course similar to that of the other tissues during the first 8–10 days, but after that time until the pre-mortal stage a residue persists which gives the Roe & Kuether reaction and which reduces indophenol.

5. Scurvy develops in guinea-pigs when the curve of the ascorbic acid tissue concentration reaches asymptotic values for long periods of depletion, and there is no definite correlation between the tissue content and the appearance of scorbutic lesions.

6. A high proportion of the ascorbic acid which is fixed by the tissues of saturated guinea-pigs and lost during the first few days of depletion on a scorbutic diet does not act as a store of vitamin C for protection against scurvy.

7. It is concluded that the first appearance of a

continuous elimination of ascorbic acid by the kidney of man is a result of 'supersaturation', the true saturation dose being lower than that ascertained by observation of urinary excretion, and consequently the elimination of ascorbic acid by the kidney is in all probability the extreme expression of overdosage. It is also suggested that, owing to the highly efficient gastric absorption of ascorbic acid by man, no advantage is gained under ordinary circumstances by parenteral rather than oral medication.

8. The specificity of the Roe & Kuether method as applied in this investigation is discussed.

One of us (J.R.P.) is indebted to the Medical Research Council for a whole-time grant. Our thanks are also due to Roche Products Ltd. for a gift of *l*-ascorbic acid.

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Spectrophotometric Study of the Excretion Products of Mepacrine compared with Synthetic Acridine and Diphenylamine Derivatives

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(Received 22 May 1946)

In the present investigation an attempt has been made to characterize some of the excretion products of mepacrine in human urine obtained from subjects on continuous mepacrine therapy. Scudi & Jelinek (1944) isolated four main fractions from dog urine. One of these they identified as unchanged mepacrine by chemical and spectrophotometric means, and they attributed acridine structures to the other three.

Extraction of large volumes of human urine with ethylene dichloride, and subsequent purification and chromatographic distribution, has yielded seven fractions which have been characterized spectrophotometrically. The general plan of fractionation is shown in Fig. 1. Some clue as to structure has been obtained by comparing the absorption curves of these purified fractions with those obtained from synthetically prepared acridine derivatives and related substances of known structure. The absorption spectra of the latter are also of interest from the point of view of acridine chemistry.

EXPERIMENTAL

Extraction of urine (Fig. 1). Large volumes of combined samples of urine from military personnel on a mepacrine regime were used. They were extracted in several lots of about 10 l. each, as follows. The urine was brought to pH 10 by addition of NaOH, and was shaken thoroughly with 2.5 l. of pure ethylene dichloride, allowed to separate, and the yellow ethylene dichloride layer removed. The residue was extracted with a mixture of 1 l. benzene, 500 ml. light petroleum and 500 ml. ether. This extract was only very slightly yellow and was not investigated further.

The ethylene dichloride extract was washed with water, centrifuging to break the gel, and the water discarded. It was then washed twice with 2.5*N*-NaOH, and twice with water. These aqueous alkaline washings were combined and designated the NaOH Fraction.

The remaining ethylene dichloride extract was shaken with three lots of 0.5*N*-HCl, which removed all its colour; the organic layer was then discarded. The acid solution was washed twice with ether to remove traces of ethylene dichloride, and the ether discarded. It was brought to pH 8.4–8.8 with NaOH and extracted with benzene. It was

made strongly alkaline and extracted once more. The colourless aqueous layer was discarded and the combined benzene extracts filtered, dried over Na_2SO_4 , and passed through a column of Brockmann's alumina, 6 mm. in diameter and 25 cm. long, under gentle suction.

Fig. 1. Fractionation of urine from subjects receiving mepacrine.

The NaOH fraction was brought to pH 9.5 with HCl, and extracted several times with ethylene dichloride. The basic material was then taken into 0.5N-HCl, washed twice with ether, made alkaline and extracted into benzene, dried and chromatographed as described above.

Chromatograms. A very pale yellow solution passed through the first alumina column. This was known as Fraction 1. There was so little coloured material present that it was not possible to investigate it further. The chromatogram which formed consisted of a thin brown band at the top, below which was a fairly narrow, intensely coloured orange band, and below this again a wide diffuse yellow band. Elution with benzene containing 10% (v/v) amyl alcohol gave Fraction 2, a very intense solution of relatively small volume which appeared to be produced by removal of part of the colour from the orange layer, and which leap-frogged down the column over the wide yellow layer. This was followed by Fraction 3, a pale, rather brownish solution, formed from a pinkish layer which had followed Fraction 2 down the column. Next came Fraction 4, a yellow solution of greater volume and medium intensity of colour, formed by elution of the wide yellow band. There remained on the column the brown band, and the orange

band, the rest of the column being nearly colourless. Prolonged elution with the same solvent gave two further Fractions 5 and 6, very pale solutions apparently coming from the nearly colourless part of the chromatogram. A further fraction was obtained by removing mechanically from the column the Orange Band.

The NaOH fraction gave a chromatogram consisting of a heavy brown band at the top with an orange band below. Elution with benzene containing 50% (v/v) amyl alcohol removed the orange band, giving a deep yellow solution.

A chromatogram of authentic mepacrine in benzene solution upon the same alumina gave a small brown layer at the top (possibly impurity), and a diffuse long yellow band below, which was in appearance the same as that of Fraction 4 and behaved in the same manner on elution.

Amounts of fractions. Fractions 2 and 4 represent the greater amount of the total; the NaOH fraction was present in a moderate amount, while only very small quantities of Fractions 3, 5 and 6 were found.

Further purification of fractions. At this stage, the solutions had to be left in the ice-chest for two months. After this time it was found that they all contained a small amount of coloured material which was not acid-soluble. Since this was produced by standing it was not included in the investigation.

Fractions 2, 3 and 4 were each extracted into 0.5N-HCl, the acid extract washed with ether, made alkaline and extracted into benzene, dried and chromatographed as before. On this occasion activated alumina (British Drug Houses Ltd.) was employed, and it was observed that Fraction 2, which had formerly given a narrow intense orange band on Brockmann alumina, now gave a long yellow band, while Fraction 4, which had formerly produced a long yellow band, now gave an intense orange band near the top. Each fraction contained very small amounts of the others, which were eliminated by collecting only the central portion of the main eluate. The fractions were then extracted into dilute HCl and washed well with ether for spectrophotometry.

Fractions 5, 6 and the NaOH Fraction were not put through a second chromatogram. They were extracted into 0.5N-HCl, washed with ether, and their absorption spectra investigated.

The Orange Band was eluted from the separated portion of the chromatogram with 0.5N-HCl, washed with ether and spectrographed directly. It is therefore likely to be less pure than the other fractions.

