Biochemical Significance of Serum Glycoproteins. III. Hepatic Production of a_1 and a_2 Globulins Responding to Injury.* (29547)

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Changes in the proportions of rat serum proteins occurring in response to injury have been studied by electrophoresis and the determination of those proteins which are soluble in 0.6 M $HClO_4$ (seromucoid fraction). Because of the heterogeneity of such fractions, it is impossible to determine whether a given constituent protein is elevated while a second is depressed. The alpha$_1$ globulin zone of rat serum, whether measured as protein or hexosamines, does not change in response to injury(2). Recent findings of Williamson showed a marked decrease in the proportions of hexoses in the a_1 zone thereby indicating a significant change in its composition(3). Darcy, on the other hand, reported an increase in a specific alpha$_1$ glycoprotein of rat serum accompanying tissue necrosis(4,5). The complexity of these changes emphasizes the need to measure individual proteins responding to injury.

In this paper the presence of the alpha$_1$ glycoprotein of Darcy and of haptoglobin is confirmed in the seromucoid fraction of rat serum. Specific methods were used to compare the changes in serum levels of these two proteins with the seromucoid fraction following laparotomy and partial hepatectomy.

Methods. Male, white rats of the Sprague-Dawley (Holtzman) strain, weighing 200 to 250 g, were used throughout. Partial hepatectomies were performed under ether anesthesia according to the method of Higgins and Anderson(6); approximately 70% of the liver was removed. Control injuries were produced by a sham operation (laparotomy) imitating in as much as possible every detail of the hepatectomy including the physical disturbance of the liver. Blood samples were obtained from anesthetized animals by heart puncture.

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Seromucoid fraction: The seromucoid levels were measured by a modification of Winzler's method(7). A 0.5 ml aliquot of serum, 4.5 ml of 0.85% saline solution and 2.5 ml of 1.8 M $HClO_4$ were combined and mixed in a 50 ml polypropylene centrifuge tube. After standing at room temperature for 10 minutes, the suspension was centrifuged at 12,000 $\times$ *g* for 15 minutes at 20°C. Two 1 ml-aliquots of the supernatant were removed and each was combined with 0.2 ml of phosphotungstic acid (5% in 2 N HCl). The precipitated proteins were separated by centrifugation and then were dissolved in 1 ml of 0.1 N NaOH. The content of protein was determined by the method of Lowry and co-workers(8). For the immunoelectrophoretic studies, the seromucoid fraction was prepared by increasing all volumes 10-fold. The supernatant from the $HClO_4$ precipitation was decanted and adjusted to a pH of 8 to 8.5 with 10% KOH. The $KClO_4$ was allowed to settle and the clear supernatant was decanted, dialyzed against distilled water, and lyophilized.

Electrophoretic methods: Immunoelectrophoresis was performed by the micro method of Scheidegger (LKB apparatus)(9) using Ionagar No. 2 (Oxoid Co.). The immunoelectropherograms were developed by means of a rabbit antiserum prepared against total rat serum or by using a specific antiserum for Darcy's glycoprotein. The proteins which have been identified in rat serum to date are labeled in Fig. 4 in accordance with an earlier publication(10). The hemoglobin-haptoglobin arc was visualized by staining with benzidine reagent(11) following the electrophoretic separation of a hemolyzed serum.

Haptoglobin: Haptoglobin was measured routinely by the peroxidase method of Connell and Smithies using hydrogen peroxide as

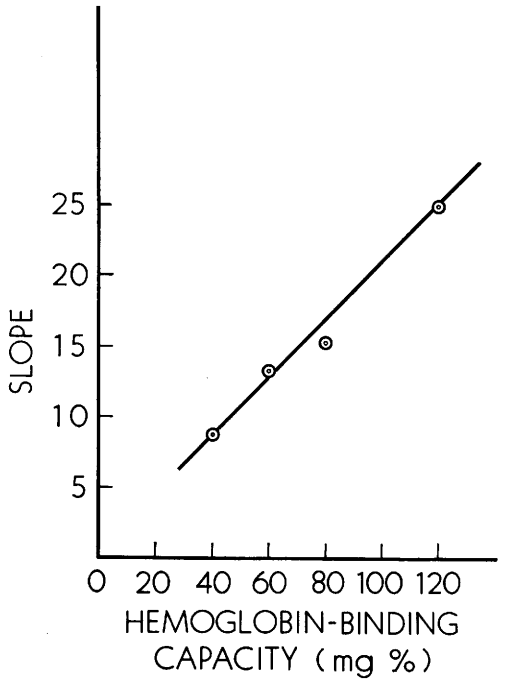

FIG. 1. Calibration curve for the guaiacol-peroxidase method for measuring level of haptoglobin. Slope is the rate of color increase as measured in a recording spectrophotometer (470 mμ) using dilutions of a pooled rat serum having an original hemoglobin-binding capacity of 120 mg%.

