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Amino Acid-Carbohydrate Coordinated Compounds.* II. The Synthesis and the Stability of 3-*O*-Aminoacyl-D-glucoses**

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3-*O*-Aminoacyl-D-glucoses (amino acids; Gly, L-Ala, L-Lys, L-Asp (α and β) and L-Glu (α and γ)) have been prepared by the condensation of 1, 2; 5, 6-di-*O*-isopropylidene-D-glucose with suitably protected amino acid derivatives in the presence of *N*, *N'*-dicyclohexylcarbodiimide, followed by the removal of the protected groups. The effect of free amino and carboxyl groups on the stability of these compounds has been clarified.

In the investigations of the mode of the linkage of amino acids to carbohydrates in glycoproteins, a few reports[1,2] on the synthesis of 6-*O*-aminoacyl-D-glucoses have been presented, with it taken as a low molecular model compound for the examination of the stability of the glycopeptide linkage. Suzuki et al.[1] synthesized 6-*O*-(*N*-benzyloxycarbonyl=Cbz)aminoacyl-D-glucoses by the condensation of 1, 2 ; 3, 5-di-*O*-benzylidene-D-glucose with *N*-Cbz-amino acids (Gly, L-Lys, L-Asp-α-benzyl and L-Asp-β-benzyl) in the presence of *N*, *N'*-dicyclohexylcarbodiimide (the DCC method), followed by the hydrolytic removal of benzylidene groups. Kochetkov et al.[2] developed the direct synthesis of the compounds by the condensation of unprotected glucose with *N*-Cbz-amino acids (Gly, DL-Ala, DL-Val, DL-Norleu, β-Ala and ε-amino caproic acid) by the DCC method. The removal of the Cbz-group from these compounds by catalytic hydrogenolysis in neutral media causes the ester bond to break; only in acidic media it is successfully accomplished. Kochetkov et al.[3] clarified the effect of the amino group in the aminoacyl group on the stability of these compounds.

* Part I: N. Muramatsu, *J. Chem. Soc. Japan, Pure Chem. Sect.* (*Nippon Kagaku Zasshi*), **84**, 861 (1963).

** Presented at the 17th annual Meeting of the Chemical Society of Japan, Tokyo, April, 1964.

1) T. Ukita and S. Suzuki, *J. Pharm. Soc. Japan*, **81**, 222 (1961).

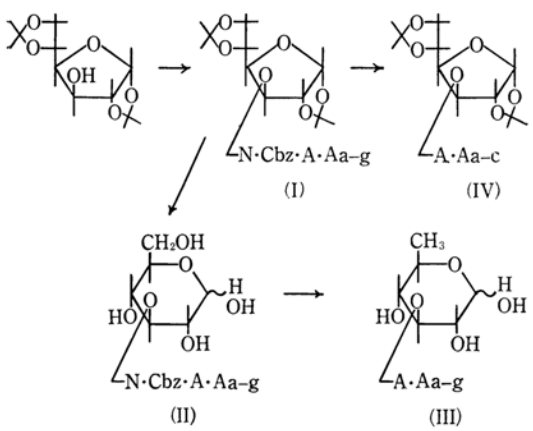
2) N. K. Kochetkov, V. A. Derevitskaya, L. M. Likhosherston, N. V. Moldtsov and S. G. Kara-Murza, *Tetrahedron*, **18**, 273 (1962).

3) N. K. Kochetkov, B. A. Dereviskaya and L. M. Likhosherston, *Izvest, Akad, Nauk SSSR*, **33**, 688 (1963).