Spectrophotometry. These products as well as substances of known structure which might serve to identify the fractions were examined in the 2300–4800 Å range by means of a Hilger E316 quartz spectrograph and a Spekker ultra-violet photometer (H324), using a tungsten steel high tension spark. Where possible, an absorption curve was obtained for each in both acid and alkaline solutions, and in a buffered solution near pH 7. (Palitzsch's boric acid-borax buffer was used in all cases except one.) It was found useful in some cases to obtain additional curves, working with slightly alkaline or acid buffered solutions. Water was used as the solvent, or, where the substance was not sufficiently soluble, dilute ethanol (up to 50%).

Picrates. Picrates of pure mepacrine and of Fractions 2 and 4 were prepared and mixed melting points taken.

RESULTS

Mepacrine (Fig. 2)

The absorption spectrum of mepacrine was first re-examined. The curves obtained were in substantial agreement with those given by Scudi & Jelinek (1944). The spectrum of mepacrine may be divided into three parts, a strong 's.w.' band in the 'short' wave-length region (2500–3000 Å.), (not covered by Scudi's work), with millimolecular extinction coefficients ranging from 47 to 61, a 'medium' wave-length band of low extinction which will be referred to as the 'm.w.' band (in the

Fig. 2. Spectral absorption curve of mepacrine. Abscissae: wave-length in Angström units. Ordinates: millimolecular extinction coefficients.

region of 3200–3700 Å., with $\epsilon \times 10^{-3}$ between 3.7 and 4.6) and a diffuse 'L.w.' band in the 'long' (4000–4750 Å.) wave-length region with millimolecular extinction coefficients 7–9. For the sake of brevity this type of curve will be referred to as the 'acridine-type'. The spectra of this and all other 'acridine-type' compounds are reproduced in two parts, the portion below 3000 Å. being plotted separately with the extinction values on a smaller scale. Where the bands are exceptionally high the peaks are shown on the graphs in insets. The peak of the short wave-length s.w. band which is at 2825 Å. in 0.5N-HCl solution is displaced to 2700 Å. in alkali. This change appears to be a gradual one, with the absorption maximum at pH 7.6 halfway between those of the acid and the alkaline solution, and having a lower extinction coefficient. In the medium wave-length region the solutions in acid and at pH 7.6 have identical m.w. absorption maxima, while in alkaline solution (at pH 9) a double peak appears. In the l.w. band the acid and pH 7.6 curves again show great similarity, with the alkaline curve distinctly different, but here it is the acid and pH 7.6 absorption heads that have double maxima. Thus the tautomeric change postulated by

Scudi & Jelinek which gives rise to the differences in absorption in the medium and long wave-length regions and is also responsible for the three isobestic points (2950, 3540 and 4140 Å.), occurs beyond the neutral point, between pH 7.6 and 9.0. Scudi & Jelinek observed the change to be complete at pH 8.55. The short wave-length s.w. band changes gradually with changing hydrogen ion concentration, and might indicate some other more gradual structural change.

Mepacrine picrate. Equal volumes of aqueous mepacrine dihydrochloride (5 mg./ml.) and of a saturated solution of picric acid in water were mixed at 70°. An intense yellow precipitate formed immediately. The solution was left overnight and the supernatant liquid was sucked off. The product, a crystalline yellow powder, melted at 154–158° when recrystallized from aqueous acetone or dilute ethanol. Thrice recrystallized from dry acetone and once more from absolute ethanol with some added drops of acetone it had m.p. 207–8° (decomp.) (Found: N, 13.9%. Calc. for $C_{29}H_{33}O_8N_8Cl$: N, 13.4%). A solution of the picrate in aqueous pyridine was titrated against an aqueous millimolecular methylene blue solution, using Bolliger's (1939) method. (Found: mol.wt.—630.2. Calc. for the monopicate: mol.wt.—628.5).

Urine fractions (Fig. 3)

The products of extraction were next examined spectrophotometrically. Here the 'apparent millimolecular extinction coefficients' were determined by comparing the colorimetric intensity of the acid solutions at 4200 Å. with that of an acid mepacrine solution and expressing the extinction values in terms of the molecular weight of mepacrine.

Fractions 2 and 4. Two of the extraction products, Fraction 2 and Fraction 4, have absorption curves closely similar to that of mepacrine with which they are, in all probability, identical. It would seem that the difference in their chromatographic behaviour cannot be due to any significant difference in structure; and although chromatographically Fraction 4 resembled mepacrine more closely than Fraction 2, this is not confirmed by the spectrophotometric curves.

Picrates were prepared from approximately 10 mg. of Fraction 2, and 4 mg. of Fraction 4, using the procedure described for mepacrine. The picrates were yellow powders which did not depress the melting point of mepacrine picrate when mixed with authentic samples.

Fraction 3. The absorption curves of Fractions 3 and 5 and the ones described as the Orange Band and the NaOH Fraction are substantially different from the mepacrine spectrum. They may be referred to as of the 'degradation-type' and can best be described as distorted acridine curves. They

all have strong s.w. bands with a very small m.w. peak and a larger l.w. peak occurring on the right-hand downward slope from the s.w. band. There are also indications of intense bands with maxima below 2200 Å., which, however, were too far into the ultra-violet to be spectrographed.

The s.w. band of Fraction 3 has $\lambda_{\max}$ displaced from 2775 Å. in acid solution to 2675 Å. in alkali, the change being accompanied by an increase in apparent $\epsilon \times 10^{-3}$ from 98 to 125. The acid curve has an m.w. maximum at 3425 Å. and a l.w. peak at 4310 Å., whereas the alkaline solution only shows one further peak at 3975 Å. Taking into account the movement of the s.w. band towards the shorter wave-lengths, as well as on the analogy of the acridine curves, it seems likely that the 3975 Å. peak is that of the l.w. band.

NaOH Fraction. The NaOH Fraction has an acid curve with m.w. and l.w. bands closely similar to those of Fraction 3. The changes in alkaline solution are also closely parallel. From the 3000–4800 Å. spectrum one would say that Fraction 3, which was present only in very small quantities, is identical with the NaOH Fraction, probably representing a small residue of the latter not extracted from the original ethylene dichloride solution by strong NaOH. The s.w. bands of the two fractions however, though similar in type, are not identical either in wave-length, in apparent extinction coefficient, or in reaction to change of pH. This may be because the NaOH Fraction has been less thoroughly purified than Fraction 3, but it is possible that it indicates some very small structural difference between the two compounds.

