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Biochemical Studies on Carbohydrates

CLXXIV. Analysis of Mutarotation of N-Glycosides

First Paper
On Aniline-N-Lactoside

By

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Regarding rotation change of free sugars, the opinion has prevailed that it is due to anomerization. On the contrary, however, that of N-glycosides has been often ascribed to isomerization of the lactol ring, for instance, Howard *et al.*[1] said that, of the two modifications of 2-nitro-4,5-dimethyl-aniline-L-arabinoside which Kuhn & Ströbele[2] had prepared and assumed as furanosides, one must be in fact a pyranoside. Berger & Lee[3] could obtain two isomers of aniline-D-riboside by condensation of aniline and D-ribose under different conditions and considered one of them to be a furanoside and the other to be a pyranoside. More recently, Weygand *et al.*[4] expressed a similar claim regarding two isomers of *p*-nitroaniline-D-xyloside.

As early as 1918, Haworth & Leitch[5] observed mutarotation of the anilide of 2,3,4,6-tetramethyl-galactopyranose. In this case, isomerization of the type pyranoside⇄furanoside can not occur, and therefore that of the $\alpha\beta$-type appears most probable. However, the analysis of the change of rotation of N-glycosides of free sugars is not an easy problem in general, because the N-glycosidic link is extremely unstable against the chemical agents which serve the purpose of establishing the ring structure, and hence, with the aim in view, the writer picked out at first aniline-N-lactoside which is blocked at C_4 of the glucose residue.

In 1888, Sorokin[6] prepared aniline-lactoside by heating its components with 96% ethanol. The product had $[\alpha]_D$ in water of $-14.19°$. The writer could not prepare in all his attempts the lactoside identical with Sorokin's but a product (**I**) with F.P. of 199–201° and $[\alpha]_D^{20}$ in dry pyridine of $-82.1°$ by the procedure of Weygand[7], and a second (**II**) with F.P. of 159° and $[\alpha]_D^{20}$ in dry pyridine of $-13.5°$ by rapid precipitation with dry

ether of a methanolic solution of the components heated for a long time. On standing for many days in air, or when recrystallized, whether from dry or aqueous methanol, without use of ether, **II** changed into **I**. Thus **I** proved to be the more stable isomer than **II**, but **I** also changed into **II** when carried down with dry ether after long heating in dry methanol. Hence, **I** and **II** are interconvertible. Next, Ellis & Honeyman[8] pointed out that the more dextrorotatory isomer of aniline-riboside (Berger and Lee's aniline-N-α-ribofuranoside[3]) is obtained, only when water is strictly avoided throughout the whole course of preparation. To synthesize **II** above, however, it was requisite not only to condense the components in perfectly water-free medium but also to add a large volume of dry ether at one time for crystallizing out of the product.

Since aniline-N-lactoside can not take furanoid structure of the glucose residue, its rotatory change takes place by simple anomerization, provided that hydrolysis and Schiff base formation are excluded. Schiff base can be detected by polarography, and its formation was disproved in any of the solvents employed in this work (0.01 *N* NaOH, buffers of pH 5.6, 7.0 and 9.6). (Regarding the polarography, details will be described in Second Paper.[8a]) In 0.01 *N* NaOH, hydrolysis of the lactoside was found also not to occur by determination of free aniline (See Experimental). In this solvent, **I** and **II** rotated to the end value of $[\alpha]_D^{20}$ of $-42°$ from the original $-65.7°$ and $-7.3°$ respectively in about 3 hours (Tables IA and IB and Fig. 1). According to Hudson[9], the mutarotation velocity of the $\alpha\beta$ interconversion of free sugar pyranoses follows the equation of the first order reversible reaction:

$$k_1+k_2=\frac{1}{t}\log\frac{r_o-r_\infty}{r_t-r_\infty},$$

where k_1 and k_2 are the respective reaction constants of $\alpha\rightarrow\beta$ and inverse rearrangements, and r_o, r_t and r_∞ the respective rotations at the beginning, at time t and at equilibrium. If the mutarotation of the writer's materials in 0.01 *N* NaOH owes its entire to establishment of $\alpha\beta$-isomerization, k_1+k_2 values calculated from the rotation changes of the isomers must equal each other, and $\log(r_t-r_\infty)$ values plotted against t fall on two parallel straight lines. In fact, the equation above accurately held true here, as is revealed by k_1+k_2 values (0.0085) in Tables IA and IB and the $\log(r_t-r_\infty)$ (r signifies $[\alpha]_D$) curves in Fig. 2, in other words, **I** is concluded to be the β isomer and **II** the α isomer. k_1 was calculated at 0.0046 and k_2 at 0.0039.

