The Amino-acid Composition of Tyrocidine


(Received 3 March 1943)

Tyrocidine hydrochloride, a crystalline antibacterial substance obtained, with gramicidin, from cultures of certain bacteria [Hotchkiss & Dubos, 1941] has been shown [Hotchkiss, 1941; Christensen, Edwards & Piersma, 1941] to be of polypeptide nature. A limited study of the constituent amino-acids has been made by these authors, and by Lipmann, Hotchkiss & Dubos [1941]. Dubos [1942] and Dubos & Hotchkiss [1942] have reviewed its biological properties.

We have studied the products of acid hydrolysis of tyrocidine by partition chromatography [Gordon, Martin & Synge, 1943 a, b]. The extraction of the acetylation mixture with organic solvents has been rendered almost quantitative, and the use of pelargonic chloride instead of methyl orange has practically eliminated 'leaching' of indicator, facilitating continuous observation of the more slowly moving 'bands'. These changes have extended the quantitative applicability of the method to acetaminobacids, whose bands move more slowly than that of acetylanaline, and although further investigation is necessary before the method can be used for the simultaneous determination of the eight common amino-acids of this group, the present procedure has proved useful with tyrocidine which seems to contain only three of them. Using a variety of solvent systems, we have not yet achieved a satisfactory chromatographic separation of the acetyl derivatives of glutamic and aspartic acid, and have made use of the differential evolution of CO₂ in the presence of ninhydrin from the corresponding free amino-acids [Van Slyke; Dillon, MacFadyen & Hamilton, 1941] for estimating their relative proportions.

From tyrocidine hydrolysates we have isolated and identified as their acetyl derivatives, phenylalanine, leucine, valine, proline, tyrosine, glutamic acid and ornithine, and have obtained further evidence for the presence of tryptophan and aspartic acid [Hotchkiss, 1941]. Alanine, glycine, lysine and hydroxyproline appear to be absent. Christensen et al. [1941] claim to have isolated alanine (as its dioxyprydate) but give no details.

All these amino-acids have been obtained predominantly as the l-isomers, except phenylalanine, which we find predominantly d-, and tryptophan, whose optical configuration was not determined. Many of the specimens isolated were considerably racemized. Our estimate of the total amount of d-amino-acids in the hydrolysate is consistent with that of Lipmann et al. [1941]. This seems to be the first report of d-phenylalanine in nature. The simultaneous incorporation by the same organism of d-leucine residues (in gramicidin) and l-leucine residues (in tyrocidine) has biological interest.

Ornithine was isolated as its di-acetyl derivative which proved identical with that obtained from l-ornithine, described here for the first time. Ornithine has previously been isolated only from hydrolysates of protein or polypeptide material that had been treated in such a way as probably to modify the guanidino group of arginine residues [Kossel & Weiss, 1913]. The negative Sakaguchi reaction of tyrothricin [Hotchkiss & Dubos, 1941] excludes arginine as an immediate precursor, although it must be borne in mind that tyrothricin is obtained from autolysed cultures. The presence of both proline and ornithine in the hydrolysate suggested that citrulline might be a common precursor for both [Wada, 1933], particularly since tyrocidine, like most proteins, gives a positive Fearon [1939] reaction. However, l-citrulline, when subjected to the same conditions of acid hydrolysis, acetylation and chromatographic analysis, gave at most 8% of a mol. of proline and 22% of a mol. of ornithine. Since under the same conditions of acid hydrolysis citrulline evolved no CO₂, while urea, another possible product of the reaction postulated by Wada, evolved 42-53% of a mol. of CO₂, and also in view of his inadequate characterization of his product, we disbelieve Wada's statement that proline is the main product formed by boiling citrulline with acid. We believe that ornithine and proline residues exist in the intact molecule of tyrocidine, although the presence of some citrulline is not excluded. Further work on the Fearon reaction of proteins is desirable.

The satisfactory results with the estimation of ornithine given below confirm our view [Gordon et al., 1943 a] that partition chromatography should provide a satisfactory micro-analytical procedure for the direct determination of lysine.

Quantitative analyses of tyrocidine hydrolysates were carried out and controlled by parallel analyses of a known mixture of the constituent amino-acids in similar quantity and proportion (Table 1). We were struck by the very low and variable recovery
of tryptophan, as in similar control experiments with a different amino-acid mixture [Gordon et al., 1943b] higher and less variable recoveries of tryptophan were obtained. Homer [1915] and Onslow [1921, 1924] suggested that other amino-acids might be involved. Gortner and collaborators (for references cf. Gortner & Norris [1923]) studied the role of tryptophan in humin formation. In our experiments humin formation is negligible although destruction of some component not present in our first mixture.

