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Studies on N-Glycosides
The Isolation, Synthesis, and Application

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I. Introduction

Our studies on N-glycosides were undertaken with the intention of solving the problem of carbohydrates in protein or the manner of their linkage with protein molecule and their significance in protein chemistry. The presence of carbohydrates even in purified protein molecule has long been recognized. It was Seegen[1] who first made report of this fact, and in opposition to his view Osborne *et al.*[2] said that carbohydrates were mere impurities. But many investigators have, since then, isolated carbohydrates from various proteins as shown in Table 1, and it is now widely recognized that all simple proteins, not to speak of glycoproteins, contain carbohydrates except a few.[3]

Table 1

Carbohydrates isolated from proteins	Source	References
glucosamine-mannose	egg albumin	1937[4]
glucosamine-dimannose	egg albumin	1929[5]
glucosamine-dimannose	blood serum	1920, 1931[6]
galacto-glucosamine-mannose	horse blood serum	1934[7]
4 mannose residues-2 glucosamine residues	egg albumin	1938[8]
glucosamine-mannose-galactose	ovomucoid	1940[9]
glucosamine-mannose-mannose	egg albumin	1941[10]
7 N-acetylglucosamine residues, 3 mannose residues, galactose	ovomucoid	1942[11] 1946

In spite of these reports on isolation, no attention has been so for paid to the manner of linkage of carbohydrates with protein. It is quite reasonable to assume that these carbohydrates in protein, if they are not mere impurities, may exist in some protein-bound forms. According to Blix[12], carbohydrates in protein are classified into the following two groups: one which exists in the form of acid salts and can be taken away by mild treatment and the other which can be split off only by a drastic procedure such as baryta or enzymic hydrolysis.

Morgan and Partridge[13] say that from serum and egg white proteins carbohydrate can be obtained only through almost perfect disintegration of protein molecule, and yet they can not completely remove the last traces of amino acids attached to isolated carbohydtate. They could not, however, elucidate its rôle in protein, though they admitted it to be an integral part of protein molecule. Rimington[14] also suggested the presence of "protein bound carbohydrate," and Krebs[15] mentioned that polysaccha-

rides and glucosamine in blood serum occurred "in combination with protein". These papers which were made public after our studies had started corroborated our hypothesis on the combination of carbohydrate with protein.

Although all efforts, by means of dissociating proteins, have been directed to the isolation and determination of carbohydrates found in proteins, no investigation has yet been carried out in order to confirm the general manner of protein-carbohydrate linkage in protein molecules. The manner of linkage of carbohydrates in protein molecule should be, if clarified, considered as an integral part of protein structure as well as of its biochemical meanings.

Our studies on this point have led us to the conviction that the carbohydrates in protein molecule usually exist in the form of N-glycoside with amino acids or polypeptides, even in the presence of hydroxy amino acids. Consequently, we shall be able to ascertain the rôle of carbohydrates in protein structure and its biochemistry. From this point of view we have performed the isolation of carbohydrates from proteins in the form of N-glycoside.

On the other hand the synthesis of N-glycosides of several amino acids and amines were made in order to investigate the chemical and biochemical nature of N-glycosidic linkage and their application.

II. Isolation of N-Glycosides from Proteins

Several compounds consisting of carbohydrates and amino acids or polypeptides were isolated from a number of proteins, and it has been found that the combination occur between amino groups of amino acids and carbonyl groups of carbohydrates, the compounds being N-glycosides, as shown in Table 2.