Effect of H and OH ions upon the rotation change of aniline-N-lactoside. Mutarotation of free sugars is catalyzed by OH ion more intensively than by H ion, but the relation between the ions is said to be the con-

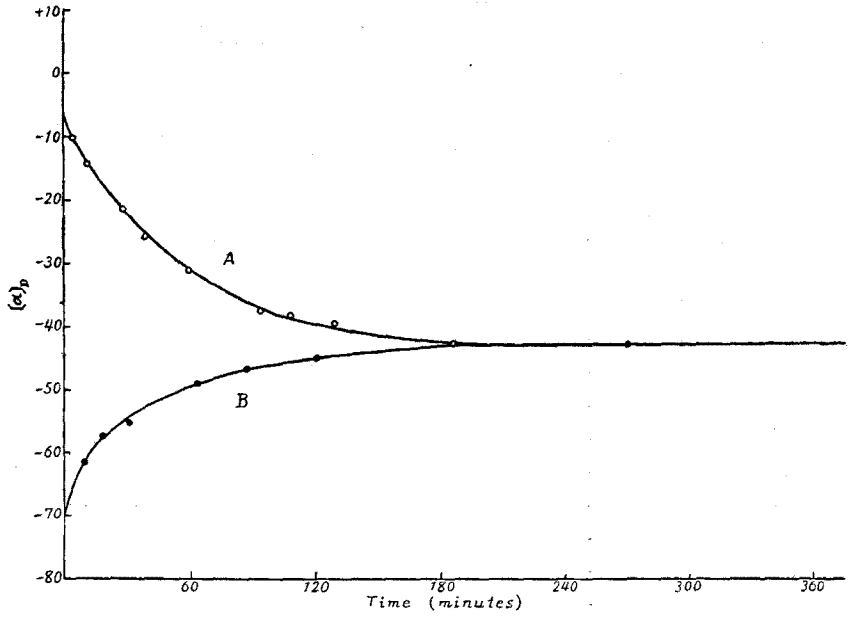

Fig. 1. Showing mutarotation of the α (A) and β (B) isomers of aniline-N-lactoside in 0.01 *N* NaOH (c 2.0) at 20°C.

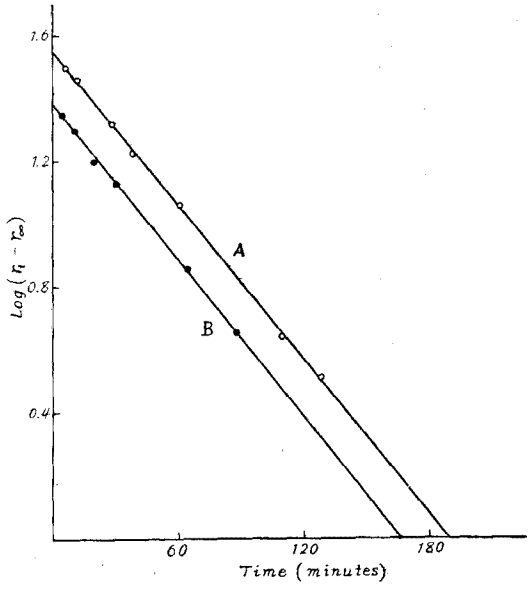

Fig. 2. $Log(r_t - r_\infty)$ (r signifies $[\alpha]_D$) of 2% solutions of the α (A) and β (B) isomers of aniline-N-lactoside in 0.01 *N* NaOH at 20°C, expressed as a function of time. Rotation tube 1 dm.

TABLE I

Mutarotation of Aniline-N-lactoside in 0.01 *N* NaOH at 20°C (c 2.0; rotation tube 1 dm.)

Time after dissolution	$[\alpha]_D$ (°)	log $(r_t - r_\infty)$ $(r = [\alpha]_D)$	k $(=k_1+k_2)$
A. The β isomer			
0 min.	-65.7*		
4½	-64.6	1.354	
9½	-61.7	1.295	0.0084
18	-57.3	1.185	0.0106
31	-55.3	1.124	0.0081
64	-49.0	0.845	0.0083
87	-46.5	0.653	0.0083
120	-44.8	0.447	0.0077
180	-42.0		Avg.: 0.0085
270	-42.1		
360	-42.0		
1480	-41.3		
B. The α isomer			
0 min.	- 7.3†		
6	-10.4	1.502	0.0087
11	-14.2	1.462	0.0083
28	-21.4	1.318	0.0085
38	-25.7	1.218	0.0081
60	-30.8	1.057	0.0093
93	-37.4	0.681	0.0084
108	-37.9	0.634	0.0080
129	-39.0	0.505	Avg.: 0.0085
186	-42.3		
270	-42.2		
360	-42.2		
1480	-41.9		

