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Received March 30, 1953. P.S.E.B.M., 1953, v82.

An Aldoheptose Phosphate in a Polysaccharide Isolated from *Shigella flexneri*. (20231)

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The polysaccharide of the somatic antigen of *Shigella flexneri*, type 3, has been shown to consist mainly of D-glucose, L-rhamnose and D-glucosamine(1-3). Approximately 1% of the dry weight of the polysaccharide preparation was found to consist of non-dialyzable, acid-stable, organically bound phosphorus. After acid hydrolysis of the polysaccharide and isolation of the water soluble barium salts of the phosphate esters, the latter were found to contain some hexose-6-phosphate and a heptose phosphate(1,3).

Materials. The degraded polysaccharide corresponding to fraction 3 or 4 was used as a source of the phosphate esters(3). Dialyzed human seminal plasma was used as a source of acid phosphatase. Amberlite IR-120 cation exchange resin was used after charging with HCl and IR-4B anion exchange resin was charged with Na_2CO_3. We are indebted to Dr. N. K. Richtmyer at the National Institutes of Health for generous gifts of several heptoses.

General methods. Phosphorus was determined by the method of Fiske and Subbarow (4). Heptose determinations were made by the sulfuric acid-cysteine procedure (CyR) described by Dische for other sugars(5).* We have found it possible to characterize all 7-carbon sugars which have been made available to us by comparing spectra of each sugar after CyR4 and CyR10 reactions (*i.e.*, after heating for 4 and 10 minutes, respectively). The ketoheptoses produce spectra which differ from each other only slightly, but have a much greater absorption at 510 mμ than do the aldoheptoses. The latter produce spectra which are similar in the visible region, but vary in their ultraviolet portions. However, aldoheptoses having mirror-image configurations about the first five carbon atoms produce very similar CyR spectra. Results with the CyR10 reaction are shown in Fig. 1. B and C which have enantiomorphic configurations about the first 5 carbons have similar spectra. In like manner, the pair F and G show a marked resemblance in the ultraviolet

* Dr. Dische suggested in April, 1952 that the pink color having a maximum absorption at about 510 mμ which we obtained with our phosphate ester fraction in his CyR10 reaction might be due to the presence of a 7-carbon sugar. A paper by Dr. Dische on color reactions of heptoses is in press for the *J. Biol. Chem.*

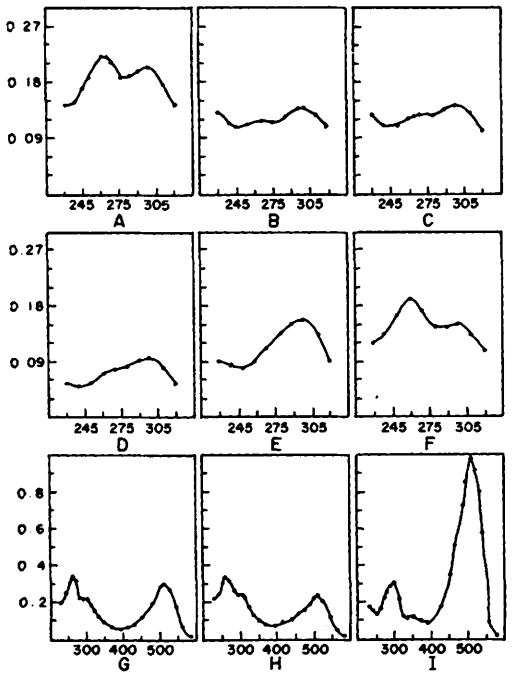

FIG. 1. Comparison of CyR10 spectra of several heptoses. In each case approximately 50 μg of sugar were present in 5.6 ml. The spectra were measured with a 1 cm light path in a Beckman spectrophotometer about 4 hr after the addition of cysteine. A, D-gluco-D-guloheptose; B, D-manno-D-gala-heptose; C, D-gulo-L-galaheptose; D, D-altro-D-glucoheptose; E, D-gala-L-glucoheptose; F, D-altro-D-mannoheptose; G, D-gala-L-manno-heptose; H, heptose from phosphate fraction of polysaccharide hydrolysate; I, sedoheptulosan. Ordinates: optical density, abscissae: wavelength in $m\mu$.

