[CONTRIBUTION FROM THE KERCKHOFF LABORATORIES OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

Preparation of 1-Amino-1-deoxy-2-ketohexoses from Aldohexoses and α-Amino Acids. I[1]

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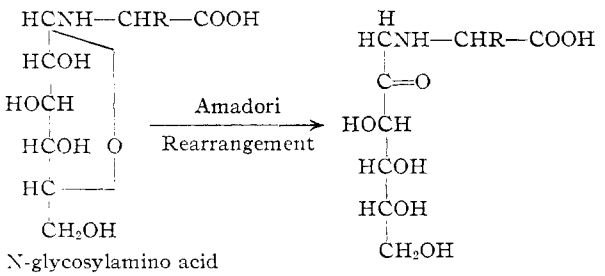
The preparation of a number of N-substituted 1-amino-1-deoxy-2-ketohexoses is described. The parent sugars were galactose, glucose and mannose; the amino acids were L-alanine, L-aspartic acid, L-glutamic acid, glycine, L-leucine, L-phenylalanine, L-serine, L-threonine and L-valine. Compounds of this type reduce ferricyanide in 0.1 *N* alkali rapidly at room temperature; in boiling alkaline solution they reduce ferricyanide nearly to the same extent as an equivalent amount of the parent sugar; on the other hand they give much lower values than the free sugar in tests depending on transformations by strong sulfuric acid as in the determination of sugar by the anthrone reagent. All the compounds of this type so far tested stimulated the incorporation *in vitro* of labeled amino acids into the proteins of rabbit reticulocytes.

Compounds of the type N-substituted 1-amino-1-deoxy-2-ketohexose, isolated from hog liver, were found to stimulate incorporation *in vitro* of amino acids into the proteins of rabbit reticulocytes.[3] The sugar residue in the isolated compounds had the configuration of fructose, and amino acids were released on hydrolysis, hence the compounds were called fructose–amino acids. Fructose–glutamic acid, and a mixture of fructose–alanine and fructose–glycine were isolated, purified, and their structure determined by comparison with the corresponding synthetic compounds, whose preparation was not described. The present communication describes the methods we have used to synthesize and isolate a number of fructose–amino acids.

The name fructose–amino acid is not strictly correct but is used for the sake of convenience. The correct name, for example, for the compound we have called fructose–glycine, is N-(1′-carboxy)-methyl-1-amino-1-deoxy-D-fructose.

Fructose–amino acids may be viewed as the Amadori rearrangement products of the N-glycosylamino acids. A property which distinguishes them from the N-glycosylamino acids is that they can, in 0.1 *N* alkali, reduce ferricyanide or oxidation–reduction dyes rapidly at room temperature. On heating



N-glycosylamino acid — N-substituted 1-amino-1-deoxy-2-ketose

with acid they decompose rather than hydrolyze, with the result that the yield of free amino acid is quite low and the sugar moiety gives decomposition and rearrangement products.[4]

Kuhn, *et al.*,[5–8] who demonstrated the nature of the Amadori rearrangement, depended for the identification of the product on its being a relatively insoluble crystalline substance, and this led them to the opinion that the Amadori rearrangement can occur only with glycosides of the aromatic primary amines. Hodge and Rist[9] showed that this restriction was too narrow; they presented evidence that N-D-glucosylglycine ethyl ester can undergo Amadori rearrangement; but they did not isolate the pure product. The one ketose–amino acid that has been synthesized and isolated (purity 75–90%) is Gottschalk's preparation of fructose–DL-phenylalanine.[4] The synthetic reaction Gottschalk used was based on the recipe of Kuhn, *et al.* and is a general one, but his isolation procedure, which depends on fructose–phenylalanine being more soluble in absolute methanol than phenylalanine, obviously is not.

The synthesis reaction we have used was essentially that used by Gottschalk.[4] Our isolation procedure was chromatographic; it is general to the extent that it served to isolate fructose–amino acids from liver and required, with nine different amino acids, changes only in the strength of the eluting acid.

In the Amadori rearrangement the hydrogens on carbons 1 and 2 of the sugar are involved; accordingly glucose and mannose glycosides of the same amino acid give the same compound after rearrangement, *i.e.*, the fructose–amino acid; galactose, by analogy, presumably gives the tagatose amino acid. Thus, it was found[3] that the optical rotation of the compound obtained from *glucose* and glycine, $[\alpha]^{25}D$ −65°, was essentially the same as that obtained from *mannose* and glycine, $[\alpha]^{25}D$ −66°. Similarly, the optical rotation of the compound obtained from *glucose* and alanine, $[\alpha]^{25}D$ −52° was essentially the same as that from *mannose* and alanine, $[\alpha]^{25}D$ −55°. The optical rotation of the corresponding compounds obtained with *galactose* were as follows: $[\alpha]^{25}D$ −13.5° (tagatose–glycine) and $[\alpha]^{25}D$ −10.3° (tagatose–alanine).