Table 1. Analysis of a known mixture which had been heated with acid under the conditions of hydrolysis employed with tyrocidine

<table>
<thead>
<tr>
<th>Known mixture</th>
<th>Amount</th>
<th>Analytical results (recovery %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp. 1</td>
<td>Exp. 2</td>
</tr>
<tr>
<td></td>
<td>mg. N</td>
<td>(batch 'E' gel)</td>
</tr>
<tr>
<td>Acetyl-l-phenylalanine</td>
<td>0·348</td>
<td>106</td>
</tr>
<tr>
<td>Acetyl-l-leucine</td>
<td>0·290</td>
<td>98·5</td>
</tr>
<tr>
<td>Acetyl-l-valine</td>
<td>0·248</td>
<td>102</td>
</tr>
<tr>
<td>Acetyl-l-tryptophan</td>
<td>0·430</td>
<td>7</td>
</tr>
<tr>
<td>Acetyl-l-proline hydrate</td>
<td>0·158</td>
<td>91</td>
</tr>
<tr>
<td>Acetyl-l-tyrosine</td>
<td>0·187</td>
<td>99</td>
</tr>
<tr>
<td>Total dicarboxylic acids</td>
<td>0·343</td>
<td>79·5</td>
</tr>
<tr>
<td>L-Glutamic acid HCl</td>
<td>0·172</td>
<td>45</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>0·171</td>
<td>113</td>
</tr>
<tr>
<td>Diacetyl-l-ornithine</td>
<td>0·493</td>
<td>93·5</td>
</tr>
<tr>
<td>Ammonium sulphate</td>
<td>0·349</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Analysis of a glutamic-acid-tryptophan mixture which had been heated with acid in the presence of, or in the absence of, air

<table>
<thead>
<tr>
<th>Known mixture</th>
<th>Amount</th>
<th>Air present during heating</th>
<th>Amino-acids</th>
<th>Carbon dioxide formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg.</td>
<td>ml. at N.T.P.</td>
<td>Found mg. %</td>
<td>mg.</td>
</tr>
<tr>
<td>L-Glutamic acid HCl</td>
<td>2·9</td>
<td>0·6</td>
<td>2·46</td>
<td>84</td>
</tr>
<tr>
<td>Acetyl-l-tryptophan</td>
<td>6·7</td>
<td>0·0</td>
<td>2·67</td>
<td>92</td>
</tr>
<tr>
<td>Diacetyl-l-ornithine</td>
<td>0·493</td>
<td></td>
<td>4·69</td>
<td>70</td>
</tr>
</tbody>
</table>

Table 3. Amino-acid analysis of tyrocidine

<table>
<thead>
<tr>
<th>(N as % of total N unless otherwise stated)</th>
<th>Present results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preparation analysed</td>
<td>A</td>
</tr>
<tr>
<td>mg. N taken for analysis</td>
<td>1·56</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>12·2</td>
</tr>
<tr>
<td>Leucine</td>
<td>8·6</td>
</tr>
<tr>
<td>Valine</td>
<td>7·1</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>9·5</td>
</tr>
<tr>
<td>Proline</td>
<td>6·4</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>6·4</td>
</tr>
<tr>
<td>Total dicarboxylic acids</td>
<td>12·2</td>
</tr>
<tr>
<td>Glutamic acid (by diff.)</td>
<td>1·8</td>
</tr>
<tr>
<td>Aspartic acid (ninhydrin)</td>
<td>6·1</td>
</tr>
<tr>
<td>Ornithine</td>
<td>9·3</td>
</tr>
<tr>
<td>Ammonia</td>
<td></td>
</tr>
<tr>
<td>Batch of gel used in analysis</td>
<td>E</td>
</tr>
</tbody>
</table>

* Determined after brief hydrolysis.

increase the destruction of tryptophan in hot acid, while Holm & Gortner [1920] studied the destruction of tryptophan heated alone with acid. All these workers allowed free access of air during hydrolysis, which is known to influence the destruction of tryptophan. In our experiments air was excluded, and we provisionally attribute the increased destruction to the interaction of tryptophan with tryptophan is almost complete. If this loss is due to interaction with other amino-acids, some destruction of these might be expected, and the results in Table 1 suggest that proline or glutamic acid might be involved. Preliminary experiments described below with tryptophan-glutamic acid mixtures were not decisive, although the evolution of CO₂ when air was present (Table 2) is of interest,
yet a fresh uncertainty into the deduction of the amino-acid composition of intact molecules from the composition of hydrolysates as usually prepared. With the average protein, of low tryptophan content, the percentage errors introduced in this way would not be as great as with tyrocidine. Nevertheless, they might invalidate stoichiometric calculations for a large molecule.