*, † Values obtained by extrapolation of the observed $[\alpha]_D$ values. * The actual value might be −70.2° which is obtained in a similar manner from the rotations at pH 9.6, because acceleration of the rotatory change at the start which occurs remarkably at pH 9.6, is conceivable also here (S. the text). † The actual value might be −9.2° because of probable initial retardation of the rotation change (S. the text).

verse in case of N-glycosides (Cf. Isbell and Frush[10]). The writer followed up the rotatory chnage at 20°C of aniline-N-lactoside in buffer solutions at pH 5.6, 7.0 or 9.6, estimating simultaneously the liberated aniline in solutions, composed similar to those above and stood at the same tempera-

ture, from time to time. 84.3% aniline became free in 30 hours at pH 5.6, and 59.3% aniline in 100 hours at pH 7.0 (Table II, Fig. 3) and no hydrolysis occurred at least within 24 hours at pH 9.6. Isbell and Frush[10] found that glycosylamine is split pretty quickly in weak acid solutions

TABLE II

Hydrolysis of Aniline-N-β-lactoside in 2% solutions at 20°C

Time after dissolution	hydrolysis (%)	k_h
A. At pH 5.6 (acetate buffer 1/5 *M*)		
11 min.	4.1	0.0016
$1\frac{7}{10}$ hrs.	22.0	0.0011
$4\frac{1}{2}$	45.2	0.0010
10	66.5	Avg.: 0.0012
30	84.3	
33	84.9	
B. At pH 7.0 (phosphate buffer 1/15 *M*)		
1 hr.	2.0	0.00015
2	4.5	0.00016
$3\frac{1}{2}$	6.5	0.00014
6	11.8	0.00015
22	32.3	Avg.: 0.00015
42	44.2	
84	55.9	
100	59.3	

(pH 3–7) but only slowly in a strongly acidic (2.5 *N* HCl). Contrary to glycosylamine, the lactoside was cleft completely in a few minutes in 2 *N* HCl. In Figs. 4A & 4B are plotted the mutarotations with the figures in Tables III and IV. In the β isomer, rapid increase of dextrorotation occurred at pH 5.6, $[\alpha]_D^{20}$ reaching $+25.7°$ from the initial value of $-70.2°$ in 25 hours, but the velocity was so slowed down at pH 7.0 that the increase of $[\alpha]_D^{20}$ to $+11.0°$ took even 4 days. On the other hand, $[\alpha]_D^{20}$ curve of the α isomer at pH 5.6 fell swiftly at first to $-32°$ from $-9.2°$ and thereupon made a sharp bend upwards to join the corresponding curve of the β isomer. The curve of rotation change at pH 7.0 followed a similar but slower course with a very blunt turn. That the decrease of dextrorotation of the α isomer is due to $\alpha\beta$-isomerization and the increase to hydrolysis within these pHs is self-evident. The consistent increase of dextrorotation of the β isomer must be a combined result of those structural changes.

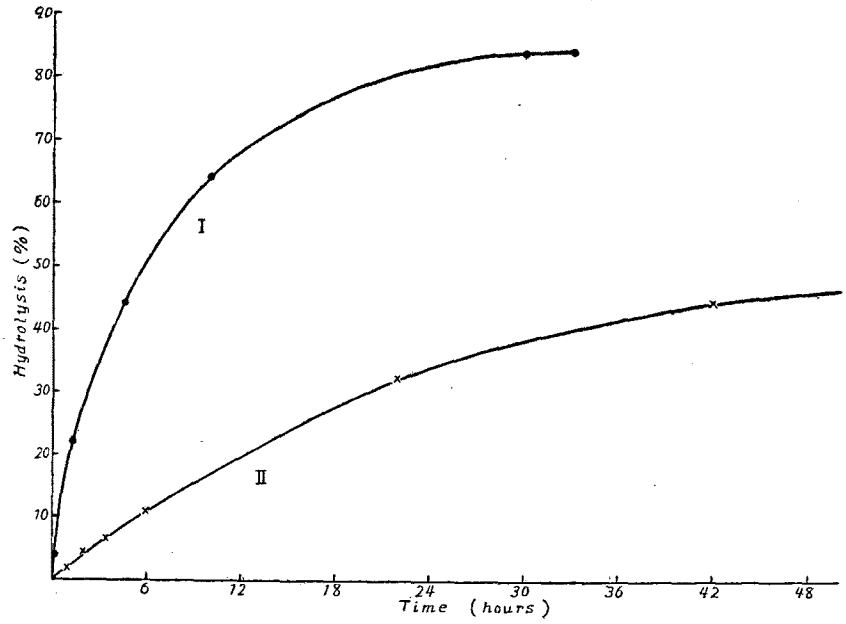

Fig. 3. Progress of hydrolysis of aniline-N-β-lactoside in 2% solutions in acetate buffer (1/5 *M*) pH 5.6 (I) and phosphate buffer (1/15 *M*) pH 7.0 (II) at 20°C, expressed as a function of time.

