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The Non-identity of Thudichum's 'Glycoleucine' and *nor*Leucine

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The natural occurrence of *nor*leucine is generally considered in the literature as having been first reported by Thudichum (1901), who obtained an isomer of leucine, originally called 'glycoleucine', from the proteins of ox brain by baryta hydrolysis. When Abderhalden & Weil (1912) first applied Fischer's 'ester' method to the proteins of nervous tissue they were familiar with the passage in Thudichum's (1901) book on the chemistry of brain, in which occurs (pp. 257, 301) the description of this amino-acid C₆H₁₃O₂N. The acid, differing charac-

teristically from ordinary *l*-leucine by the insolubility of its Cu salt and of the free acid, had a sweet taste, and was therefore called 'glycoleucine' in distinction from the tasteless ordinary leucine. Although Thudichum considered 'glycoleucine' as identical with the aminocaproic acid which he had obtained from caproic acid by the well-known bromine process, he cautiously refrained from drawing a conclusion as to its constitution on account of the insufficiently characterized commercial caproic acid used for the synthesis.

In their first paper on the subject Abderhalden & Weil (1912) paid special attention to the isolation of Thudichum's acid and, guided largely by the above-mentioned characteristics, succeeded in isolating small amounts of a dextrorotatory acid with the properties of 'glycoleucine' from the leucine fractions of hydrolyzed nervous tissue and of spinal cord. Abderhalden relied on fractional recrystallization for the separation of the mixture of isomeric leucines, but reverted more recently (Abderhalden & Heyns, 1933) to Thudichum's original method of first fractionating the Cu salts, a procedure followed also by Czarnetzky & Schmidt (1932).

In their second paper Abderhalden & Weil (1913*a*) considered the acid as identical with the *l*(+)- α -aminocaproic acid which Fischer & Hagenbach (1901) had obtained from *n*-caproic acid by the bromine process followed by resolution of the resulting *dl*- α -aminocaproic acid. The name 'glycoleucine' was discarded by Abderhalden as unsuitable and replaced by 'caprine' and subsequently by 'norleucine' (Abderhalden, Froehlich & Fuchs, 1913).

The last name has since passed into the literature, and so has the reference to Thudichum's (1901) book as the original place of publication of 'glycoleucine'. For the sake of historical accuracy, however, it should be pointed out that the paragraph referring to 'glycoleucine' in this book is merely a German translation from Thudichum's earlier (1884) book, *Physiological Chemistry of the Brain*. A somewhat fuller account of the properties of the two isomeric leucines, referring also to the synthesis of 'glycoleucine' from caproic acid, appeared two years later (Thudichum, 1886). All three accounts, however, are based (as indicated by Thudichum in the prefaces of his books) on his *Reports to the Local Government Board*, published as Blue Books. The original work *On Glycoleucin, the first Chemolytic Isomer of Leucin, its Properties and Combinations* appeared in 1882 on p. 305 of the 11th *Annual Report of the Local Government Board*, 1881-2 (H.M. Stationery Office, London).

In this publication the specimens of the Cu salt and of the pure 'glycoleucine' prepared from it are given numbers (3220 and 3222) which appear in Thudichum's own handwriting, on the labels of his preparations, which form part of the complete collection of Thudichum's preparations brought to light some time ago (Rosenheim, 1930) and which is now preserved at the National Institute for Medical Research. The collection includes synthetic glycoleucine (no. 153) and ordinary leucine from brain (no. 150), the numbers coinciding with those in a list of his preparations recorded by Thudichum in the pages of his notebooks now in the possession of one of us (O. R.).

Our examination of the 'glycoleucine' prepara-

tions made it clear from the outset that only racemic leucine isomers could be present, for none of them showed a significant optical rotation (Table 1). Further study left no room for doubt that 'glycoleucine' is not identical with *norleucine*, but consists of *dl*-leucine. This conclusion is based on the following evidence.

(1) Comparison of the Thudichum specimens with authentic leucine and *norleucine* by the 'mixed chromatogram' method, employing partition chromatography with paper (Consdén, Gordon & Martin, 1944) showed that all three preparations differed from *norleucine* but agreed with leucine.

(2) Selective oxidation by the *l*-amino-acid oxidase of *Proteus vulgaris* (Bernheim, Bernheim & Webster, 1935; Stumpf & Green, 1944) yielded *d*-leucine, identified by its optical rotation in water and HCl solution. The α -ketoisocaproic acid formed at the same time was isolated as its dinitrophenylhydrazones, which has a characteristically higher m.p. than the corresponding derivative of α -keto-*n*-caproic acid expected to arise from *norleucine*.

