SOME ASPECTS OF THE PHARMACOLOGY OF *PARA*-AMINOSALICYLIC ACID

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Para-Aminosalicylic acid has now become of such general interest that it is hardly necessary to quote in detail the many references in the literature to its chemistry, pharmacology and clinical value. Most of the studies on the pharmacology of this substance have been carried out in Sweden[1,2,3,4,5] and America [6,7,8,9,10] and little has so far been published in this country. The object of the present series of experiments was to confirm, if possible, certain aspects of the published work and to extend the study of the pharmacology of *p*-amino-salicylic acid in other directions.

The acute and chronic toxicity of the drug in the strain of mice used in this laboratory was first determined, together with the pathological effects of prolonged administration. Blood levels and rate of excretion in mice were studied and following the work of Beyer[11,12] the possible effect of 4-carboxy-phenylmethanesulphonanilide (Caronamide) in retarding excretion was investigated. Erdei and Snell[13], Nagley and Logg[14] and other clinical workers have commented on the fall in temperature produced in tuberculous patients treated with *p*-aminosalicylic acid and it seemed desirable to study the possible antipyretic effect of the drug. O'Connor[15], bearing in mind the known action of salicylates, referred to the possibility of causing hypoprothrombinæmia by the use of *p*-aminosalicylic acid, and it was felt that experimental evidence on this point, also, would be useful.

The chemotherapeutic action on *Mycobacterium tuberculosis* in mice was investigated, using a technique very similar to that described by Youmans and Mc Carter[16]. A report by Woody and Avery[17] that potassium iodide potentiated the action of streptomycin in guinea-pigs infected with tubercle suggested that this work should be repeated using *p*-amino-salicylic acid as the tuberculostatic drug.

Experimental Methods

Toxicity. White Swiss mice of both sexes weighing 20 to 25 g. were used. In acute experiments, the drug was administered intravenously, subcutaneously, intraperitoneally and orally as an aqueous solution of the sodium salt, and observations of mortality were continued for 7 days after injection. Oral doses were given by stomach tube under light ether anaesthesia. In chronic experiments, the drug was given dissolved in the drinking water and the amount consumed daily per cage of 10 animals was measured. The solution was provided in drinking bulbs with constricted outlets, so that the animals were able to obtain liquid by licking the ends of the tubes but no loss of the solution by spilling occurred.

Blood levels. Blood samples were obtained at intervals after administration, by decapitation of the mice. Estimations were carried out by the method described below.

Antipyretic effect. Rabbits of a mixed stock and both sexes, weighing 2 to 3 kg., were used. Pyrexia was induced by intravenous injections of a solution of pyrogen prepared from *Pseudomonas æruginosa* by the method of Welch *et al.*[18]. Rectal temperatures were determined with clinical thermometers. *p*-Aminosalicylic acid and known antipyretics were administered intravenously and orally.

Prothrombin Times. Prothrombin times on rabbits were determined by Quick's method[19]. Blood samples were removed from the ear vein by syringe containing 0·1 ml. of 0·10M sodium oxalate per ml. of blood.

Chemotherapy. Mice were used of the same strain as those used for the toxicity experiments. They were infected with *M. tuberculosis* H37 Rv. These organisms were cultured in a modified Dubos medium containing NaH_2PO_4 1·0 g.; Na_2HPO_4, $12H_2O$ 6·25 g.; Sodium citrate 1·5 g.; $MgSO_4,7H_2O$ 0·6 g.; Casein hydrolysate 2·0 g.; Tween 80 0·5 g .; tap water 1000 ml. Cultures for mouse inoculation were grown in the above medium for 10 days at 37°C. Most of the clear supernatant was poured off and the deposit harvested. A 5 ml. sample of the thick suspension was assayed for moist weight of organism per ml. by centrifuging, resuspending in a small amount of alcohol (50 per cent.) and centrifuging again in a tared tube. The supernatant liquid was poured off and all excess of moisture was removed with a small cotton wool swab. This method appeared to give reasonably reproducible results when several assays were made on one suspension. The assayed suspension was standardised to twice the concentration required finally, using the modified Dubos medium.

Fresh egg yolks were separated and shaken with an equal volume of sterile saline and strained through several layers of muslin. Material for inoculation was prepared by mixing equal parts of egg yolk mixture and double strength culture suspension. 0·25 ml. of this suspension containing 0·3 mg. moist weight of organisms (occasionally 0·1 or 1·0 mg.) was injected intravenously into each mouse.

The survival time of untreated mice infected with 0·3 mg. of organisms was usually between 15 and 30 days and survival time over a period of 28 days was used as the main criterion of the protective action of drugs. Post-mortem examinations were carried out on the heart, lung, liver, spleen, kidney and gut for macroscopic lesions and in certain cases microscopical examinations of fixed sections were made. We have not found the close correlation between post-mortem appearance and dose of drug reported by Raleigh and Youmans.[20]

The drug under examination was administered either subcutaneously, twice a day, into the interscapular region or orally in the drinking water, as described for the toxicity experiments. In the latter case, measurements were made of the daily consumption per cage of 5 mice. Dosage was commenced within an hour or two of infection, with the exception of the curative experiments, where the drug was given 14 days after infection.

Estimation of *p*-Aminosalicylic Acid

After trials of the numerous published methods, we find the following modification of the method described by Klyne and Newhouse[21] the most useful for our purpose.

(i) *Determination in Blood.*

Reagents: Solution of *p*-toluenesulphonic acid 20 per cent. in 0·2N hydrochloric acid.

Hydrochloric acid, 10 per cent. v/v.

0·75M disodium hydrogen citrate prepared by mixing 39·4 g. of citric acid with 16·8 g. of sodium hydroxide and diluting to 250 ml.

Solution of *p*-dimethylaminobenzaldehyde 2 per cent. in alcohol (95 per cent.).