substrate and guaiacol† as the chromogenic compound(12). In this method only the hemoglobin-haptoglobin complex reacted with the hydrogen peroxide; excess, free hemoglobin was unreactive at a pH of 4.0. Dilutions of a pooled rat serum (hemoglobin-binding capacity, 120 mg%), to which were added a known excess of a standardized solution of human hemoglobin(13), were used to measure the rate of color development in the hydrogen peroxide-guaiacol system. A Beckman Model DB spectrophotometer was used to record the color development at 470 mμ. The slopes of these curves were plotted against the actual hemoglobin-binding capacity, Fig. 1.

The pooled serum, used in the above procedure, was previously standardized for its hemoglobin-binding capacity by means of an electrophoretic procedure(14). Fig. 2 illustrates the manner in which the value of 120 mg% was established. When higher concentrations of hemoglobin were added to the serum, the capacity to bind it in the α_2 globulin region was exceeded and free hemoglobin appeared at the origin. Preliminary experiments confirmed the data of Betsuyaku (15) showing equal complexing of human and rat hemoglobin by rat serum. The advantages of solubility and stability to storage made the use of human hemoglobin preferable.

The electrophoretic method was also used to confirm the binding capacities of sera from experimental rats having extremely low or high values by the guaiacol method. The values obtained by the two procedures did not differ significantly.

Alpha$_1$ glycoprotein of Darcy: The concentration of this α_1 glycoprotein was measured using a double diffusion Ouchterlony technique(4,16,17). Six ml of a 1% solution of Difco Certified agar, prepared in 0.85% saline solution (1:5000 merthiolate), were pipetted into Petri dishes of 2½ inches diameter. The dishes were maintained on a level table until the gel solidified. A pattern

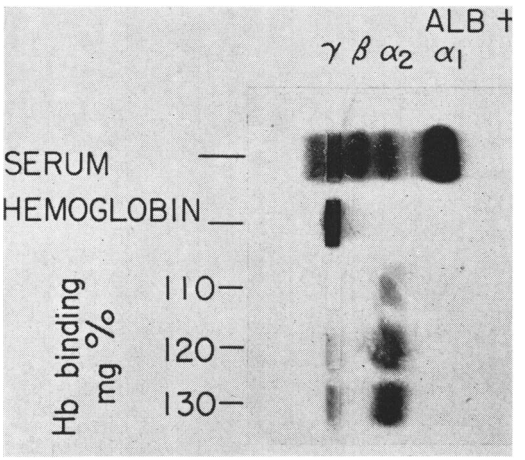

FIG. 2. Electrophoretic determination of hemoglobin-binding capacity of pooled serum from rats 24 hr after laparotomy. Increasing amounts of a standard human hemoglobin solution were added to samples of serum. Hemoglobin complexed with haptoglobin migrated as the α_2-region while excess hemoglobin remained at the origin. Hemoglobin-binding capacity was taken as the concentration of hemoglobin which just yielded a faint benzidine reaction at the origin.

† 1.86 g guaiacol (analytical grade) and 50 ml of 1 M acetic acid were added to 400 ml of water. The pH was carefully adjusted to 4.0 and final volume was made to 500 ml.