Table 2

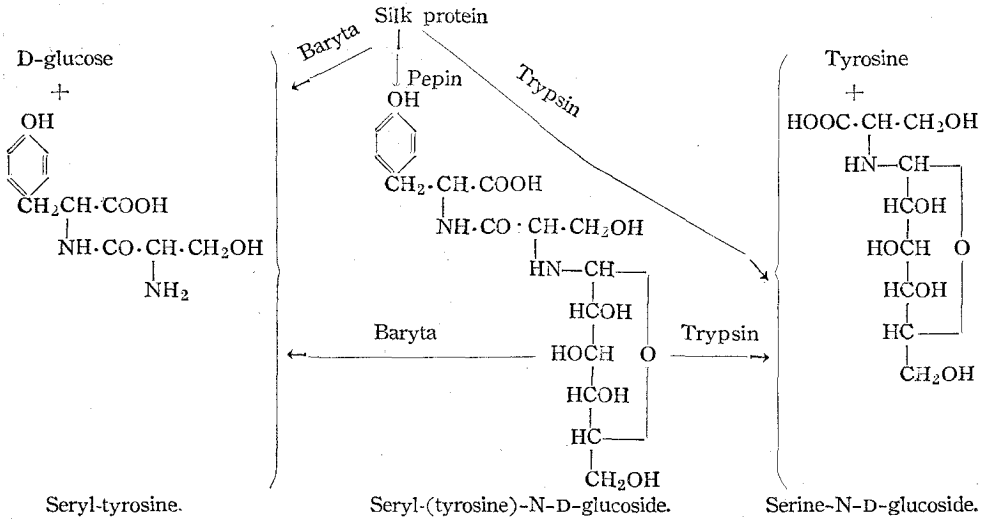
N-Glycosides isolated from proteins	Source	References
Seryl-(tyrosine)-N-D-glucoside	Silk floss	16)
Arginyl-(tryptophanyl-tyrosine)-N-D-glucoside	Casein	17)
Serine-N-D-glucoside	Herrig-roe	18)
Methionyl-(leucine)-N-L-arabinoside-5-phosphate	Earth-Worm	19)
Arginyl-(histidine)-N-D-fructoside-6-psosphate	Penicillium notatum Q176	20)
Histidinyl-(cystine)-N-D-glucoside-6-phosphate	Placenta	21)
Alanine-N-D-glucoside	Rabbit hair	22)
Aspartic acid-disaccharide (consisting of D-glucose and L-arabinose)	Soy bean	23)

Given below is the case of seryl-(tyrosine)-N-D-glycoside from silk floss to illustrate the procedure for the isolation of N-glycosides.

The estimation of carbohydrate in 20 different kinds of silk cocoon showed average value 1.46% calculated as glucose, and silk floss and fibroin contain 2.40% and 0.30%, respectively. Silk floss (230 gm.) was put into 3 L. of diluted HCl, adjusted to pH 3, and incubated with 0.5 gm. of pepsin at 38°C for 6 weeks keeping pH at 3. The solution was neutralized with Na_2CO_3, condensed *in vacuo* to 1/3 volume and treated with basic lead acetate and NH_4OH to give a precipitate, which was

purified after dissolving and condensed *in vacuo*. White precipitates were obtained from the solution by adding 8 times the volume of anhydrous methanol and dried ether, which was submitted to dialysis through bladder membrane, and the outer solution was condensed and treated with methanol and ether, giving white amorphous powder, with the yield of 0.5 gm. This powder showed negative tests in Ninhydrin and Fehling reactions and positive in Molisch reaction, but after hydrolysis it became positive in Ninhydrin and Fehling reactions. Tyrosin, serine, and D-glucose were isolated from acid hydrolysate. On the other hand, amorphous products were obtained by treating silk floss with baryta and trypsin, and their constitutions were established and the following scheme was obtained.

Fig. 1. Constitution of N-glucoide isolated from silk protein.



This result proves that carbohydrates in protein actually exist at least in the form of N-glycoside, and this corroborates our assumption. There may be other possible linkages, but it is certain that carbohydrate contributes to the completion of protein construction in the form of N-glycosidic linkage.

III. Synthesis of N-Glycosides

Studies on systematic synthesis of N-glycosides have been carried out to help determining the constitution of isolated N-glycosides and to investigate the chemical