In addition to the details of their preparation we

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(2) Supported by a grant from Eli Lilly and Co. Department of Biochemistry, University of Colorado School of Medicine, Denver, Colorado.

(3) H. Borsook, A. Abrams and P. H. Lowy, *J. Biol. Chem.*, **215**, 111 (1955).

(4) A. Gottschalk, *Biochem. J.*, **52**, 455 (1952).

(5) R. Kuhn and A. Dansi, *Ber.*, **69**, 1745 (1936).

(6) R. Kuhn and F. Weygand, *ibid.*, **70**, 769 (1937).

(7) R. Kuhn and L. Birkofer, *ibid.*, **71**, 621 (1938).

(8) F. Weygand, *ibid.*, **73**, 1259, 1284 (1940).

(9) J. E. Hodge and C. E. Rist, THIS JOURNAL, **75**, 316 (1953).

TABLE I

SOME DETAILS OF THE PREPARATION OF AMADORI REARRANGED N-GLYCOSYLAMINO ACIDS AND ELEMENTARY ANALYSES OF THE PRODUCTS

Compound	Parent sugar	Reaction time, hr.	Trichloroacetic acid used for elution, *M*	Elementary anal. of final product, % Carbon Calcd.	Carbon Found	Hydrogen Calcd.	Hydrogen Found	Nitrogen Calcd.	Nitrogen Found
Fructose–L-alanine	Glucose	2	0.10	43.1	42.9	6.83	6.90	5.58	5.53
Fructose–L-alanine	Mannose	2	.10	43.1	42.3	6.83	7.44	5.58	5.30
Fructose–L-aspartic acid	Glucose	3	.05	40.8	40.2	5.82	6.00	4.75	4.88
Fructose–L-glutamic acid	Glucose	3	.05	42.7	42.6	6.20	6.80	4.53	4.67
Fructose–glycine	Glucose	3	.10	40.6	40.8	6.38	6.90	5.92	5.90
Fructose–glycine	Mannose	3	.10	40.6	40.55	6.38	6.57	5.92	5.78
Fructose–L-leucine	Glucose	2.5	.35	49.2	48.9	7.92	7.18	4.78	4.92
Fructose–L-phenylalanine	Glucose	1.5	.50	55.1	53.2	6.47	6.70	4.30	4.55
Fructose–L-serine	Glucose	3	.20	40.5	40.2	6.42	6.80	5.25	5.10
Fructose–L-threonine	Glucose	2.2	.20	42.7	42.9	6.87	7.10	4.99	4.98
Fructose–L-valine	Glucose	3	.20	47.3	47.6	7.60	8.20	5.00	5.10
Tagatose–L-alanine	Galactose	2	.10	43.1	43.5	6.83	6.74	5.58	5.53
Tagatose–glycine	Galactose	3	.10	40.6	40.55	6.38	6.57	5.92	5.78

present below some of the chemical and biological properties of the ketose–amino acids prepared.

Experimental

Synthesis Procedure.—The following procedure was typical of the synthesis reaction. Three hundred and fifty mg. of amino acid was suspended in 140 ml. of absolute methanol and refluxed in a boiling water-bath for five to ten minutes. Five grams of anhydrous D-glucose was then added and the refluxing resumed. In about ten minutes the solution became clear. The formation of the ketose–amino acid was followed by the test previously described[3] which consists of reduction of ferricyanide in 0.1 *N* alkali at room temperature under specified conditions, conversion of the ferrocyanide formed to Prussian Blue, which is then measured at 690 mμ. This test was carried out on 0.1-ml. aliquots of the reaction mixture at 15-minute intervals. A maximum value was obtained usually in two to three hours. The refluxing was continued 15 minutes longer. The solution was then evaporated *in vacuo* to dryness.

A slight acidity or proton donating catalyst is required for the Amadori rearrangement.[8] With monoaminomonocarboxylic amino acids, the acidity generated by the condensation of the amino group in the formation of the N-glycosylamino acid is sufficient for this purpose. Poor yields of the Amadori rearranged products were obtained with aspartic acid and glutamic acid until one mole of alcoholic KOH per mole of amino acid was added to the reaction mixture. Evidently the acidity is too great, otherwise, for the initial glycoside formation and subsequent rearrangement.