1·0 ml. of oxalated blood is added to 13 ml. of distilled water with shaking. After 3 minutes, 6 ml. of *p*-toluenesulphonic acid reagent is added slowly with shaking. The mixture is filtered through a Whatman No. 4 paper and refiltered if necessary to give a clear solution. To 5 ml. of the filtrate, add 1·0 ml. of the citrate buffer, 0·4 ml. of 10 per cent. hydrochloric acid and 1·0 ml. of *p*-dimethylaminobenzaldehyde reagent. Make up to 10 ml. and read on the Spekker absorptiometer after 15 minutes, using 0 B1 filters and a 1 cm. cell. A blank is similarly prepared from normal blood.

(ii) *Determination in Urine.*

Reagents:—Trichloracetic acid, 10 per cent. w/v.

Solution of sodium hydroxide 1 per cent.

Hydrochloric acid, 10 per cent. v/v.

Ehrlich's reagent (1 g. of *p*-dimethylaminobenzaldehyde in 3·3 ml. of concentrated sulphuric acid diluted to 50 ml. with distilled water).

(*a*) *Procedure for free* p-*aminosalicylic acid.*

The urine is diluted to 50 volumes with distilled water and 1 ml. of the diluted urine added to 9·0 ml. of the following mixture:—Trichloracetic acid 10 per cent. w/v 32 ml.; solution of sodium hydroxide 1 per cent. 30 ml.; Ehrlich's reagent 10 ml.; distilled water 18 ml. The colour is read on the Spekker absorptiometer using 0B1 filters and 1 cm. cells.

(*b*) *Procedure for total* p-*aminosalicylic acid.*

To 1·0 ml. of urine 2 ml. of hydrochloric acid 10 per cent. v/v is added and the whole diluted to 10 ml. with distilled water. This diluted urine is heated on a water-bath at 100°C. for 1 hour, when all the acid free, and conjugated, is decarboxylated to *m*-aminophenol. The solution is then diluted to 25 ml. and the *m*-aminophenol estimated by the method described for free acid, substituting the 1 : 25 acid solution for the 1 : 50 aqueous dilution. Standard curves are used to convert the extinction coefficients into *p*-aminosalicylic acid concentrations in all estimations.

RESULTS

Toxicity.

(*a*) *Acute Experiments.* The sodium salt was administered but the doses in Table I are expressed as the acid. Mortality figures give the number of mice dead 7 days after administration.

TABLE I

ACUTE TOXICITY OF *p*-AMINOSALICYLIC ACID IN MICE

Dose in mg./g.	6·0	5·0	4·0	3·5	3·25	3·0	2·5	2·0
Mortality intraperitoneally	5/5		1/5	0/5		0/5		
Mortality intravenously ...			2/2	3/3	5/5	5/5	2/5	0/5
Mortality subcutaneously	5/5	5/5	2/5			0/5		
Mortality orally		5/5	2/5			1/5		

From these figures, the approximate LD50 for the various routes appear to be as follows:—Intraperitoneal 4·5 mg./g.; Intravenous 2·5 mg./g.; Subcutaneous 4·0 mg./g.; Oral 4·0 mg./g.

(*b*) *Chronic Experiments.*

TABLE II

CHRONIC TOXICITY OF *p*-AMINOSALICYLIC ACID IN MICE

Concentration in Drinking water	Average Consumption in mg./mouse/day	Limits of Average Consumption mg./mouse/day	Mortality after 12 weeks
per cent.			
1·0	53·3	22·0—70·0	7/10
0·75	40·6	27·0—50·0	5/9
0·5	24·6	13·4—40·0	0/10

From these figures, assuming the average weight of a mouse to be 20 g., it would appear that the approximate LD50 for daily administration over a period of 3 months is slightly less than 2·0 mg./g. The maximum tolerated dose under the same conditions would be rather more than 1·25 mg./g.

Subcutaneously, 1·25 mg. and 2·5 mg./mouse given twice daily for 8 weeks caused no deaths in groups of 5 mice.

Comparable figures are not readily available in the literature, the majority of workers being content to quote the concentration of drug in the diet. McClosky *et al.*[10] found the maximum tolerated intravenous dose to rats and rabbits to be more than 2·0 mg./g. and 0·5 mg./g. respectively. In guinea-pigs, single oral doses of 3 mg./g. produced 30 per cent. mortality. The same authors found that daily oral doses of 0·5 mg./g. to guinea-pigs produced a mortality of 70 per cent. after 32 doses. It would seem, therefore, that mice are rather more resistant than guinea-pigs to prolonged oral administration of *p*-aminosalicylic acid. Other workers, [2,7,8] have reported toxicity figures based on diet concentrations but, in the absence of records of consumption, it is not possible to compare them with those obtained in the present experiments. Levaditi *et al.*[22] appear to be the only other workers who have administered

oral doses to mice over a long period (75 days) and their figures of 2·5 mg./g. for a toxic dose and 1·0 to 1·5 mg./g. for a tolerated dose agree well with those given above.

A histological examination of the tissues of the mice used in the chronic series of experiments was made either at the time of death or, if the animals survived the test period, at the end of the test. Many of the kidney sections showed a cloudy swelling with some congestion of the tubules and the appearance of a deposit in the lumen. The glomeruli appeared normal. Many of the liver sections showed a loss of cell outline, with granulation of the cytoplasm and signs of nuclear degeneration. The hearts, lungs and small intestines appeared normal in nearly all cases.

Blood levels. Following oral doses of 0·5 mg./g to mice, the blood level rose rapidly to about 30 mg./100 ml. in 1 hour and fell quickly to zero after about 4 to 5 hours. This is in agreement with earlier reports using rabbits and guinea-pigs[2,10]. Caronamide given simultaneously with *p*-aminosalicylic acid in oral doses of 0·125 to 0·5 mg./g. had no effect on the peak blood level but appeared to delay somewhat the fall in blood levels. (Fig. 1.)