(3) Asymmetric resolution by *Penicillium roqueforti* Thom (cf. Schulze & Likiernik, 1893) yielded *d*-leucine, identified as in (2) above.

(4) The preparations yielded acetyl derivatives agreeing in m.p. with acetyl-*dl*-leucine and not depressing its m.p., which is more than 40° higher than that of acetyl-*dl*-*norleucine*.

Thudichum obtained his 'glycoleucine' by alkaline hydrolysis of brain proteins, whereas by acid hydrolysis of the same material and of other 'animal albuminous matters' he obtained 'leucine' having the usual properties. We have examined a leucine specimen of his (no. 150) and found it to agree in optical rotation in water and HCl solution with *l*-leucine (Table 1). It is clear from present-day knowledge of the behaviour of amino-acids that the baryta hydrolysis racemized the leucine present. The difference in taste of 'leucine' and 'glycoleucine' noted by Thudichum is consistent with Fischer & Warburg's (1905) observations on the sweet taste of *dl*-leucine and *d*-leucine.

Thudichum's failure to recognize the identity of his glycoleucine with racemic leucine may be readily understood when it is recalled that in 1882 two relevant facts were still unknown, i.e. that natural leucine is optically active and that its racemization is brought about by baryta, altering both its solubility (Schulze & Likiernik, 1891) and taste (Fischer & Warburg, 1905).

The divergence in properties from *norleucine* of Thudichum's synthetic product fully justifies his scepticism as to the nature of the caproic acid he used as starting material.

In view of these results it seemed to us of interest to examine an acid hydrolysate of ox spinal cord for the presence of *norleucine*. Most workers sub-

sequent to Thudichum who claim to have isolated *norleucine* have used this tissue. Examination of the hydrolysate by the method of Consden *et al.* (1944) failed to reveal any *norleucine*, whereas the addition to the hydrolysate of authentic *dl-norleucine* (the added *norleucine* N being 0.03 % of the total N of the hydrolysate) could be clearly detected.

Optical rotation has been the criterion of identification most frequently employed by past workers in reporting isolations of *norleucine*. Unfortunately the optical rotation of *l*(+)-*norleucine*, both in water and HCl solution, is intermediate between those of the authentic and widely distributed protein constituents *l*(-)-*leucine* and *l*(+)-*isoleucine*. Most of the other criteria have been physical properties of the free amino-acid crystals, or of crystals of derivatives (Abderhalden & Weil, 1912, 1913 *a, b*; Czarnetzky & Schmidt, 1932; Yaginuma, Arai & Hayakawa, 1932; Abderhalden & Heyns, 1933; Nuccorini, 1934). In these studies little, if any, effort was made to exclude the possibility of preparations being mixed crystals or 'molecular compounds' of (say) *leucine* and *isoleucine* derivatives. F. Ehrlich (1904) specifically mentions the difficulties of separating these two compounds by crystallization from water of the free amino-acids or their Cu salts. In what is often regarded as the most convincing identification of *norleucine*, the gas titration of Czarnetzky & Schmidt (1932), it is unfortunate that the optically active natural product was compared with racemic synthetic material.

Of characteristic properties in the dissolved state for differentiating *norleucine* from its naturally occurring isomers the most suitable recorded by previous workers seems to have been the velocity of trimethylamination of the corresponding bromo-

(cf. Vickery, 1941). The absence of *leucine*, *isoleucine* and other probable contaminants from such preparations should also be demonstrated. A considerable number of microbiological and chromatographic methods suitable for both these purposes has recently become available, and some of these are also capable of greatly facilitating the isolation of *norleucine* from natural material.

EXPERIMENTAL AND RESULTS

Authentic preparations employed for comparisons

dl-Leucine I. The preparation of Synge (1939). Resolved to yield *d*-*leucine* $[\alpha]_D^{20}$, -13.2° (6N-HCl, $l=2$, $c=5$), and its acetyl derivative as there described.

dl-Leucine II. A synthetic preparation, kindly put at our disposal by Dr A. Neuberger. $[\alpha]_D$, $\pm 0.0^\circ$ (water or 6N-HCl).

dl-norleucine I. The preparation of Synge (1939). Resolved to yield *d*-*norleucine* and its acetyl derivative as there described.

dl-norleucine II. Synthesized by Dr M. S. Dunn, Los Angeles, and kindly given by Prof. C. L. A. Schmidt, Berkeley University, California.

