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The Properties of Two Glycoproteins Isolated from the Plasma of Normal and Tumor Bearing Mice[1]

BY J. S. NISSELBAUM[2] AND PETER BERNFELD

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Both α-globulins, isolated from the plasma of normal and tumor bearing mice, respectively, are glycoproteins containing hexose, hexosamine, sialic acid and small amounts of methylpentose. These two glycoproteins are distinct from one another since they contain different amounts of the above products, and because of differences with respect to their nitrogen content, biuret reaction, extinction coefficient and isoelectric point. The protein preparation from the tumor bearing animals behaves on electrophoresis like a nearly uniform substance over a wide range of *p*H, while that from normal mice is a mixture of several proteins. It appears likely that the α-globulin isolated from tumor bearing mice is one of the regular constituents of normal mouse plasma α-globulin, the concentration of which increases during the tumor growth.

The occurrence of glycoproteins in normal and pathological sera has been described by many investigators. Some of these glycoproteins have been isolated as crystalline products and, according to ultracentrifugal and electrophoretic studies, some were obtained as uniform compounds.[3-7] Most of the serum glycoproteins are α_1- or α_2-globulins.

With the technique of zone electrophoresis on starch gel as supporting medium, it was possible to isolate two glycoproteins from mouse plasma.[8] Both are α-globulins; one was obtained from normal mice of a pure, inbred strain, the other from animals of the same strain bearing an implanted tumor. The tumor bearing mice have been found to possess about twice as much α-globulin as normal animals of the same strain.[9]

It is the purpose of the present paper to describe the properties of these two glycoproteins, and to compare them to each other.[10]

Experimental

Isolation of the Glycoproteins.—Two samples of plasma pooled from separate groups of mice were used. One group consisted of normal C57BL/6 mice, the other of Sarcoma 180 bearing animals of the same strain. The blood from the tumor bearing mice was drawn 12 to 14 days after implantation of the tumor. The fractionation of the plasma proteins was carried out by zone electrophoresis on a starch gel as supporting medium.[8] The α-globulin fractions isolated from the plasma of both groups of animals were used for the analyses described in the present paper.

Analyses.—All analyses were carried out on aliquots of stock solutions, containing about 10 mg. per ml. of the protein under investigation.

Dry weight was determined on a sample which had been extensively dialyzed against 0.01 *M* NaCl, then lyophilized and dried to constant weight at 78° under 0.05 mm. pressure. The weight of NaCl contained in the sample was calculated from the volume and subtracted from the dry weight.

Biuret reaction was carried out according to the method of Robinson and Hogden[11] with crystalline ovalbumin (Armour) as the reference protein.

Total nitrogen was determined by micro-Dumas.[12]

Optical density at 278 mμ was determined with a Beckman DU spectrophotometer.

Total hexose was determined by the orcinol method of Sörensen and Haugaard.[13]

Since 0.6 *M* perchloric acid did not produce any precipitate in the α-globulin samples, the analyses were carried out after precipitation of the glycoproteins with phosphotungstic acid, according to Winzler, *et al.*[14]

Pentose was measured by Bial's method, as described by Mejbaum.[15]

Methylpentose (deoxyhexose) was determined according to Dische and Shettles,[16] with L(−)-fucose (Mann Lab.) as a standard.

Hexuronic acid determinations were carried out both by the naphthoresorcinol method[17,18] and by the carbazol method of Dische.[19] During the latter reaction a yellow color was observed due to charring of the large excess of protein by concd. H_2SO_4. This resulted in a continuous increase in the optical density of the reaction mixture between 675 and 475 mμ, without showing a maximum in this range. An optical density at 525 mμ due to the typical reaction of hexuronic acid with carbazol was, therefore, considered to be negligible in our mixtures.

Hexosamine was determined by the Rimington modification[20] of the Elson and Morgan method.[21]

Sialic acid was determined with Ehrlich's *p*-dimethylaminobenzaldehyde reagent as described by Werner and Odin.[22] The spectrum between 470 and 650 mμ of the color obtained during this reaction was compared to that produced by a sample of crystalline sialic acid from bovine submaxillary mucin containing two acetyl groups per molecule, kindly supplied by Dr. Blix.[23]

Phosphate analyses were carried out by the Fiske-SubbaRow method[24] as modified by King.[25]

Sulfate esters were measured nephelometrically as $BaSO_4$ with the Fisher Nefluoro Photometer after 6 hours hydrolysis of a sample with 1 *N* HCl at 110° in a sealed tube.

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(2) Post-doctoral Research Fellow of the National Institutes of Health, Public Health Service.

(3) K. Schmid, THIS JOURNAL, **75**, 60 (1953); **77**, 742 (1955).

(4) H. E. Weimer, J. W. Mehl and R. J. Winzler, *J. Biol. Chem.*, **185**, 561 (1950).