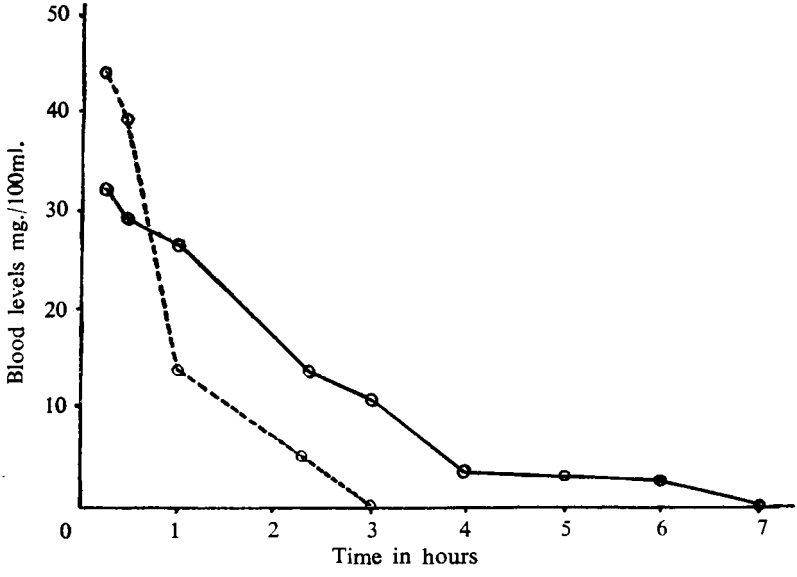

FIG. 1.—Effect of Caronamide on *p*-Aminosalicylic Acid blood levels in mice

O———O { *p*-Aminosalicylic acid 0·5 mg./g. orally
Caronamide 0·5 mg./g. orally

O------O *p*-Aminosalicylic acid 0·5 mg./g. orally

This result was not obtained regularly and, in any case, did not appear to be of the same magnitude as that obtained with penicillin (Fig. 2). However, it seemed worthy of trial in human volunteers and Figure 3. shows the negligible effect exerted by caronamide on the blood levels of *p*-aminosalicylic acid in two normal men.

Apart from the blood concentration produced in mice by one large

dose, it appeared desirable to examine the blood levels occurring under the conditions of the therapeutic test. Accordingly, determinations were made on mice which had been receiving 0·125 per cent. of the acid in their drinking water (=0·25 mg./g./day) for 4 days. The value, 1·2 mg./100 ml., was almost too low to measure with any degree of accuracy. Nevertheless, this level, as will be seen, is adequate to protect mice for a considerable time against tuberculosis infection, and this fact would seem to suggest that high blood levels may not be essential in clinical treatment, or that the acid is converted *in vivo* into a more active compound.

Antipyretic effect. *p*-Aminosalicylic acid and aspirin were administered orally to rabbits, simultaneously with an intravenous injection of bacterial pyrogen. Rectal temperatures were taken hourly for 5 hours thereafter,

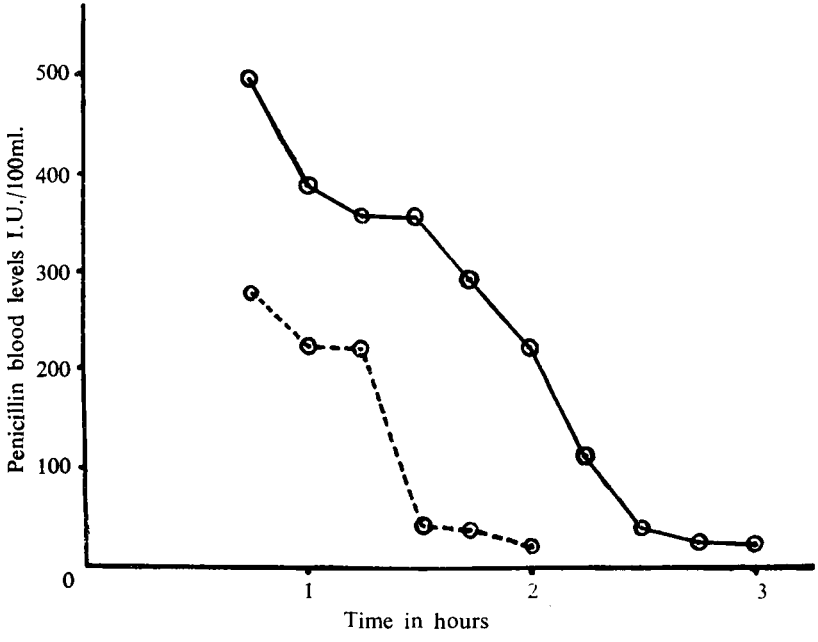

FIG. 2.—Effect of Caronamide on Penicillin blood levels in mice

O———O { Penicillin 25 I.U./g. subcutaneously
Caronamide 0·5 mg./g. orally

O------O Penicillin 25 I.U./g. subcutaneously

and the average maximum rise of temperature calculated. No significant difference was noted between the control group and that receiving the acid, whereas a significant fall in temperature occurred in those animals treated with aspirin. It seems clear that *p*-aminosalicylic acid, under these conditions, has no antipyretic action.

Effect on Prothrombin Times. Two experiments on rabbits were carried out. In experiment 1, normal prothrombin times were determined twice at an interval of 1 week, followed by daily oral doses of 0·5 g. After 3 days' treatment no significant change of prothrombin time had

TABLE III

ANTIPYRETIC EFFECT OF *p*-AMINOSALICYLIC ACID

Number of Rabbits	Dosage	Average maximum rise in temperature °F.
12	Standard Pyrogen 1 : 250 Dilution 5 ml./rabbit intravenously	1·91
11	Standard Pyrogen as above + 1·0 g./kg. of acid orally	1·7
12	Standard Pyrogen as above + 200 mg./kg. of aspirin orally	1·27

occurred. In experiment 2, only one normal level was determined, followed by daily intravenous doses of 0·5 g. After 5 days, a statistically significant increase in prothrombin time was observed, but this fell again to a non-significant level after 12 days. Table IV gives the results of these experiments and it would appear that, on the whole, *p*-aminosalicylic acid, in the dose used, has very little effect on the prothrombin times of rabbits.