Preparation of 'glycoleucine' from Thudichum's Cu salt

The blue crystalline powder (no. 3220) was insoluble in cold or boiling methanol, benzyl alcohol or acetone; this indicates the absence of *isoleucine*. The salt (5.4 g.) was suspended in 500 ml. water and H_2S passed into the suspension, which was kept boiling gently for several hours. The colourless solution obtained after removal of CuS (*norit*) was taken to dryness on the water-bath. The fluffy residue of white shiny crystals was dissolved in 150 ml. boiling water and diluted with 75 ml. of ethanol. Shiny leaflets deposited on cooling (F1, 3.13 g.). The filtrate was concentrated *in vacuo* to 50 ml. and the addition of an equal volume of ethanol produced a further crop of crystals (F2, 0.51 g.).

Table 1. Optical rotation of Thudichum's preparations

No.	Preparation	Solvent	$[\alpha]_D$	T°	c (% w/v)
150	'Leucine'	Water	-10.7°	25	0.9
		6N-HCl	$+14.7^\circ$	24	1.0
3222	'Glycoleucine'	Water	-0.6°	25	1.0
		6N-HCl	-0.6°	27	0.9
	From Cu salt: F1	Water	$+0.74^\circ$	17	1.0
		6N-HCl	$+0.24^\circ$	17	2.0
		6N-HCl	$\pm 0.0^\circ$	17	2.0
		6N-HCl	$\pm 0.0^\circ$	20	2.0
153	Synthetic 'glycoleucine'	Water	$\pm 0.0^\circ$	20	1.0
		6N-HCl	$\pm 0.0^\circ$	20	2.0

All rotations were measured in a 4 dm. tube.

acids (Abderhalden & Beckmann, 1932). However, Abderhalden & Heyns (1933) applied this reaction only to crude fractions of natural origin, and not to their purified preparations.

In view of our results it seems desirable that before *norleucine* be accepted as occurring in nature, the amino-acid isolated should be characterized by methods less equivocal than those hitherto employed

Partition chromatography of Thudichum's preparations

The method used is described by Consden *et al.* (1944). A few mg. of each sample were stirred with a drop of water. The copper salt suspensions were treated with H_2S and the resulting mixtures used without filtering. About 3-4 μ l. of each solution

were put on a strip of Whatman no. 1 paper, which was then hung from a trough containing benzyl

sprayed with 0.1% (w/v) ninhydrin in *n*-butanol, dried again and heated to 105° for 5 min.

Fig. 1. Diagram of chromatogram of Thudichum's preparations. The solutions were applied along the broken line in the positions indicated by the dotted circles. The shaded areas represent the spots obtained after development of the chromatogram for 48 hr. in benzyl alcohol saturated with water, drying, and treatment with ninhydrin.

alcohol saturated with water, in an atmosphere saturated with water and benzyl alcohol and containing HCN. After 48 hr., the strip was dried,

A preliminary experiment showed that each of the Thudichum preparations gave only one spot and hence each could be considered as consisting of

one amino-acid only. Further, the R_F values (Consden *et al.* 1944) were consistent with their all being leucine. In a second run, each sample was applied to the paper as described above, with and without the addition of *dl*-norleucine I. As a control, a mixture of leucine, *isoleucine* and *norleucine* was used. Fig. 1 shows the results obtained after running and treating with ninhydrin. Whereas each of the Thudichum samples gave single spots corresponding to leucine, those which had the addition of *norleucine* gave two spots corresponding to leucine and *norleucine*.

dl-norLeucines I and II were examined as above and shown to be indistinguishable.

Action of l-amino-acid oxidase on 'glycoleucine'

(a) *d*-Leucine from 'glycoleucine'. Suspensions of 'resting' *Proteus vulgaris* (Strain X19) were prepared according to Stumpf & Green (1944) and aged for 14 days at 0°. Ten ml. of a suspension (5.5 mg. dry wt./ml.) were added to a solution of 1 g. glycoleucine (no. 3222) in 100 ml. phosphate buffer of pH 7.4 and the mixture aerated for 5 hr. at 37°. After centrifuging, the supernatant solution was

resulting hydrazone was freed from non-acidic hydrazone (resulting from partial decarboxylation of the keto-acid) by washing its solution in ethyl acetate with a dilute solution of NaHCO_3 , and finally recrystallized from a mixture of ethyl acetate and petrol. The 2:4-dinitrophenylhydrazone of α -ketoisocaproic acid crystallized in rhombic crystals, m.p. 155–156° (found: N (Dumas), 18.5%; calc. for $\text{C}_{12}\text{H}_{14}\text{O}_6\text{N}_4$: N, 18.1%). A mixed melting-point with the hydrazone prepared in the same way from *dl*-leucine showed no depression. The highest m.p. recorded for the dinitrophenylhydrazone of α -keto-*n*-caproic acid is 148° (cf. Stumpf & Green, 1944; Blanchard, Green, Nocito & Ratner, 1944).