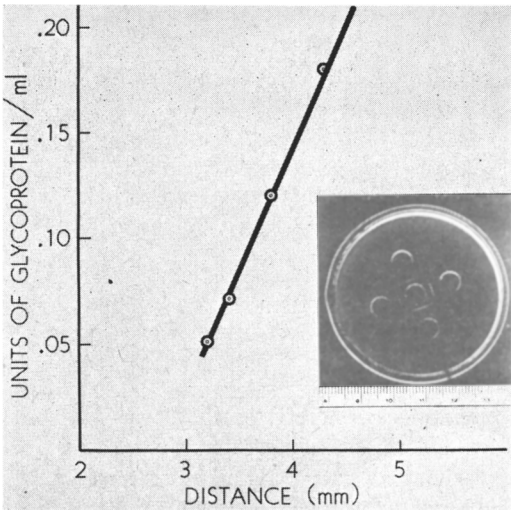

FIG. 3. Relaton of units of α_1 Darcy globulin to distance of the precipitin arc from the antigen well. Insert shows double diffusion Ouchterlony system used in determination.

of holes, each 5 mm in diameter, Fig. 3, was punched into the agar with the help of a plastic die. The 4 peripheral sample holes were separated from the centrally located antiserum well by 5 mm. The latter was filled with the specific antiserum for the α_1 protein, while each of the 4 outer wells was filled with a suitable dilution of the sample, usually 1:5 or 1:10. After incubating at 38°C for 18 hours, the distance from the leading edge of the precipitin arc to the edge of the antigen well was measured using a microscope with a 5 mm micrometer disc in the eye piece. A calibration curve, Fig. 3, was prepared using a previously standardized serum.‡

Results. Preliminary studies by zone electrophoresis, using both paper and agar media, showed the presence of approximately equal quantities of α_1 and α_2 globulins with some beta globulins and albumin in the seromucoid fraction. Immunoelectrophoresis, Fig. 4, demonstrated the presence of at least 7 proteins, albumin (trace), 2 alpha$_1$ globulins, 3 alpha$_2$ globulins and 1 beta globulin. Frequently traces of other proteins, especially in the alpha$_2$ region were also present. One of the alpha$_1$ proteins, the alpha$_1$ glycoprotein described by Darcy(5), was visualized only by its specific anti-serum, Fig. 4. The haptoglobin (alpha$_{2a}$) band was visualized by staining an electropherogram of hemolyzed serum with benzidine reagent(11). These observations are in accord with those of Goa (18), Biserte(19) and Schultze(20) using human serum and those of Benjamin and Weimer(21) for rat serum.

Normal serum levels of the seromucoid fraction, alpha$_1$ of Darcy and haptoglobin sometimes differed between groups of rats. In comparing the effects of laparotomy and hepatectomy on the levels of these proteins, each operation was performed on the same series of rats which included 4 to 6 control animals. The final data, however, always showed the same magnitude of change from control levels. The mean concentrations in

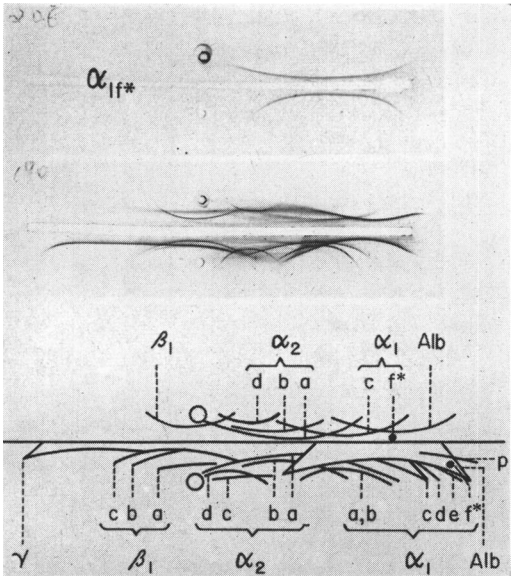

FIG. 4. Immunoelectrophoretic patterns of seromucoid fraction (upper part of each electropherogram) and total serum (lower portion of each electropherogram). The top most electropherogram was developed with the specific antiserum for Darcy's α_1 glycoprotein; the center one was developed using anti total rat serum. Lower diagram is a schematic representation of some of the proteins identified in the immunoelectrophoretic system(10), as follows: p, prealbumin (lipoprotein); Alb, albumin; α_1, a, macroglobin; b, unknown; c, glycoprotein (S 3.5)?; d, ceruloplasmin; e, unknown; f, globulin of Darcy (visualized by specific antiserum). α_2, a, haptoglobin; b, unknown; c, lipoprotein; d, "pi" protein. β_1, a, unknown; b, seromucoid; c, siderophilin. γ, gamma.