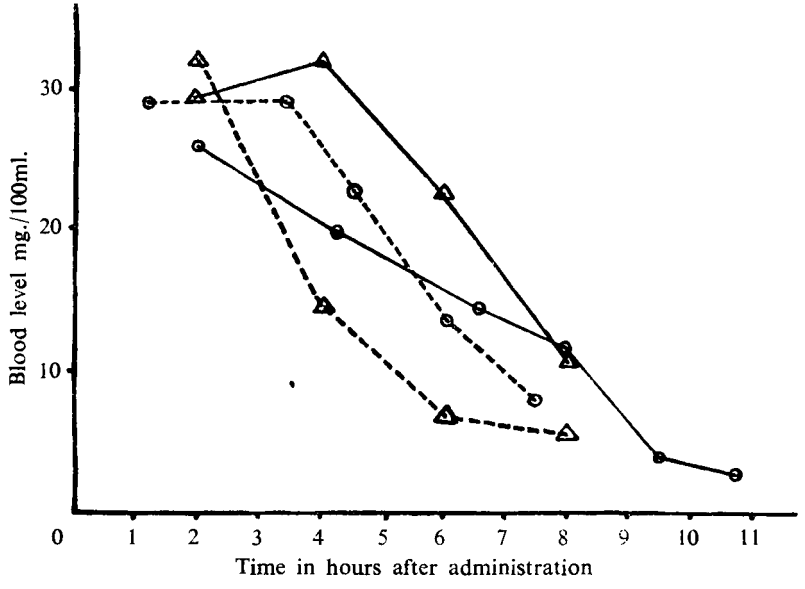

FIG. 3.—Effect of Caronamide on *p*-Aminosalicylic Acid blood levels in human subjects

(All doses administered orally)

Δ————Δ Subject A 20 g. of *p*-aminosalicylic acid + 3 g. of caronamide; 4 hours later 3 g. of caronamide.

Δ------Δ Subject A 20 g. of *p*-aminosalicylic acid

O————O Subject B 20 g. of *p*-aminosalicylic acid + 3 g. of caronamide; 4 hours later 3 g. of caronamide.

O------O Subject B 20 g. of *p*-aminosalicylic acid

TABLE IV

EFFECT OF *p*-AMINOSALICYLIC ACID ON THE PROTHROMBIN TIMES OF RABBITS

Experiment	Number of Rabbits	Daily Dose	Average Prothrombin times in seconds			
			Normal	After 3 days treatment	After 5 days treatment	After 12 days treatment
1	4	0·5 g. of acid per rabbit orally	10·5 ; 9·25	9·95		
2	5	0·5 g. of acid per rabbit intravenously	8·1		10·5	9·5

Therapeutic action. The first experiments were directed towards determining the sensitivity of the test to graded doses of drug and infective organisms. Table IV gives the average survival rates of fairly large numbers of mice under varying conditions, treated with graded oral doses.

TABLE V

EFFECT OF *p*-AMINOSALICYLIC ACID ON SURVIVAL RATES OF MICE INFECTED WITH *M. tuberculosis*

Concentration in drinking water	Acid consumed mg./mouse/day	Infecting dose of organism H37 RV (intravenously)		
		1·0 mg.	0·3 mg.	0·1 mg.
per cent.		per cent.	per cent.	per cent.
0·25	10·0 approx.	68 (15)	90 (20)	90 (20)
0·125	5·0 approx.	30 (20)	83 (35)	100 (20)
0·0625	2·5 approx.	20 (15)	55 (40)	87 (15)
nil	nil	0 (25)	18 (65)	56 (25)

Figures in brackets indicate the number of mice used.

It will be seen that the test offers a reasonable degree of discrimination, particularly in the group receiving 0·3 mg. of organism. Streptomycin was used as a standard of comparison and Figure IV shows the survival rates of mice infected with 0·3 mg. H37 RV and treated with twice-daily subcutaneous injections of streptomycin or oral doses of *p*-aminosalicylic acid.

Taking the area of each curve as a measure of the comparative action of the respective doses of the two drugs, we found that streptomycin administered subcutaneously was between 3 and 6 times more active than *p*-aminosalicylic acid orally.

TABLE VI

COMPARATIVE THERAPEUTIC ACTION OF *p*-AMINOSALICYLIC ACID AND STREPTOMYCIN SUBCUTANEOUSLY

Daily dose	Mortality after 8 weeks	Average survival times	Average grade of lung lesions
2·5 mg. of acid	0/5	56 days (max.)	3·75
750μ g. of streptomycin	0/5	56 days (max.)	2·6
375μ g. of streptomycin	2/5	44 days	4·0
Nil	5/5	18·2 days	4·0

When both drugs were given subcutaneously, the results were as shown in Table VI. Giving some weight to the observations on the lung lesions, it would appear that streptomycin is about 5 times more active than *p*-aminosalicylic acid when both drugs are given subcutaneously.

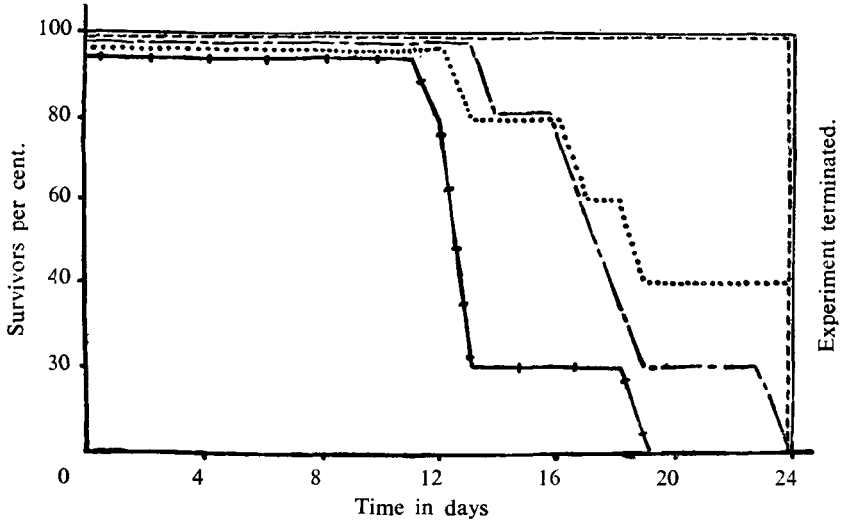

FIG. 4.—Comparison of *p*-Amineosalicylic acid (orally) with Streptomycin (subcutaneously) against experimental tuberculosis in mice