The action of *Penicillium* spp. on glycoleucine

The classical experiment of Schulze & Likiernik (1891, 1893) on the asymmetric biological resolution of synthetic *dl*- α -amino-isocaproic acid by a green mould from cheese, *Penicillium glaucum* Link, has, as far as we are aware, never been repeated. The isolation for the first time of *d*-leucine thus enabled these authors to establish the constitution of natural *l*-leucine. Since that time the designation *P. glaucum*

Table 2. Products of action of l-amino-acid oxidase of *Proteus vulgaris* on 'glycoleucine' and *dl*-leucine

	$[\alpha]_D^{20}$ ($c=1$, $l=4$)		N (Dumas) (%)	
	Water	6N-HCl	Found	Calc. for $\text{C}_6\text{H}_{13}\text{O}_2\text{N}$
From 'glycoleucine'	+11.4°	–15.6°	10.4	10.7
From <i>dl</i> -leucine II	+11.1°	–15.4°	—	—

treated with norite and taken to dryness *in vacuo*. Approximately 60% of the unattacked amino-acid was recovered (cf. Stumpf & Green, 1944), identical with *d*-leucine obtained in the same yield from a similar control experiment with *dl*-leucine II (see Table 2).

(b) α -Keto-isocaproic acid from 'glycoleucine'. The keto-acid produced by the selective oxidation of the laevorotatory moiety of 'glycoleucine' was characterized as its 2:4-dinitrophenylhydrazone. After the removal of *d*-leucine the remaining solution was concentrated to 40 ml. and, after dilution with 20 ml. of 6N-HCl, an excess of a solution of 2:4-dinitrophenylhydrazine in 2N-HCl was added. The

Link, now known to cover many distinct species, has been discarded by mycologists, who recognized, in agreement with Thom (1910), that Link 'lumped into his species *P. glaucum* every kind of green *Penicillium*'. As it is no longer possible to identify the species used by Schulze & Likiernik, we selected for exploratory experiments with *dl*-leucine three *P.* species at random, i.e. *P. notatum*, strain B592, *P. terrestre*, no. 763, National Collection of Type Cultures (N.C.T.C.) and *P. roqueforti* Thom no. 588, N.C.T.C. Of these, the last species, originally isolated from cheese, like Schulze's species, proved to be the most suitable. It may be noted that the strain of *P. terrestre* had been received by the

Table 3. The action of *Penicillium* spp. on 'glycoleucine' and *dl*-leucine

<i>P.</i> species employed	Substrate	Length of incubation period (days)	Final colour of medium	Yield of <i>d</i> -leucine		$[\alpha]_D$	
				g.	% of theory	In water*	In 6N-HCl*
<i>P. roqueforti</i>	'Glycoleucine'	78	None	0.279	56	+10.9°	–15.1°
	<i>dl</i> -Leucine II	76	None	0.291	58	+10.9°	–15.2°
<i>P. notatum</i>	<i>dl</i> -Leucine II	84	Deep yellow	0.160	33	+11.7°	–14.8°
<i>P. terrestre</i>	<i>dl</i> -Leucine II	84	Brown	0.095	19	—	–15.1°

* The optical rotations were measured in a 4 dm. tube, $c=1$ (± 0.1).

National Collection of Type Cultures as *P. glaucum* from the late Sir Arthur Harden and is presumably identical with the strain used by McKenzie & Harden (1903) in their research on the resolution of racemic acids by the biological method.

(a) *Cultivation of the moulds.* The moulds were grown on Czapek's (1902) solution of inorganic salts, 'glycoleucine' and *dl*-leucine serving as the sole source of carbon and nitrogen. Solutions of 1.0 g. of the respective amino-acid in 200 ml. medium were introduced into sterile conical flasks of 750 ml. capacity, which were autoclaved for 1½ hr. at 15 lb./sq.in. pressure. After inoculation with the spores of the moulds, obtained from fresh subcultures, the flasks were incubated at 24° in the dark for periods of from 2 to 3 months.