TABLE I

Compd.	Mol. Formula	Yield %	M. p.	α_D	Analysis N, % Found	N, % Calcd.	C, % Found	C, % Calcd.	H, % Found	H, % Calcd.	Remarks
I-a	$C_{22}H_{29}O_9N$	53	82—83	−16.5 MeOH $c=1$, 23°	3.18	3.10	58.06	58.53	6.50	6.47	
I-b	$C_{23}H_{31}O_9N$	60	89—90	−29.9 MeOH $c=1$, 22°	3.29	3.01	59.44	59.34	6.97	6.71	
I-c	$C_{34}H_{44}O_{11}N_2$	80	79—80		4.57	4.29	62.38	62.56	7.08	6.79	
I-d	$C_{31}H_{37}O_{11}N$	71	syrup	−22.84 MeOH $c=1$, 19°	2.27	2.33	62.18	62.09	6.42	6.22	
I-e	$C_{31}H_{37}O_{11}N$	43	68—71	−17.3 MeOH $c=1$, 19°	2.51	2.33	61.85	62.09	6.36	6.22	
I-f	$C_{32}H_{39}O_{11}N$	73	83—85	−35.2 MeOH $c=0.2$, 20°	2.45	2.28	62.76	62.62	6.71	6.40	
I-g	$C_{32}H_{39}O_{11}N$	66	80—81	+6.0 MeOH $c=0.8$, 23°	2.45	2.28	62.93	62.62	6.47	6.40	
II-b	$C_{17}H_{23}O_9N$	51	79—80	28.7 MeOH $c=0.4$, 26°	3.30	3.04	52.65	52.98	6.20	6.02	
II-c	$C_{28}H_{36}O_{11}N_2$	48	89—90	50.7 H_2O $c=0.2$, 26°	4.57	4.86	58.45	58.32	6.32	6.29	
III-a	$C_9H_{16}O_9N$	74	90—92	41.6 H_2O $c=0.6$, 25°	4.54	4.72	40.33	40.54	6.81	6.72	1/2 Oxalate
III-b	$C_{10}H_{18}O_9N_1$ 1/2 H_2O			23.1 H_2O $c=0.5$, 26°	4.15	4.58	39.17	39.34	6.29	6.27	1/2 Oxalate 1/2 Hydrate
III-c	$C_{14}H_{28}O_{12}N_2$	54	101—105	71.9 H_2O $c=0.1$, 26°	6.64	7.03	42.05	42.20	6.64	6.58	1 Oxalate
III-d	$C_{10}H_{21}O_{11}N$	60	108—111	28.1 H_2O $c=0.2$, 22°	4.48	4.47	38.68	38.33	5.80	6.11	1 Hydrate
III-e	$C_{10}H_{21}O_{11}N$	66	105	28.1 H_2O $c=0.2$, 22°	4.60	4.47	38.68	38.33	5.80	6.11	1 Hydrate
III-f	$C_{11}H_{23}O_{11}N$	52	amorph.	15.8 H_2O $c=0.2$, 26°	4.27	4.28	40.72	40.36	6.13	6.46	1 Hydrate
III-g	$C_{11}H_{23}O_{11}N$	84	amorph.	34.7 H_2O $c=0.7$, 22°	4.13	4.28	39.90	40.36	6.15	6.46	1 Hydrate
IV-a	$C_{14}H_{26}O_9N$	82	180—181°	−26.3 MeOH $c=1$, 22°	4.02	3.43	47.25	47.19	6.31	6.18	1 Oxalate
IV-b	$C_{17}H_{27}O_{11}N$	85	185—188	−24.9 MeOH $c=1$, 20°	3.31	3.32	48.12	48.45	6.52	6.45	1 Oxalate
IV-c	$C_{22}H_{36}O_{15}N_2$	66	193—195	−17.1 H_2O $c=1$, 22°	4.88	4.93	45.96	46.47	6.55	6.38	2 Oxalate

This paper will deal with the preparation and the stability of 3-*O*-aminoacyl-D-glucoses (amino acids; Gly, L-Ala, L-Lys, L-Asp-α-benzyl, L-Asp-β-benzyl, L-Glu-α-benzyl and L-Glu-γ-benzyl). According to Kochetkov's report[2] 3-*O*-*N*-Cbz aminoacyl-D-glucose was formed as a minor product of these condensations. It was identified with the authentic sample by a synthesis using the condensation of 1, 2 ; 5, 6-di-*O*-isopropylidene-D-glucose with *N*-Cbz-glycine, followed by the removal of the isopropylidene groups. The procedure employed in one of the author's reports[4] was used here. Equal moles of 1, 2 ; 5, 6-di-*O*-isopropylidene-D-glucose[5] and suitably protected *N*-Cbz-amino acid derivatives were condensed in the presence of DCC in absolute pyridine.