———— 2 × 375 μg. of streptomycin daily.
– – – – – 0·125 per cent. *p*-aminosalicylic acid (*c.* 5·0 mg./day)
— — — — — 2 × 187·5 μg. of streptomycin daily
..... 0·0625 per cent. *p*-aminosalicylic acid (*c.* 2·5 mg./day)
—+—+—+— Controls

Curative Action. It was realised that the above type of experiment, although quite useful for screening purposes, did not reproduce the conditions occurring clinically, where an established infection has to be treated. Some experiments were, therefore, carried out using mice in which the infection had been allowed to develop, as judged by the histological examination of control animals, before treatment with *p*-aminosalicylic acid was commenced. Figure V gives the result of such an experiment, from which it will be seen that the acid has some curative effect, although, naturally, it is not so effective as when given from the commencement of the infection. Further experiments on these lines are being carried out.

Development of resistance. Evidence that resistance does not develop either experimentally or clinically has already been reported [4,14,23,24,25]. Our own experiments, so far, confirm these reports and it is unnecessary to report them in detail. Briefly, the experiments were of two types, one in which the infective organism (H37 RV) had been grown in medium containing the acid and had then been used to infect mice which were subsequently treated with *p*-aminosalicylic acid, and the second in which the infective organism was obtained from mice which had been treated with *p*-aminosalicylic acid for a considerable period.

In the first case, the organism had been grown in a medium containing 1·0 mg./ml. of *p*-aminosalicylic acid and mice infected with this strain were found to be quite as responsive to the therapeutic action of the acid as mice infected with a similar strain grown in a normal medium.

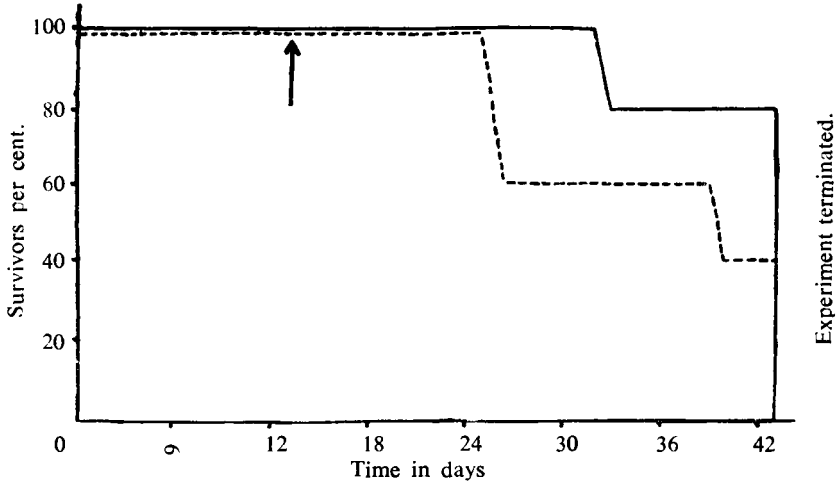

FIG. 5.—Effect of *p*-aminosalicylic acid against an established tubercular infection in mice.

———— 0·125 per cent. *p*-aminosalicylic acid (*c.* 5·0 mg./day)
- - - - - - - - - - Controls
↑ Dosage of *p*-aminosalicylic acid started

In the second case, the strain of organism was isolated from mice which had received 10 mg./day orally for 42 days and, here again, mice infected with this strain showed no sign of resistance. These results would appear to support previous work that resistance does not occur to any large extent, but, in view of the suggestion[26] that it is not easy to demonstrate resistance to streptomycin experimentally in mice, it was thought advisable to repeat the second type of experiment using more passages through *p*-aminosalicylic acid-treated animals and with streptomycin controls. These experiments are still in progress and will be reported at a later date.

TABLE VII

EFFECT OF POTASSIUM IODIDE ON THE TUBERCULOSTATIC ACTION OF *p*-AMINOSALICYLIC ACID

Concentration in drinking water	Mortality	Average survival times
0·0625 per cent. of acid	7/10	31·3 days
0·0625 per cent. of acid + 0·04 per cent. of potassium iodide ...	7/10	39·3 days
0.04 per cent. of potassium iodide	8/10	19.0 days
Nil	5/5	18·2 days

Potentiation by Potassium Iodide. Woody and Avery[17] have reported that potassium iodide has a marked potentiating effect on the action of streptomycin in tuberculous guinea-pigs. It was decided to investigate the effect of potassium iodide on the therapeutic action of *p*-aminosalicylic acid, and Table VII gives the results. Statistically, the difference in survival time is not significant. The effect of potassium iodide is not so marked as reported by Woody and Avery for streptomycin, but the above experiment is being repeated using increased concentrations.

Discussion

The experiments in which *p*-aminosalicylic acid was given in the drinking water are of a more quantitative character than those previously described, inasmuch as the daily drug consumption has been measured. This has enabled us to determine a rather more accurate maximum oral tolerated dose over a period of 3 months and to make a comparison of the tuberculostatic action of streptomycin and *p*-aminosalicylic acid. Parenterally or orally, *p*-aminosalicylic acid appears to be much less active than streptomycin parenterally.