The three species showed characteristic differences on incubation. Surface growth was fairly rapid with *P. notatum* and less so with *P. terrestre*. Only relatively slight submerged growth took place with *P. roqueforti*, isolated small patches of white mycelium appearing on the surface only after 8–10 weeks, and the solution remained completely colourless. The colour of the solution in the *P. notatum* flasks gradually deepened from a pale to an intense yellow, and became deep brown in the media inoculated with *P. terrestre*.

(b) *Recovery of unattacked leucine.* At the end of the arbitrarily chosen incubation period, the mycelium was removed by filtration through paper, the solution decolorized with norite when necessary and concentrated until crystallization began. The colourless shiny crystals were collected and dried at 100°, after washing with water and ethanol. A small second crop was usually obtained by carefully adding ethanol to the filtrate of the first crop. The final solution was freed from ethanol and reserved for further examination (see below).

The results, presented in Table 3, again demonstrate the identity of 'glycoleucine' with *dl*-leucine. All three *Penicillia* had attacked preferentially the natural antipode, *l*-leucine, but the amounts of the remaining dextrorotatory isomer varied to a considerable extent. The yield of *d*-leucine in the case of *P. roqueforti* was of the same order (two-thirds of theory) as in Schulze & Likiernik's (1893) experiment with *P. glaucum*. The results are in agreement with the conclusion reached by Pringsheim (1910; cf. McKenzie & Harden, 1903) that moulds attack both components of racemic amino-acids simultaneously but at different rates.

(c) *α-Ketoisocaproic acid from 'glycoleucine' by the action of P. roqueforti.* Only traces of α-keto-isocaproic acid were found amongst the reaction products of *P. notatum* and *P. terrestre* (1.5–3 and 4–5 mg. respectively, as 2:4-dinitrophenylhydrazones). In the case of *P. roqueforti*, however, copious precipitates of hydrazones were obtained in the experiments with 'glycoleucine' and *dl*-leucine. On purification (see above) and crystallization from ethyl

acetate-petrol they yielded well-formed prisms, m.p. 154–155°, unchanged in mixed melting-point with an authentic specimen of 2:4-dinitrophenylhydrazone of α-ketoisocaproic acid and had N, 18.2%.

Fig. 2. Diagram of chromatogram of spinal cord hydrolysate, carried out as described under Fig. 1.

These results indicate that the growing submerged mycelium of *P. roqueforti* synthesizes an active *l*-amino-acid oxidase, giving rise to α -ketoisocaproic acid. The inability of this mould to make use of the acid would thus explain the scanty development of surface growth and therefore the limiting factor under the conditions of the experiment may be an insufficient supply of carbon.

Preparation of acetyl derivatives

100 mg. each of Thudichum's natural (no. 3222) 'glycoleucine', the product F1 (above) regenerated from Thudichum's Cu salt, and Thudichum's synthetic (no. 153) 'glycoleucine' were acetylated by the addition, in portions and with cooling, of 0.18 ml. acetic anhydride and 1.8 ml. 2N-NaOH. After 10 min., 10N-H₂SO₄ equivalent to the NaOH was added and an acetyl derivative crystallized out. After keeping at 0° overnight it was filtered off, washed with a little water and dried. All three preparations yielded 60–70% of the theoretical of acetyl derivative, having m.p. 157–158°, and in no case depressing the m.p. of authentic acetyl-*dl*-leucine of m.p. 158° (cf. Fischer, 1901).

Acetyl-*dl*-norleucine was prepared in the same way and in the same yield from *dl*-norleucine I. It had m.p. (constant on recrystallization from water) 108°. (Found: C, 55.6; H, 8.65; N (Kjeldahl), 8.2%; acid equiv. wt. 171. C₈H₁₅O₃N requires C, 55.5; H, 8.67; N, 8.1%; acid equiv. wt. 173.) (Cf. Snyder, Shekleton & Lewis (1945).)

Mixtures of acetyl-*dl*-leucine and acetyl-*dl*-norleucine melted unsharply at temperatures intermediate between the m.p.'s of the pure substances.

Partition chromatographic examination of hydrolysate of ox spinal cord

Spinal cord from a freshly slaughtered ox was refluxed for 24 hr. with 6N-HCl. After removal of most of the HCl *in vacuo* and filtration from fat and humin, the hydrolysate was diluted with water to give a solution containing 11.35 mg. N/ml. (Kjeldahl).