‡ Serum contained 36 units of α_1 globulin of Darcy per 100 ml.

TABLE I. Levels of Seromucoid Fraction, a_1 Darcy, and Haptoglobin 24 Hours After Laparotomy and Partial Hepatectomy.

Treatment	Seromucoid, mg %	a_1 Darcy, units/100 ml	Haptoglobin,* mg %
Control (9)‡	193 ± 60†	27 ± 7†	62 ± 12†
Laparotomy (13)	452 ± 96	135 ± 13	111 ± 23
Hepatectomy (13)	207 ± 92	60 ± 12	27 ± 10

* Hemoglobin-binding capacity.
† Mean levels ± standard deviations.
‡ Values in parentheses are numbers of rats used.

mg or units per 100 ml of serum for all of the animals are presented in Table I. It is apparent from these data that the alpha$_1$ globulin was more sensitive to laparotomy than either haptoglobin or the seromucoid fraction itself. The a_1 of Darcy was increased by 400% compared with 135% for the seromucoid and 80% for haptoglobin. Partial hepatectomy abolished or reduced the response attributed to laparotomy. The a_1 of Darcy was reduced to a level which was still 120% above the normal, the seromucoid was reduced to normal and haptoglobin to 43% of its normal value.

Discussion. The increased seromucoid fraction of rat serum in response to injury is primarily the result of a synthetic process occurring in the liver(22). This fraction was shown by immunoelectrophoresis to consist of 7 components of which the a_1 of Darcy and the a_2 globulin, haptoglobin, were chosen to be typical of the proteins responding to trauma.

The a_1 of Darcy is known to be elevated in pregnancy, tumor bearing rats, and in rats injured by subcutaneous injection of turpentine(4,5). This protein is probably of hepatic origin(17). Of the a_2 globulins, haptoglobin is frequently associated with the response to trauma(23-29). Experimental injury to animals also evokes a response in the haptoglobin level(30,31). Jayle(28), Schumacher(27) and Betsuyaku(23) concluded, on the basis of clinical evidence, that haptoglobin is normally produced in the liver. Betsuyaku arrived at the same conclusion using carbon tetrachloride intoxicated rats(15). Despite the clinical evidence, the paucity of experimental data has made it difficult to localize the source of the haptoglobin with any degree of certainty (28,32). Murray and Connell have considered the possibility that the site of injury is the source of haptoglobin. The exudate produced at the site of a subcutaneous injection of turpentine in rabbits did not, however, contain high levels of haptoglobin, thereby making the injury an unlikely source of this protein(33).

The data presented in Table I show that the 2 a-globulin constituents of the seromucoid fraction, the a_1 of Darcy and haptoglobin, responded to injury in the same manner as did the gross fraction. Partial hepatectomy, furthermore, resulted in a striking reduction in serum levels of these proteins. These observations are consistent with the earlier conclusions(22,34) that the liver is the major source of the a-globulins produced in response to injury.

Summary. Seromucoid fraction of rat serum was shown by immunoelectrophoresis to contain albumin (a trace), 2 a_1-globulins, including the a_1 of Darcy, 3 a_2 and 1 β-globulin. The changes in serum levels of the a_1-globulin of Darcy and haptoglobin (an a_2-globulin) were compared with the seromucoid fraction in rats 24 hours following laparotomy and partial hepatectomy. Both proteins, as well as the seromucoid fraction itself, were elevated following injury; these increases were either abolished or considerably reduced by partial hepatectomy. These observations are consistent with the concept that the liver is the major source of the a-globulins produced in response to injury.

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1. Neuhaus, O. W., Liu, A., *Fed. Proc.,* 1962, v23, 525.

2. Neuhaus, O. W., Balegno, H. F., Chandler, A. M., PROC. SOC. EXP. BIOL. AND MED., 1961, v107, 960.

3. Williamson, M. B., Di Lallo, L., Haley, H. B., *Clin. Chem.,* 1963, v9, 274.

4. Darcy, D. A., *Brit. J. Cancer,* 1960, v14, 524.

5. ———, *Protides of the Biological Fluids,* H. Peeters, ed., Elsevier, Amsterdam, 1963, v10, p. 131.

