idly than Sr^{85}, in another, Sr^{85} entered more rapidly, and in 2 cases, both radioisotopes entered at the same rate. Both ions passed more rapidly into and out of the peritoneal cavity than the fluid accumulated, but passage of ions was slower than flow of water. Rate of passage varied in different patients as did ratio of rates for Sr^{85} and Ca^{45}. The high protein content indicated that peritoneal fluid was not an ultrafiltrate although the Sr^{85}/Ca^{45} ratio corresponded to that of an ultrafiltrate in some cases. The ratio in pleural fluid approximated that of an ultrafiltrate. Sr^{85} entering more rapidly. The ratio of passage of Sr^{85} and Ca^{45} into interstitial fluid studied in one patient with massive edema indicated increased permeability of the capillaries, both isotopes entering the fluid at about the same rate.

1. Samachson, J., Lederer, H., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 867.

2. Samachson, J., Kabakow, B., Spencer, H., Laszlo, D., *ibid.*, 1959, v102, 287.

3. Bellin, J., Laszlo, D., *Science,* 1953, v117, 331.

4. Clark, E. P., Collip, J. B., *J. Biol. Chem.,* 1925, v63, 461.

5. Fiske, C. H., SubbaRow, Y., *ibid.,* 1925, v66, 375.

6. McFarland, H., Hein, R. E., *U.S.A.E.C. Rep.,* April 1953, TID-5098, p27.

7. Lewin, R., Rosoff, B., Hart, H. E., Stern, K., Laszlo, D., *Advances in Radiobiology,* p298, Oliver & Boyd, Edinburgh, 1957.

8. Hyatt, R. E., Smith, J. R., *Am. J. Med.,* 1954, v16, 441.

9. Prentice, T. C., Siri, W., Joiner, E. E., *ibid.,* 1952, v13, 668.

10. McKee, F. W., Wilt, W. G., Hyatt, R. E., Whipple, G. H., *J. Exp. Med.,* 1950, v91, 115.

11. Irving, J. T., *Calcium Metabolism,* p38, Methuen & Co., London, 1957.

12. Malm, O. J., *Calcium Requirement and Adaptation in Adult Men,* p59, Oslo Univ. Press, Oslo, 1958.

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Lipid Bound Glutamic Acid Deficiency in Aging Arteriosclerotic Subjects.*† (25600)

R. E. HAMILTON AND L. O. PILGERAM

St. Barnabas Hosp. Research Foundation and Dept. of Physiology, University of Minnesota School of Medicine, Minneapolis

A previous report(1) described chromatographic identification of bound glutamic acid in the lipid fraction of rat liver. Our object was to determine whether this factor also exists in human plasma lipoproteins and whether the compound shows variation in the aging arteriosclerotic human subject.

Methods. Twenty ml of blood were introduced into a centrifuge tube containing 1.5 ml acid citrate dextrose solution. Fractionation of alpha and beta lipoproteins was carried out according to method of Lever *et al.*(3). Following fractionation, the tubes containing alpha- and beta-lipoproteins received 75 ml of ethyl alcohol:ethyl ether (3:1) extraction mixture. The suspension was stirred 3 hours at room temperature. Then the clear supernatant was decanted into 125 ml Erlenmeyer flasks. To the precipitate were added another 75 ml of lipid extracting media, suspension stirred, then stored overnight at −25°C(4). After removal from deep freeze, stirring was maintained 3 hours. The tubes were then centrifuged and this second extraction was pooled with the previous one. This extraction procedure was repeated using anhydrous ethyl ether. Pooled extracts were brought to anhydrous state over sodium hydroxide pellets *in vacuo* at 40°C. Phospholipids were extracted from the residue with three 15 ml portions of anhydrous petroleum-ether (b.p. 30°-60°C). If residue and solvent are anhydrous, there is no contamination from amino acids