Orange Band Fraction. The fraction known as the Orange Band exhibits s.w.-maxima which are similar to Fraction 3 in their wave-lengths, shift in wave-lengths, and in that apparent $\epsilon \times 10^{-3}$ of the solution in alkali is higher than that of the acid solution; the actual figures, however, 53 and 73, are much lower than for Fraction 3. There is a distinct m.w. band at 3420 Å. which decreases with increasing pH and all but disappears in N-NaOH solution, with an accompanying shift in $\lambda_{\max}$ to 3450 Å. The maximum of the diffuse l.w. band shifts from 4250 Å. in acid to 4120 Å. in alkaline solution. Thus the picture from 3000 to 4800 Å. is similar in type to that of Fraction 3 but has very distinct differences.

Fraction 5. The s.w. maxima of Fraction 5 show great similarity to Fraction 3 in respect of wave-lengths but differ markedly in their maximum apparent extinction values. Not only are they substantially lower than in Fraction 3, but here the absorption maximum is higher in acid than in alkaline solution as is the case with the NaOH Fraction. The more weakly alkaline solution (at pH 9.0) has a maximum at an intermediate wave-

length and a lower extinction. The acid curve shows a double-peaked m.w. band (at 3230 and 3440 Å.). Only one peak is visible (at 3400 Å.) at pH 9.0. The l.w. band of the acid curve is also decreased by increasing pH, while both m.w. and l.w. bands completely disappear in strongly alkaline solution, leaving the bare downward slope of the s.w. peak. Fraction 5 appears to be of the same general type of compound but is not identical with any of the other fractions.

Fraction 6. No conclusions could be reached about Fraction 6 which was available in such small quantities that only the s.w. band could be obtained. Its apparent millimolecular extinction coefficient in terms of mepacrine is unusually, and probably fictitiously, high, reaching 216 in 0.5N-HCl solution and 163 at pH 9.6–10. The wave-lengths of these maxima, 2825 Å. in acid and 2750 Å. in weak alkali, indicate that Fraction 6 is not identical with any of the other fractions. The available data are insufficient to decide whether it belongs to the 'acridine' or to the 'degradation' type of compound.

None of the 'degradation-type' spectra had points that could with reasonable certainty be described as isobestic.

Four of the fractions thus have absorption spectra widely differing from mepacrine, but all essentially similar to each other, so it may be assumed that there are no great structural differences between these four fractions. It may be noted that the differences between corresponding wave-length maxima are slight, probably indicating that the molecular weights of these fractions are of the same order.

Acridine derivatives (Fig. 4)

Absorption spectra were obtained of acridine and a number of its derivatives in order to ascertain whether any of these give spectra of the 'degradation' type. The millimolecular extinction coefficients of these compounds are true values, calculated on the basis of their own molecular weights.

A few of these, notably acridine and 5-amino-acridine, have already been spectrographed by Charlampowiczowna & Marchlewski (1930), Radulescu & Ostrogovitch (1931), and Dima & Pogângeanu (1939) and, quite recently, by Craig & Short (1945) and Turnbull (1945). A good deal of this work is not strictly comparable with our results because different solvents or pH's were used, or the wave-length range was different. In the cases, however, where a comparison is possible, satisfactory agreement was found.

In most of these derivatives isobestic points could be detected, indicating that at our lowest pH the compounds were present mainly in the form of univalent ions, and no significant formation of

Fig. 3. Spectral absorption curves of urinary excretion products of mepacrine. Abscissae: wave-length in Angström units. Ordinates: apparent millimolecular extinction coefficients. (Since the formulae of these compounds are unknown, the extinction coefficients were calculated arbitrarily in terms of mepacrine, as follows. The concentration of the solution was assessed by comparison of its yellow colour with the yellow colour of a standard mepacrine solution under similar conditions, and apparent ϵ calculated using this concentration and the molecular weight of mepacrine.)

Fig. 4. Spectral absorption curves of acridine derivatives. Abscissae: wave-length in Angström units. Ordinates: millimolecular extinction coefficients.

Fig. 5. Spectral absorption curves of acridones and diphenylamine derivatives. Abscissae: wave-length in Angström units. Ordinates: millimolecular extinction coefficients.

divalent ions had taken place (cf. Albert & Goldacre, 1943).

Acridine. Acridine has s.w. and l.w. but no m.w. bands. As might be expected from its molecular weight as compared with mepacrine, and the absence of the bathochromic chloro-, methoxy- and amino-groups, the entire spectrum of acridine is displaced towards the shorter wave-lengths. It is further characterized by the exceptionally high intensity of its absorption bands as expressed by high extinction coefficients and very low half-widths. A strongly alkaline solution and one at pH 7.6 have identical s.w.-maxima (2500A., $\epsilon \times 10^{-3} = 179$), which change in acid solution to 2560A., with $\epsilon \times 10^{-3}$ decreased to 125. The l.w.-maximum is invariable around 3540A. but undergoes a marked change in intensity, $\epsilon \times 10^{-3}$ being 7.7 in alkaline solution and 15.9 in acid, with the lowest value near the neutral point. (At pH 7.6 $\epsilon \times 10^{-3} = 6.1$.) Both the acid and alkaline solutions have an 'inflection' on the right-hand side. There are isobestic points at 2520, 2790 and 3730A. Thus, unlike the case of mepacrine, it is the s.w. band

which seems to change more rapidly with changing pH, while the change between the acid and alkaline forms of the l.w. band may be more gradual and goes through a minimum at an intermediate pH. The acid curve, which has the lowest extinction value in the s.w. band, has the highest in the l.w. band. The higher extinction values of the s.w. bands on the alkaline side may be due to the high resonance energy of unionized acridine, while in acid solution the acridinium ion is responsible for the lower s.w. maximum and the characteristic shape of the l.w. band.

5-Aminoacridine has s.w. bands substantially lower than acridine itself but only slightly shifted to the right. There is a continuous decrease in absorption intensity with increasing pH, the acid solution having $\epsilon \times 10^{-3} = 91$, the solution at pH 7.6, 66, and the alkaline solution 48. This last change is accompanied by a slight shift in the $\lambda_{\max}$ to 2630A. (The acid and pH 7.6 solutions have the same $\lambda_{\max}$ at 2610A.) The inclusion of the 5-amino-group is responsible for a small and diffuse m.w. band in acid solution. This band still exists at pH 7.6

and even has a suggestion of a double maximum, but completely disappears in alkaline solution. The L.W. band is strongly displaced to the right of the acridine L.W. band and has two well resolved peaks in acid solution which, however, do not compare with the acridine peaks in intensity. There are isobestic points at 2850 Å. and 4320 Å. It is doubtful whether the point at 3730 Å. is isobestic.