region. The results with D and E are not as striking. Since no heptoses of the L-series were available, it is not certain whether the same results would be obtained with them. Hexoses corresponding to these pairs do not give ultraviolet spectra similar to those of the heptoses. If the complete spectra for the CyR4 and CyR10 reactions of all these sugars had been reproduced here, it would be seen that even those which have similar ultraviolet spectra differ in some characteristic manner such as whether or not the CyR4 and CyR10 spectra intersect at any point or to what extent the maxima change in intensity when the sugars are heated with acid for 10 minutes instead of 4 minutes. The *altro-sugars* were supplied as their hexaacetates. Since the acetyl groups are hydrolyzed during the heating with strong sulfuric acid in the CyR procedure, the spectra represent those which would be obtained with the free sugars. Sedoheptulosan (Fig. 1, I) is representative of the ketoheptoses. It may be seen that the spectrum for the heptose obtained from the phosphate fraction of the hydrolyzed polysaccharide (Fig. 1, H) is very similar to that obtained with D-gala-L-mannoheptose (Fig. 1, G).

It was possible to adapt the well-known oxidation of aldo-sugars by bromine to a micro-scale procedure for distinguishing aldo- and keto-sugars:

.10 ml sugar (50 μg)
.35 ml 0.5M phosphate, pH 7.5
.05 ml 0.5M Br_2 in ethanol (or ethanol alone)

The above mixtures were incubated for 20 minutes at about 35°C. The pH dropped to about 6.9 during this time. To the bromine-treated sugar solution was added 0.5 ml of a similar mixture containing buffer, water and ethanol. To the control sugar solution (without bromine) was added 0.5 ml of a solution of water, buffer and bromine which had been preincubated 20 minutes to destroy the bromine. These 1 ml samples were then carried through the CyR10 procedure and the optical densities were measured at 410 $m\mu$ (for hexoses) or 510 $m\mu$ (for heptoses) about 2 hours after the addition of cysteine. It was necessary to compensate the bromine-treated and control sugar samples in the above manner with reagent blanks in order to equalize the effects of bromide ions on the CyR color reaction, since bromide may either augment or diminish the color depending on what sugar is being studied. The results obtained with several hexoses and heptoses are presented in Table I. It may be seen that the free heptose obtained from the phosphate-ester fraction of the hydrolyzed polysaccharide is oxidized to the extent of about 75% which is similar to results obtained with the other aldoses.

The bromide which forms during the incubation affects the color development in the CyR reaction in another manner. The yellow color given by hexoses is stabilized and does not shift to a blue on long standing such as occurs with most hexoses in the absence of

TABLE I. Effect of Bromine on Aldo- and Keto-sugars as Shown by the CyR Colorimetric Procedure. See *General methods* for details of procedure. Values are averages of duplicate determinations.

Sugar	Bromine	Wave-length (mμ)	Optical density
D-Glucose	—	410	.715
"	+	"	.058
D-Fructose	—	"	.996
"	+	"	.969
D-Gala-L-mannoheptose	—	510	.180
"	+	"	.016
L-Glucoheptulose	—	"	.272
"	+	"	.253
Heptose from polysaccharide	—	"	.176
"	+	"	.043

bromide ions. Similarly with the heptoses, a gradual shift in color to a purer pink is delayed in the presence of bromide. *Crystalline ethyl mercaptals* and their hexaacetates were prepared on a microscale similar to the procedure described by Wolfrom and Karabinos(6). It was possible to obtain a fair amount of crystalline derivatives with as little as 3 mg of heptose. In the case of the acetylated mercaptals, the extraction with chloroform was omitted and the derivatives were directly crystallized by the addition of water at the end of the acetylation reaction. The mercaptals were recrystallized from water and the acetylated mercaptals were recrystallized from ethanol by the addition of water. *Melting points* (uncorrected) were obtained with a Fisher-Johns melting point apparatus.