Whatever general significance for amino-acid analysis our observations may have, the errors encountered have entirely prevented the stoichiometric calculation of a 'minimum molecule' of tyrocidine hydrochloride, since molecules of a composition within the accuracy of our determinations can easily be postulated having two or any larger number of Cl' groups, while we cannot rigidly exclude a molecule having only one Cl' group.

Nevertheless, if we combine Hotchkiss's tryptophan and NH₃ figures with those now obtained for other constituents, we can say that 88–105% of the N of tyrocidine has been identified in its hydrolysate. A reliable estimate of the molecular weight by physical means might throw considerable further light on the data presented here.

**EXPERIMENTAL**

**General analytical procedure**

[cf. Gordon et al., 1943 a, b]

Unless otherwise stated, preparations were hydrolysed by heating for 24 hr., at the temperature of boiling 6 N HCl, with 0·8 ml. acetic acid and 3·6 ml. 6 N HCl in sealed tubes which had been freed from air by boiling the mixture in vacuo before sealing [Gordon et al., 1943 b].

The acetylation and extraction procedure has been modified as follows: 5 ml. 4 N NaOH were used in place of 10 ml. 2 N NaOH. After acidification the solution was immediately absorbed in 16 g. silica gel. After thorough
mixing this was made to a slurry with 17% butanol-CHCl₃ (half-saturated with water), and made into a column in the usual way. No indicator was used. The vessels were washed through three times with small quantities of solvent, which were then added separately to the top of the column and allowed to drain in. Finally, fresh solvent to a total of 11. was run through the column. This multi-plate ‘chromatographic’ extraction utilizes solvent much more efficiently than the single-plate separating funnel procedure previously employed.

The scheme of fractionation shown above was employed.

The initial fractionation with 17% butanol-CHCl₃ was introduced to ensure complete solution of the material for analysis, which does not occur when 1% butanol-CHCl₃ is used first. The silica was saturated with a 0-05% aqueous solution of pelargonin chloride [Gordon et al., 1943a].

In calculating N values from the titrations, the following factors were used: ornithine and tryptophan, 2 atoms N; glutamic and aspartic acids, ½ atom N; other fractions 1 atom N per acid equivalent.

Determination of glutamic and aspartic acids

After titration, fraction (2-1 + 3-1) was hydrolysed with 6N HCl for 6 hr. at 100°, evaporated to dryness in vacuo and dissolved in water. A suitable portion was taken for ninhydrin-CO₂ determination by the method of Christensen, West & Dimick [1941] with 10 min. heating. Calibration experiments showed that under these conditions glutamic acid evolved 1-02 mol. CO₂, and aspartic acid 1-88 mol. CO₂, and the relative amounts of the two amino-acids present were calculated from this.

CO₂ evolved during hydrolyses

Before analysis of their contents the hydrolysis tubes were connected (still sealed) to the Van Slyke-Neill manometric apparatus through pressure tubing; the tip of the tube was then broken off inside the rubber, the CO₂ transferred to the chamber and determined as described by Van Slyke et al. [1941]. Control experiments showed that no CO₂ was evolved by the HCl-acetic acid mixture during 24 hr. heating.

Preparation of tyrocidine hydrochloride

The material studied was obtained from the residues of our preparation of gramicidin from tyrothricin [Gordon et al., 1943b]. Preparation A was obtained by three recrystallizations according to Hotchkiss & Dubos [1941]. After the 1st recrystallization, the material had (α)D +20°-15° = -98°, after the 2nd, -100°, and after the 3rd, -100-5° (95% ethanol, c = 1-1-3). Prep. A had N = 14-5% (Kjeldahl, with or without P-H pretreatment). Prep. B was obtained from A by one further recrystallization.