5-Amino-3-methylacridine was spectrographed next, as it was expected that the new group would affect the absorption spectrum at least by destroying the symmetry of the molecule. The s.w. band of this compound shows a gradual increase in $\lambda_{\max}$ and decrease in $\epsilon_{\max}$ as the pH is increased. As in 5-aminoacridine, the m.w. band is present in acid solution and at pH 7.6. The changes in the L.W. band are interesting: there is no double maximum in acid or alkaline solution and it is the solution at pH 7.6 that has two peaks. There are also inflexions on both sides of the three L.W. bands and isobestic points at 2850, 3370 and 4410 Å.

2-Chloro-5-aminoacridine has intense s.w. bands at about 2700 Å.; the neutral curve has the lowest intensity. In the m.w. and L.W. range, the absorption of the acid solution is the most differentiated. It has a significant m.w. band with two maxima, and an L.W. band with three clearly defined peaks, two of which bear a marked resemblance to the corresponding two maxima of 5-aminoacridine. The m.w. band is present in a much diminished form at pH 7.6 and disappears in alkali. The L.W. band of the pH 7.6 solution is a single-peaked band with inflexions on both sides, while that of the alkaline solution has several well developed inflexions as well as a suggestion of a second maximum. There seem to be isobestic points at 2700, 2810 and 3610 Å., and one near 4350 Å. may be within the experimental error in that part of the wave-length scale.

2-Chloro-5-diacetylaminacridine. Diacetylation, in the 5:5- or the 5:10-position, alters the spectrum quite appreciably, as can be expected from the limitation on the number of possible structural isomers. The s.w. band of 2-chloro-5-diacetylaminacridine is much the same as that of the unacetylated compound, but this as well as the L.W. band shows a general leftward shift. Another difference is that the alkaline solution alone has two clear maxima in the m.w. region. The L.W. region of the alkaline solution corresponds to that of 2-chloro-5-aminoacridine, but in acid solution the shape of this band, and the total absence of absorption bands in the m.w. region, closely correspond to the acridine spectrum at the corresponding pH.

2-Chloro-5-amino-7-methoxyacridine. The absorption spectrum of 2-chloro-5-amino-7-methoxyacridine, a possible cleavage product of mepacrine, has two characteristic features. One is the relatively lower extinction of the L.W. band: s.w. 52, m.w. 4.6,

L.W. 5.6 at pH 2.9; s.w. 17.5, m.w. 2.4, L.W. 4 at pH 8.9. The other is the uniform decrease in absorption intensity over the whole range of the spectrum, as the pH is gradually increased, leading to an unusually low curve at pH 8.9 (the most strongly alkaline curve obtained). This change is limited to extinction values. There is nearly no change in the wave-lengths of the absorption maxima; neither is there any significant change in the general shape of the curve with the exception of the second L.W. peak in acid and neutral solution (all s.w. and m.w. peaks are single) which is toned down to an inflexion at pH 8.9. Three isobestic points could be detected at 2950, 3525 and 4525 Å.

2-Chloro-5-amino-7-hydroxyacridine. Hydrolysis of the methoxy-substituent introduces a phenolic group capable of ionization. 2-Chloro-5-amino-7-hydroxyacridine, postulated by Hammick & Firth (1944) on chromatographic evidence to be one of the urinary excretion products of mepacrine, shows considerable deviation from the spectrum of its methoxy analogue. All extinction values are increased in proportion. The unusual depression of the alkaline curve is absent. The acid s.w. band has a single absorption peak at 2720 Å. ($\epsilon \times 10^{-3} = 102$), which is lowered as the pH is increased and again rises to 85 in 2.5N-NaOH solution, without any appreciable change in $\lambda_{\max}$. A second peak is developed in alkaline solution. There is a strongly developed m.w. band with an inflexion on the left-hand side; this develops with increasing alkalinity to give a second peak at pH 8.5; in strongly alkaline solution a third peak appears. In the case of the L.W. band it is the acid and neutral solutions which have suggestions of a second peak, while the alkaline solutions give smooth single bands. There is some displacement to the longer wave-lengths of the entire spectrum in alkaline solution. There are isobestic points at 3250 and 3675 Å.

2-Chloro-7-methoxyacridone (Fig. 5) was spectrographed as being a possible product of hydrolytic fission of mepacrine. It has single s.w. bands of which the acid one is slightly higher and the alkaline one is somewhat displaced to the right. Two maxima could be detected in the m.w. region of the strongly alkaline spectrum, and the L.W. band of this curve contains a distinct inflexion. The acid solution and the one at pH 7.8 give curves with smooth, single L.W. bands, and no definite m.w. peaks could be found in the spectrum of either; it is significant, however, that the rather flat minima in this region have higher extinction values than the two m.w. maxima of the strongly alkaline solution. The two isobestic points are at 2975 and 4275 Å.

2-Chloro-7-hydroxyacridone (Fig. 5). Hydrolysis of the methoxy-group in this compound would give 2-chloro-7-hydroxyacridone. This has lower s.w.

bands than the methoxy-derivative, and, as in the hydroxyamino-compound, the introduction of the phenolic group has resulted in a double peaked absorption head in alkaline solution. The left side of this curve is a straight line which was traced up to $\epsilon \times 10^{-3} = 186.5$ at 2275 Å. without reaching the maximum. There is no m.w. peak in acid solution nor at pH 7.6, but there is one in alkaline solution, as in the case of the methoxy-ketone. Unlike the latter, this is a single peak and passes well above the other two curves. The l.w. bands of the acid and alkaline curves have indistinct single maxima and it is in the solution of pH 7.6 that the l.w. band is resolved into two peaks of equal magnitude, thus resembling 5-amino-3-methylacridine. The l.w. bands of the acid and the pH 7.6 curves coincide in wavelength and extinction, but the alkaline band is somewhat lower and is displaced from 4170 Å. to 4600 Å. The points at 2530, 2780, 3680 and 4340 Å. are isobestic. No exact analogy can thus be drawn between the hydrolysis of the 7-methoxy-group in the acridone and the 5-aminoacridine, but the general nature of the change seems to be on the same lines, and as might be expected, primarily affects the alkaline solutions; this is indicated by the double s.w. maxima and the rightward shift of the l.w. band in both acridone and amino-compounds in alkaline solution.