Preliminary tests with the hydrolysate as de-

scribed in the section dealing with the chromatography of Thudichum's preparations gave no indication of a spot corresponding to *norleucine*. For the purpose of a semi-quantitative estimation, various amounts of *norleucine* were run in benzyl alcohol as already described and it was found that as little as 0.5 µg. of *norleucine* gave a faint but distinct spot. The spinal cord hydrolysate (16 µl.) was run for 48 hr. with and without the addition of 0.5 µg. of *norleucine*, and 0.5 µg. of *norleucine* alone served as a control. The result is shown in Fig. 2. Whereas spinal cord alone gave no spot corresponding to *norleucine*, such a spot appeared quite clearly when *norleucine* was added to the hydrolysate. It is therefore concluded that if *norleucine* is present in spinal cord, the *norleucine* N must be less than 0.03% of the total N.

SUMMARY

1. Thudichum's original specimens of 'glycoleucine', the amino-acid isolated by him in 1882 from the proteins of brain, have been examined (a) for optical activity, (b) by partition chromatography, (c) by treatment with the *l*-amino-acid oxidases of *Proteus vulgaris* and of *Penicillium roqueforti*, and (d) by the preparation of acetyl derivatives.

2. The results proved conclusively that 'glycoleucine' is not identical with *norleucine*, as hitherto assumed, but consists of *dl*-leucine.

3. A partition chromatographic examination of an acid hydrolysate of ox spinal cord indicated the absence of *norleucine*. The need is emphasized of characterizing this amino-acid by less equivocal methods than hitherto employed before accepting it as occurring in nature.

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A Colorimetric Method for the Determination of Cinchona Alkaloids

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During determinations of the concentrations of cinchona alkaloids in the blood of chicks, difficulty was experienced in finding a sufficiently sensitive method for the non-fluorescent alkaloids, cinchonine and cinchonidine. Young chicks up to 4 weeks old were used, so that samples of not more than 1 ml. of blood and plasma were obtainable. The oral doses of alkaloids were only 50 mg./kg. body weight. It was therefore necessary to find a method of determination sensitive to at least 1 μ g. of alkaloid.

The silico-tungstate nephelometric method of Kyker, Webb & Andrews (1941) was tried, but even after modification to suit our conditions, it was not possible to detect less than 10 μ g. of alkaloid. Previously our attention had been attracted to the method of Prudhomme (1940), in which quinine combines with eosin to form a coloured compound extractable with chloroform. This method was rejected mainly because the eosin colours were too weak, and the coloured compound in chloroform solution was readily adsorbed on to the walls of the tube.

Our interest in this reaction was revived when it was discovered by one of us (E. W. R.) that bromothymol blue formed a similar compound with quinine. If to a solution of quinine bromothymol blue indicator was added and the mixture allowed to stand, a yellow colour could be extracted by shaking with chloroform. The depth of colour extracted was proportional to the concentration of quinine. This reaction was given by the four principal alkaloids of cinchona bark, quinine, quinidine, cinchonine and cinchonidine, by certain derivatives of these alkaloids, and by some other nitrogen-containing compounds (see later). It was decided to investigate the possibilities of this reaction as a quantitative method for determining cinchona alkaloids in biological material.

EXPERIMENTAL

Factors influencing colour production

Optimum time for bromothymol blue alkaloid reaction. Mixtures of quinine and bromothymol blue solutions were allowed to stand for varying intervals of time before extraction. The amount of colour extracted reached a constant level in 60 min.

Choice of solvent for colour extraction. Although chloroform was used by Prudhomme (1940) to extract the eosin-quinine compound, its use had certain objections. Being heavier than water, it was difficult to pipette off the lower chloroform layer into colorimeter cells without contamination with the aqueous layer. Chloroform readily formed emulsions on shaking with aqueous solutions, which were difficult to break down even by centrifuging. Other organic solvents

Fig. 1. The influence of pH on the extraction of bromothymol blue cinchona compound by (A) chloroform, and (B) benzene.

were therefore tried, including light petroleum, carbon disulphide, carbon tetrachloride, *n*-butyl and amyl alcohols, benzene, toluene and xylene. Petroleum failed to extract any colour, while *n*-butyl and amyl alcohols extracted bromothymol blue itself. The other solvents were about equally efficient, and benzene was finally chosen.

Effect of pH on extraction. Decrease of pH was found to increase the amount of colour extracted by chloroform.