–A·A.

a, $-OCCH_2NH_2$

b, $-OCCHCH_3$ (NH$_2$)

c, $-OCCH(CH_2)_4NH_2$ (NH$_2$)

d, $-OCCHCH_2COOH$ (NH$_2$)

e, $-OCCH_2CHCOOH$ (NH$_2$)

f, $-OCCHCH_2CH_2COOH$ (NH$_2$)

g, $-OCCH_2CH_2CHCOOH$ (NH$_2$)

After the filtration, the precipitated *N*, *N'*-dicylohexylurea was washed with excess benzene. The combined mixture of the filtrate and the washings was washed with dilute hydrochloric acid, aqueous sodium bicarbonate and water, and then dried over sodium sulfate. The benzene layer was evaporated under reduced pressure to a thick syrup, and crystallized by trituation with petroleum ether. The 1, 2 ; 5, 6-di-*O*-isopropylidene-3 - *O* - *N* -Cbz-aminoacyl-D-glucoses (I) thus obtained gave negative silver ammonia and ninhydrin reactions, and exhibited absorption bands characteristic of ester carbonyl at 1740—1755 cm^{-1} and the bands characteristic of urethane carbonyl at 1675—1710cm^{-1} in the infrared spectra. The hydrolysis of isopropylidene groups of I in 75% acetic acid at 80°C for two or three hours gave 3-*O*-*N*-Cbz-aminoacyl-D-glucoses (II). Although II was purified by cellulose powder chromatography, only a few compounds could be obtained as crystals. These compounds gave positive silver ammonia and negative ninhydrin reactions. The compound II was converted to 3-*O*-aminoacyl-D-glucoses (III) by hydrogenolysis in aqueous methanol in the presence of Pd-C. In the case of neutral and basic aminoacyl-derivatives, III was obtained as oxalate. The compound III gave positive silver ammonia and ninhydrin reactions. The structure of III was confirmed by periodate oxidation, in which the consumption of the periodate was three moles per mole of III. 1, 2 : 5, 6-di-*O*-isopropylidene - 3 - *O* - aminoacyl-D-glucose (IV) was obtained from I by the procedure described above. The physical properties of I, II, III and IV are summarized in Table I.

The stability of these compounds was investigated at various pH values under the experimental conditions shown in Table II.

Table II. Conditions on hydrolysis for the investigation on the stability of ester bond

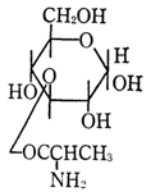
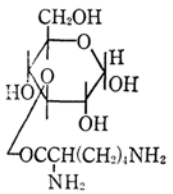
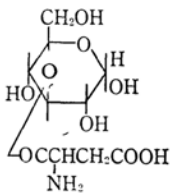
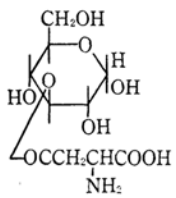
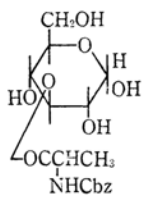
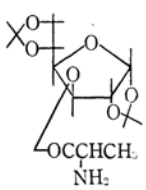
Temperature: 40°C
Concentration: 0.02 mol.
PH: 1, 2.2, 3, 4, 5, 6, 8.
Buffer: McIlvain's Buffer.
Separation of the degradation products:
Paper chromatography: Solv. *n*-BuOH sat. with H_2O
Paper electrophoresis: Solv. Pyridine : HOAc : H_2O (2 ml. : 4 ml. : 1000 ml.)
Determination of the degradation products:
D-Glucose: Anthrone method
Amino acids: Bioassay

The hydrolysis was carried out in the concentration of 0.02 mmol. in order to obtain the half-life periods and rate constants as the first order reaction.[3] As an example, diagrams of reaction time-liberated glucose in the hydrolysis of 3-*O*-L-alanyl-D-glucose at pH 6 and 1 are shown in Fig. 1. In the case of pH 1, the half-life period was calculated by the extrapolation of the data. The half-life periods of various compounds at pH 6 and 1 are listed in Table III.