None of the absorption spectra described above deviated unduly from the general 'acridine-type' of curve and it was concluded that, unlike the assumed nature of Scudi & Jelinek's fractions, the degradation products isolated by our method no longer possessed the resonance of the acridine nucleus. Two ways suggested themselves as leading to the destruction of acridine resonance: hydrogenation of the ring in the 5:10-positions would yield acridanes, while the more drastic hydrolytic cleavage at the 5-position would result in the formation of ortho-substituted diphenylamine derivatives. These two types of compounds have already been shown to have very similar optical properties (Auwers & Kraul, 1925), and they could be expected to give similar absorption curves. Diphenylamine derivatives, the more stable of the two types of compound, were then investigated.

Diphenylamine derivatives (Fig. 5)

5-Chloro-4'-methoxydiphenylamine-2-carboxylic acid. The absorption spectrum of 5-chloro-4'-methoxydiphenylamine-2-carboxylic acid was determined, varying the concentration of the solutions as little as possible since the absorptions of these were found to deviate appreciably from Beer's law. In contrast to the yellow fluorescent solutions of acridine derivatives this compound gave colourless and non-fluorescent solutions.

The spectrum of the diphenylamine derivative consists of an absorption band at about 2290 Å., with two smaller peaks on its right-hand downward slope, at about 2810 and 3550 Å. respectively. The curve is of the 'degradation-type' and can best be compared with Fraction 3 and the NaOH Fraction but differs from both of these, and from all the fractions, in that the entire spectrum is considerably displaced towards the ultra-violet. This corresponds to the similar difference between acridine and mepacrine and, as in that case, could be accounted for by a difference in molecular weights, and the absence of several bathochromic groups. As in the 'degradation-type' curves, no isobestic points can be located definitely. There is little difference in the wave-length maxima of the individual peaks at different pH's. The much enhanced alkaline s.w. band ($\epsilon \times 10^{-3} = 62$ in 0.1N-NaOH solution as against 32 in 0.5N-HCl) cannot be observed with any of the fractions, from which one might infer that the acidity of the latter is of a lesser order. On the whole, however, the curves support the view that the fractions obtained by our extraction from alkaline urine are compounds belonging to one of the two classes described above. The fact that they are coloured and fluorescent may favour their belonging to the acridane rather than the diphenylamine class.

5-Chlorodiphenylamine-2'-carboxylic acid. It was hoped that by comparison with the spectrum of another diphenyl derivative of known composition the above conclusion might be elaborated further, but this was not found to be the case. 5-Chlorodiphenylamine-2'-carboxylic acid was chosen as representing a degradation product of acridine in which cleavage has taken place on the other side of the 5-carbon atom. This compound shows s.w.-absorption at wave-lengths higher than the ones described in the foregoing paragraph, and has a further band which is in the m.w. region; this latter band shifts from 2910 Å. in alkaline (and even weakly alkaline) solutions to 3470 Å. in acid, a change normally attributable to the usual changes in the carboxyl-group, but one which cannot be discerned with any certainty in the case of the other diphenylamine-derivative or in any of the fractions. As in the last case, the spectrum is of the 'degradation-type' and Beer's law is not obeyed. The improbably high extinction maxima given for the s.w. band are those of solutions that were freshly prepared and spectrographed as soon as was practicable. The values increased with time, and with solutions 18 days old the absorption peaks could not be reached. It is suggested that this is due to the formation of a loose molecular complex arising from interaction of the carboxylic and amino-groups. The change, which would depend on the strengths of these two groups, does not take

place with 5-chloro-4'-methoxydiphenylamine-2-carboxylic acid where the considerable electron-donating (+*M*) effect of a methoxy-group in *p*'-position sufficiently enhances the +*T* effect of the *o*-amino-group to reduce the acid strength of this compound.

In Table 1 the maxima with their millimolecular extinction coefficients and half-widths for all the curves are collected for reference.

Table 2 gives the colours of fluorescences in acid and in alkaline solutions.

DISCUSSION

On spectrographic evidence as well as from the mixed melting points of their picrates Fractions 2 and 4, constituting the major part of our urine extract, consist of unchanged mepacrine. The peculiarities of chromatographic behaviour might possibly be accounted for by varying degrees of solvation of the mepacrine producing varying strengths of adsorption upon the alumina column.

The other fractions are likely to be acridanes or diphenylamine derivatives. More work on the

Table 1. *Wavelengths, millimolecular extinction coefficients and half-widths* of absorption maxima of urinary fractions and known compounds*

Mepacrine (dihydrochloride)			
s.w. band:	0.5N-HCl	pH 7.6	pH 9.0
$\lambda_{\max}$	2825 A.	2765 A.	2700 A.
$\epsilon_{\max}$	58	47	61
Half-width	290 A.	310 A.	300 A.
m.w. band:			
$\lambda_{\max}$	3480 A.	3480 A.	3440, 3600 A.
$\epsilon_{\max}$	4.6	4.6	3.75, 4.1
Half-width	410 A.	280 A.	290, 240 A.
L.w. band:			
$\lambda_{\max}$	4300, 4450 A.	4300, 4450 A.	4210 A.
$\epsilon_{\max}$	9.1, 8.6	8.7, 7.8	7.1
Half-width	700 A.	725 A.	730 A.
Fraction 2			
s.w. band:			
$\lambda_{\max}$	2810 A.	2790 A.	2710 A.
$\epsilon_{\max}$	47.5	45	50
Half-width	300 A.	360 A.	310 A.
m.w. band:			
$\lambda_{\max}$	3450 A.	3460 A.	3430, 3620 A.
$\epsilon_{\max}$	4.3	3.65	3.3, 4.25
Half-width	370 A.	340 A.	
L.w. band:			
$\lambda_{\max}$	4330 A.	4280 A.	4210 A.
$\epsilon_{\max}$	8.7	7.1	6.3
Half-width	580 A.	700 A.	760 A.
Fraction 4			
s.w. band:			
$\lambda_{\max}$	2810 A.	2760 A.	2675 A.
$\epsilon_{\max}$	46	41.5	50
Half-width	370 A.	360 A.	330 A.
m.w. band:			
$\lambda_{\max}$	3460 A.	3450 A.	3460, 3610 A.
$\epsilon_{\max}$	4.5	4.1	4.1, 4.3
Half-width	320 A.	280 A.	430 A.
L.w. band:			
$\lambda_{\max}$	4280, 4430 A.	4270 A.	4210
$\epsilon_{\max}$	7.7, 7.3	7	6.2
Half-width	700 A.	730 A.	820 A.

* This term is used for the approximate width (in angströms) of the absorption band where $\epsilon = \frac{1}{2}\epsilon_{\max}$. Where necessary, it is obtained by extrapolation.

Table 1 (cont.)