(5) R. K. Brown, W. H. Baker, A. Peterkofsky and D. L. Kauffman, THIS JOURNAL, **76**, 4244 (1954).

(6) D. M. Surgenor, L. E. Strong, H. L. Taylor, R. S. Gordon and D. M. Gibson, *ibid.*, **71**, 1223 (1949).

(7) T. L. McMeekin, *ibid.*, **62**, 3393 (1940).

(8) P. Bernfeld and J. S. Nisselbaum, *J. Biol. Chem.*, in press.

(9) P. Bernfeld and F. Homburger, *Cancer Research*, **15**, 359 (1955).

(10) Presented in part before the Division of Biological Chemistry at the 126th Meeting of the American Chemical Society at New York, Sept., 1954. See Abstracts of this Meeting, J. Nisselbaum and P. Bernfeld, p. 6C.

(11) H. W. Robinson and C. G. Hogden, *J. Biol. Chem.*, **135**, 727 (1940).

(12) The analyses were carried out by Schwarzkopf Microanalytical Lab., Woodside 77, N. Y.

(13) M. Sörensen and G. Haugaard, *Biochem. Z.*, **260**, 247 (1933).

(14) R. J. Winzler, A. W. Devor, J. W. Mehl and I. M. Smyth, *J. Clin. Invest.*, **27**, 609 (1948).

(15) W. Mejbaum, *Z. physiol. Chem.*, **258**, 117 (1939).

(16) Z. Dische and L. B. Shettles, *J. Biol. Chem.*, **175**, 595 (1948).

(17) B. Tollens and F. Rorive, *Ber.*, **41**, 1783 (1908).

(18) J. A. Mandel and C. Neuberg, *Biochem. Z.*, **13**, 148 (1908).

(19) Z. Dische, *J. Biol. Chem.*, **183**, 489 (1950).

(20) C. Rimington, *Biochem. J.*, **34**, 931 (1940).

(21) L. E. Elson and W. T. J. Morgan, *ibid.*, **27**, 1824 (1933).

(22) I. Werner and L. Odin, *Acta Soc. Med. Upsal.*, **57**, 230 (1952).

(23) G. Blix, *Z. physiol. Chem.*, **240**, 43 (1936); *Skand. Arch. Physiol.*, **80**, 46 (1938); *Acta Chem. Scand.*, **2**, 467 (1948).

(24) C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, **66**, 375 (1925).

(25) E. J. King, *Biochem. J.*, **26**, 292 (1932).

Only the increase in turbidity upon addition of $BaCl_2$ was taken into account to estimate sulfate-S. Blanks with pure samples of methionine or cysteine were low and showed that the interference of these amino acids in the sulfate analysis was negligible.

Isoelectric points were determined by moving boundary electrophoretic analyses of 4-mg. protein samples in a fused quartz cell of the Antweiler-Boskamp Microelectrophoresis apparatus. Aliquots of 0.4 ml. of 1% protein solutions were dialyzed overnight in Visking dialysis tubes of 1/4-inch diameter against 50 ml. of "tris"-citrate buffers, ranging in *p*H from 3.3 to 8.8. All buffer solutions were made up to have the same electric conductivity, *i.e.*, 6.75×10^{-4} mho/cm. at 0°, a 0.01 *M* KCl solution with a known conductivity (7.76×10^{-4} mho/cm. at 0°) was used as a reference. The buffer solutions were prepared by adding a 2 *M* solution of trishydroxymethylaminomethane to 6 ml. of 1 *M* citric acid until the desired *p*H was reached, as measured with a glass electrode, and diluting the solution with water to a constant conductivity. The electrophoretic analyses were carried out as reported earlier, and each experiment at a given *p*H was repeated twice with the same protein sample, as previously described.[9] The initial protein-buffer interface was shifted a short distance into the visible part of the ascending channel of the cell and the protein patterns before and after the electrophoretic migration were photographed by means of a Schlieren-cylindrical lens optical system.

The electrophoretic mobilities were calculated from the distance of the protein peak after 6 and 12 minutes migration from the starting point. The results from 3 experiments at both 6 and 12 minutes were averaged. A sample of whole human plasma in veronal citrate buffer of *p*H 8.6 and ionic strength 0.1 was run in the same cell. The distance migrated by the albumin was used as a basis for the calculation of mobilities; the mobility of human plasma albumin under these conditions is 5.9×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}.

Results and Discussion

Electrophoretic analyses at *p*H 8.6 of the α-globulin fractions gave the following results: preparation 1 from tumor bearing mice contained 84.4% of a component migrating as a uniform substance in an electric field at this *p*H, it further contained 12.6% α_3-globulin and 3% albumin; preparation 2 had a similar composition; the preparation from normal mice consisted of 70.1% α-globulin, 15.1% α_3-globulin and 14.8% albumin.