In order to compare the stability of the neutral and basic amino acid esters, the half-life periods of 3-*O*-L-alanyl-D-glucose and of 3-*O*-L-lysyl-D-glucose are plotted against the individual pH values in Fig. 2. Figure 3 shows the half-life period of the acidic amino acid esters; 3-*O*-α-L-aspartyl-D-glucose and 3-*O*-β-L-aspartyl-D-glucose.

4) N. Muramatsu, *J. Chem. Soc. Japan, Pure Chem. Sect.* (*Nippon Kagaku Zasshi*), **84**, 861 (1963).
5) O. Schmidt, "Method in Carbohyd.," Vol. II, Academic Press Inc., New York. (1963), p. 320.

TABLE III

Compd.	Structure	$\tau_{1/2}$ (pH, 1)	$\tau_{1/2}$ (pH, 6)	$k\times10^3$ (pH, 6)
III-b		107	33/4	2.1
III-c		192	33/4	2.2
III-d		∞	31/2	2.1
III-e		∞	57	0.08
II-b		100	101/3	0.83
IV-b		—	31/4	1.1

Discussion

The neutral and the basic 3-*O*-aminoacyl-D-glucoses gave similar curves, as is shown in Fig. 2. In the region of pH 4—7, their stabilities are almost identical. This reflects the effect of the α-amino group mainly, while the ε-amino group in the L-lysyl ester has almost no effect. In the region of pH 1—3, however, 3-*O*-L-lysyl-D-glucose is more stable than 3-*O*-L-alanyl-D-glucose. This may be understood in terms of Kochetkov's interpretation[3] that the protonation on the free amino

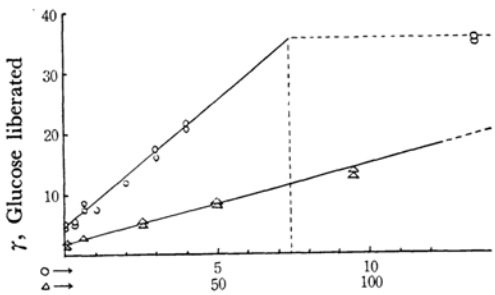


Fig. 1. The diagram of reaction time - liberated D-glucose of 3-*O*-L-alanyl-D-glucose at pH 6 and 1.
○: at pH 6 △: at pH 1

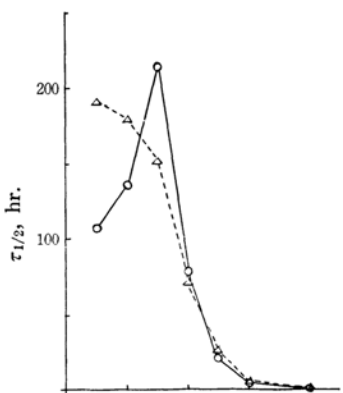


Fig. 2. The half life periods of 3-*O*-L-alanyl-D-glucose (○) and 3-*O*-L-lysyl-D-glucose (△) at pH 1—8.

TABLE IV. R_f VALUES (PPC) AND MOBILITY OF PAPER ELECTROPHORESIS

Compd.	D-Glucose	L-Ala	II-b	IV-b	III-b	L-Lys	II-c	III-c	IV-c	L-Aps	II-d	II-e
R_f values	0.08	0.08	0.29	0.04	0.68	0.03	0.33	0.67	0.02	0.01	0.31	0.25
Mobl. of PEP*	−1.5	−2.5	−7.5	−7.6	−1.6	−11.5			−15.8	5.5		
Compd.	III-d	III-e	IV-d	IV-e	L-Glu	II-f	II-g	III-f	III-g	IV-f	IV-g	
R_f values	0.72	0.73	0.01	0.01	0.02	0.31	0.34	0.75	0.75	0.01	0.02	
Mobl. of PEP*			−0.8	−0.8	2.7	−2.0	−3.5	−1.5		−1.0	−1.2	

* cm./2 hr.