Hydrolysis proceeded with velocity constant k_h of 0.0012 at pH 5.6 and with that of 0.00015 at pH 7.0, as derived from the amounts of aniline liberated at the initial stage (11th to 270th minute at pH 5.6, and 60th to 360th minute at pH 7.0) by equation

$$k_h=\frac{1}{t}\log\frac{\text{Total aniline}}{\text{Total aniline}-\text{Free aniline at } t} \quad \text{.....(Eq. a)}$$

The constant of the velocity at pH 5.6 can be also calculated out from the rotation change at the early stage of the period where the α and β anomers are in equilibrium (37th to 330th minute after dissolution) by equation

$$k_h=\frac{1}{t_2-t_1}\log\frac{r_{t1}-r_\infty}{r_{t2}-r_\infty} \quad \text{.....(Eq. b),}$$

where r_∞ denotes the rotation after assumed complete hydrolysis, namely, +43.9°. This manner of calculation can not be applied for the constant of hydrolysis at pH 7.0 because the mutarotations of the both isomers take even 7 hours until their courses join together and moreover the inverse reaction is remarkable.

If, in Eq. b, k_h is substituted with 0.0012 or 0.00015, r_{t2} with the rotation at αβ equilibrium, for instance, −32.6° at the 37th minute on the curves at pH 5.6 or −30.0° at the 450th minute on the curve at pH

