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Immunochemical Studies on Blood Groups. XIV. The Separation and Quantitative Estimation of Glucosamine and Galactosamine in Blood Group Substances[1]

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A method has been developed for the separation and estimation of glucosamine and galactosamine as the 2,4-dinitrophenylaminohexitols; about 0.5 mg. of hexosamine is required. Hog and human blood group A substances were found to have glucosamine–galactosamine ratios averaging 1.5 and 1.6, respectively; group O(H) hog and human preparations 2.2 and 2.5; and human B preparations 2.8. Horse and bovine materials had varying ratios, apparently unrelated to their A, B, O specificities.

Introduction

Following the discovery of galactosamine (chondrosamine) as a second hexosamine in blood group substances,[2,3] it became of importance to develop a method for the quantitative separation and estimation of glucosamine and galactosamine as part of the continuing effort to elucidate the structure of the blood group substances in relation to their unique immunological specificity. Identification of these amino sugars has been accomplished as the hydrochloride (glucosamine), the N-carbobenzyloxy derivative,[4] as various Schiff bases[5,6] and by X-ray diffraction.[7]

Paper chromatography has been used by Aminoff, Morgan and Watkins[3] for the separation and identification of the hexosamines in blood group substances, but the necessity for using basic solvents such as collidine resulted in poor recoveries, of the order of 30%. A separation of the N-2,4-dinitrophenyl derivatives of the hexosamines on paper has also been reported.[8] Gardell, *et al.*,[9] oxidized glucosamine and galactosamine with ninhydrin and separated the resulting arabinose and lyxose on paper.

The first quantitative estimation of the two hexosamines in blood group substance was done by Annison, James and Morgan[10] who prepared the N-2,4-dinitrophenyl derivatives and separated them on a borate-buffered celite column with amyl alcohol–chloroform mixtures. Using 100-mg. portions of blood group A and H substances and 500 mg. of Le^a substance, they obtained on single samples glucosamine–galactosamine ratios of 0.9 for human ovarian cyst A substance,[10] 1.1 for human ovarian cyst H substance[11] and 2.7 from human ovarian cyst Le^a substance.[12] Since quantities of blood group substance of this order were not available to us, attempts were made to adapt the method of Morgan, *et al.*,[10] to 2–3-mg. samples of material. In this range the color of the DNP–glucosamine band was observed to disappear continuously during chromatography by their procedure, resulting in variable and consistently low recoveries of DNP–glucosamine as compared to DNP-galactosamine which appeared to be more stable.

More recently Gardell[13] has obtained a useful separation of the two hexosamines on a Dowex-50 ion-exchange resin eluting with 0.3 *N* hydrochloric acid. Foster and Stacey[14] have reported the separation of N-acetylglucosamine from N-acetylgalactosamine by ionophoresis on filter paper. The enzyme hexokinase isolated from yeast, has been reported[15] to phosphorylate glucosamine in the presence of ATP, galactosamine being unaffected. A method for the estimation of glucosamine and galactosamine based on this enzymatic reaction has been reported[16] utilizing the decrease in Elson–Morgan[17a,b] color after precipitation of the hexosamine phosphorylated by the enzyme.

Method.—It is believed that the irreversible disappearance of hexosamine in alkaline solution is explicable in terms of reaction between the reducing end group and the amino group on carbon 2. Lobry de Bruyn[18] has postulated a bimolecular condensation between two glucosamines to give 2,5-bis-(1,2,3,4-tetrahydroxy-*n*-butyl)-pyrazine. To eliminate this type of complication with alkali, an attempt was made to reduce the end aldehyde group to an hydroxymethyl group, using sodium borohydride which has been shown[19] to be a useful reagent for this purpose in aqueous solution. Since sodium borohydride reacts slowly with water to give alkaline solutions, reduction of the hexosamines was done at 0° with a large excess of sodium borohydride in an effort to favor the reduction over the alkaline condensation. Excess borohydride was decomposed with acid and the hexosaminitols were dinitrophenylated by the method of Sanger,[20] no precautions as to temperature or alkalinity being

(1) This investigation was carried out under grants from the National Institutes of Health, Public Health Service (RG-34) and the William J. Matheson Commission.

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(18) C. A. Lobry de Bruyn, *Rec. trav. chim.*, **18**, 77 (1899).