Experimental procedure. Polysaccharide material obtained from the somatic antigen of cells harvested from 16 carboys of a broth culture of *Sh. flexneri*, type 3, was refluxed for 4 hours at 100° C in N HCl. The solution was chilled and the pH was adjusted to about 8.5 by the addition of NaOH. Barium acetate was added and a small precipitate was removed. The supernatant solution was treated in the cold with 3 volumes of ethanol, the precipitate was washed with 80% ethanol and most was redissolved by the addition of a small amount of water and HCl to about pH 4. The insoluble residue was discarded and the supernatant fluid was readjusted to about pH 8.5 and the resulting precipitate was removed. The supernatant fluid was then treated with 3 volumes of ethanol and the precipitate was washed with 80% ethanol. This material was suspended in water and Ba was removed by the addition of H_2SO_4. The solution of phosphate esters was buffered with acetate at pH 5 and treated with seminal phosphatase. Approximately 50% of the phosphorus was released which represented about 65% of the heptose. Protein, inorganic phosphate and unsplit phosphate esters were removed by treatment with $ZnSO_4$ and Ba $(OH)_2$(7). The yellowish supernatant fluid was adjusted to about pH 5 by the addition of HCl and the solution was deionized by means of ion exchange resin columns. The deionized solution was adjusted to about pH 5 and was concentrated by vacuum distillation. The resulting solution was almost colorless and contained approximately 12 mg of heptose based on a comparison of the CyR color with that of D-gala-L-mannoheptose. The spectrum given by this material in the CyR10 reaction is shown in Fig. 1, H. Only one spot was obtained in one dimensional paper chromatograms in several solvents although the CyR4 spectrum showed the presence of a small amount of hexose which had previously been demonstrated in the phosphate ester fraction (1,3).

Failure to obtain a visible reaction of the heptose on paper chromatograms sprayed with orcinol(8) indicated the absence of a ketoheptose. That we were concerned with an aldoheptose was also shown by its oxidation by bromine (see *General methods*).

The concentrated HCl used in the prepara-

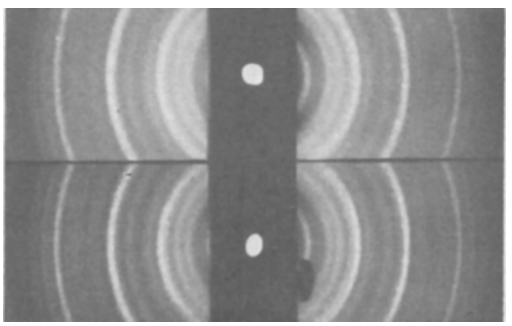

FIG. 2. X-ray diffraction patterns of crystalline hexaacetyl ethyl mercaptal of D-gala-L-mannoheptose (top) and the corresponding derivative of the heptose isolated from the polysaccharide of *Sh. flexneri* (bottom).

tion of mercaptals destroys ketoheptoses. We were able to prepare the crystalline ethyl mercaptal (plates) and hexaacetate of the ethyl mercaptal (needles) after drying the heptose solution obtained from the phosphate esters. Since the CyR spectra of the unknown heptose resembled that of D-gala-L-mannoheptose, the corresponding derivatives of the latter were also prepared. The ethyl mercaptals melted at about 200-203° C (uncorr.), but the mixed melting point was at 193-199°C. Similarly with the thrice crystallized hexaacetates of the ethyl mercaptals, the individually determined melting points were about 141-143° C, while the mixed value was lowered to about 133-138°C. The values for the separate derivatives agree fairly well with those reported for authentic samples of the D-gala-L-mannoheptose derivatives: ethyl mercaptal (204-205°C, corr.), acetylated ethyl mercaptal (145-146°C, corr.) (9).

Although D-altro-D-mannoheptose gives CyR spectra similar to those obtained with the heptose of the polysaccharide and with D-gala-L-mannoheptose (Fig. 1, F, G and H), the melting point of its ethyl mercaptal (151-153°C) is different(10).

X-ray powder diffraction patterns (Fig. 2.) show that the hexaacetyl ethyl mercaptal of the aldoheptose isolated from the polysaccharide is identical with that of gala-mannoheptose. However, such patterns do not differentiate between D and L configurations. Since we had very little material, it was not feasible to measure optical rotations, but the fact that the mixed melting points of both the mercaptals and the hexaacetyl mercaptals were lower than the individual melting points is a good indication that we are dealing with the enantiomorph, L-gala-D-mannoheptose.

Since ketoheptoses in the CyR reaction have a much higher absorption coefficient at 510 mμ than aldoheptoses (Fig. 1), it should be possible to detect isomerization of heptoses by measuring a change of optical density at this wavelength. Preliminary experiments with dialyzed sonic extracts of *Sh. flexneri* failed to reveal any isomerase activity with either the aldoheptose phosphate isolated from the bacterial polysaccharide or with sedoheptulose-7-phosphate kindly supplied by Dr. B. L. Horecker of the National Institutes of Health.