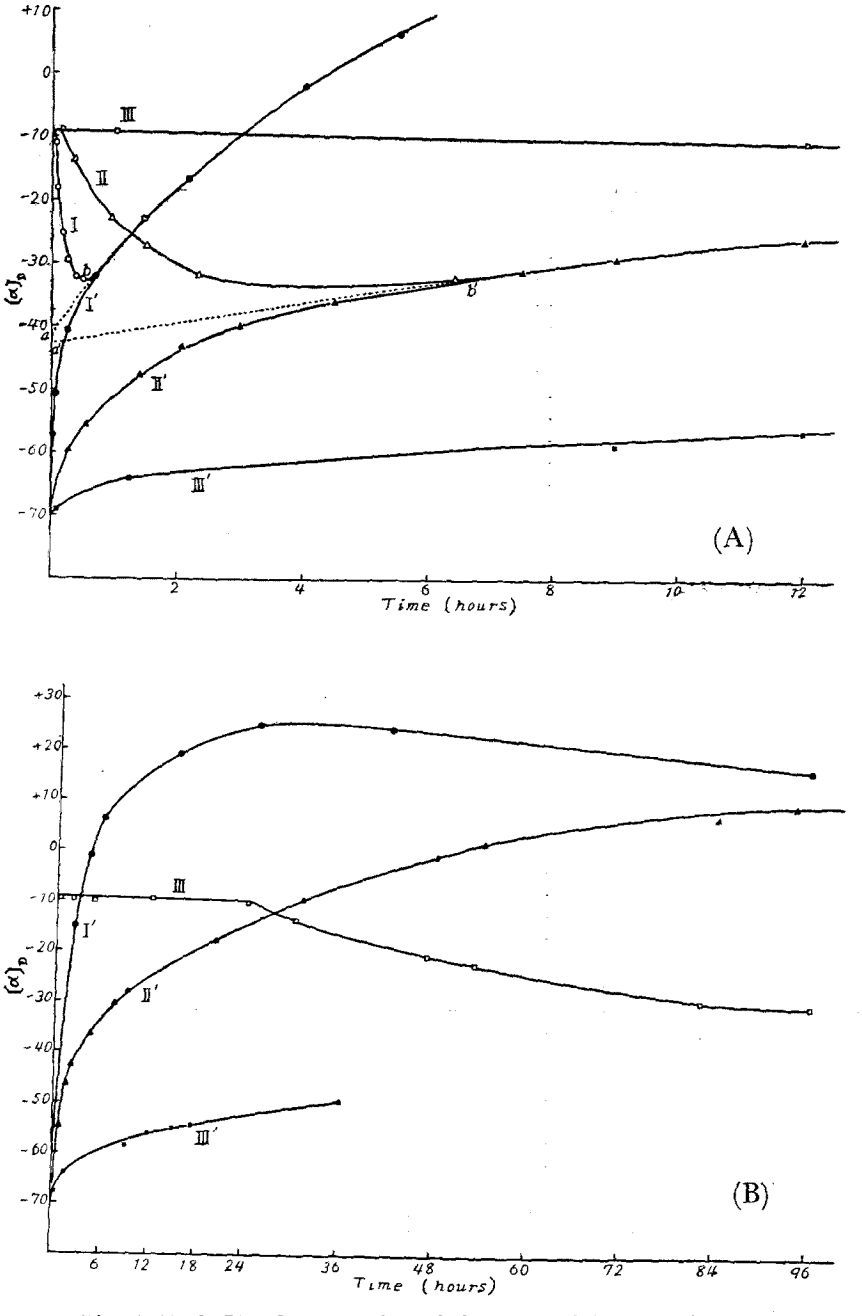

Fig. 4 (A & B). Mutarotation of the α and β isomers of aniline-N-lactoside in 2% solutions in acetate buffer (1/5 *M*) pH 5.6 (I & I′), phosphate buffer (1/15 *M*) pH 7.0 (II & II′) and carbonate buffer (1/10 *M*) pH 9.6 (III & III′) at 20°C, expressed as a function of time. Regarding the dotted lines a–b and a′–b′, see the text.

TABLE III

Mutarotation at 20°C of Aniline-N-β-lactoside at pH 5.6, 7.0 and 9.6 (c 2.0)

Time after dissolution	$[\alpha]_D^{20}$ (°)	K*	k_h*
A. In acetate buffer (1/5 *M*), pH 5.6			
2 min.	−57.3		
5½	−50.9	0.052	
15	−40.6	0.058	
37	−32.6	Avg.: 0.055	
2⅙ hrs.	−16.5		0.0011
4	− 1.7		0.0011
5½	+ 6.8		0.0011
15	+19.2		Avg.: 0.0011
25	+25.7		
40	+24.5		
50	+23.1		
96	+18.0		
B. In phosphate buffer (1/15 *M*), pH 7.0			
6 min.	−64.9		
17	−59.7		
35	−55.7	0.0053	
1⅗ hrs.	−47.6	0.0056	
2⅕	−43.3	0.0055	
3	−39.9	Avg.: 0.0055	
4½	−35.5		0.00013
7½	−30.0		0.00011
9	−28.0		0.00010
20	−17.2		Avg.: 0.00011
26	−11.9		
31	− 8.3		
48	− 0.7		
54	+ 2.2		
84	+ 8.1		
94	+11.0		
124	+11.2		
C. In carbonate buffer (1/10 *M*), pH 9.6			
0 min.	−70.2†		
5	−69.7		
1¼ hrs.	−64.0		
2	−62.7		
9	−58.8		
12	−56.3		
15	−55.1		
17½	−54.1		
36	−49.9		

* See the text. † Extrapolated initial rotation.

7.0, r_∞ with +43.9°, t_2 with 37 or 450 minutes and t_1 with 0 minute in the two cases, r_{t_1} values of −41.7° and −42.4° are given which are rotations at hypothetical equilibrium of the α and β anomers without hydrolysis at pH 5.6 and 7.0 respectively. These values nearly coincide with the

TABLE IV

Mutarotation at 20°C of Aniline-N-α-lactoside at pH 5.6, 7.0 and 9.6 (c 2.0)

Time after dissolution	$[\alpha]_D^{20}$ (°)	K*
A. In acetate buffer (1/5 *M*), pH 5.6		
3 min.	−11.1	
5½	−18.3	0.055
10	−25.5	0.051
15	−29.5	Avg.: 0.053
23	−32.1	
29	−32.6	
40	−31.6	
1 9/10 hrs.	−22.9	
4½	+ 2.0	
B. In phosphate buffer (1/15 *M*), pH 7.0		
7½ min.	− 9.1	
20½	−13.8	
36	−19.2	0.0056
55	−23.0	0.0060
1½ hrs.	−24.2	0.0057
2⅓	−31.9	Avg.: 0.0058
4	−33.1	
6½	−31.7	
16	−20.9	
C. In carbonate buffer (1/10 *M*), pH 9.6		
0 min.	− 9.2†	
5	− 9.2	
1 1/6 hrs.	− 9.0	
4½	−10.1	
12	− 9.9	
24	−10.8	
30	−13.7	
47	−20.1	
53	−21.8	
82	−27.5	
96	−28.7	

* See the text. † Extrapolated initial rotation.

equilibrium rotation $-42.1\pm0.1°$ of the solution in 0.01 *N* NaOH (See Fig. 1). They are also obtained by extension to t_0 of the curves at $\alpha\beta$ equilibrium, because the rotation values at the points where the extensions (dotted lines in Fig. 4A) interesect the ordinate represent them. Next, let R_t and r_t stand for the rotation on one of the extensions and the actual rotation respectively at time t, and d_t for (r_t-R_t), the relative velocity of occurrence of the α and β isomers may be expressed by equation

$$K=\frac{1}{t_2-t_1}\log\frac{d_{t1}}{d_{t2}},$$

where K is the velocity constant of the interconversion of the both anomers.