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(20) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

TABLE I

ANALYSIS OF MIXTURES OF GLUCOSAMINE AND GALACTOSAMINE

Starting mixture	Glucosamine found, μg.	Recovery, %	Galactosamine found, μg.	Recovery, %	Glucosamine / Galactosamine Found	Glucosamine / Galactosamine Calcd.
1 mg. glucosamine + 0.5 mg. galactosamine	890	89	420	84	2.1	2.0
1 mg. glucosamine + 0.5 mg. galactosamine	920	92	505	101	1.8	2.0
0.5 mg. glucosamine + 0.5 mg. galactosamine	450	90	440	88	1.0	1.0
0.5 mg. glucosamine + 1.0 mg. galactosamine	440	88	830	83	0.5	0.5
0.4 mg. glucosamine + 0.4 mg. galactosamine	355	89	225	75	1.6	1.3
0.5 mg. glucosamine + 0.5 mg. galactosamine	435	87	410	84	1.1	1.0
		av. 89		av. 86		

TABLE II

RECOVERY OF ADDED GLUCOSAMINE AND GALACTOSAMINE

Starting mixture	0.95 mg. hog 20 + 250 μg. glucosamine + 250 μg. galactosamine	0.95 mg. hog 20[a]	1.0 mg. hog 42 (8%) + 250 μg. glucosamine + 250 μg. galactosamine	1.0 mg. hog 42 (8%)[a]	0.98 mg. hog 17 + 250 μg. glucosamine + 250 μg. galactosamine	0.98 mg. hog 17[a]
Glucosamine found, μg.	395	180	390	155	380	165
	−180		−155		−165	
Added glucosamine recovered, μg.	215		235		215	
Recovered, %	86		94		86	
Galactosamine found, μg.	330	110	285	80	295	100
	−110		− 80		−100	
Added galactosamine recovered, μg.	220		205		195	
Recovered, %	88		82		78	
Ratio of recovered Glucosamine / Galactosamine	1.0		1.1		1.1	

[a] Determination actually made on twice this amount of material and result divided by 2.

required. The DNP-aminohexitols were separated from the bulk of colored impurities by passage through a silicic acid column with 10% ethanol–chloroform (v./v.); and then resolved by passage through a borate buffered column of silicic acid–celite with 20% ethanol–chloroform. Morgan, *et al.*,[10] achieved a separation of the DNP-hexosamines, because in the pyranose form only the DNP-galactosamine had a pair of *cis*-hydroxyl groups with which to form a charged borate complex preferentially soluble in the aqueous phase and hence moving more slowly with organic solvents than did the uncharged DNP-glucosamine.

Upon reduction to the open chain aminohexitol both DNP-glucosaminitol and DNP-galactosaminitol could theoretically give a variety of borate complexes. It was, therefore, fortunate that whichever borate complexes did form were sufficiently different to give a useful separation under the conditions employed. It is of interest that the order of movement on the borate-buffered column is DNP-galactosaminitol (R = 0.4) > DNP-glucosaminitol (R = 0.1), the reverse of the order observed by Morgan, *et al.*,[10] for the DNP-hexosamines.

To establish the precision of the method, a number of runs were made on mixtures prepared from pure glucosamine and galactosamine. From the results listed in Table I, it can be seen that in the range from 0.7 to 1.5 mg. of the mixed hexosamines the recoveries were of the order of 85%, with the glucosamine–galactosamine ratios differing from the theoretical by an average of 0.15 unit. To exclude excessive or uneven rates of destruction by the other constituents of blood group substances, recovery determinations were carried out adding known amounts of amino sugars to known amounts of blood group substance. Three blood group substances were hydrolyzed and analyzed for glucosamine and galactosamine both alone and with known amounts of hexosamine added before hydrolysis (Table II). The glucosamine in the blood group substance alone was subtracted from that found with blood group substance plus added hexosamines. The value so obtained represents the recovery of the added glucosamine, and a similar calculation gives the galactosamine recovery. The results of three such determinations shown in Table II give an average recovery of added glucosamine of 89% and an average recovery of added galactosamine of 83%. Moreover, the ratios of recovered glucosamine to recovered galactosamine are in close agreement with the ratios initially added. These results are taken to indicate the absence of any extensive or selective destruction of either glucosamine or galactosamine in the determination.