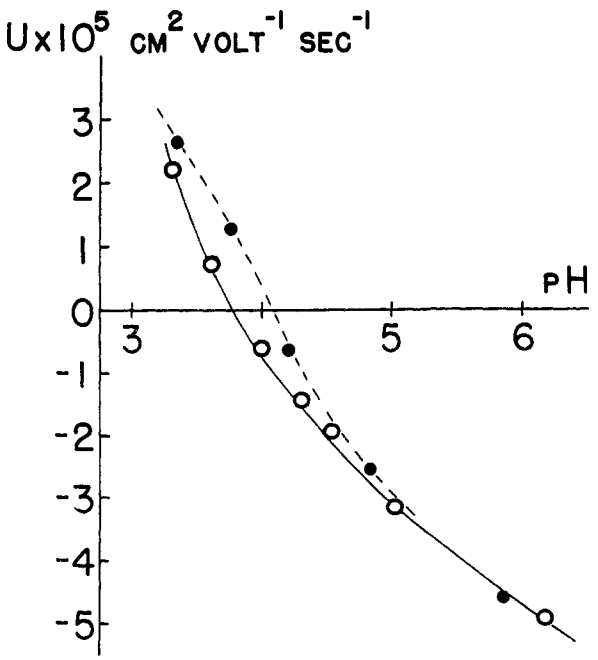

Fig. 1.—Electrophoretic mobility as a function of the *p*H of two α-globulin preparations: from normal mouse plasma (black dots and dotted line), and from the plasma of tumor bearing mice (open circles and solid line).

Preparation 2 from tumor bearing mice has been shown to migrate as an almost uniform substance over a wide range of *p*H (*p*H 3.6 to 8.6). In contrast, the α-globulin preparations from normal mice were found to be much less uniform in an electric field, in particular at lower *p*H values. This is not surprising, since it is well known that, at least in human plasma, α-globulin is a mixture of numerous individual proteins. The finding of a rather high degree of uniformity in the α-globulin preparations from tumor bearing mice seems to indicate, however, that the increase of α-globulin during tumor growth is due to the appearance of a single individual protein in that plasma.

The data on some properties of the α-globulins from both normal and tumor bearing mice are presented in Table I. The electrophoretic mobilities of the two proteins as a function of the *p*H are plotted in Fig. 1. The values given for the preparation from normal animals are those of the main component.

TABLE I

PROPERTIES OF α-GLOBULINS ISOLATED FROM THE PLASMAS OF NORMAL C57BL/6 MICE AND C57BL/6 MICE BEARING SARCOMA 180

	Mouse plasma α-globulin		
	Normal	Tumor	
		Prepn. 1	Prepn. 2
Hexose, %	1.6	5.0	5.4
Pentose, %		<0.2	
Methylpentose, %	0.20		0.54
Hexuronic acid, %	0	0	0
Hexosamine, %	3.3		4.9
Sialic acid, %	2.3	4.4	4.6
Sulfate S, %	0.074		0.072
Phosphorus, %		0.26	
Nitrogen, %	12.05		11.71
Biuret reaction[a]	0.806		1.07
Extinction coefficient ($E^{1\%}_{1cm.}$ 278 mμ)	8.8		11.6
Isoelectric point	*p*H 4.1 ± 0.05[b]		*p*H 3.8 ± 0.05

[a] Biuret value of crystalline ovalbumin = 1.0. [b] Main component.

From these results, three main points appear evident. First, both α-globulins are to be classified as glycoproteins, due to their content of hexosamine and hexose. Second, there are marked differences between the properties of α-globulins from normal and tumor bearing mice, respectively. This confirms the opinion expressed above that one individual protein appears to be increased in the plasma of tumor bearing mice; the properties of this protein appear to be different from the properties of the mixture of proteins, contained in α-globulin of normal mice. Last, although marked differences exist between the properties of the two α-globulins, one can find, at least qualitatively, certain similarities. Both contain hexose, hexosamine, sialic acid and small amounts of methylpentose and sulfate esters. The nitrogen content is low in both and neither of them contains hexuronic acid. These qualitative similarities make it unlikely that a

glycoprotein like that isolated from the plasma of tumor bearing mice is completely absent in the plasma of normal mice, and it seems probable that it is one of the constituents of α-globulin which increases in concentration during the tumor growth.

The observation that the addition of sulfate ester containing polysaccharides to normal human plasma gives the illusion of an increased α-globulin[26] made it necessary to analyze our α-globulins for sulfate esters. Both globulins were found to contain only very small amounts of sulfate sulfur, and the phenomenon of the increase of α-globulin during tumor growth appears to be unrelated to the interaction of polysulfates with normal plasma proteins.