Characterization of tyrocidine constituents

244 mg. tyrocidine HCl (Prep. A) was hydrolysed and prepared for chromatography as described above, using double the quantity of each reagent. After making the hydrolysate alkaline, NH₄ was removed in vacuo before addition of acetic anhydride. The chromatographic fraction was as above, with columns of suitably enlarged diameter. Pelargonic chloride was used as indicator throughout. Significant quantities of this were eluted by

30% propanol-cyclohexane though not by 17% butanol-CHCl₃. In these cases the contaminated fraction, dissolved in a little 17% butanol-CHCl₃, was filtered through a layer of 2 g. silica gel saturated with water. By washing with a suitable volume of fresh solvent the acetamino-acid was eluted free from pelargonic.

Acid equivalent weight (A.E.W.) and N (Kjeldahl) determinations were carried out as before [Gordon et al., 1943b]; in the former, the first faint persistent pink of phenolphthalein was the end-point. C and H determinations were made by Dr G. Weiler, Oxford. Melting-points are given uncorrected unless otherwise stated. Optical rotations were observed in a 2 dm. tube.

1.1.1. Acetyl-d-phenylalanine. Crystallization from CHCl₃ gave a product (m.p. 160°) which on recrystallization yielded 39 mg. having m.p. 164°, which was depressed to 150-158° on admixture of authentic acetyl-d-phenylalanine (m.p. 169-171°). (α)D +9°-37° (ethanol, c = 1-1). Du Vigneaud & Meyer [1932] record for acetyl-d-phenylalanine m.p. 172° (corr.), (α)D +5°-51° (ethanol). (Found: C, 63-6; H, 6-48; N, 6-53%; A.E.W. 211. Calc. for C₈H₁₅O₄N: C, 63-7; H, 6-28; N, 6-77%; A.E.W. 207.)


1.2.2. Acetyltryptophan. This would be expected in this fraction [Gordon et al., 1943b]. Crystallization from CHCl₃-cyclohexane followed by recrystallization from CHCl₃ yielded 4-7 mg. having m.p. 170-190°, not depressed on admixture of acetyl-l-tryptophan. (α)D -17°-13° slightly positive (< +3°) (ethanol, c = 0-15). After evaporation from ethanol solution N content was 9-1% (F-HI pretreatment), and A.E.W. = 205. (Calc. for C₁₁H₁₄O₄N₂: N, 11-4%; A.E.W. 246.) Perhaps this fraction was a mixture of acetyl-leucine and acetyltryptophan. In two of the three quantitative analyses described below this fraction was also very small; in the other a substantial acid fraction was found.

1.3.1. Acetyl-l-valine. Crystallization from CHCl₃ yielded 13 mg. having m.p. 158°, not depressed on admixture of authentic acetyl-l-valine. The crystals were contaminated by a water-insoluble gummy material, possibly derived from tryptophan, which proved difficult to eliminate. (α)D +2°+ 2°+ 10° (ethanol, c = 0-35). Gordon et al. [1943a] record for acetyl-l-valine m.p. 164°, (α)D +4°-4° (ethanol, c = 2). (Found: C, 54-8; H, 8-22; N, 8-0%; A.E.W. 176. Calc. for C₉H₁₄O₂N: C, 52-8; H, 8-17; N, 8-8%; A.E.W. 159.)

1.3.3. Acetyl-l-proline (monohydrate). Crystallization from wet ethyleacetate yielded 23 mg. having (air-dry) m.p. 76-78°, not depressed on admixture of authentic acetyl-l-proline hydrate. (α)D -12°-5° (H₂O, c = 0-7) (rotation calc. for anhydrous compound). Martin & Synge [1941] record m.p. 78°, (α)D -114°-0° (H₂O, c = 4-2). Analyses were on the air-dry hydrate. (Found: C, 47-2; H, 7-26; N, 7-7%; A.E.W. 184. Calc. for C₁₀H₁₄O₂N•H₂O: C, 48-9; H, 7-43; N, 8-0%; A.E.W. 175.)

1.4.1. Acetyl-l-tyrosine. Treatment with ethanol-light petroleum eliminated much gummy material, after which
were slowly deposited 17 mg. crystals (slightly contaminated by gum) having m.f. 145°, not depressed on admixture of authentic acetyl-l-tyrosine. (a) Δp = 41° (H₂O, c = 0.4). Du Vigneaud & Meyer [1932] record m.f. 152-154° (corr.), (a) Δp = 47-5° (H₂O, c = 0). (Found: C, 57.6; H, 5.53; N, 5.9%; a.e.w. 252. Calc. for C₁₁H₁₀O₃N: C, 59.3; H, 5.83; N, 6.3%; a.e.w. 223.)