Results and Discussion

A large number of blood group substances prepared by previously described methods from hog,[21]

(21) A. Bendich, E. A. Kabat and A. E. Bezer, *J. Exp. Med.*, **83**, 485 (1946).

human,[22] horse[23] and cattle[24] sources and characterized were available and were analyzed for glucosamine and galactosamine. The results are tabulated according to species and blood group in Table III. To allow more ready comparison hexosamine values are given per mg. of blood group substance, although the actual determinations were generally done on 2–3 mg. of sample. When enough material was available more than one determination was made and the results were averaged for tabulation. Hexosamine values are given to the nearest 5 μg., and ratios to only one decimal point since it is felt that the method is not more precise than this. Some idea of the reproducibility of the method may be seen by the values obtained on some representative samples. For example, on duplicate 2-mg. samples of hog 42 (8%) run at different times, the glucosamine found was 295 and 310 μg. and the galactosamine was 150 and 160 μg. For A. K. 10% the duplicate values obtained on 2-mg. samples were 360 and 350 μg. of glucosamine and 170 and 175 μg. of galactosamine; and for B. K. C_6H_5OH insol. (2 mg.) 255 and 270 μg. of glucosamine and 150 and 155 μg. of galactosamine.

TABLE III

GLUCOSAMINE AND GALACTOSAMINE IN HOG AND HUMAN BLOOD GROUP SUBSTANCES

Sample	No. of determ.	Glucosamine, μg./mg. B.G. S.[a]	Galactosamine, μg./mg. B.G. S.[a]	Elson–Morgan total hexosamine, μg./mg. B.S. S.	Yield, %	Ratio glucosamine/galactosamine
Preparations with A activity from individual hog stomach linings						
Hog 9	1	175	125	330	91	1.4
Hog 16	1	125	95	330	67	1.3
Hog 17	2	175	105	290	96	1.7
Hog 18	2	180	95	310	89	1.9
Hog 20	2	185	115	310	97	1.6
Hog 21	3	145	115	330	79	1.3
Hog 24	3	135	85	280	79	1.6
Hog 26	1	165	110	320	86	1.5
Hog 28	2	155	90	330	74	1.7
Hog 30	3	145	105	320	78	1.4
Hog 31	3	155	110	310	86	1.4
Hog 55(8%)	1	145	95	290	83	1.5
					av.	1.5
Preparations with O(H) activity from individual hog stomach linings						
Hog 23	1	220	85	320	95	2.6
Hog 25	1	185	75	330	79	2.5
Hog 27	2	155	70	300	75	2.2
Hog 29	3	170	75	350	70	2.3
Hog 38(4%)[f]	1	145	65	270	78	2.2
Hog 38(8%)[f]	2	130	65	320	61	2.0
Hog 42(4%)[f]	1	155	70	290	78	2.2
Hog 42(8%)[f]	3	145	75	270	82	1.9
					av.	2.2
Preparations with A activity from human A_1 saliva						
W.H.$_2$ 10%	3	150	95	300	82	1.6
W.H.$_1$ C_6H_5OH ins.	2	115	70	290	64	1.6
B.K. 10%	1	105	65	210	81	1.6
B.K. C_6H_5OH ins.	2	130	75	240	85	1.7
G.C. 10%	1	145	95	280	86	1.5
A.B.$_4$ 10%[b]	1	205[b]	125[b]	290		1.6
					av.	1.6
Preparations with A activity from human A_2 saliva						
W.G. C_6H_5OH ins.	2	140	65	290	71	2.2
A.K. 10%	2	180	85	290	92	2.1
Preparations with B activity from human B saliva						
E.J. 10%	1	165	65	300	77	2.5
E.J. C_6H_5OH ins.	1	140	45	230	81	3.1
S.J. 10%	1	130	50	300	60	2.6
J.M. 10%	1	155[b]	60[b]	260		2.6
J.J. C_6H_5OH ins.	3	140	50	250	76	2.8
J.C. 10%	1	140	50	280	68	2.8
J.C. C_6H_5OH ins.	1	145	45	210	90	3.2
P.M. 10%	1	135	60	290	67	2.3
P.M. C_6H_5OH ins.	1	130	45	200	88	2.9
S.E. C_6H_5OH ins.	1	130	45	190	92	2.9
					av.	2.8
Preparation from human saliva of individuals of group 0						
Bd 10%[c]	1	165	65	240	96	2.5
F.P. C_6H_5OH ins.[d]	1	140	55	220	89	2.5
Preparations from human pseudomucinous cyst fluids						
Morgan cyst A	2	115	100	30	72	1.2 (0.9)[e]
Morgan cyst H	2	130	75	28	73	1.7 (1.1)[e]
Morgan cyst Le[a]	2	130	55			2.4 (2.7)[e]
Morgan human B	1	125	45	25	68	2.8

[a] Average of determinations if more than one. [b] Amount of sample not measured quantitatively. [c] This preparation tested by Dr. W. T. J. Morgan showed Le[a] but no O(H) activity. [d] This preparation tested by Dr. W. T. J. Morgan showed Le[a] and O(H) activity. [e] Ratio in ( ) obtained by Morgan, *et al.*[10–12] [f] C. Howe and E. A. Kabat, unpublished data.