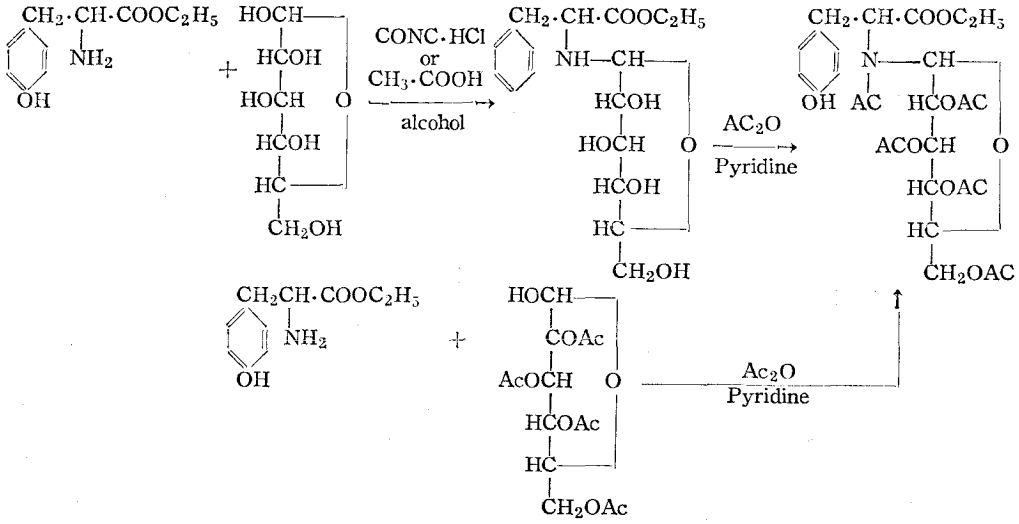
Table 3

Amino acid-N-glycosides synthesized	m.p.	$[\alpha]_D$	References
L-Tyrosine-ethylester-N-D-glucside pentaacetate	140- 1°	−2.7°	24)
„ -N-L-rhamnoside	110- 2°	6.7°	„
„ -N-D-galactoside	118-20°		„
L-tyrosine-methylester-N-D-glucoside	113°		„
L-Glutamic acid-diethylester-N-D-glucoside	149-50°		25)
„ -N-D-galactoside	162- 3°		„
„ -N-L-rhamnoside	97°		„

and biochemical properties of the linkage, using several kinds of amino acids, aromatic amines as aglycones, and reducing sugars including glucosamine. Synthesized amino acid-N-glycosides are shown in Table 3.

N-glycosides described above were synthesized by condensation in absolute alcohol with addition of a small amount of conc. HCl or glacial acetic acid as catalyser. The reaction scheme is shown in Fig. 2.

Fig. 2. Synthesis of L-tyrosine-ethylester-N-D-glucoside pentaacetate.



The amino acid-N-glycosides synthesized are negative in the Nynhydrin reaction before hydrolysis, and positive after it.

N-glycosides of p-aminobenzoic acid and its related compounds including sulfanilamide were synthesized as shown in Table 4.

N-acetyl-D-glucosamine as well as D-glucosamine were detected as the component in the physiologically significant polysaccharides which were isolated from proteins[4-11], microorganisms[28-30], and blood group factors[31-38] and, consequently, we synthesized N-glycosides of N-acetyl-D-glucosamine as shown in Table 5.

Table 5

N-glycoside of N-Acetyl-D-glucosamine.

N-Glucosaminides synthesized	m. p.	References
Aniline-(N-acetyl)-N-D-glucosaminide	193°	35)
p-Toluidine- „	176- 7°	„
o-Toluidine- „	169-70°	„
p-Anisidine- „	172- 3°	„
p-Aminobenzoic acid- „	159°	„
Anthranilic acid- „	171- 2°	„

Synthesis of amino acid-(N-acetyl)-N-D-glucosaminides also is in progress at our laboratory.

Table 4

N-glycosides of p-Aminobenzoic acid and Its Related Compounds.