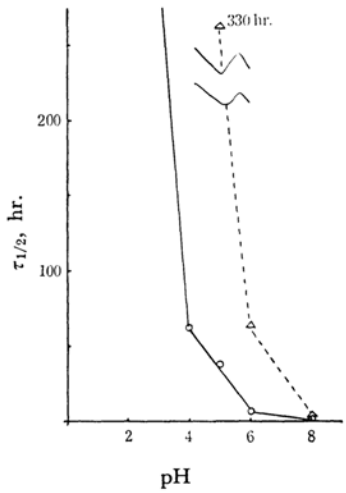


Fig. 3. The half life periods of 3-*O*-α-L-aspartyl-D-glucose (△) and 3-*O*-β-L-aspartyl-D-glucose (○) at pH 1—8.

group prevents the attack of the proton on the ester bond. This tendency is more prominent in the case of the L-lysyl ester because it has two amino groups.

The acidic amino acid esters, as described in Fig. 3, show almost the same stability as the neutral and the basic amino acid esters in the region of pH 5—7. The difference in stability between 3-*O*-β-L-aspartyl-D-glucose and 3-*O*-α-L-aspartyl-D-glucose in the region of pH 4—6 is caused by the interrelation of distance between the ester bond and the amino group. In more acidic media below pH 4, these acidic amino acid esters are extremely stable. This may be explained by the hydride ion formation of the intramolecular carbonyl group with the amino group, which reduces the effect of the amino group. The effects of both the stability and the instability of the free amino group on these compounds are unambiguous in view of the fact that the half-life periods of 3-*O*-L-alanyl-D-glucose and 3-*O*-*N*-Cbz-L-alanyl-D-glucose at pH 1 and 6 (shown in Table III) are interchanged alternately.

On the other hand, the interaction of the amino and the carbonyl groups in these aminoacyl sugars must be considered to be one of the factors causing instability, in addition to the effect of the amino group mentioned above. The half-life period of 1, 2 ; 5, 6-di-*O*-isopropylidene-3-*O*-L-alanyl-D-glucose at pH 6 listed in Table III is almost the same value as that of 3-*O*-L-alanyl-D-glucose, this seems to be negative evidence for the deduction above. However, such an estimation should be based upon a comparison of the corresponding derivatives with the same conformations. This aspect is now under examination.

Experimental

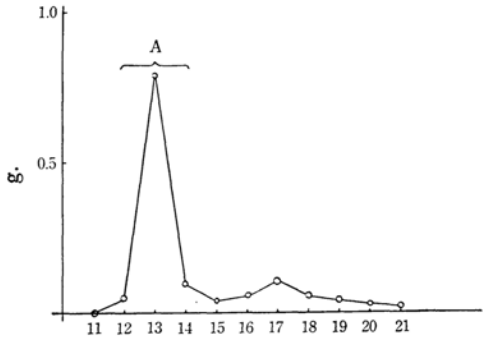
For paper chromatography and paper electrophoresis "Toyo Roshi filter paper No. 51A" was used. The paper chromatography technique used was the ascending procedure using the buffer cited in Table II. Paper electrophoresis was carried out using the buffer of pH 4 cited in Table II, the condition selected was 26.7 V./cm. The R_f values for paper chromatography and the mobilities for electrophoresis may be summarized as follows: These spots were detected with silver ammonia[3] (free carbonyl), ninhydrin (amino) and Rydn reactions[6] (imino). The melting points are not corrected.