Fraction 3			
	0.5 N-HCl	0.5 N-NaOH	
s.w. band:			
$\lambda_{\max}$	2775 A.	2675 A.	
$\epsilon_{\max}$	98	125	
M.W. band:			
$\lambda_{\max}$	3425 A.	—	
$\epsilon_{\max}$	13.4	—	
L.W. band:			
$\lambda_{\max}$	4310 A.	3975 A.	
$\epsilon_{\max}$	10	7.6	
NaOH Fraction			
	0.5 N-HCl	pH 9.0	
s.w. band:			
$\lambda_{\max}$	2610 A.	2640 A.	
$\epsilon_{\max}$	152	117	
Half-width	440 A.	480 A.	
M.W. band:			
$\lambda_{\max}$	3425 A.	—	
$\epsilon_{\max}$	14.6	—	
L.W. band:			
$\lambda_{\max}$	4410 A.	4130 A.	
$\epsilon_{\max}$	7.5	8.2	
Orange Band			
	0.5 N-HCl	pH 5.0	N-NaOH
s.w. band:			
$\lambda_{\max}$	2750 A.	2760 A.	2675 A.
$\epsilon_{\max}$	57	53	73
M.W. band:			
$\lambda_{\max}$	3420 A.	3450 A.	3610 A.
$\epsilon_{\max}$	15.1	13.1	11.6
L.W. band:			
$\lambda_{\max}$	4230 A.	4250 A.	4120 A.
$\epsilon_{\max}$	7.5	7.9	7.75
Fraction 5			
	0.5 N-HCl	pH 9.0	N-NaOH
s.w. band:			
$\lambda_{\max}$	2770 A.	2690 A.	2650 A.
$\epsilon_{\max}$	63	47	57.5
M.W. band:			
$\lambda_{\max}$	3230, 3440 A.	3400 A.	—
$\epsilon_{\max}$	12.3, 12	6	—
L.W. band:			
$\lambda_{\max}$	4260 A.	4230 A.	—
$\epsilon_{\max}$	8.5	5	—
Fraction 6			
	0.5 N-HCl	pH 9.6-10	
s.w. band:			
$\lambda_{\max}$	2825 A.	2750 A.	
$\epsilon_{\max}$	216	163	
Half-width	430 A.	410 A.	
Acridine			
	0.5 N-HCl	pH 7.6	0.5 N-NaOH
s.w. band:			
$\lambda_{\max}$	2560 A.	2500 A.	2500 A.
$\epsilon_{\max}$	125	179	179
Half-width	85 A.	90 A.	90 A.
L.W. band:			
$\lambda_{\max}$	3550 A.	3540 A.	3520 A.
$\epsilon_{\max}$	15.9	6.1	7.7
Half-width	240 A.	480 A.	350 A.

Table 1 (cont.)

5-Aminoacridine				
s.w. band:	0.5N-HCl	pH 7.6	0.1N-NaOH	
$\lambda_{\max}$	2610 A.	2610 A.	2630 A.	
$\epsilon_{\max}$	91	66	48	
Half-width	175 A.	125 A.	200 A.	
M.w. band:				
$\lambda_{\max}$	3225 A.	3140, 3260 A.	—	
$\epsilon_{\max}$	1.9	1.0, 1.1	—	
L.w. band:				
$\lambda_{\max}$	4060, 4250 A.	4025 A.	4075 A.	
$\epsilon_{\max}$	9, 8.9	7	7.3	
Half-width	570 A.	650 A.	700 A.	
5-Amino-3-methylacridine				
s.w. band:				
$\lambda_{\max}$	2460 A.	2640 A.	2700 A.	
$\epsilon_{\max}$	102	85	54	
Half-width	—	250 A.	170 A.	
M.w. band:				
$\lambda_{\max}$	3300 A.	3300 A.	—	
$\epsilon_{\max}$	2.2	2.5	—	
L.w. band:				
$\lambda_{\max}$	4060 A.	4050, 4300 A.	4000 A.	
$\epsilon_{\max}$	8.3	6.8, 5.8	6.7	
Half-width	680 A.	650 A.	750 A.	
2-Chloro-5-aminoacridine				
s.w. band:				
$\lambda_{\max}$	2660 A.	2690 A.	2650 A.	
$\epsilon_{\max}$	123	101	141	
Half-width	200 A.	150 A.	240 A.	
M.w. band:				
$\lambda_{\max}$	3160, 3300 A.	3180, 3320 A.	—	
$\epsilon_{\max}$	3.6, 4.5	1.5, 1.6	min. 1.8	
L.w. band:				
$\lambda_{\max}$	3810, 4010, 4240 A.	4100 A.	3950, 4130 A.	
$\epsilon_{\max}$	7.2, 9.1, 8.7	8.5	6.8, 7.6	
Half-width	650 A.	700 A.	770 A.	
2-Chloro-5-diacetylaminocridine				
s.w. band:				
$\lambda_{\max}$	2640 A.	2490 A.	2640 A.	
$\epsilon_{\max}$	122	73	183	
Half-width	170 A.	—	230 A.	
M.w. band:				
$\lambda_{\max}$	—	($\lambda_{\min}$ 3060 A.)	3200, 3400 A.	
$\epsilon_{\max}$	—	($\epsilon_{\min}$ 1.2)	7.3, 8.7	
L.w. band:				
$\lambda_{\max}$	3690 A.	3670 A.	3660, 3940 A.	
$\epsilon_{\max}$	18.8	12	10.4, 11	
Half-width	310 A.	400 A.	Approx. 850 A.	
2-Chloro-5-amino-7-methoxyacridine				
s.w. band:	pH 2.9	pH 4.1	aq. dest.	pH 8.9
$\lambda_{\max}$	2720 A.	2750 A.	2740 A.	2700 A.
$\epsilon_{\max}$	52	52	42.5	17.5
Half-width	310 A.	310 A.	330 A.	350 A.
M.w. band:				
$\lambda_{\max}$	3410 A.	3410 A.	3410 A.	3430 A.
$\epsilon_{\max}$	4.6	4.6	3.9	2.4
Half-width	250 A.	210 A.	320 A.	270 A.
L.w. band:				
$\lambda_{\max}$	4120 A.	4140 A.	4150 A.	4140 A.
$\epsilon_{\max}$	5.6	5.6	5.7	4
Half-width	700 A.	680 A.	670 A.	720 A.

Table 1 (cont.)