6. Higgins, G. M., Anderson, R. M., *A.M.A. Arch. Path.,* 1931, v12, 186.

7. Winzler, R. J., *Methods of Biochemical Analysis,* D. Glick, ed., Interscience, New York, 1955, v2, p. 279.

8. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem.,* 1951, v193, 265.

9. Scheidegger, J. J., *Intern. Arch. Allergy,* 1955, v7, 103.

10. Neuhaus, O. W., Havez, R., Biserte, G., *Arch. Biochem. Biophys.,* 1964, v106, 159.

11. Uriel, J., in *Analyse Immuno-Electrophoretique,* P. Grabar and P. Burtin, ed., Masson et Cie, Paris, 1960, p. 33.

12. Connell, G. E., Smithies, O., *Biochem. J.,* 1959, v72, 115.

13. Crosby, W. H., Munn, J. I., Furth, F. W., *U. S. Armed Forces Med. J.,* 1954, v5, 693.

14. Neuhaus, O. W., Sogoian, V. P., *Nature,* 1961, v192, 558.

15. Betsuyaku, I., *J. Jap. Soc. Int. Med.,* 1962, v51, 119.

16. Darcy, D. A., *Immunology,* 1960, v3, 325.

17. ———, personal communications.

18. Goa, J., *Acta Chem. Scand.,* 1960, v14, 1790.

19. Biserte, G., Havez, R., Hayem-Levy, A., *Clin. Chim. Acta,* 1960, v5, 272.

20. Schultze, H. E., Heide, K., Haupt, H., *ibid.,* 1962, v7, 854.

21. Benjamin, D. C., Weimer, H. E., *J. Immunol.,* 1963, v91, 331.

22. Chandler, A. M., Neuhaus, O. W., *Am. J. Physiol.,* 1964, v206, 169.

23. Betsuyaku, T., Yachi, A., Fukuda, M., Anzai, T., Wada, T., Carpenter, C. M., PROC. SOC. EXP. BIOL. AND MED., 1964, v115, 267.

24. Schumacher, G., Schlumberger, H. D., *Deut. Med. Wochensch.,* 1963, v88, 645.

25. ———, *Klin. Wochensch.,* 1962, v40, 81.

26. Jayle, M. F., Moretti, J., Mouray, H., *Les Haptoglobins,* M. F. Jayle, ed., Masson et Cie, Paris, 1962, p. 41.

27. Schumacher, G., Schlumberger, H. D., *Klin. Wochensch.,* 1962, v40, 67.

28. Jayle, M. F., Moretti, J., *Prog. in Hematology,* 1962, v3, 342.

29. Nyman, M., *Scand. J. Clin. Lab. Invest.,* 1959, v11, Supp. 39.

30. Robert, L., Mombelloni, P., Crosti, P., PROC. SOC. EXP. BIOL. AND MED., 1961, v107, 499.

31. Robert, B., Robert, L., Jayle, M. F., *Experientia,* 1959, v15, 385.

32. Laurell, C. B., Grönvall, C., *Adv. Clin. Chem.,* H. Sobotka and C. P. Steward, ed., Academic Press, New York, 1962, v5, 135.

33. Murray, R. K., Connell, G. E., *Nature,* 1960, v186, 86.

34. Neuhaus, O. W., Balegno, H. F., Milauskas, A. T., *Am. J. Physiol.,* 1962, v202, 257.

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Mechanism of Aminonucleoside-Induced Nephrosis in the Rat. IV. Hepatic Mitochondrial Oxidative Phosphorylation.* (29548)

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Demonstration of significant reduction in the disappearance of inorganic orthophosphate associated with the oxidation of several tricarboxylic acid cycle intermediates by mitochondria prepared from the kidneys of rats treated with the nephrotogenic aminonucleoside, 6-dimethylamino-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl)-purine(1,2,3), has raised several questions concerning the mechanism of action of this agent. *A priori,* if this finding is indicative of a direct effect of aminonucleoside on some aspect of the enzymatic reaction sequence involved in mitochondrial oxidative phosphorylation, then induction of similar effects in other tissue mitochondria should be demonstrable. Reported herewith are studies of oxidative phosphorylation in rat liver mitochondria during the course of induction of aminonucleoside-disease and in normal rat liver mitochondria incubated in the presence of added aminonucleoside.

Methods. The procedures and methods em-

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