 groups. One group was placed on a lipotropic-deficient diet, the other on the same diet supplemented with choline chloride. The deficient diet had the following composition: vitamin-free, casein 100 g, sucrose 653 g, salt mixture (Wesson) 27 g, celluflour 20 g, coconut oil 200 g, plus 1 g of a vitamin supplement supplying the following amounts per kg of diet: thiamine HCl 4 mg, riboflavin 3 mg, pyridoxine HCl 6 mg, Ca pantothenate 14 mg, niacinamide 14 mg, biotin 5 mg, folic acid 5 mg, menadione 10 mg, and PABA 150 mg. Vitamins A, D, and E were fed in adequate amounts twice weekly. Control animals were fed the same diet supplemented with 3 g of choline chloride per kg of diet. All animals were on their respective diets for a minimum of 3 weeks prior to sacrifice. In some experiments, after it had been established that the deficient animals had a low concentration of low-density lipoproteins, some of the animals were fed the choline supplemented diet for 5-9 days and then sacrificed. In some cases the animals were allowed food up to the time of sacrifice, in others no food was allowed for approximately the last 22 hours. All animals were sacrificed by decapitation, the blood collected and allowed to clot. To obtain enough serum, the blood of 4 or 5 rats was pooled. Chylomicra were removed. The low-density lipoproteins were precipitated with dextran sulfate and calcium chloride following the procedure of Sakagami and Zilversmit(2). Four ml of the infranate were usually taken. Heavy walled glass-stoppered centrifuge tubes of 12 ml capacity were used. For precipitation 0.04 ml of a 5% solution of dextran sulfate and 0.1 ml of an 11.1% solution of calcium chloride per ml of infranate were used. The tubes, after standing overnight in a refrigerator, were centrifuged at about 2,500 r.p.m. for 30 minutes. The supernatant fluid was decanted, the tube inverted on filter paper and allowed to drain. Most of the inside of the tube then was dried with a roll of filter paper.

Determination of cholesterol and triglycerides in dextran sulfate precipitate. The precipitate in the bottom of the tube was spread by light tapping. Approximately 1 g of a synthetic zeolite† was added and mixed with the lipoprotein precipitate. Ten ml of reagent grade chloroform were added and the tube shaken thoroughly. If all the precipitate did not break away from the sides of the tube during approximately the first half hour, it was separated by means of a fine tipped rod. The tube was set aside with occasional shaking and allowed to stand overnight. Cholesterol was determined by a previously described procedure(3) modified for use in a Klett-Summerson photoelectric colorimeter. Color development was carried out at 22° for a 20-minute period. Although matched colorimeter tubes were used, each served as its own blank by taking a colorimeter reading prior to addition of the sulfuric acid. Triglycerides were measured on another aliquot using a procedure previously described(4). The extraction procedure has been shown to give good reproducible results in the case of both rat and human serum (unpublished work). It has also been shown in the case of human serum that the sum of the cholesterol or triglyceride values of the dextran sulfate precipiated material plus that of the supernatant solution agreed well with the values obtained on the serum itself. The size of the zeolite particles, though important when these lipids are being measured in rat serum or infranate, is not important when the lipids are being extracted from the dextran sulfate precipitable lipoproteins.

Triglycerides and cholesterol were deter-

† Obtained from A. H. Taylor Co., Baltimore, Md.

TABLE I. Effect of a Choline-Deficient Diet on the Low-Density Lipoproteins of Rat Serum.

	Low-density lipoproteins		Liver		
No. of groups*	Cholesterol, mg %	Triglyceride, mg %	Cholesterol, %	Triglyceride, %	Remarks
11	20 ± 1.7†	23 ± 2.1†	.78	23.0	Deficient, unfasted
5	25 ± 2.6	27 ± 6.9	1.10	24.0	Deficient, fasted
8	46 ± 3.5	61 ± 3.4	.28	3.7	Controls, unfasted
5	51 ± 6.0	53 ± 3.9	.40	2.6	Controls, fasted
5	56 ± 2.3	59 ± 7.8	1.00	21.00	Deficient, choline added last 5-9 days

* Each group represented the serum of 4 or 5 rats. † Standard error between groups.