1, 2 ; 5, 6-Di-*O*-isopropylidene-3-*O*-*N*-benzyloxycarbonyl-aminoacyl-D-glucoses (I).—DCC (0.011 mol.) was added to a solution of *N*-Cbz-amino acid derivatives (0.01 mol.) in dry pyridine (100 ml.) which had been cooled to 0°C, 1, 2 ; 5, 6-di-*O*-isopropylidene-D-glucose (0.01 mol.) was added to the mixture a few minutes later, and then the solution was kept at room temperature overnight. The precipitated *N, N'*-dicyclohexylurea was filtered and washed with benzene (150 ml.). The mixture of the filtrate and the washings was washed several times with 5% hydrochloric acid, 2% sodium bicarbonate solution and water, then dried over anhydrous sodium sulfate, and evaporated to a syrup under reduced pressure. The syrup thus obtained was dissolved in ether and treated with petroleum ether until it became turbid; then it was left in a refrigerator. The crude crystals were recrystallized from ethyl acetate and water. The physical constants of 1, 2 ; 5, 6-di-*O*-isopropylidene-3-*O*-*N*-Cbz-aminoacyl-D-glucoses (I) prepared by this method are presented in Table I.

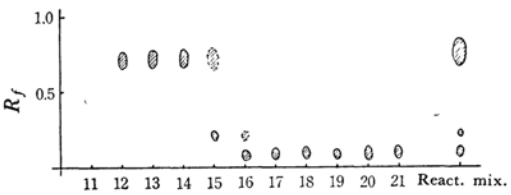
3-*O*-*N*-Benzyloxycarbonyl-aminoacyl-D-glucoses (II).—A solution of 1, 2 ; 5, 6-di-*O*-isopropylidene-3-*O*-*N*-Cbz-aminoacyl-D-glucose (I) (0.01 mol.) in 75% acetic acid (40 ml.) was refluxed for three hours, and then the solution was evaporated to a syrup under reduced pressure. In this syrup, 3-*O*-*N*-Cbz-aminoacyl-D-glucose, D-glucose resulting from the hydrolysis of the ester bond, and an unknown substance which is perhaps the partial-hydrolysed product "1, 2-*O*-isopropylidene-3-*O*-*N*-Cbz-aminoacyl-D-glucose" were detected by paper chromatography. The purification of 3-*O*-*N*-Cbz-aminoacyl-D-glucose was attempted by partition chromatography over cellulose powder (Tokyo Roshi: Grade B) as an adsorbent and *n*-butyl alcohol saturated with water as the effluent. Each fraction was detected by paper chromatography. The elution pattern is shown in Fig. 4. The fractions of the peak of A were collected and evaporated under reduced pressure, and a syrup of 3-*O*-*N*-Cbz-aminoacyl-D-glucose was obtained. Among these a few compounds were crystallized from ethyl acetate. The physical constants of 3-*O*-*N*-Cbz-aminoacyl-D-glucoses are presented in Table I.

3-*O*-Aminoacyl-D-glucoses (III).—*a) The Case of Neutral and Basic Amino Esters.*—To a solution of 3-*O*-*N*-Cbz-aminoacyl-D-glucose (0.005 mol.) in methanol (50 ml.), oxalic acid (0.01 mol.) and 10% Pd-C (2 g.) suspended in water were added; the mixture was then subjected to hydrogenolysis. Hydrogen gas was bubbled into the stirred mixture at room temperature until no carbondioxide was detected in the exhaust. This

6) H. N. Rydon and P. W. G. Smith, *Nature*, **169**, 922 (1952).



Elution pattern of 3-*O*-*N*-Cbs-L-alanyl-D-glucose.
Column: cellulose powder "Toyo Roshi: Grade B" ϕ4 cm. × 55 cm.
Effluent: *n*-BuOH saturated with H_2O



Paper chromatography of each fractions.

Fig. 4.

took about 4—5 hr. The catalyst was removed by filtration and washed with water. The filtrate and the washings were combined and evaporated to a syrup under reduced pressure below 40°C. The syrup was dissolved in methanol and crystallized with the addition of acetone. Recrystallization was then repeated with the same procedure. The yields of these products were about 60—70%.

b) The Case of Acidic Amino Acid Esters.—In this case oxalic acid is not necessary. The other procedures were conducted with the method described above. The yields were 55—85%. The physical constants of 3-*O*-aminoacyl-D-glucoses are presented in Table I.