Average of K values of the α and β isomers at pH 5.6, calculated as above, amounts to 0.055 and that at pH 7.0 to 0.0057. Next, K equals (K_1+K_2), and $\frac{K_1}{K_2}$ the β anomer in % of an equilibrated mixture divided by the α anomer likewise expressed. Furthermore, $[\alpha]_D$'s of these isomers themselves are $-70.2°$ and $-9.2°$ respectively and those of the mixtures at the hypothetical equilibrium ($t=0$) $-41.7°$ and $-42.4°$ at pH 5.6 and 7.0 respectively. Hence, to the solution of pH 5.6 at the hypothetical equilibrium applies the equation

$$(-9.2)x+(-70.2)y=-41.7\times 100\ ,$$

where x is the percentage of α anomer and y that of β anomer.

Therefore, $x=46.7$, $y=53.3$

and $\frac{K_1}{K_2}=\frac{53.3}{46.7}$ or $K_1=\frac{53.3}{46.7}K_2$

Hence, the values of K_1 and K_2 calculated by combining the last equation with $K=K_1+K_2$ amount:

at pH 5.6, $K_1=0.030$
$K_2=0.025$
at pH 7.0, $K_1=0.0031$
$K_2=0.0026$

The ratio of the amounts of α and β anomers at any moment is obtained from $[\alpha]_D$ values of the α and β isomers and of the hypothetical mixture at t_0 and the difference between $[\alpha]_D$'s at t on the actual mutarotation curves and respective dotted lines in Fig. 4, which is equal to the difference between the $[\alpha]_D$ at t and that at equilibrium when no hydrolysis is assumed to occur, namely, $-41.7°$ or $-42.4°$. The amounts of the anomers in per cent of the total lactoside including the hydrolyzed were reckoned by employing those ratios and hydrolysis percentages, and the figures thus obtained for the case when the α isomer was maintained at those pH's are shown in Table V, in which the percentages of the anomers at pH 9.6 and in 0.01 N NaOH are also given. The amount of the β anomer at pH 7.0 was larger than those at pH's 5.6 and 9.6 after 3 hours from preparation of the solutions.

At pH 9.6, aniline-N-α-lactoside differed somewhat from the β isomer in behaviour of rotation change. The latter showed slow increase of $[\alpha]_D$ from the commencement up to $-49.9°$ in 36 hours, which is ascribed to simple $\alpha\beta$-isomerization because of no occurrence of hydrolysis. In the α-lactoside, however, the rotation remained constant for the first 24 hours, followed by gradual decrease of the dextrorotation due to the change of this anomer into the other. In connection with the initial constancy of the

TABLE V

Amounts of the two Anomers of Aniline-N-lactoside, as expressed in per cent of the Total Lactoside, at Various Time after Dissolution of the α-Lactoside (c 2.0) in Solvents of Various pH's

The solutions were maintained at 20°C.

pH of the solutions	Anomer	Time in hrs.							
		0	1	2	4	10	20	36	at equil.
5.6 (Solv. acetate buffer)	α	100.0	37.1	33.1	26.7	16.2	9.7	6.9 (eq.)	6.9
	β	0.0	43.2	38.9	31.3	18.9	11.3	8.0	8.0
7.0 (Solv. phosphate buffer)	α	100.0	73.6	59.8	42.5	36.7	32.2	26.9	18.2 (100 hrs. after dis.)
	β	0.0	24.1	36.2	49.8	44.3	37.8	31.6	21.6
9.6 (Solv. carbonate buffer)	α	100.0	100.0	100.0	100.0	100.0	100.0	79.3	no eq. even after 96 hrs.
	β	0.0	0.0	0.0	0.0	0.0	0.0	20.9	
ca. 12.5 (Solv. 0.01 *N* NaOH)	α	100.0	64.3	51.7	46.0 (eq.)				46.0
	β	0.0	35.7	48.3	54.0				54.0