N-glycosides synthesized	m.p.	$[\alpha]_D$	References
P-Aminobenzoic acid-N-D-glucoside	132°	−64°	26)
„ -N-D-mannoside	182°	38°	„
„ -N-D-galactoside	154- 6°	—	„
„ -N-D-arabinoside	156- 8°	—	„
„ -N-D-xyloside	172°	59°	„
„ -N-L-rhamnoside	169-70°	—	„
P-Aminobenzoic acid-ethylester-N-D-glucoside	178°	−28°	26)
„ -N-D-mannoside	179-80°	7°	„
„ -N-L-arabinoside	180- 1°	−11°	„
„ -N-D-xyloside	116- 7°	−28°	„
„ -N-L-rhamnoside	194°	14°	„
Anthranilic acid-N-D-glucoside	137- 8°	68°	„
„ -N-D-glucoside pentaacetate	184- 6°	77°	„
„ -N-D-mannoside	syrup	—	„
„ -N-D-galactoside	152°	−16°	„
„ -N-L-arabinoside	168°	11°	„
„ -N-D-xyloside	167°	−11°	„
„ -N-L-rhamnoside	165- 6°	52°	„
„ -N-D-fructoside	139-40°	−18°	„
Anthranilic acid-methylester-N-D-glucoside	126- 7°	− 5°	27)
„ -N-D-mannoside	177- 8°	−54°	„
„ -N-D-galactoside	152°	−102°	„
„ -N-D-xyloside	170°	−22°	„
„ -N-L-rhamnoside	syruy	—	„
m-Aminobenzoic acid-N-D-glucoside	109°	6°	„
„ -N-D-mannoside	136°	−10°	„
Sulfanilamide-N-D-glucoside	210°	−122°	„
„ -N-D-mannoside	204°	−103°	„
„ -N-D-galactoside	160- 1°	11°	„
„ -N-L-arabinoside	197°	12°	„
„ -N-D-xyloside	155- 7°	46°	„
„ -N-L-rhamnosid	178-81°	95°	„
„ -N-D-fructoside	170°	—	„
N′-Acetylsulfanilamide-N-D-glucoside	159°	−59°	27)
„ -N-D-xylosid	121°	−58°	„
Sulfanilic acid-N-D-glucoside	>300°	—	„
„ -N-D-mannoside	>300°	—	„
„ -N-D-galactoside	>300°	35°	„
„ -N-L-arabinoside	>300°	—	„
„ -N-D-xyloside	>300°	—	„
„ -N-L-rhamnoside	>300°	25°	„
„ -N-D-fructoside	>300°	—	„

IV. Chemical Properties of N-Glycosidic Linkage

On the assumption that the N-glycosides will show chemical properties different from those of usual O-glycosides, we conducted a number of experiments, and obtained the following results.

(a) Synthesized N-glycosides were found, contrarily to O-glycosides, to be labile in conc. alkali, but when concentration is relatively low, it is rather stable in alkali, and is easily split off in mild acidic condition.[36] Acetylation of N-glycosides is, therefore, best achieved by treating with acetic anhydride and pyridine, and deacetylation is accomplished by treating with sodium methylate[37] without cleavage of N-glycosidic linkage.[38]

(b) One of the characteristic properties of N-glycosides is the transglucosidation of aglycone among some N-glycosids. When N-glycoside was heated in alcohol with another kind of compound as aglycone, a new N-glycoside with another kind of aglycone was formed.[39] Of many N-glycosides tested, the following revealed this property.

1. p-Toluidine-N-D-glucoside + aniline →
   aniline-N-D-glucoside + p-toluidine.
2. p-Toluidine-N-D-glucoside + p-nitroaniline →
   p-nitroaniline-N-D-glucoside + p-toluidine.
3. p-Toluidine-N-D-glucoside + m-nitroaniline →
   m-nitroaniline-N-D-glucoside + p-toluidine.

(c) Some N-glycosides, e. g. p-toluidine-N-D-glucoside, were shown by Amadori[40] to have two modifications; one that melts at 115° and the other at 152°, and the other that is obtained by heating the former at near its melting point as well as by melting the components in the absence of solvent. Kuhn *et al.*[41] have explained this mechanism, named it "Amadori rearrangement", and described the three transformed compounds of p-toluidine, p-phenetidine, and p-anisidine-N-D-glucoside.[42]

We took up investigation of this intramolecular rearrangement, and demonstrated that the trace of acetic acid, p-toluenesulfonic acid, and NH_4Cl can work as catalyser. And we added two more transformed compounds of o-toluidine- and aniline-N-D-glucoside to the above mentioned three.[43] Their melting points and rotatory powers are given in Table 6.

Table 6

N-glucosides	Amadori rearranged compounds	
	m.p.	$[\alpha]_D$
o-Tlouidine-N-D-glucoside (m.p. 84°)	123- 5°	−34.2°
Aniline-N-D-glucoside (m.p. 131°)	127-30°	−37.8°

The transformed compounds can be identified by their melting point and rotatory power which are different from those of the original compounds, and also by the characteristic color reactions for them. We then successfully prepared glucosone

starting from transformed p-toluidine-N-D-glucoside (p-tolyl-isoglucosamine).[44-45] This method of preparation is easier, and gives more yield (19%) than the usal method which starts from glucosazone does.