Biological Properties.—The growth of transplanted Sarcoma 180 in mice of the strain C57BL/6 was affected neither by injection of the host with α-globulin from tumor bearing mice (simultaneously or prior to the transplantation), nor by addition of this α-globulin to the tumor cell suspension before implantation.

On the other hand, the α-globulin, isolated from tumor bearing mice, did not induce progressive growth of a transplantable tumor (Sarcoma I) in a host otherwise resistant to this tumor (mice of the C57BL/6 strain), *i.e.*, the α-globulin obtained from Sarcoma 180 bearing mice was not identical with or did not contain the "enhancing" or "XYZ" factor.[27,28]

Acknowledgment.—We wish to thank Professor Blix for his generous gift of crystalline sialic acid.

(26) P. Bernfeld, *Federation Proc.*, **13**, 183 (1954); **14**, 182 (1955).

(27) S. Flexner and J. W. Jobling, *Proc. Soc. Exper. Biol. Med.*, **4**, 156 (1907).

(28) G. D. Snell, A. M. Cloudman, E. Failor and P. Douglass, *J. Nat. Cancer Inst.*, **6**, 303 (1946).

BOSTON, MASSACHUSETTS

COMMUNICATIONS TO THE EDITOR

THE SODIUM-DIBORANE REACTION

Sir:

Stock[1] by direct reaction between sodium and diborane obtained $Na_2B_2H_6$. More recent investigators[2] obtained NaB_2H_6 and showed by X-ray analysis that it contained sodium borohydride. Studies in this laboratory have clarified the nature of the sodium–diborane reaction and have established the identity of another product, the new compound NaB_3H_8.

Early in the present investigation it was discovered that ethers promote the reaction of sodium and diborane. In ethyl ether sodium, as amalgam, and diborane reacted completely in two days at room temperature, forming a product of composition NaB_2H_6. Rate studies indicated that the reaction in the initial stages involved two gram atoms of sodium per mole of diborane and that $Na_2B_2H_6$ was an intermediate. X-Ray and infrared analysis of NaB_2H_6 confirmed the presence of sodium borohydride and revealed an unidentified constituent, soluble in ethyl ether. Upon evaporation of ether solutions, white solids of composition NaB_3H_8 remained. Calcd. for NaB_3H_8: Na, 36.1; B, 51.1; hydrolyzable hydrogen, 141 mmole/g. Found: Na, 34.5 and 35.6; B, 52.6 and 51.5; hydrolyzable hydrogen, 137 and 142 mmole/g. The X-ray pattern of NaB_3H_8 was unique and well-defined. Further instrumental and chemical analyses of NaB_2H_6 gave no evidence of constituents other than sodium borohydride and NaB_3H_8. The experimental facts quite satisfactorily support the relationship

$$2Na + 2B_2H_6 \longrightarrow NaBH_4 + NaB_3H_8$$

On this basis, an 80% yield of NaB_3H_8 was obtained in one experiment. The relatively small quantity of product compared to reactor volume and quantity of amalgam made quantitative isolation difficult.

The compound NaB_3H_8 is quite soluble in water and in ammonia and is more resistant toward hydrolysis than is sodium borohydride. The solid is thermally stable at least to 200°. Solution of NaB_3H_8 in ethyl ether is accompanied by solvation; at 0° a hemi- and a mono-etherate were identified.

The sodium–diborane reaction is undoubtedly of greater complexity than that indicated by the equation, which does not specify probable reaction intermediates. However, the new compound, NaB_3H_8, may be expected as a product of reactions employing a variety of conditions. It is suggested that NaB_3H_8 be designated as sodium triborohydride. A detailed description of this and related investigations of the reaction of sodium with diborane will be submitted at a later date.

WILLIAM V. HOUGH
CALLERY CHEMICAL COMPANY LAWRENCE J. EDWARDS
CALLERY, PENNSYLVANIA ALBERT D. McELROY

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(1) A. Stock, "Hydrides of Boron and Silicon," Cornell University Press, Ithaca, N. Y., 1933.

(2) J. S. Kasper, L. V. McCarty and A. E. Newkirk, THIS JOURNAL, **71**, 2583 (1949).

LIGHT CATALYZED ORGANIC REACTIONS. IV.[1] THE OXIDATION OF OLEFINS WITH NITROBENZENE

Sir:

As a consequence of the previously reported synthesis of oxetanes by light induced addition of carbonyl compounds to substituted olefins,[2] we have investigated the reaction of nitrobenzene with

(1) Part of a program of research supported by a grant from the Godfrey L. Cabot Fund, Publication No. 61, M.I.T. Solar Energy Conversion Project.

(2) E. Paterno and G. Chieffi, *Gazz. chim. ital.*, **39**, 341 (1909); G. Büchi, C. G. Inman and E. S. Lipinsky, THIS JOURNAL, **76**, 4327 (1954).