Isolation Procedure.—The dry residue was dissolved in 10 to 15 ml. of water and placed on a column 22 × 450 mm. of Dowex-50 H^+. Twenty hold-up volumes of water were sent through and discarded, thus removing all the free sugar and color. Then 20 hold-up volumes of the following molarities of trichloroacetic acid were sent through in succession: 0.05, 0.10, 0.20, 0.35 and 0.50. The rate of flow, obtained with pressure, was two hold-up volumes per hour; the fractions were one-half a hold-up volume.

The reduction test on 0.1-ml. aliquots located the ketose–amino acid. In every case it was a band extending over about three hold-up volumes. Sometimes there was a front band about five hold-up volumes before the main band; its total reducing value was 20%, or less than that of the main band; it was discarded.

The pooled fraction comprising the main band, after extraction of trichloroacetic acid with ether, was lyophilized. The dry residue was dissolved in a minimum amount of water and rechromatographed as above on the same resin in a column 9 × 450 mm. The dry product after lyophilization was taken three times through solution in absolute methanol and precipitation with dry ether, after which it was dried *in vacuo*. The usual yield, beginning with 350 mg. of amino acid, was about 200 mg. of ketose–amino acid. The product was a fluffy, non-crystalline, white powder, which was slightly hygroscopic, and withstood heating at 80° for two hours. With longer heating at 80°, or at higher temperature there was decomposition with browning.

At times the batch size was four times as large as that described above. The reaction time was the same as with the smaller batch, the chromatographic column was the same height and four times the diameter.

The following tests were carried out on all the compounds prepared: total sugar by reduction of ferricyanide in boiling alkaline solution and by the anthrone reagent, reduction of ferricyanide in 0.1 *N* alkali at room temperature, and stimulation of amino acid incorporation *in vitro* into the proteins of rabbit reticulocytes. The procedures were the same as previously described.[3]

Table I gives the molarity of trichloroacetic acid used in the different preparations for their elution from the Dowex-50, and the calculated and found elementary analyses. There is a small uncertainty in the elementary analyses of some of these compounds because of the difficulty of removing the last traces of water.

All ketose–amino acid compounds we have prepared so far gave values for sugar close to that of an equivalent amount of the parent sugar when determined by reduction of ferricyanide in boiling alkaline solution; and very much lower values than the parent sugar by methods which depend on the action of strong sulfuric acid, forming products which condense to give a colored compound with a reagent such as anthrone (Table II).

A more useful test which distinguishes the Amadori rearrangement products from both the N-glycosylamino acids and free sugar, is the ability of the former to reduce ferricyanide in 0.1 *N* alkali at room temperature. The N-glycosylamino acids and the free sugars give very low values by this test. There are small differences between the different fructose–amino acids; tagatose–alanine and tagatose–glycine gave much higher values than any of the fructose–amino acids (Table II). This test also has been adapted for use in paper chromatography.[3]

All fructose–amino acids and both the tagatose–amino acids stimulated incorporation *in vitro* of labeled C^{14}-leucine into the proteins of rabbit reticulocytes. Similar results were obtained with labeled glycine, histidine and lysine. The values given in Table II are the averages of six or more tests of each compound at 5×10^{-4} *M*, which was the minimum concentration giving a maximum effect, and at 1×10^{-4} *M*. In all of these tests iron (in the form of ferrous ammonium sulfate) was added to a final concentration in the reaction mixture of 0.5 μg. per ml. None of these compounds have any stimulating effect without added iron.

Discussion

Fructose–amino acids have been isolated from dried and fresh hog liver.[3] Gottschalk claimed that a compound of this type was released by an influenza virus enzyme from mucoproteins; the nitrogenous constituent contained lysine but whether it consisted only of the free amino acid or

TABLE II

COMPARISON OF SOME CHARACTERISTIC PROPERTIES OF FRUCTOSE– AND TAGATOSE–AMINO ACIDS[a]

Compound	Total sugar: Parent sugar	Total sugar: Hot reduction of ferricyanide, glucose	Total sugar: Anthrone, % of glucose	Reduction of ferricyanide at room temp.,[3] % of fructose–glycines	Stimulation of incorporation *in vitro* of leucine into reticulocyte proteins, % of blank: Concn., *M* 5×10^{-4}	Stimulation of incorporation *in vitro* of leucine into reticulocyte proteins, % of blank: Concn., *M* 1×10^{-4}
Fructose		90	95	3	108	..
Glucose		100	100	≈0	109	..
Galactose		74	71	≈0	100	..
Fructose–L-alanine	Glucose	99	20	129	132	117
Fructose–L-alanine	Mannose	100	21	129	139	115
Fructose–L-aspartic acid	Glucose	82	45	89	135	132
Fructose–L-glutamic acid	Glucose	81	35	114	118	108
Fructose–glycine	Glucose	103	21	100	139	126
Fructose–glycine	Mannose	104	20	103	139	127
Fructose–L-leucine	Glucose	81	11	104	126	120
Fructose–L-phenylalanine	Glucose	70	13	108	127	121
Fructose–L-serine	Glucose	100	23	142	140	128
Fructose–L-threonine	Glucose	81	6	143	135	118
Fructose–L-valine	Glucose	87	12	110	132	118
Tagatose–L-alanine	Galactose	68	11	225	143	131
Tagatose–L-glycine	Galactose	71	16	210	143	130