2.1.3.1. Dicarboxylic amino-acids. A preliminary determination on a portion of this fraction (R (chromatogram band rate) = 0.2 (approx.) on both 17% butanol-CHCl₃ and 30% propanol-cyclohexane columns [Gordon et al., 1943a]) showed exactly 2 acid equiv./atom N, suggesting the presence of dicarboxylic acids only. Since attempts at crystallization led only to mixed products, the material was refractionated on a 17% butanol-CHCl₃ chromatogram, and the front and back portions of the resulting broad band were collected separately. Crystallization of the former material from ethanol-CHCl₃ yielded 6 mg. having m.f. 190°, not depressed on admixture of authentic acetyl-l-glutamic acid. Bergmann & Zervas [1928] record m.f. 199° (corr.). (Found: C, 44.9; H, 5.71; N, 8.4%; a.e.w. 91. Calc. for C₆H₁₀O₂N: C, 44.4; H, 5.82; N, 7.4%; a.e.w. 94.5.) The material available did not suffice for optimum analytical accuracy.

In a second analysis, the corresponding fraction from the hydrolysate of 210 mg. of Prep. A was hydrolysed (6 hr. in 6N HCl at 100°), and evaporated to small volume. The dicarboxylic acids were separated in the usual way. Saturation with HCl gas at 0° gave slow deposition of glutamic acid HCl crystals (16 mg.), having (a) Δp = 13° (H₂O, c = 0.3) (rotation calc. for glutamic acid). Wood [1914] records for l-glutamic acid in water +1 mol. HCl (a) Δp = 27°. (Found: N, 7.3%; a.e.w. 98. Calc. for C₅H₈O₄N.HCl: N, 7.6%; a.e.w. 92.)

The mother liquors from the above, after most of the HCl had been removed in vacuo, were treated with copper acetate in 2 ml. water. The crystalline Cu salt deposited was filtered off and decomposed in the usual way with H₂S, yielding material (5-2 mg.) which did not readily crystallize from water, having N content (7%) lower than the theoretical for aspartic acid. After treatment with excess HCl followed by evaporation to dryness, the material had (a) Δp = 13° (H₂O, c = 0-2) (rotation calc. for dry wt. before HCl treatment). Wood [1914] records for l-aspartic acid in H₂O +0.94 mol. HCl (a) Δp = 18-4°. A ninhydrin-CO₂ determination showed the same CO₂/N ratio as aspartic acid (see above). From this identity, the insolubility of the Cu salt, the —COOH/N ratio, and the fact that the rate and appearance of the dicarboxylic acid chromatogram band from tyrocidine hydrolysates could be closely simulated by mixtures of aspartylglutamic and aspartylaspartic acids, we conclude that the fraction under consideration was an impure specimen of aspartic acid, and that tyrocidine hydrolysates contain l-glutamic and l-aspartic acids, the former considerably racemized.

3.2. NN-diacetyl-l-ornithine. Crystallization from ethylacetate and from ethanol-ether successively yielded 25 mg. needles having m.f. 156°, not depressed on admixture of authentic diacetyl-l-ornithine (see below). (a) Δp = 4° (ethanol, c = 0.4). (Found: C, 50.6; H, 7.77; N, 12.9%; a.e.w. 224.) On 17% butanol-CHCl₃ and 30% propanol-cyclohexane chromatograms the band-rate R was approx. 0.1, and the material was chromatographically indistinguishable from authentic diacetyl-l-ornithine on a butanol-

CHCl₃ column, while readily separable from diacetyl-dl-lysine [cf. Gordon et al., 1943a].

Acetylation of l-ornithine

l-Ornithine HCl prepared from l-arginine HCl by the action of liver arginase [Hunter, 1939] and having (a) Δp = 10° (H₂O, c = 0.8) was subjected to acetylation and extraction as described above, and yielded material similar in appearance and crystallization properties to the product from tyrocidine, having m.f. 156° (constant on recrystallization), (a) Δp = 6° (ethanol, c = 0-8). (Found: C, 49.9; H, 7.68; N, 12.7%; a.e.w. 218. C₅H₈O₄N₄ requires: C, 50.0; H, 7.40; N, 13.0%; a.e.w. 216.)

Quantitative analyses of tyrocidine hydrolysates, etc.

Three analyses were performed, according to the general scheme given above, on 10-20 mg. tyrocidine HCl with 3 g. silica gel in 1 cm. tubes for the butanol-CHCl₃, and 2 g. for the other fractionations. Methyl orange was substituted for pelargonin in those columns where the development was begun with 1% butanol-CHCl₃ or 5% propanol-cyclohexane, because of its superior sensitivity.