2-Chloro-5-amino-7-hydroxyacridine				
	0.02N-HCl	pH 7.0	pH 8.5	2.5N-NaOH
s.w. band:				
$\lambda_{\max}$	2720 A.	2740 A.	2750, 2860 A.	2740, 2935 A.
$\epsilon_{\max}$	102	70	65, 59	85, 82
Half-width	320 A.	280 A.	430 A.	510 A.
M.W. band:				
$\lambda_{\max}$	3425 A.	3420 A.	3320, 3470 A.	3325, 3480, 3630 A.
$\epsilon_{\max}$	8.7	8.5	5.8, 6.8	6, 9, 5.9
Half-width	270 A.	290 A.	—	—
L.W. band:				
$\lambda_{\max}$	4200 A.	4210, 4410 A.	4530 A.	4510 A.
$\epsilon_{\max}$	12.3	12, 10.1	9.5	11.7
Half-width	680 A.	700 A.	870 A.	830 A.
2-Chloro-7-methoxyacridone				
	N-HCl	pH 7.8	N-NaOH	
s.w. band:				
$\lambda_{\max}$	2725 A.	2700 A.	2810 A.	
$\epsilon_{\max}$	86	75	73	
Half-width	290 A.	350 A.	320 A.	
M.W. band:				
$\lambda_{\max}$	($\lambda_{\min}$ 3400 A.)	($\lambda_{\min}$ 3400 A.)	3325, 3500 A.	
$\epsilon_{\max}$	($\epsilon_{\min}$ 2.5)	($\epsilon_{\min}$ 3.0)	2.3, 1.8	
L.W. band:				
$\lambda_{\max}$	4200 A.	4140 A.	4180 A.	
$\epsilon_{\max}$	8	7.7	6.8	
Half-width	580 A.	650 A.	720 A.	
2-Chloro-7-hydroxyacridone				
	N-HCl	pH 7.6	N-NaOH	
s.w. band:				
$\lambda_{\max}$	2700 A.	2670 A.	2660, 2920 A.	
$\epsilon_{\max}$	62.5	53	45, 49	
Half-width	310 A.	360 A.	—	
M.W. band:				
$\lambda_{\max}$	—	—	3430 A.	
$\epsilon_{\max}$	—	—	4.7	
L.W. band:				
$\lambda_{\max}$	4170 A.	4040, 4230 A.	4600 A.	
$\epsilon_{\max}$	7.1	6.6, 6.7	6.4	
Half-width	650 A.	650 A.	840 A.	
5-Chloro-4'-methoxydiphenylamine-2-carboxylic acid				
	0.5N-HCl	pH 7.6	0.1N-NaOH	
s.w. band:				
$\lambda_{\max}$	2290 A.	—	2230 A.	
$\epsilon_{\max}$	32	—	62	
M.W. band:				
$\lambda_{\max}$	2810 A.	2410, 2880 A.	2820 A.	
$\epsilon_{\max}$	13	16.7, 15.3	15.5	
Half-width	420 A.	450 A.	600 A.	
L.W. band:				
$\lambda_{\max}$	3550 A.	—	—	
$\epsilon_{\max}$	7.4	—	—	
Half-width	480 A.	—	—	
5-Chlorodiphenylamine-2'-carboxylic acid				
s.w. band:				
$\lambda_{\max}$	2400 A.	2400 A.	—	
$\epsilon_{\max}$	408	497	—	
M.W. band:				
$\lambda_{\max}$	3470 A.	2920 A.	2910 A.	
$\epsilon_{\max}$	8.5	22.5	15.0	

Table 2. *Fluorescence of urinary fractions and known compounds*

Substance	Colour of fluorescence	
	In acid	In alkali
Mepacrine	Intense green	Intense green
Fraction 2		
" 3	Pale blue "	Pale blue "
" 4	Intense green	Intense green
" 5	Not done	Not done
" 6		
NaOH Fraction	Green "	Yellow "
Orange Band	"	Green
Acridine	Intense whitish blue	Intense violet-blue
5-Aminoacridine	" " "	Intense whitish blue
5-Amino-3-methylacridine	" " "	" " "
2-Chloro-5-aminoacridine	" " "	" " "
2-Chloro-5-diacetylaminoacridine	Pale greenish blue	" " "
2-Chloro-7-methoxyacridone	Intense whitish blue	" " "
2-Chloro-7-hydroxyacridone	Greyish green	Yellow
2-Chloro-5-amino-7-methoxyacridine	Pale greyish blue	Pale blue
2-Chloro-5-amino-7-hydroxyacridine	Green	Yellow

acridanes would appear to be necessary before final conclusions can be reached. The results of such work will be reported later.

On the whole, the formation *in vivo* of acridanes seems a more likely process, but acridanes are known to be unstable (Jensen & Friedrich, 1927; Albert & Linnell, 1936) and to revert easily to acridines on exposure to atmospheric oxygen. It seems improbable, therefore, that an acridane, produced by reduction of mepacrine, would survive the several manipulations of our extraction process without being oxidized back to the corresponding acridine. The greater stability of diphenylamines, once they are formed, favours the second alternative. In this latter case ring-cleavage at the 5-position might well be preceded by the intermediate formation of the unstable 5:10-dihydro-acridine (acridane). If these fractions are, in fact, diphenylamines it should be pointed out that they must have retained several of the chromophores present in mepacrine with, possibly, part or all of the side-chain intact, since they absorb in approximately the same wave-length region as mepacrine. It follows from their method of extraction that, with the exception of the NaOH Fraction, they do not possess a free carboxylic acid group. One possibility would be an acid amide produced by the first stage of hydrolysis at the 5-carbon atom. Investigation of a series of diphenylamine derivatives might provide further indications as to their individual structures.

In addition to its original purpose, the spectrophotometry of the various acridine derivatives has provided useful data concerning the relationship between absorption spectra and chemical constitution in the acridine series. Some of these points have already been mentioned in the Results.

In particular, it has been noted that acridine has two absorption bands while 5-aminoacridines have three, and that the additional band, the m.w.-band,

is generally strongest in acid solution. It seems plausible to conclude that the presence of this band is due to a quinonoid structure in 5-aminoacridines (I), the 5-iminoacridane (II) form of which is responsible for the m.w.-band which is most pronounced at acid pH's. This is probably due to the electron shift from the 5- towards the 10-position which occurs in acid solution, while under alkaline conditions any electron shift that might occur would tend to be in the reverse direction.