rotation may be mentioned the article of Worley & Andrews[11], who observed that D-glucose and D-galactose don't mutarotate according to the monomolecular equation at 0°C undergoing retardation or acceleration of the change at the start. They tried to explain the fact by assuming the accumulation of the intermediate substance at the initial stage. Whether the interpretation may hold true or not, the constant rotation of aniline-N-α-lactoside mentioned above is a phenomenon possible to occur at pH's where the mutarotation velocity of the lactoside is comparable with that of galactose at the low temperature. The cause of the adjoining gradual decrease of dextrorotation must be mere anomerization, because of no occurrence of hydrolysis as above. For a similar reason, acceleration of the mutarotation at the very beginning is accepted for the β-lactoside (See also the note of Table I).

Absorption spectra. Ultraviolet absorption was examined in a Hitachi EPB-U type spectrophotometer. The spectra of the α- and β-lactoside in 0.01 *N* NaOH were identical with each other with a maximum at the wave length 237.5 mμ and were not altered by $\alpha\beta$-isomerization on standing. The spectra here resemble that of aniline closely in shape but is shifted towards longer waves with increase of extinction (batho- and hyperchromic effect of the glycosidic sugar) (Table VI, Fig. 5). The α-lactoside in the acetate buffer pH 5.6 showed also the same spectrum as above im-

mediately after dissolution, which, however, became nearly identical with that of aniline (λ_{max1} 230 mμ, ε_{max1} 5996; λ_{max2} 280 mμ, ε_{max2} 944; λ_{min} 257.5 mμ, ε_{min} 281) in 25 hours, approaching to the latter by and by with time due to hydrolysis of the substance (Table VI).

TABLE VI

Ultraviolet Absorption of Aniline-N-lactoside

2% solutions of the α- and β-lactoside in various solvents were stood at 20°C and examined at various time spectrophotometrically with portions 1000-fold diluted with the respective solvents. Figures express wave lengths in mμ. In parentheses are given the respective molecular extinctions (ε)

Anome		Time after dissolution in hrs.			
		0	2	10	25
A. Solvent 0.01 *N* NaOH					
β-Anomer	λ_{max1}	237.5 (12940)	237.5 (12971)	237.5 (12916)	
	λ_{max2}	285.0 (1593)	285.0 (1599)	285.0 (1591)	
	λ_{min}	262.5 (815)	262.5 (826)	262.5 (808)	
α-Anomer	λ_{max1}	237.5 (11408)	237.5 (11398)	237.5 (11388)	
	λ_{max2}	285.0 (1361)	285.0 (1359)	285.0 (1356)	
	λ_{min}	262.5 (653)	262.5 (653)	262.5 (651)	
B. Solvent acetate buffer (1/5 *M*), pH 5.6					
α-Anomer	λ_{max1}	237.5 (11112)			232.0 (6380)
	λ_{max2}	285.0 (1190)			280.0 (968)
	λ_{min}	262.5 (350)			258.0 (286)

EXPERIMENTAL

Preparation of aniline-N-β-lactoside (***I***). 2.6 g. of lactose and 1 cc. of aniline(redistilled) were taken up in 1.5 cc. of water and shaken vigorously in boiling water. The mixture became homogeneous and light brown after 20 minutes and solidified after 30 minutes. Then it was stood in a refrigerator overnight, brought onto a suction filter and washed with aqueous methanol ($CH_3OH : H_2O = 1 : 1$ by volume) and then with abs. methanol. Yield 2.5 g. (80% of theory), F.P. 187–190°. Recrystallized three times from the aqueous methanol, it was obtained in thin needles with F.P. of 199–201°. $[\alpha]_D^{20}$: −82.1°

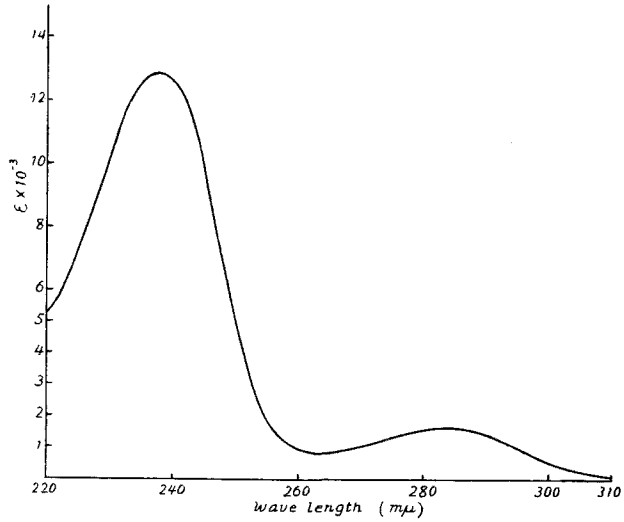

Fig. 5. Ultraviolet absorption spectrum of aniline-N-β-lactoside in 0.01 *N* NaOH (at 20°C).