Caronamide has been shown to have little or no action on *p*-aminosalicylic acid blood levels and it seems likely therefore that the renal mechanism for the excretion of the acid is different from that obtaining in the case of penicillin. This antibiotic has been shown to be excreted largely *via* the tubules and the experimental result suggests that *p*-aminosalicylic acid may be excreted mainly by the glomeruli.

p-Aminosalicylic acid has been shown to have no effect on the prothrombin times of rabbits, and it would appear therefore that it does not form any derivative of salicylic acid capable of affecting the prothrombin times and that prolonged use is unlikely to lead to hæmorrhagic states.

The contrast between the lack of antipyretic effect shown by *p*-aminosalicylic acid experimentally and the reports of such an effect clinically may be due to the difference in sensitivity of rabbits and man to antipyretic drugs. The dose of acetylsalicylic acid necessary to exert a marked antipyretic effect in rabbits is considerably larger, weight for weight, than for a similar effect in man, and other workers[27] have reported similarly large doses of other antipyretics as being required by rabbits. Co Tui and Schrift's report[28] of the relative insensitivity of rabbits to pyrogen compared with man is probably another demonstration of this disparity. Brownlee[29] has very recently shown that *p*-aminosalicylic acid has a peripheral vaso-dilating action in the human subject, and suggests that the heat loss so produced may explain the drug's antipyretic effect. In a fur bearing animal, such as the rabbit, the vascular effect would be unlikely to produce such a marked heat loss and this may be an alternative explanation of the different action of the drug in rabbits and man. It is proposed to investigate further the antipyretic action of *p*-aminosalicylic acid using partially depilated rabbits.

Resistance to *p*-aminosalicylic acid does not seem to occur, nor does

potentiation by potassium iodide, but further work on both these points is desirable.

SUMMARY

1. Acute and chronic toxic doses of *p*-aminosalicylic acid to mice have been determined by various routes of administration.

2. Prolonged oral and subcutaneous administration to mice produces some pathological effects on the liver and kidney.

3. A method is described for the estimation in blood and urine, and blood levels curves are given in mice and man.

4. Caronamide has no effect on the blood levels in man.

5. It appears to have no hypoprothrombinæmic or antipyretic effect in rabbits.

6. It exerts a protective effect in mice infected with *M. tuberculosis* H37 Rv, but it is not so effective an antitubercular drug as streptomycin.

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THE SEPARATION AND IDENTIFICATION OF ERGOT ALKALOIDS BY PAPER PARTITION CHROMATOGRAPHY

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During the long history of ergot much time has been devoted to the study of the alkaloids, to which the drug owes its therapeutic and toxic properties. Many workers have contributed to our knowledge of this subject and, in particular, the problem of assaying ergot has been a major topic, engaging the attention of laboratories all over the world. A good summary of analytical work up to 1937 has been given by Barger[1] and a later account has more recently been published by the American Pharmaceutical Association[2]. In spite of the effort already expended on this project, however, available methods still lack specificity; biological assays measure the total potency due to the alkaloids in the preparation under examination, while colorimetric and other chemical methods estimate the total alkaloids, the water-insoluble or the water-soluble alkaloids. The results may be stated in terms of alkaloid calculated as ergotoxine, ergotamine or ergometrine but no method so far available will allow the actual amounts of these alkaloids in a specimen of ergot to be determined.

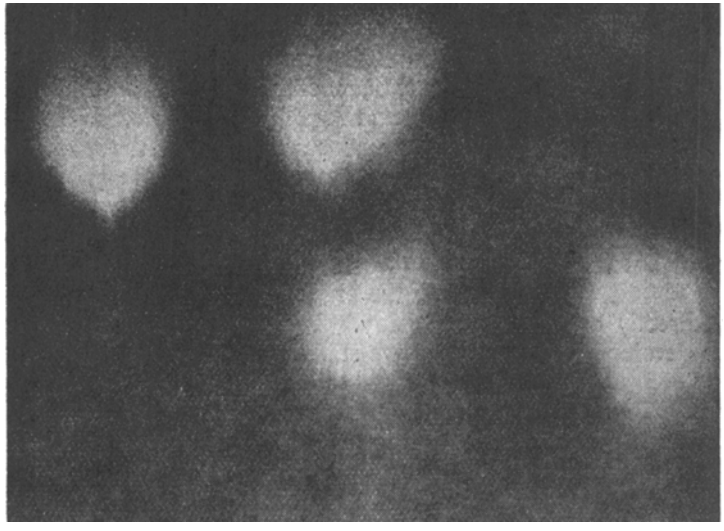
It was the purpose of the work, described in the present communication, to apply the technique of paper partition chromatography[3] to this problem for, in view of the remarkable success of this new technique in other fields, there was good reason to believe that results of interest would emerge.

Separation of Alkaloids

The separation of amino-acids on paper chromatograms using a water saturated solvent, first described by Consden, Gordon and Martin[3], is well known. These workers used strips of filter paper, on which were placed spots of the solutions under test, the upper end of the paper being immersed in a horizontal trough containing the water-saturated solvent. The strips were hung in an airtight chamber in an atmosphere saturated with water and solvent. The solvent from the trough gradually passed down the paper causing separation of the amino-acids which were subsequently located on the paper by use of the ninhydrin reagent. A suitable chamber was provided by using a stoneware drain-pipe standing vertically and closed by a lead tray at the bottom and a sheet of plate glass at the top. Water saturated with solvent was placed at the bottom of the chamber in order to maintain the required atmosphere. Full details are given in the original paper, to which the reader is referred.