In most cases studied, the total hexosamine yield by the above described method ran to from 70–90% of the total determined by the Elson–Morgan color reaction.[17a,b] Since the Elson–Morgan reaction in our hands had a precision of about ±10%[25] and since mixtures of amino acid and reducing sugars (both present in blood group substance) are reported[26a,b] to give non-specific color in this test, it might well be that the yields of hexosamine obtained by the chromatographic method are even better than indicated. Indeed, the yields obtained on weighed samples of pure hexosamine (with one exception) (see Table I) represented 85–95% of theoretical, somewhat better than the yields based on the Elson–Morgan color given by the blood group substances.

Some basis of comparison of this method with the previously described one by Morgan, *et al.*,[10] was possible on three samples of blood group substances (A, H and Le[a]) isolated from human pseudomucinous ovarian cysts and kindly sent to us by Dr. W. T.

(22) E. A. Kabat, A. Bendich, A. E. Bezer and S. M. Beiser, *J. Exptl. Med.*, **85**, 685 (1947).

(23) H. Baer, E. A. Kabat and V. Knaub, *ibid.*, **91**, 105 (1950).

(24) S. M. Beiser and E. A. Kabat, *J. Immunol.*, **68**, 19 (1952).

(25) N. F. Boas, *J. Biol. Chem.*, **204**, 553 (1953).

(26) (a) C. Vasseur and J. Immers, *Arkiv. Kemi*, **1**, 253 (1949); (b) H. N. Horowitz, M. Ikawa and M. Fling, *Arch. Biochem.*, **25**, 226, (1950).

J. Morgan. From the results listed at the bottom of Table III, it may be seen that for the cyst A and cyst H substance the ratios (done on 100-mg. samples) by Morgan, *et al.*, were somewhat lower and for the cyst Le^a substance (done on a 500-mg. sample) somewhat higher than the results obtained in the present investigation. This discrepancy might have its basis in the previously noted selective destruction of DNP glucosamine during chromatography, which would be more evident with smaller samples.

One of the most striking features of this survey is the almost complete parallelism between the ratio of glucosamine to galactosamine found and the serological specificity of the hog and human substances. The 12 hog group A preparations studied, each from a single hog stomach lining, have an average ratio of 1.5 with a range of values from 1.3 to 1.9. This is significantly different from the 8 hog preparations, each from a single hog stomach with group O(H) specificity which have an average ratio of 2.2 with a range of 1.9 to 2.6. Moreover, human preparations with group A specificities which are known[27] to be serologically very similar to the hog A preparations have an average glucosamine to galactosamine ratio of 1.6, identical within experimental error with the hog A ratio.

Not enough materials from saliva of humans of group O were available to make any generalization, but the two substances studied (Bd. 10%, F. P. C_6H_5OH ins.) have ratios of 2.5 quite different from the human A ratios and very close to the hog O(H) ratios. These two preparations also showed considerable Le^a activity.

Of some interest is the finding on two preparations (W. G. C_6H_5OH ins. and A. K. 10%) which were obtained from individuals of blood group A_2. Although the data are fragmentary at best, it is interesting that the glucosamine to galactosamine ratios of 2.2 found for these human A_2 preparations were between the ratios for the human A_1 preparations (1.6) and the human preparations with Le^a activity from O saliva (2.5). A_2 erythrocytes are known to be agglutinated by anti-O(H) sera and Hirzfeld[28] has postulated that A_2 and A_1 represent successive mutations from O along one line and B represents mutation from O along another line. The differences in the glucosamine to galactosamine ratios may merely be a chemical reflection of these mutations.

One further complicating factor in the study of the relationship between blood group and hexosamine ratio must be mentioned. In general, except for hexosamine ratios and some variation in fucose content, certain of the blood group substances (A, B, O(H), Le^a) are similar in gross chemical composition (reducing sugar, hexosamine, nitrogen). Hence any preparation isolated, for example, from human saliva might contain all the blood group substances secreted by the individual. Since the Le^a substance is seen in Table III to have a higher ratio (2.7), the presence of variable amounts of this material in human A or O preparations might give a greater spread in glucosamine–galactosamine ratios. This might especially apply to the two preparations from individuals of blood group O, both of which have been shown to have Le^a activity and one of which had no O(H) activity, as kindly tested by Dr. W. T. J. Morgan.