Discussion. This seems to be the first report of the occurrence of an aldoheptose phosphate in nature. Ketoheptose esters of phosphate have been reported previously(11-13), and free sedoheptulose (D-altroheptulose) and D-mannoheptulose were shown to be present in plants(15,16). It has recently been found that the polysaccharide of the somatic antigen of *Sh. sonnei,* phase II, contains a rather large amount of an aldoheptose apparently in non-phosphorylated form(14).

Summary. An aldoheptose phosphate has been isolated from the polysaccharide of the somatic antigen of *Sh. flexneri,* type 3. The heptose moiety is identical with gala-mannoheptose and appears to be the enantiomorph of D-gala-L-mannoheptose.

The authors wish to express their gratitude to Dr. Nelson K. Richtmyer of the National Institutes of Health for his interest in the problem of identification of the aldoheptose, to Mr. William White, also of the National Institutes of Health, for kindly preparing the X-ray diffraction patterns, and to Mr. William D. Hann for isolating the crude polysaccharide and other valuable assistance.

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Received March 12, 1953. P.S.E.B.M., 1953, v82.

Study with Fluorescent Antibody of Fate of Intradermally Injected Proteins in Rabbits.* (20232)

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The fate of intravenously injected antigenic substances has been followed with interest because of its bearing on the mechanism of destruction of foreign materials in relation to the stimulation of antibody formation, and the distribution of the lesions of experimental serum disease obtained with different antigens(1-4). The present report presents data concerning the sequence of events following intradermal injection of two antigenic proteins in normal and passively sensitized rabbits and in rabbits sensitized with homologous antigen plus Freund's adjuvants.

Methods. 2.5-3.0 kg rabbits were used throughout. Antiserum pools, containing 1.21 and 0.50 mg N/ml of antibody against Armour's crystalline ovalbumin (Ea) or Armour's bovine Fraction II (BGG), were obtained from animals injected with these antigens in combination with Freund's adjuvants. Some rabbits were passively sensitized the day before the test by intravenous injection of 4.0 mg of antibody N(5). Others were sensitized 3 weeks earlier by inoculating the two front foot pads with 0.05 ml of an emulsion containing 20 mg protein antigen and 3 mg heat-killed tubercle bacilli per ml of mineral oil (Bayol F). Test injections of 2 or 10 mg of 3 times recrystallized Ea(6) or BGG in 0.10 ml of physiological saline were made intradermally on the shaved outer aspect of the hind leg above the heel. Animals were sacrificed 1, 6, 24, and 48 hours after the test injection. The injected area of skin and the homolateral popliteal lymph node, and sometimes other tissues, were removed and quick frozen. 4-6 μ cold-box sections of these tissues were stained with fluorescein-labelled, homologous antibody, obtained from the serum pools described above, and examined under the fluorescence microscope. The technic of labelling antibody with fluorescein and the staining methods are described by Coons *et al.*(7-9). Controls demonstrating the specificity of the fluorescent staining included: absence of staining by heterologous labelled antibody, and inhibition of specific staining in sections treated first with unlabelled antibody. Sections stained by the Giemsa technic, were also examined.

Results. The normal rabbits showed minimal microscopic changes. Those sensitized passively or with the aid of adjuvants presented typical Arthus reactions, 10 to 25 mm in diameter, maximal at 6 or 24 hours, and remaining conspicuous at 48 hours in the adjuvant group. The microscopic picture included edema and polymorphonuclear exudation, marked at 1 hour in the deeper skin layers, involving all layers at 6 and 24 hours, and massive around vessels and hair follicles. Deep areas of hemorrhage were seen, particularly in the responses to Ea. At 48 hours, there was an increase in fixed cells and other mononuclears. The adjuvant sensitized rabbits showed more intense and prolonged reactions, including marked fixed cell activation in the papillary layer, some infiltration of lymphoid elements at 24 and 48 hours, and necrosis,

* The expenses of this study were defrayed in part by grants from the Kresge Foundation and the National Multiple Sclerosis Society. This is publication No. 133 of the Robert W. Lovett Memorial Foundation for the Study of Crippling Diseases.

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