(c 0.5) in dry pyridine; −70.2°[12] (c 2.0) in carbonate buffer (0.1 *N*) of pH 9.6. The product was difficultly soluble in pyridine, methanol and dioxane and insoluble in ethanol, acetone and ether.

N (micro Kjeldahl)

$C_{18}O_{10}H_{27}N$: Calc. 3.36%; found 3.33%.

Preparation of aniline-N-α-lactoside (***II***). 2 g. of lactose and 0.6 cc. of aniline were refluxed, avoiding moisture, with 100 cc. of dry methanol for 15 hours. A small insoluble part of lactose was filtered off and to the clear filtrate were added 8 volumes of dry ether. Crystals were deposited in thin needles, which were filtered off, washed with 3 changes of dry ether and dried in vacuo over $CaCl_2$. The substance obtained melted at 158–159°. Yield here 1.2 g. (49% of theory). It was recrystallized twice without elevation of the melting point. The product was hygroscopic. $[\alpha]_D^{20}$: −13.5° (c 0.5) in dry pyridine; −9.2°[12] (c 2.0) in carbonate buffer of pH 9.6. Its solubilities in organic solvents resembled those of the β-isomer.

N (micro Kjeldahl): Found 3.37%.

From the mother fluid of the crude product, placed in a refrigerator, came out gradually large needles amounting in total to 0.2 g., which showed F.P. of 189° and $[\alpha]_D^{20}$ in carbonate buffer pH 9.6 of −65.8°[12] (c 1.5), proving the crude β anomer.

Conversion of ***II*** *into* ***I***. 1) The α anomer (**II**) was stood in air for 2 months. The crystals, which became tinted light brown, had F.P. of 185–187° and $[\alpha]_D^{14}$ in carbonate buffer pH 9.6 of −62.7°[12] (c 1.0). 2) 0.1 g. of **II** was dissolved in 1 cc. of 50% methanol by warming and allowed to cool, whereby needles came out. Yield 0.08 g. F.P. was 195–197° and $[\alpha]_D^{15}$ in carbonate

buffer of pH 9.6—67.2°[12] (c 1.0). 3) 0.1 g. of **II** was dissolved in 50 cc. of dry methanol and evaporated in a vacuum desiccator containing $CaCl_2$. The crystalline residue (needles) had F.P. of 191° and $[\alpha]_D^{16}$ in carbonate buffer pH 9.6 of −67.3°[12] (c 1.2).

*Conversion of **I** into **II**.* 0.6 g. of the β-lactoside (**I**) was suspended in 100 cc. of dry methanol and refluxed for 6 hours avoiding moisture. A small insoluble part of **I** was filtered off and 7 volumes of dry ether were added to the filtrate. The crystalline deposit was collected on a filter, washed with dry ether and placed in a vacuum desiccator containing $CaCl_2$. It was twice reprecipitated by the aid of dry ether after heating each time with 90 cc. of dry methanol for 5 hours, and dried similar to above. Yield 0.3 g. F.P. 158°. $[\alpha]_D^{20}$ of it in 0.01 *N* NaOH changed to −42.2° in 3.5 hours from the initial −9.2° (c 2.0) (Comp. with Fig. 1).

Measurement of hydrolysis. The degree of hydrolysis of the lactoside was expressed with the amount of liberated aniline in per cent of total aniline including the bound. Free aniline could be determined spectrophotometrically without cleavage of the lactoside as follows: 3 cc. or less of a solution containing 0.06–24 mg. free aniline is pipetted out of a lactoside solution into a stoppered (ground joint) flask, made up to 3 cc. with water, when necessary, and quickly alkalized with 0.15 cc. of 10% NaOH, followed by shaking for just 1 minute. The mixture is then added to with 0.015 cc. of the sat. $CaOCl_2$ solution (freshly prepared), shaken again accurately for 1 minute and stood for 1 hour at room temperature (20°C) to measure thereupon the extinction at the wave length 365 mμ. The amount of free aniline is read from the standard curve.

Summary

1. The α and β isomer of aniline-N-lactoside were prepared.

2. The mutarotation of them in buffer solutions of pH 5.6 and of pH 7.0 is combined result of hydrolysis and anomerization, and that in a buffer pH 9.6 and 0.01 *N* NaOH is caused by mere anomerization.

3. The progress of anomerization and hydrolysis is analyzed mathematically.

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