The problem is even more complex when the data on horse and cattle preparations are analyzed (Table IV). From the materials studied, there seems to be no apparent relationship between the hexosamine ratios and the ABO specificities of these preparations. This is not altogether surprising, however, since these materials have been shown to be far more complex than those of the hog or the human. Purified materials from individual horse stomachs may show either A or B or AB activity or may be inactive and show neither A, B nor O(H) activity.[23] Bovine materials have been found with A, B, O, combinations of these activities, or with no

TABLE IV

GLUCOSAMINE AND GALACTOSAMINE IN HORSE AND BOVINE BLOOD GROUP SUBSTANCES

Sample	No. of determ.	Glucosamine, μg./mg. B.G.S.	Galactosamine, μg./mg. B.G.S.	Elson–Morgan total hexosamine, μg./mg. B.G.S.	Yield, %	Ratio, glucosamine / galactosamine
Horse stomach preparations with A activity						
Horse 1-15%	1	140	75	230	94	1.9
1-25% from 15%	1	165	85	270	93	2.0
Horse stomach preparations with B activity						
Horse 2-15%	1	75	65	150	92	1.2
2-25%	2	140	95	290	81	1.5
4-15%	1	140	90	220	105	1.6
4-25%	1	155	90	280	88	1.7
Horse stomach preparations with minimal B activity						
5-15%	1	160	85	270	91	1.9
5-25%	1	85	75	230	70	1.0
Inactive horse stomach preparations						
Horse 3-25%	1	135	90	270	83	1.5
5-C_6H_5OH ins.	1	70	30	110	91	2.3
6-15%	1	155	70	230	98	2.2
6-25%	1	115	75	210	90	1.5
Bovine stomach preparations with A activity						
Cow 18.10%	1	65	45	140	79	1.4
31.10%	1	170	100	310	87	1.7
35.10%	1	170	75	300	82	2.3
36.10%	1	150	85	270	88	1.8
38.10%	1	200	115	320	98	1.7
47.10%[a]	1	120	40	190	84	3.0
Bovine stomach preparations with B activity						
Cow 21.10%	1	150	70	310	71	2.2
23.10%	1	140	80	290	76	1.8
25.10%[a]	2	185	120	230	130	1.5
26.10%[a]	1	170	95	310	85	1.8
27.10%[a]	1	185	105	280	103	1.8
Inactive bovine stomach preparations						
Cow 22.10%	1	185	80	340	78	2.3
28.10%	1	115	85	230	87	1.4
34.10%	1	150	80	280	82	1.9
37.10%	1	210	95	330	93	2.2
C_6H_5OH insol. preparations from bovine stomach						
18 C_6H_5OH ins.	1	120	75	220	88	1.6
21 C_6H_5OH ins.	1	175	85	260	100	2.1
23 C_6H_5OH ins.	1	150	55	260	79	2.7
22 C_6H_5OH ins.	1	190	70	310	84	2.7

[a] Weak activity.

(27) E. A. Kabat, H. Baer, R. L. Day and V. Knaub, *J. Exp. Med.*, **91**, 433 (1950).

(28) R. R. Race and R. Sanger, "Blood Groups in Man," Charles C Thomas, Inc., Springfield, Illinois, 1950, pp. 23–27.

activity.[24] Furthermore, bovine substances have been shown to possess a common bovine specificity[24] and to contain the so-called "J" factor of cattle.[29a,b] Cross reaction between the horse and bovine A substances with hog or human anti-A shows them to be much more distantly related.[24,27] It is not surprising, therefore, that these more complex materials cannot yet be fitted into the pattern, in terms of their glucosamine–galactosamine ratios, found for the hog and human substances. Indeed, their cross reactivity with the hog and human A, B or O substances may merely involve a small number of the total residues of amino sugar in their respective molecules.

The data on the hog and human blood group substances provide the first indication, on a significant number of individual samples, of chemical differences between the blood group A, B and O substances which appear to correlate with their immunological specificity. However, complications due to the presence of multiple specificities in certain preparations must be considered. This is especially apparent in the materials from horse and bovine sources.