[a] The following sizes of aliquots were used: hot reduction of ferricyanide, 1.0 ml. of 4×10^{-5} *M*; anthrone 1.0 ml. of 4×10^{-3} *M*; room temperature reduction of ferricyanide, 0.1 ml. of 4×10^{-3} *M*. The tests on the incorporation of leucine were carried out at 38° for four hours.

was in peptide or other combination was not determined.[10,11] Evidently the 1-amino-1-deoxy-2-ketose configuration may occur in some mucoproteins. Hodge and Rist[9] have implicated this type of linkage of sugar with proteins in the browning of dried foods during storage.

(10) A. Gottschalk and P. E. Lind, *Nature*, **164**, 232 (1949).

(11) A. Gottschalk, *ibid.*, **167**, 845 (1951).

It is interesting in view of the finding that fructose– and tagatose–amino acids stimulate amino acid incorporation into rabbit reticulocyte proteins that Rogers, *et al.*,[12] found that N-D-glucosylglycine stimulated the growth of *Lactobacillus gayoni*.

(12) D. Rogers, T. E. King and V. H. Cheldelin, *Proc. Soc. Exptl. Biol. Med.*, **82**, 140 (1953).

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[CONTRIBUTION FROM THE DEPARTMENT OF MEDICINE, COLUMBIA UNIVERSITY COLLEGE OF PHYSICIANS AND SURGEONS AND THE EDWARD DANIELS FAULKNER ARTHRITIS CLINIC OF THE PRESBYTERIAN HOSPITAL]

Structural Studies on Chondroitin Sulfuric Acid. II. The Glucuronidic Linkage[1,2]

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The structure of chondrosine, the repeating unit of chondroitin sulfate of cartilage, tendon and umbilical cord and of chondroitin, a sulfate-free analog, has been established previously as β-D-glucopyranosyluronic acid 2-deoxy-2-amino-D-galactose. In this paper the conversion of the disaccharide into a glucopyranosyluronic acid pentitol *via* the crystalline methyl ester hydrochloride by oxidative deamination with ninhydrin is described. This was converted to a glucopyranosylpentitol, presumably a β-D-glucopyranosyl-D-lyxitol isolated as a crystalline octaacetate. Periodate studies on the amorphous free sugar demonstrated the consumption of four moles of periodate with the formation of two moles of formic acid and one mole of formaldehyde. This indicated a 1,2-link for the glucosidolyxitol, necessitating a 1,3-glucuronidic linkage in chondrosine and thereby in chondroitin sulfate and other polymers which contain chondrosine as the major repeating unit. The hexosaminidic linkage is discussed.

The reaction of amino sugars with ninhydrin has been made the basis of their qualitative and quantitative estimation and separation.[3] Conversion of glucosamine and chondrosamine to arabinose and lyxose, respectively, is accomplished rapidly and in good yield.

The repeating unit of umbilical cord hyaluronic acid, hyalobiuronic acid, has been established as β-D-glucopyranosyluronic acid-2-deoxy-2-amino-D-glucose.[4] This was based on conversion to β-D-glucopyranosyl)-D-arabinose *via* the corresponding hexosaminic acid which was oxidatively deaminated with ninhydrin.[5]

(1) This work was supported by grants from the National Institutes of Health and the New York Chapter of the Arthritis and Rheumatism Foundation.

(2) Taken in part from a thesis to be submitted by Eugene A. Davidson in partial fulfillment of the requirements for the Ph.D. degree, Faculty of Pure Science, Columbia University.

(3) S. Gardell, F. Heijkenskjold and A. Rochnorlund, *Acta Chem. Scand.*, **4**, 970 (1950).

(4) B. Weissmann and K. Meyer, THIS JOURNAL, **76**, 1753 (1954).

(5) It has been pointed out to us that a choice between the structure proposed by us[4b] and that proposed by Wolfrom, *et al.*,[6] may appear ambiguous. We wish to re-emphasize that the experimental data presented by the latter are compatible with both structures. However, the data presented in our previous paper are only compatible with the structure proposed by us. Furthermore, Prof. Wolfrom according to a personal communication has obtained additional evidence which supports the structure proposed by us.