(d) Chitin is a polymer of N-acetyl-D-glucosamine and widely distributed in nature. Treated with conc. alkali, chitin gives chitosan which has been assumed to be a polymer of D-glucosamine. Chitosan has been found to give free D-glucose by liberating nitrogen on deamination by the action of nitrous acid. However, as the condition under which the experiments were done[46-47] is acidic, so we are afraid that the cleavage of O-glycosidic linkage may happen. We investigated, therefore, the use of a neutral deamination agent, such as Barium hypobromite, expecting that polyhexosan might be obtained. Contrary to our expectation, we found free D-glucose formed as a deamination product, instead of polyhexosan. Consequently, it is concluded that some parts, at least, of intermolecular linkage of D-glucosamine in chitosan might be concerned with nitrogen atom.[48]

V. Biochemical Activities of N-Glycosides

Some of the biochemically important compounds in organism are known to contain N-glycosidic linkage in their molecules, e. g. some B-vitamins including B_{12}[49] and nucleosides, and though no investigation has been made as to the biochemical significance of this linkage, it is possible that the linkage has a certain biological effect in organism owing to its reactive properties. Little has been reported on the biochemical aspect N-glycosides except some of natural origin, and so we have made research, using sulfanilamide-N-D-glucoside as first case.[50] Some researcher[51,52] reported its higher solubility in water and lesser toxicity. Our experiments were made on B. coli communis in equimolecular concentration to compare its own action with the original compound. The experimental conditions were at 37°C and pH 7.6 and bacterial growth was estimated by using an electrophotometer. The results are summarized in Fig. 3.

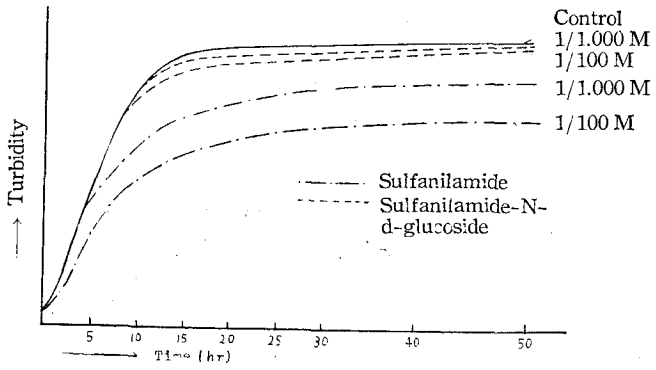


Fig. 3. Effect of Sulfanilamide-N-d-glucoside on B. coli.

As shown in Fig. 3, sulfanilamide-N-D-glucoside under this condition lessens the inhibitory action of sulfanilamide. This effect has not yet been ascertained on other microorganisms, and under other experimental conditions some different results have

been obtained, but we should seek the beneficial effect of glycosidizing sulfanilamide in the increase of solubility and in the decrease of toxicity. Bioassays of other N-glycosides on other microorganisms under various conditions are now under way.

For so many centuries the extracts of the earth-worm have been widely known in this country to have an antifebrile effect; this has been ascertained by the fact that methionyl-(leucine)-N-L-arabinoside-5-phosphate isolated from the organism is responsible for this effect in the case of artificially fevered rabbits.[53] The antifebrile effect has been found also in N-glycoside isolated from Penicillium.[54]

An interesting report on the biochemical action of the protein-bound carbohydrate was published[55]; B. dysenterial antigen is antigenic, only when it is in a bound form, but not when dissociated into its components, peptide and polysaccharide.

VI. Prospect and Conclusion

We started our studies on N-glycosides as described above to investigate the manner of protein-carbohydrate linkage in protein molecule, and succeeded in isolating many kinds of carbohydrates in the form of N-glycosides. These are the first contribution in this field. We conclude that some carbohydrates in protein exist in the form of N-glycoside, but we have failed to elucidate the rôle of carbohydrate as integral part of protein molecule as well as to establish a general rule.

The systematic synthesis of N-glycosides is still far from the completion, and our efforts will continue along this line. With regard to their chemical properties we have discovered some interesting characters, which we expect to open a big field of application.

Investigation of the biochemical activities of synthesized N-glycosides are still at the preliminary stage, and experiments are expected to be extended to carbohydrates in protein, which are considered to have some physiological significances. Studies along this line are related to the biochemistry of nucleic acids, which is one of the most important fields of the biochemistry of the present day, and toward which our concern is now being directed.

Is the significance of carbohydrates in protein, the study of which has so long been neglected in protein chemistry, so small as generally presumed? Is our effort to correlate so disconnected groups of substance as carbohydrate and protein, after all, in vain? These questions will be answered only when we have obtained enough results.

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