Experimental[30]

N-2,4-Dinitrophenylglucosaminitol.—Glucosamine·HCl (1.0 g.) was dissolved in water (20 ml.) and cooled in an ice-bath. To this was added dropwise with stirring 10 ml. of a cold solution of sodium borohydride (0.5 g.). The mixture was allowed to sit in an ice-bath for 30 minutes and then at room temperature for 2 hours. It was then carefully acidified with 6 *N* hydrochloric acid to decompose the excess sodium borohydride. After addition of sufficient sodium bicarbonate to make the solution alkaline, 2,4-dinitrofluorobenzene (2.1 ml.) in ethanol (75 ml.) was added and the mixture shaken for 2 hours at room temperature. The resulting deep-orange solution was evaporated to dryness at reduced pressure and taken up in dilute hydrochloric acid. Continuous ether extraction for 2 days and removal of the ether under reduced pressure gave 1 g. of a yellow-orange residue, which was purified by passage through a silicic acid column (14 g. silicic acid +7 ml. aqueous phase) using 25% (v./v.) methanol–chloroform saturated with water as the developer. The heavy yellow band having R = 0.5 gave on evaporation yellow needles, m.p. 163–164°, yield 58%.

Anal. Calcd. for $C_{12}H_{17}O_9N_3$: C, 41.5; H, 5.0; N, 12.0. Found: C, 41.7; H, 5.1; N, 11.5 (Kjeldahl).

N-2,4-Dinitrophenylgalactosaminitol.—Chondrosamine·HCl (140 mg.) prepared as described by Meyer and Smyth[31] was carried through essentially the same procedure as that used with glucosamine. Purification by chromatography on silicic acid as before gave a glossy yellow material (R = 0.45) which on solution in ethanol and treatment with petroleum ether gave an amorphous yellow powder, m.p. 141.5–142°, yield 73%.

Anal. Calcd. for $C_{12}H_{17}O_9N_3$: C, 41.5; H, 5.0; N, 12.0. Found: C, 41.8; H, 5.4; N, 12.2.

Preparation of the Dinitrophenylaminohexitol from Blood Group Substances.—Amounts of blood group substance containing 0.3–1.0 mg. of total hexosamine (1–3 mg. of blood group substance) in a volume of 2 ml. were made up to 2 *N* by the addition of 0.4 ml. of concentrated hydrochloric acid in Pyrex test-tubes (15 × 150 mm.). The tubes were sealed with self sealing rubber caps, held in place by rubber bands, and immersed in a boiling water-bath for 2 hours. (Pressure inside the tubes was released by piercing the caps with hypodermic needles several times during the first few minutes of boiling.) The tubes were then chilled in an ice-bath uncapped and placed at an angle on a manifold connected to a Cenco hyvac pump through a Dry Ice–ethanol trap and a potassium hydroxide tube. The solutions were usually evaporated to dryness in about 4–5 hours of continuous operation of the pump.

The dry residues in the tubes were dissolved in 2 ml. of water and cooled in an ice-bath. An aqueous solution of sodium borohydride (containing 25 mg./ml.) was made by dissolving the powder in ice-cold water. Two ml. of this solution was immediately pipetted into the tubes containing the hexosamine samples and the mixture kept in an ice-bath for 90 minutes, then at room temperature for an additional 30 minutes. At the end of this time the excess sodium borohydride was decomposed by the careful, dropwise addition of 6 *N* hydrochloric acid.

The solution was transferred quantitatively with water and ethanol washings to 50-ml. round-bottom flasks and made alkaline by addition of solid sodium bicarbonate. Then 0.3 ml. of 2,4-dinitrofluorobenzene in 5 ml. of ethanol was added, followed by enough ethanol to give a homogeneous solution, and the mixture kept at room temperature overnight.

The next morning the flasks were placed in a water-bath at 37° for one hour; the deep yellow-orange solutions were then acidified by addition of 1 ml. of concentrated hydrochloric acid and distilled to dryness under reduced pressure with several additions of absolute ethanol to ensure removal of all acid. The remaining orange solid was chromatographed first on silicic acid to remove impurities, and then on silicic acid–celite to separate DNP-glucosaminitol from DNP-galactosaminitol.