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which may have been absorbed to initial precipitate and carried over in alcohol-ether extraction(5). Pooled petroleum-ether extracts were evaporated from reflux flasks at 65°C. The lipids were hydrolyzed with 25 ml of 6 N HCl at 108°C for 22 hours. After chilling, the hydrolysate was quantitatively filtered to remove insoluble fatty acids. After filtration and drying *in vacuo* at 45°C-50°C, the residue was taken into solution with 10 ml of warm, distilled water and hydrolysis products were separated on a Dowex-50 (400 mesh) cation exchange column (0.9 x 55 cm). The flow was adjusted to 7 ml/hour and 10 ml samples of 1.5 N HCl eluate were collected. Glutamic acid and serine were eluted in the vicinity of 90 ml of eluate. All collection vials were dried *in vacuo* with inside desiccator temperature of 40°C. Glutamic acid and serine were separated *via* descending paper chromatography using a solvent system of butanol-acetic acid-water (21:50:100) for 30 hours. It is not necessary to precede paper chromatography with column chromatography. It was used here because of simultaneous need for adequate amounts of phospholipid bases for other analyses not concerned with this report. After the paper was removed from chromatography chamber and dried at room temperature for at least 3 hours, it was rapidly dipped in a methanolic-ether-potassium hydroxide solution (3 parts of methanol, 1 part ether, 1% KOH), which removed an erratic background during color development. After drying, the paper was dipped in 0.1% ninhydrin acetone solution (pH 5) and dried at room temperature 30 minutes. Amino acid spots become visible but instead of both glutamic acid and serine giving typical blue-purple spot, serine becomes straw-colored whereas glutamic acid becomes pale blue. Equal areas were cut from the paper, including blanks as well as amino acids, and placed into flat bottom vials. One ml (pH 5) citrate buffer, 0.05 ml of 0.7 N acetic acid, and 1.5 ml of 2% ninhydrin solution (pH 5) were added. After 5 minutes shaking, vials were placed in covered, vigorously boiling water bath for 25 minutes. After chilling to room temperature the colored solutions were transferred to 10 ml volumetric flasks. Two-3 ml portions of propanol-water diluent were used to extract all color. Rinses were pooled into flasks; the flasks diluted to proper volume with diluent, mixed, and read at 570 mμ. Standards were run with each analysis. *Minimum requirements* for accepting a patient as an arteriosclerotic subject were (a) clinical and laboratory history of myocardial infarction from which the subject had recovered for at least 4 months, and (b) electrocardiographic changes characteristic of an infarction. Plasma was drawn only from subjects who fasted a minimum of 8 hours overnight prior to taking a sample. *Recovery.* One hundred or more μg of glutamic acid introduced into the analytical procedure beginning with acid hydrolysis, provided individual recovery values of 96, 107 and 100%. Serine, choline, and ethanolamine were also determined and gave recoveries of 96 ± 6%, 97 ± 4%, and 106 ± 6% respectively. Recovery of glutamic acid or serine in amounts less than or equivalent to 50 μg was low, ranging from 48 to 83%. No adequate explanation appears for this low recovery, although it is possible that some glutamic acid may have been converted to pyrrolidone carboxylic acid. The method did not extract free glutamic acid (1).

Results. The results of this study are given in Table I. It is apparent that the lipid bound, ninhydrin positive constituent identified as glutamic acid is present as a constituent of human plasma and that this new lipid factor is deficient in the aging, arteriosclerotic human subject. The average normal subject has 33.9 μM per liter of plasma as compared to 17.0 μM for the average arteriosclerotic. The change in distribution ratio of glutamic acid between alpha and beta lipoprotein fractions favors the beta fraction and appears to be a part of a defective mechanism for metabolism of these lipoprotein moieties. The normal subject has 47% in alpha fraction compared to 35% in this fraction for the arteriosclerotic. This abnormal distribution ratio correlates with the change in alpha-beta distribution ratio of cholesterol as observed by Barr *et al.*(6) in arteriosclerotic patients. In passing from the normal to the arteriosclerotic condition, alpha lipoprotein incurred, on the average, a 63% loss ($p<0.003$) in this lipid factor. The arteriosclerotic beta lipo-

TABLE I. Lipid Bound Glutamic Acid in Normal and Arteriosclerotic Subjects.

Normal subjects	H.K.	S.G.	D.S.	I.G.	G.S.	O.H.
Age	44	18	18	21	26	35
Wt	205	140	140	180	165	155
Alpha fraction	.394*	.200	.238	.164	.232	.182
Beta "	.332	.374	.172	.174	.346	.186
Total	.726	.574	.410	.338	.578	.368

Arteriosclerotic subjects	A.T. & W.L.		W.F. & P.A.		G.M.	W.M.	E.D.
Age	44	51	63	70	62	50	54
Wt	225	206	-	185	164	180	180
Date of infarction	3/57	3/57	7/56	6/56	1955	5/57	5/57
Alpha fraction	.028		.040		.156	.136	.100
Beta "	.168		.080		.154	.248	.132
Total	.196		.120		.310	.384	.232

* All values reported in mg %.

protein showed about a 37% loss with respect to the normal in lipid bound glutamic acid, however, range of variability in beta lipoprotein glutamic acid was sufficiently great as to cause this value to border on being significant.

In the differential ethanol separation of Cohn's microfractionation method No. 10 the 2 major types of lipoproteins, alpha and beta, are concentrated in separate fractions. Electrophoretic determinations of plasma fractions from normal individuals and arteriosclerotics have indicated that separation of the lipoproteins is complete. It appears reasonable to assume that the lipid bound glutamic acid is carried by the alpha and beta lipoprotein. This consideration is supported by the observed change in percentage distribution of glutamic acid between alpha and beta lipoprotein in normal and pathological subjects and correlation of this change to known changes in plasma content of alpha and beta lipoproteins with arteriosclerosis(6). Our measurements can not exclude the possibility that this factor is selectively isolated with or adsorbed to the alpha and beta lipoprotein or one of the other fractions; however, observations (3,6) that lipids recovered from fraction IV + V + VI are in combination with $alpha_1$ globulin and those recovered from fraction I + II + III are combined with $beta_1$ globulin would strongly argue for specific binding to alpha and to beta lipoprotein fractions.