In acridones the presence of this band corresponds to the keto form of these compounds, which also involves a quinone-like arrangement. The existence of the 5-iminoacridane form has been suggested on other physical and chemical grounds (Albert & Goldacre, 1943; Albert & Ritchie, 1943), but no conclusive spectrographic evidence was available. Such evidence has now been obtained in the

absorption spectrum of a 5-diacetylaminocridine, the structure of which has been established chemically by Wilkinson & Finar (1946). In alkaline solution this compound gives a spectrum containing all three bands of its unacetylated analogue, but in acid solution the m.w.-band disappears and the spectrum reverts to the very characteristic shape of the acridinium spectrum since diacetylation has rendered any further interaction between the 5-nitrogen and the ring structure, of the kind that occurs in acid solution, impossible.

Scudi & Jelinek give curves for three fractions isolated from dog-urine which are of the acridine type. None of these curves corresponds precisely to any of the acridine derivatives we have studied. However, the curves for Scudi's Fraction B1 are similar in type to those of 2-chloro-5-amino-7-hydroxyacridine; the shift in the l.w.-band to the right in alkaline solution being particularly noteworthy. It is interesting to observe that the two known compounds containing a phenolic group both show a shift of the l.w. band to the right in alkaline solution, all other known compounds showing either a leftward shift or no change in wave-length of this band in alkaline solution. We consider it possible that Scudi's Fraction B1 may consist of mepacrine with an —OH group in place of the —OMe group.

This is also the conclusion independently arrived at by Hammick & Mason (1945). Other findings of these authors, however, are not confirmed by our results. Two of their products of extraction, designated B2 and C, apparently indicating an analogy to Scudi & Jelinek's fractions, have been thought by them, on polarographic evidence, to be 2-chloro-5-amino-7-methoxy- and 2-chloro-5-amino-7-hydroxyacridine. Our absorption spectra for these synthetic compounds, however, show no resemblance to the curves published by Scudi & Jelinek for their fractions. It appears probable, therefore, that Hammick & Mason's fractions B2 and C are not identical with Scudi's, and that the postulated identity (Hammick & Firth, 1944) of 2-chloro-5-amino-7-hydroxyacridine with Scudi's Fraction C is incorrect.

The curves for Scudi's Fraction C deviate less from the mepacrine spectrum than any acridine derivative we have investigated. The change which converts mepacrine into Fraction C in the dog is probably very slight. None of our curves give any clue to the nature of his Fraction B2, the tautomerism of which is unlike anything we have studied.

It might be noted here that we should not expect to find among our fractions anything corresponding

to Fractions B2 or C, since these latter are not benzene-soluble. Our NaOH Fraction, which corresponds to Fraction B1 in so far as it was removed from the organic solvent by strong NaOH, has been shown by the spectrophotometric curves to be a completely different type of compound. It is unlikely that this is due to the extraction of different substances by ethylene dichloride and *iso*-amyl-alcohol, since both fractions are benzene-soluble, and may be the result of differences in mepacrine metabolism in man and the dog.

The limited data available for comparison of our Orange Band with the residual orange band on Scudi's chromatogram would indicate a general similarity of these two products.

SUMMARY

1. Seven basic, yellow coloured fractions have been isolated from the urine of volunteers taking mepacrine; spectral absorption curves for the range 2300 to 4800 Å. have been obtained for five of these, and picrates of two of them prepared.

2. Two of the fractions, the greater part of the material, have been identified as unchanged mepacrine.

3. The other fractions are thought to be either acridanes or derivatives of diphenylamine. No final decision is reached in the present paper, but the question is briefly discussed.

4. It has been shown that Scudi & Jelinek's Fractions B2 and C from dog urine are not identical with 2-chloro-5-amino-7-methoxyacridine, or 2-chloro-5-amino-7-hydroxyacridine.

5. A comparison of the spectra of acridine, 5-aminoacridine and a diacetylated 5-aminoacridine has yielded evidence of the existence of the 5-iminoacridanes as a contributing structure to the resonance hybrid of 5-aminoacridines, especially in acid solutions.

6. Spectral absorption curves have also been obtained for a number of other acridine and diphenylamine derivatives, and certain conclusions drawn concerning their structure in solutions at different pH's.

We should like to thank Major James Reid, R.A.M.C., for arranging for the collection of urine which made this work possible. We are also indebted to Dr Harold King, F.R.S., Dr D. Ll. Hammick, Messrs May & Baker Ltd. and Messrs Imperial Chemical Industries Ltd., for supplies of some of the known compounds studied, and to Messrs A. Hoffer, and W. Weedon for technical assistance. The costs of the investigation were defrayed by a grant from the Medical Research Council.

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Observations upon the Application of Partition Chromatography to the Determination of the Monoamino-acids in Proteins

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(Received 31 May 1946)

Synge (1939) and Martin & Synge (1941*b*) have shown that the acetamino-acids

have characteristic partition coefficients between organic solvent and water. As a result of experience gained (Martin & Synge, 1941*a*) a new form of chromatography was introduced (Martin & Synge, 1941*b*) which employs two liquid phases. The stationary phase (water) is held by a carrier (silica gel) which under ideal conditions should not adsorb the acetamino-acids. (Ryan & Brand (1944) were in error when they described the method as adsorption chromatography.) The movement of the acid bands is marked by the presence of a suitable indicator in the aqueous phase. It has been shown that each acetamino-acid will, for a particular solvent mixture, move down the column at a definite rate (see Gordon, Martin & Synge, 1943*a*) although evidence has been obtained in the present work which suggests that the *R* value (i.e. movement of band/movement of surface) may be influenced by the presence of other acetamino-acids upon the column and by the water content of the silica.

In succeeding publications the Leeds group (Gordon *et al.* 1943*a*, 1944) have improved the method and extended it to include phenyl-alanine, the leucine isomers (leucine and *isoleucine* cannot

be resolved) proline, valine, tyrosine and alanine. They also included methionine, but evidence will be presented in the present paper which suggests that its recovery is dependent upon the particular amino-acid mixture analyzed and possibly upon the nature of the silica gel.

Partition chromatography was the first method which made possible the serial micro-estimation of the above group of amino-acids. It has been suggested (Martin & Synge, 1945) that the ultimate aim of protein analysts should be the adoption of a routine procedure applicable to small quantities of protein, and realizable, as Chibnall (1946) has pointed out, only by assessing the relative merits of the various methods of analysis. It is with this object in view that considerable prominence has been given to a discussion of the comparative data so far available. Certain recommendations have also been made which it is hoped will facilitate future comparative analyses.

Apart from preliminary experiments on gelatin and wool (Gordon *et al.* 1943*a*, 1943*b*) the partition method has not been used for the analysis of the more complex proteins. The author has made extensive use of it for the analysis of many proteins, and the procedure as suggested by the Leeds workers has not been modified except in minor details. Certain anomalous results have been obtained, however, and suggestions are made to overcome such difficulties that may arise.