Preparation and Use of Columns.—Ruled chromatographic tubes (9 × 280 mm.) with standard taper (10/18) sintered glass disk bottoms were used throughout.[32]

A. **Silicic Acid Column.**—For most of the determinations silicic acid prepared from Eimer and Amend water glass by the method of Tristram[33] was used. (This was later found to be replaceable by a mixture of Mallinckrodt silicic acid (special for chromatography) and celite (2:1), which gave identical results.) Three-g. portions of the silicic acid are thoroughly triturated with 1.5 ml. of the aqueous phase of a mixture of ethanol, chloroform and water (1:9:1 v./v.). Then with several additions of the organic phase (clarified by passage through filter paper) the slurry of silicic acid is poured into the chromatographic tube and allowed to pack by running through solvent, care being taken not to let the top run dry.

With the aid of several 3–4 ml. portions of the developer (organic phase) the DNP-aminohexitols are transferred quantitatively to the column, allowing each addition to run through before the next portion is added. The column is then developed at a flow rate of about 10 drops per minute by successive additions of solvent, never allowing the column to run dry. Unreacted 2,4-dinitrofluorobenzene, 2,4-dinitrophenol and most DNP-amino acids were found to run fast on this column ($R > 1$) and were removed. DNP-glucosaminitol and DNP-galactosaminitol were removed next as a single band (R = 0.3). Salts and other colored impurities remained at the top of the column. The solvent was distilled from DNP-aminohexitol fraction at reduced pressure, the distillation apparatus rinsed down with acetone, and the acetone in turn removed in a Fisher Filtrator under reduced pressure with a stream of air directed into the flask and an infrared lamp to hasten removal of solvent by warming. The orange film remaining was then ready to be chromatographed on the silicic acid–celite column.

B. Borate-buffered Column.—A mixture of 1.5 g. of silicic acid and 1.5 g. of celite was triturated with 2 ml. of the aqueous phase of the system ethanol–chloroform–0.4 *M* potassium borate buffer *p*H 10.8 (2:8:1 v./v.). A slurry of this was then made with the organic phase and transferred to the chromatography tubes with several portions of the organic phase. The silicic acid–celite was tamped down with a glass rod (made to fit snugly the inner bore of the tubes) to give a column free of channels and air bubbles. The residue from the silicic acid column was then transferred quantitatively to the top of this column with several 3–4 ml. portions of solvent and developed at a flow rate of

(29) (a) C. Stormont, *Proc. Nat. Acad. Sci.*, **35**, 232 (1949); (b) W. H. Stone, Ph.D. dissertation, Univ. of Wisconsin, 1953.

(30) The carbon and hydrogen analyses were done by Elek Micro Analytical Labs., Los Angeles, California.

(31) K. Meyer and E. M. Smyth, *J. Biol. Chem.*, **119**, 507 (1937); an additional sample was kindly supplied by Dr. Karl Meyer.

(32) Obtainable from Scientific Glass Apparatus Co., Bloomfield, N. J.

(33) G. R. Tristram, *Biochem. J.*, **40**, 721 (1946).

about 10 drops per minute. The DNP-galactosaminitol had an $R = 0.4$ and DNP-glucosaminitol an $R = 0.1$.

Estimation of the Amino Sugars.—The bands of DNP-galactosaminitol and DNP-glucosaminitol were collected separately in flasks and evaporated to dryness *in vacuo*. The residues were dissolved in 10-ml. portions of water and suitable dilutions were made from this for reading in the Beckman spectrophotometer (model DU) at 3600 Å. The amounts of DNP-aminohexitols corresponding to these readings were then read off from a calibration curve made from known dilutions of reference material.

The absorptions of the DNP-aminohexitols were found to be proportional to concentrations within the range 2–12 μg./ml., with molar extinctions (ε) of 18.2×10^3 for DNP-glucosaminitol and 16.5×10^3 for DNP-galactosaminitol at 3600 Å. which corresponded to the absorption maximum.

NEW YORK, N. Y.

[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

Polymyxin B_1.[1] Fractionation, Molecular Weight Determination, Amino Acid and Fatty Acid Composition

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A sample of polymyxin B was hydrolyzed in order to recover the isopelargonic acid. However, countercurrent distribution showed two acids to be present, the expected 6-methyloctan-1-oic acid and an isoöctanoic acid. Purity studies by countercurrent distribution of the polymyxin B sample then gave two different polymyxin peptides, B_1 and B_2. On hydrolysis both gave α,γ-diaminobutyric acid, threonine, leucine and phenylalanine. B_1 gave only the isopelargonic acid. B_1 and B_2 thus differ by the nature of the acid component. The molecular weight of B_1 was determined by the method of partial substitution and found to be 1150 ± 10%. Quantitative isolation studies by countercurrent distribution of a hydrolysate of B_1 showed it to contain 6 moles α,γ-diaminobutyric acid (the optical rotation indicated one mole of D and five moles of L), two moles L-threonine, one mole D-phenylalanine, one mole L-leucine and one mole of isopelargonic acid.