Hecht(2) has demonstrated a highly significant inhibitory effect of glutamic acid on thrombokinase activity in the blood coagulation system. It is not known whether the lipid bound glutamic acid factor described here possesses thrombokinase inhibitory activity. If it should possess this inhibitory capacity, this fact taken together with its striking deficiency in arteriosclerotic subjects, who possess markedly disturbed blood coagulation systems(7), should warrant further work on the structure and defective metabolic relationships of this factor.

Finally, it can not be stated whether the observed difference between normals and pathologicals represents a loss of lipid bound glutamic acid in the pathological subject or whether we are dealing with molecular structural differences which convey differential solubility properties.

Summary. A ninhydrin positive, lipid bound factor chromatographically identified as glutamic acid has been found in the organic solvent extract of human plasma alpha and beta lipoproteins. This factor is grossly deficient ($p<0.006$) in plasma of the aging, arteriosclerotic subject and has an abnormal distribution pattern between alpha and beta lipoproteins of the pathological patient. This deficiency of lipid bound glutamic acid may be related to a defective blood coagulation system of the human arteriosclerotic.

1. Pilgeram, L. O., Greenberg, D. M., *J. Biol. Chem.*, 1955, v261, 465.

2. Hecht, E., *Nature*, 1951, v167, 279.

3. Lever, W. F., Gurd, F. R. N., Uroma, E., Brown, R. K., Barnes, B. A., Schmid, K., Schultz, E. L.,

J. Clin. Invest., 1950, v30, 99.
4. McFarlane, A., *Nature,* 1942, v149, 439.
5. Kirk, E., Page, I., Van Slyke, D., *J. Biol. Chem.*, 1934, v106, 203.
6. Barr, D. P., *Circulation,* 1953, v8, 641.
7. Schram, A. C., Pilgeram, L. O., *ibid.,* 1959, v20, 991.

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Effect of S-(Dichlorovinyl)-L-Cysteine on Incorporation of Precursors into Deoxypentose Nucleic Acid of Leukocytes.* (25601)

N. S. MIZUNO, G. J. HAGERTY, D. D. JOEL, J. H. SAUTTER AND M. O. SCHULTZE
Division of Vet. Pathology and Parasitology, College of Vet. Med., and Dept. of Agricultural Biochemistry, University of Minnesota, St. Paul

Continuing our investigations on the mechanism by which trichloroethylene-extracted soybean oil meal or S-(dichlorovinyl)-L-cysteine (DCVC)(1) can induce fatal aplastic anemia in calves we studied the effects of DCVC on incorporation of radioactively labeled precursors into deoxypentose nucleic acid (DNA) of leukocytes.

Procedures. Experiments with P^{32}-phosphate. Male Holstein calves, weighing 100-130 lb were conditioned at least 2 weeks prior to experiments until judged normal by clinical and hematological observations. Sodium P^{32}-phosphate, (2.5 mc, specific activity: 48-208 c/g, pH = 7.4, in 0.9% aqueous sodium chloride) was injected intravenously. A dose of DCVC (total of 3.3 mg/kg in 3 equal portions during 10 hr), previously found to induce fatal aplastic anemia in young calves(1), was injected intravenously on same day as P^{32} (first portion 5 hr before P^{32}; 2 calves) or 2 days prior to P^{32} (4 calves), or 4 days prior to P^{32} (2 calves). Four calves receiving no DCVC served as controls. All calves were kept in metabolism cages and fed, twice daily, suspension of hexane-extracted soybean oil meal in milk. Counts of thrombocytes, total and differential leukocytes were made daily. Heparinized blood (75 ml) was collected 6 hours after P^{32} injection and then daily. It was centrifuged at 5°C and 2000 g for 30 minutes, plasma removed and recentrifuged. One ml of plasma and 0.1 ml of glycerol were placed in aluminum planchets lined with filter paper and dried under infrared lamp for measurement of radioactivity. The buffy coat was lifted out, briefly washed with cold 0.16 M sodium chloride-0.01 M sodium citrate solution and DNA isolated by the method of Kay *et al.*(2). DNA-containing precipitate was suspended in 2.5 ml of 5% trichloroacetic acid, heated at 100°C for 30 minutes, cooled, diluted to 5 ml with H_2O, and centrifuged. Aliquots of clear supernatant were dried on paperlined planchets to measure radioactivity, or used for DNA determinations(3). Specific activity of leukocyte DNA was calculated as counts/minute (CPM)/μg DNA. The collected urine was filtered, 1 ml aliquots dried in paperlined planchets under infrared lamp. Radioactivity was measured with gas-flow, thin window Geiger counter and calculated with corrections for physical decay and self-absorption.

Isolated leukocyte DNA was checked for contamination by RNA and by inorganic P^{32}. RNA was determined in trichloroacetic acid extracts by the orcinol method(4). To check for inorganic P^{32} contamination, P^{32}-phosphate was added to chilled buffy coat suspension at concentrations comparable to those found in plasma, and isolation procedure for DNA was initiated at once. Radioactivity of isolated DNA was then determined. *With tritiated thymidine.* Incorporation of this compound into leukocyte DNA was studied *in vitro* with blood from normal and DCVC-treated calves. Fresh blood containing 1/10 volume disodium ethylenediamine tetraacetic

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