Using this technique and employing *n*-butyl alcohol-acetic acid-water mixture as solvent we investigated the behaviour of ergot alkaloids on Whatman No. 1 paper. The alkaloids gave little or no colour with the ninhydrin reagent but their positions on the paper were readily detected by their fluorescence in ultra-violet light. It became immediately evident

that the water-insoluble alkaloids, of the ergotoxine and ergotamine groups, passed down the paper with the solvent front. However, separation of the water-insoluble from the water-soluble alkaloids occurred and, what is more important, ergometrine and ergometrinine passed down the paper at different rates and consequently could be separated and identified. The solvent employed was prepared by shaking together *n*-butyl alcohol (4 vols.), glacial acetic acid (1 vol.) and water (5 vols.). After standing the upper layer of *n*-butyl alcohol was placed in the trough and used as the moving phase on the chromatogram, while the lower aqueous acetic acid layer was placed in a dish at the bottom of the drain-pipe. Satisfactory chromatograms resulted when the alkaloids were employed as tartrates, lactates or maleates, but when present as sulphates little movement on the paper took place. A chromatogram was prepared by placing 0·05 ml. of solution containing 5 to 10 μg. of alkaloid on the paper. After allowing the solvent to run down the paper for 12 to 18 hours the chromatogram was air dried and examined using a suitable source of filtered ultra-violet light. With samples of pure ergometrine and ergometrinine, in solution as maleates, the following R_F values were obtained:—Ergometrine, 0·59; ergometrinine, 0·68.



Left: Ergometrine. *Right*: Ergometrinine.
Centre: Mixture of Ergometrine and Ergometrinine.

Fig. 1. Section of chromatogram of ergot alkaloids in ultra-violet light, showing the relative positions of ergometrine and ergometrinine.

Figure 1 shows a typical chromatogram, photographed while exposed to ultra-violet light, and illustrates the application of the method for purposes of identification.

An obvious extension of this work is the development of a method for estimating ergometrine, the most important ergot alkaloid. In this we

have only been partially successful. The fluorescent spots corresponding to the respective alkaloids may be marked on the chromatogram with pencil and subsequently measured in area or cut out and extracted to remove the alkaloids. These methods did not yield satisfactory results, however, for the quantitative removal of the alkaloids from the paper proved surprisingly difficult and the area of the spots could not be used for quantitative work. Our most successful results were obtained by preparing a series of standard spots of ergometrine on a paper chromatogram at the same time as the sample under test was examined, and matching the fluorescence of the respective spots under ultra-violet light. As with most fluorescent methods difficulties caused by quenching of the fluorescence at high concentrations were encountered and it was necessary to use solutions containing less than 0·001 per cent. of ergometrine before the fluorescence approached a linear relationship to concentration. Matching was performed visually and the precision was not high, the experimental error being of the order of ±20 per cent. As an alternative procedure chromatograms were prepared using serial dilutions of the standard and test solutions until dilutions were reached at which the fluorescence of the alkaloidal spots disappeared. This technique failed owing to the difficulty of determining the end-point, for even at extreme dilutions the fluorescence persisted.

The Ergometrine Content of Ergot

The first attempt to determine chemically the water-soluble alkaloids in ergot was made by Hampshire and Page[4] and their method has formed the basis of the B.P. 1948 assay process, in which the water-soluble alkaloidal content is expressed in terms of ergometrine and estimated from the difference between estimations of the total and water-insoluble alkaloids. Numerous other researches on the same topic have been published but the only processes of note are those developed by Grove[5] and by Powell *et al.*[6] by which the ergometrine is extracted and determined directly by colorimetric assay. These latter processes have been further developed in a collaborative study described by Smith[7] and have also been included in a report on the assay of ergot issued by the American National Formulary Committee[8].

We have estimated the ergometrine content of ergot, using our chromatographic technique and, for this purpose, the following process was used. 5 g. of ergot, ground to No. 60 powder, was defatted by extraction with light petroleum and air dried at room temperature. The resulting powder was thoroughly mixed with 0·3 g. of sodium bicarbonate and water was added, drop by drop, with stirring until there was obtained a well damped mass, which was then placed in a percolator (made from a piece of glass tubing 1 inch in diameter) and extracted with peroxide-free ether containing 5 per cent. of alcohol. Extraction of the alkaloids was slow and was best performed by drawing off 10 ml. of percolate at hourly intervals until about 70 ml. had been collected, after which the marc and solvent were allowed to remain in contact overnight before further percolate was withdrawn. Collection of the percolate was then continued, as described above, until another 100 ml. had been withdrawn, when the

process was stopped and the marc allowed to remain in contact with the solvent overnight. The extraction was completed in the morning by drawing off portions of percolate at half-hourly intervals until the total volume of extract amounted to 200 to 250 ml. The percolate was collected in an amber glass bottle and the whole process carried out in a dark room. After transferring the ethereal extract to a separating funnel the alkaloids were removed by shaking with 6 quantities, each of 10 ml., of 5 per cent. lactic acid; the acid extracts being collected in a graduated cylinder and the volume adjusted to 100 ml. with distilled water. Portions of this extract were then suitably diluted with 1 per cent. lactic acid until 0·05 ml. placed on a No. 1 Whatman paper strip and developed with *n*-butyl alcohol-acetic acid-water mixture, as described in the first section, gave a fluorescent spot approximately equal in intensity to that obtained with an ergometrine standard containing 0·2 to 0·5 μg. of ergometrine in 0·05 ml. By running a series of standards on the same paper the ergometrine content of the ergot was estimated.

Table I summarises the results obtained on samples of ergot, which were also assayed by the process of the B.P. 1948 and by that of the American National Formulary Committee[8].

It will be seen that the N.F. and chromatographic methods, in most cases, gave results for the ergometrine contents which were in reasonable agreement, but that the B.P. process afforded figures for the water-soluble alkaloids far in excess of the ergometrine present. Accordingly some of the final tartaric acid extract containing the total alkaloids, obtained by the B.P. process, was submitted to chromatographic analysis. Besides alkaloids of the ergotoxine group ergometrine and ergometrinine were detected. In addition a slower moving band was present above the ergometrine and this we were able to identify as being due to lysergic and *iso*-lysergic acids. It thus became clear that the use of boiling ether for extraction resulted in partial hydrolysis of the alkaloids with the production of lysergic acid, which was removed together with the water-soluble alkaloids and was estimated as ergometrine. The B.P. process therefore, did not yield reliable figures for the ergometrine content of the drug.