1, 2 ; 5, 6-Di-*O*-isopropylidene-3-*O*-aminoacyl-D-glucoses (IV).—Compounds I were hydrogenolysed with the procedure mentioned above and so transformed to 1, 2 ; 5, 6-di-*O*-isopropylidene-3-*O*-aminoacyl-D-glucose (IV). In these cases, after the filtration of the catalyst, the filtrate was concentrated and crystallized from methanol or methanol and ether. The physical constants of 1, 2 ; 5, 6-di-*O*-isopropylidene-3-*O*-aminoacyl-D-glucoses are presented in Table I.

General Procedure for the Investigation of Hydrolysis.—A 0.02 mol. solution of 3-*O*-aminoacyl-D-glucoses in an appropriate buffer was soaked into a thermostat controlled at 40°C; 0.05 ml. portions of the solution were sampled intermittently; they are spotted along Line A in Fig. 5. At the same time, the standard solution of D-glucose or amino acid was spotted on the paper at Point B. The spots were developed on paper electrophoresis. The part containing the degradation product was excised along Line A and eluted with 10 ml. of water as shown in Fig. 6.[7] D-

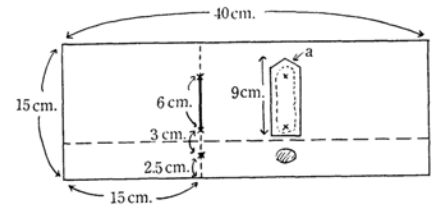


Fig. 5.

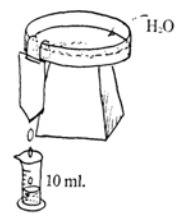


Fig. 6.

Glucose was estimated by the Anthron method[8] and amino acid by bioassay[9] using the eluted solution.[10] The recovery of the method was 100±6%, and the reference sample was made by the procedure described above using non-spotted paper.

a) Neutral and Basic Amino Acid Esters.—In these cases the hydrolysis was pursued by the estimation of D-glucose, as the mobility of D-glucose was different from that of 3-*O*-aminoacyl-D-glucoses. For example, in Table IV and Fig. 1 the progress of the hydrolysis of 3-*O*-L-alanyl-D-glucose at pH 6 and 1 is shown. In this case 3-*O*-L-alanyl-D-glucose was weighed (6.0 mg.) and dissolved in 1 ml. of a buffer. The final value of the D-glucose liberated should be 36 γ in 0.01 ml. of the solution. Figure 2 was the result of plotting the half-life period against the pH values.

b) Acidic Amino Acid Esters.—In this case the hydrolysis could not be pursued by the estimation of D-glucose, for the mobility of 3-*O*-aminoacyl-D-glucose was similar to that of D-glucose. Therefore, it was pursued by the estimation of amino acid by the bioassay method, as the mobility of the amino acid was different from that of 3-*O*-aminoacyl-D-glucose. The half-life periods at all the pH values are shown in Fig. 3.

c) The Hydrolysis of 3-O-N-Benzyloxycarbonyl-L-alanyl-D-glucose.—In this case the hydrolysis was pursued by the estimation of D-glucose after the development of the spot by paper chromatography.

d) The Hydrolysis of 1, 2 ; 5, 6-*Di-O-isopropylidene*-3-*O-L-alanyl-D-glucose.*—In this case the hydrolysis was

7) Dipping of the filter paper into water is practically effective for the estimation of amino acids.

8) T. A. Scott. Jr., and E. H. Melvin, *Anal. Chem.*, **25**, 1656 (1953).

9) T. Tsunoda, "Tanpakushitsu kagaku," Vol. I, Kyoritsu Shuppan Co., Ltd., Tokyo (1954), p. 232. The assay was essentially the same as described on it, and Microorganisms used were as follows: Lactobacillus Arabinocus 17-5 (ATCC 8014) for the assay of L-Glu. Leuconostoc Mesentaroides p-60 (ATCC 8042) for that of L-Asp. Leuconostoc Citrovoum ATCC 8081 for that of L-Ala.

10) Direct densitometry using the filter paper could not be accepted on both case of D-glucose and amino acids by its great error. And ninhydrin method for amino acids also could not be accepted.

pursued by the estimation of L-alanine after the development of the spot by paper chromatography.

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