Introduction

During the course of structural studies with the polypeptide polypeptin[2] it was found that hydrolysis with sulfuric acid gave an ether extractable acid with a molecular weight approximating 150. Because of the general similarity of polypeptin to the class of polypeptide antibiotics known as polymyxins, the question of the relationship of this acid to the 6-methyloctan-1-oic acid[3] derived from the polymyxins was raised. This prompted a comparative study with one of the polymyxins. A generous sample of polypeptide material belonging to the polymyxin B type was made available to us through the kindness of Dr. P. Regna of Pfizer and Co. In 1949 Regna and co-workers[4] succeeded in purifying crude polymyxin B by chromatography on cotton sodium succinate followed by precipitation as the salt of the dye, polar yellow and final crystallization as the salt of naphthalene-β-sulfonate. The final product and the sulfate prepared from it behaved as a single pure substance when studied by paper chromatography. Acid hydrolysis followed by paper chromatography gave spots corresponding to α,γ-diaminobutyric acid, threonine, leucine and phenylalanine. An ether extract of the hydrolysate gave the optically active 6-methyloctan-1-oic acid mentioned above.

Molecular weight studies[5] on polymyxin B purified by another method gave a value of 1280 ± 70. This figure is based on pressure–area studies with monomolecular films of the peptide.

When the sample of polymyxin B at our disposal was hydrolyzed and extracted with ether, an acid fraction was obtained as expected. However, when it was studied by countercurrent distribution two different acids, one of which was the expected 6-methyloctan-1-oic acid, were found to be present. This finding raised the question of either the purity of the sample of polymyxin B or of the accepted composition. It prompted a further study into the purity of the sample, its molecular weight and amino acid composition.

Experimental

Hydrolysis of an Unfractionated Sample of Polymyxin B.—When a sample of peptide material was hydrolyzed for 24 hours at 108° with 6 *N* hydrochloric acid the yield of fatty acid was poor. However, two-dimensional paper chromatography of the residue remaining after evaporation of the HCl, in the systems 2-butanol–ammonia and 2-butanol–formic acid[6] showed spots corresponding to α,γ-diaminobutyric acid, threonine, leucine and phenylalanine. No other spots appeared. This result showed that the sample probably was not grossly contaminated with polymyxins of the other types.

For isolation of the fatty acid fragment 10 g. of the sample was hydrolyzed for 24 hours in 100 ml. of boiling 18% sulfuric acid under an atmosphere of nitrogen. Sulfuric acid was found to give a better yield than hydrochloric acid. The dark colored oil which had separated on the surface was extracted with ethyl ether. The ether extract was dried by freezing out the water in an acetone–solid carbon dioxide bath. The ether was removed by distillation through a Widmer column. A residue of 1.1 g. of a yellow oil with a rancid odor remained.

The residue was fractionated by countercurrent distribution in an automatic all-glass apparatus[7] using the system *n*-heptane–20% aqueous pyridine as shown in Fig. 1. For analysis 1-ml. aliquots of lower phase were directly titrated against phenolphthalein with 0.01 *N* sodium hydroxide. A distribution curve at 197 transfers is shown by the upper pattern of Fig. 1. After removal of the solute in the slowest moving band which proved to be sulfuric acid, the distribution was continued to 301 transfers. Analysis at this point gave the lower pattern of Fig. 1. Cuts 1a and 1b were removed at this stage and peak 2 was recycled for 200 more transfers. The material was then isolated in two cuts 2a and 2b from shoulder and peak, respectively. This pattern is not shown.

(1) Presented in part at the A.C.S. Meeting in Miniature, Metropolitan Long Island Subsection, February 20, 1953.

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(3) S. Wilkinson, *Nature*, **164**, 622 (1949).

(4) P. P. Regna, I. A. Solomons, B. K. Forscher and A. E. Timreck, *J. Clin. Invest.*, **28**, 1022 (1949).

(5) A. V. Few and J. H. Schulman, *Biochem. J.*, **54**, 171 (1953).

(6) W. Hausmann, THIS JOURNAL, **74**, 3181 (1952).

(7) L. C. Craig, W. Hausmann, E. H. Ahrens and E. J. Harfenist, *Anal. Chem.*, **23**, 1236 (1951).