Control analyses on a known mixture after heating in acid are shown in Table 1. Similar experiments on simple mixtures of glutamic acid and acetyltryptophan were carried out in the presence and absence of air. In the former analysis air was readmitted to the 20 ml. tube to a pressure of 4 cm. Hg after the tube had been completely evacuated. The analytical procedure was simplified by omitting the propanol-cyclohexane chromatograms. Table 2 indicates the slightly higher recoveries found in the absence of air and gives the CO₂ evolution measured as described above. No CO₂ was evolved by tyrocidine or by the control mixture or by 4.0/5 and 6.1 mg. l-citrulline (Hofmann La Roche—kindly provided by Prof. H. A. Krebs) when heated in acid under the same oxygen-free conditions. 1.65 and 2.5 mg. of urea similarly treated yielded 0.565 and 0.98 mg. CO₂, respectively. After 4 mg. citrulline had been treated as above the resulting material was analysed in the same way as the glutamic acid-tryptophan mixtures, yielding at most 8% of a mol. of acids having R > 0.5 in 17% butanol-CHCl₃ (which would include acetylproline), and 22% of a mol. of diacetylmorphine. These substances were not identified; the figures are based on titration of bands having corresponding rates. (For recovery of proline and ornithine from known mixture cf. Table 1.)

Table 3 shows the results (uncorrected) of our analyses of tyrocidine together with the figures obtained by other workers.

SUMMARY

1. Technical modifications have extended quantitative analysis by partition chromatography to a further group of amino-acids.

2. From an acid hydrolysate of tyrocidine, phenylalanine, leucine, proline, valine, tyrosine, ornithine and glutamic acid have been isolated by partition chromatography, and characterized as their acetyl derivatives. Additional evidence has been obtained for the occurrence of tryptophan and
aspartic acid. This appears to be the first isolation of ornithine by acid hydrolysis of an intact protein or polypeptide.

3. The phenylalanine isolated had mainly the d- configuration; the other amino-acids were mainly L-.

4. 88–105 % of the N of tyrocidine has now been accounted for, but the variability of the analytical figures has not permitted stoichiometric calculations. The implications of this variability, which exceeds the analytical errors, are discussed.

We are grateful to Dr R. J. Dubos and to Sir H. H. Dale, P.R.S., for making a supply of tyrothricin available to us.

We wish to thank the Director and Council of the Wool Industries Research Association and the International Wool Secretariat for permission to publish this work.

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The Structure of the Galactose-phosphate Present in the Liver During Galactose Assimilation

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In this paper further support is provided for the view [Kosterlitz, 1937; 1938; 1939b] that in the galactose-phosphate present in the liver during galactose assimilation the phosphate group is attached to carbon atom 1. It has not yet been possible to separate this ester from the accompanying glycerophosphate, and from small quantities of glucose-1-phosphate and an unidentified ester. But the facts that reducing hexosemonophosphates can be removed by alkaline hydrolysis, and that Ba glycerophosphate is very resistant to acid hydrolysis and has a specific rotation of 0°, have made possible the approximate determination of the rate of acid hydrolysis of the galactose-phosphate and of its specific rotation. Since the ester is non-reducing, resistant to alkaline hydrolysis and very labile to acid hydrolysis, it is concluded that the first carbon atom is substituted. The velocity constant (k) of hydrolysis of the natural ester in 0.25 N HCl at 25° is 0.91 × 10⁻³, while that of synthetic galactose-1-phosphoric acid is 0.89 × 10⁻³.

Natural Ba galactose-1-phosphate has [α]₂₅ = 113° while the synthetic ester has [α]₂₅ = 109.5°. These values strongly suggest that the natural and synthetic esters are identical. It is of interest to note that Wolfrom & Pletcher [1941] and Wolfrom, Pletcher & Brown [1942] have succeeded in finally proving that the glucose-1-phosphate of Cori, Colowick & Cori [1937] has the structure of phosphoryl α-mono-d-glucopyranoside. Since the principle of the synthesis of galactose-1-phosphate is analogous to that devised by Cori et al. [1937] for glucose-1-phosphate, the findings of Wolfrom et al. [1942] give further support to the view expressed on other grounds [Kosterlitz, 1939a] that the synthetic galactose-1-phosphate is phosphoryl α-mono-d-galactopyranoside.

EXPERIMENTAL

The general methods were the same as those described in previous papers [Kosterlitz, 1937; 1939a]. The Ba salts of the phosphoric esters were obtained