TABLE II. Effect of Choline Deficiency on Non-chylomicra Lipids of Rat Serum.*

	Infranate		Low-density lipoproteins		
No. of groups	Cholesterol, mg %	Triglyceride, mg %	Cholesterol, mg %	Triglyceride, mg %	Remarks
4	79 ± 7.9†	52 ± 6.2†	22 ± 1.4†	25 ± 2.1†	Choline deficient
3	116 ± 4.9	113 ±18	49 ± 5.2	58 ± 7.2	Controls

* All were sacrificed unfasted. † Standard error between groups.

mined on the infranate of many of the sera. A number of these were done before it was realized that in order to obtain complete extraction of cholesterol and triglycerides from rat serum it is necessary to use zeolite of mesh 80-100 as reported previously by Cheng and Zilversmit(5). Only values obtained using 80-100 mesh particles are given here. We have compared the results obtained by this method with those using a recently published method of Carlson's(6) which uses 2:1 chloroform-methanol as the extraction media and obtained good agreement.

Results. Results recorded in Tables I and II show that deficient animals invariably showed only about one-half the concentration of cholesterol and triglycerides in the low-density serum lipoprotein compared with the control animals. Supplementation of the diet of the deficient animals raised the concentration of these lipids to normal after a few days. This took place before there was a definite drop in liver fat. The results shown in Table II show that the cholesterol content of the high-density lipoproteins (non-dextran sulfate precipitable material) changed little, if at all, as a result of the choline deficiency. The triglyceride content of this fraction, however, was much less than that found in the controls. Incidentally the control values are quite comparable to what we have found in rats fed regular Purina rat pellets.

Discussion. Our results are in good agreement with those of Olson *et al*(7) who used an ultracentrifugation technic and found practically complete disappearance of the low-density lipoproteins in rats on a choline deficient diet. It would appear that one of the first effects of choline supplementation is to increase the concentration of the low-density lipoproteins in the plasma. This would be expected if, as emphasized by Olson and Vester(8), the lipoproteins of Sf 0-12 are the bare vehicle for removal of triglycerides from the liver. Whether the increase in cholesterol content of the low-density lipoproteins following choline supplementation is a manifestation of an increase in concentration of a triglyceride transporting vehicle which contains cholesterol as an integral part of the molecule, or represents cholesterol in transit from the liver cannot be stated.

Lombardi and Schotz(9), using C^{14} palmitic acid, found no evidence of interference with triglyceride removal from the liver in short term choline deficiency experiments, possibly due to the fact that their animals had only a relatively mild deficiency state, as indicated by the fact that the liver triglyceride content based upon body weight

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.

We are indebted to Mr. E. W. Ruark, for phosphorus determinations.

1. Cornwell, D. G., Kruger, F. A., J. Lipid Res., 1961, v2, 110.

2. Sakagami, T., Zilversmit, D. B., ibid., 1962, v3, 111.

3. Forbes, J. C., J. Lab. and Clin. Med., 1931, v16, 562.

4. Forbes, J. C., Forbes, A. L., Petterson, O. M., Proc. Soc. Exp. Biol. and Med., 1960, v105, 628.

5. Cheng, A. L. S., Zilversmit, B. D., J. Lipid Res., 1959, v1, 190.

6. Carlson, L. A., J. Atherosclerosis Res., 1963, v3, 334.

7. Olson, R. E., Jablonski, J. R., Taylor, E., Am. J. Clin. Nutrition, 1958, v6, 111.

8. Olson, R. E., Vester, J. W., Physiol. Rev., 1960, v40, 677.

9. Lombardi, B., Schotz, M. C., Proc. Soc. Exp. Biol. and Med., 1963, v112, 400.

Received July 1, 1964. P.S.E.B.M., 1965, v118.

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

A. C. KIBRICK, C. Q. HASHIRO, M. I. WALTERS AND A. T. MILHORAT
Institute for Muscle Disease, Inc., New York City

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

* A preliminary report of this study appeared in Abstracts of 6th Intern. Congr. Biochem., 1964, v5, 423.

† This study was aided by grants from Nat. Inst. of Arthritis and Metab. Dis., and Muscular Dystrophy Assn. of America, Inc.