TABLE I

Sample of Ergot	B.P. 1948 Process		National Formulary Committee Process		Ergometrine determined chromatographically
	Total alkaloids expressed as ergotoxine	Water-soluble alkaloids expressed as ergometrine	Total alkaloids expressed as ergotoxine	Water-soluble alkaloids expressed as ergometrine	
	per cent.	per cent.	per cent.	per cent.	per cent.
1	0·22	0·043	0·215	0·023	0·019
2	0·10	0·0125	0·12	0·0086	0·006
3	0·19	0·038	0·195	0·025	0·026
4	0·16	0·040	0·195	0·020	0·016
5	0·20	0·045	0·21	0·022	0·024

Some preliminary experiments with liquid extract of ergot B.P. 1914 and liquid extract of ergot B.P. 1932 showed that the presence of ergometrine in these preparations could readily be confirmed by suitably

diluting with 1 per cent. lactic acid and preparing a chromatogram. Rough assays could be carried out as the colouring matter of the extracts remained almost stationary at the top of the chromatograms. Old extracts contained both ergometrinine and lysergic acid.

Ergometrine Preparations

At the British Pharmaceutical Conference 1948 Foster and Stewart[9] gave an account of the stability of ergometrine preparations. During the discussion on the paper Eastland[10] questioned the conclusion of the authors that the drop in biological potency of ergometrine maleate injection on storage was due to conversion of ergometrine into ergometrinine, and stated that this was at least partially due to hydrolysis of ergometrine to lysergic acid. Eastland supported his views by experimental data showing that while colorimetric assays of ergometrine injection, filled into ampoules under nitrogen and incubated for some months at 45°C., showed little loss of alkaloid by direct assay, a lower figure for the alkaloidal content was obtained if the injection were rendered alkaline and the alkaloid extracted before being estimated. It was suggested that the difference was due to lysergic acid.

During the past year we have carried out direct and indirect assays on ampoules of injection of ergometrine maleate B.P. 1948 which had been stored at room temperature for periods up to 10 years. For indirect assays the alkaloid was extracted with ether after making alkaline and saturating the solution with sodium chloride as described by the N.F. Committee[8]. Preliminary extraction experiments using solutions of pure ergometrine and ergometrinine maleates, of the same strength as used for the injection, showed that 90 to 95 per cent. recovery of ergometrine and 95 to 100 per cent. recovery of ergometrinine resulted. When applied to ergometrine maleate injection the process gave a recovery of 90 to 95 per cent. of the total alkaloidal content when freshly prepared ampoules were employed, but with 5-year-old ampoules the recovery was only 75 to 80 per cent. Allowing for a 10 per cent. loss during the extraction it would appear that some 15 per cent. of alkaloid in the older ampoules remains to be accounted for. The results of Eastland were therefore confirmed.

In order to study the composition of the injection more closely

TABLE II

Sample of ergometrine maleate injection B.P. 1948	Time of storage at room temperature	Components identified on chromatogram
Freshly made and unsterilised ...	—	Ergometrine
Freshly made and sterilised at 10 lb. pressure of steam for 30 minutes	—	Ergometrine Ergometrinine Traces of lysergic and *iso*-lysergic acids
Sterilised and stored at room temperature	5 years	Ergometrine Ergometrinine Lysergic and *iso*-lysergic acids

samples were examined by paper partition chromatography using *n*-butyl alcohol-acetic acid-water mixture as solvent. The results are summarised in Table II.

The general results indicated that on sterilisation of the injection some conversion, estimated to be approximately 20 per cent., of ergometrine to ergometrinine occurs. Very little further conversion appears to take place on storage at room temperature which, however, results in slow hydrolysis of the alkaloids with the formation of lysergic and *iso*-lysergic acids. The presence of the lysergic acids is better shown by using a basic solvent prepared by shaking a mixture of *n*-butyl alcohol (4 vols.), water (5 vols.) and pyridine A.R. (1 vol.), allowing to separate and using the *n*-butyl alcohol layer as the moving phase on the chromatogram, while the aqueous layer is used for saturating the atmosphere of the chamber. Under these conditions and using No. 1 Whatman paper the R_F values were as follows:—Lysergic acid, 0·2; *iso*-lysergic acid, 0·4.

The presence of lysergic acid was further confirmed by extracting the alkaloid from some old injection, the final alkaloidal extract being made with 1 per cent. lactic acid. On preparing a chromatogram with this extract ergometrine and ergometrinine were identified but the lysergic acids, present in the chromatogram of the original injection, had disappeared.

It was of interest to examine chromatographically tablets of ergometrine maleate which had been stored at room temperature and, for this purpose, the tablets were extracted with, or dissolved in, 1 per cent. lactic acid. Very little, if any, formation of ergometrinine or lysergic acid was detected in tablets which had been stored for periods up to 5 years.

Identification of Water-Insoluble Alkaloids

The water-insoluble alkaloids of ergot are distinguished from ergometrine by the presence in their molecular structures of certain amino-acids. Jacobs and Craig[11] found among the products of alkaline hydrolysis of ergotinine the lactam of a dipeptide, derived from L-phenylalanine and D-proline, while Smith and Timmis[12] showed that ergosine gave a similar lactam of the dipeptide of L-leucine and D-proline. Stoll, Hofmann and Becker[13] showed that ergocornine, ergocristine and ergokryptine, isolated from the ergotoxine group of alkaloids, also contained amino-acids of the L-series in addition to D-proline.

TABLE III

ALKALOIDS CHARACTERISED BY STRUCTURES DERIVED FROM LYSERGIC OR *iso*-LYSERGIC ACID, AMMONIA, A KETO ACID, D-PROLINE AND ONE OTHER AMINO-ACID

Additional amino acid	Ergotamine group (pyruvic acid group)	Ergotoxine group (Dimethyl-pyruvic acid group)
L-phenylalanine	Ergotamine Ergotaminine $C_{33}H_{35}O_5N_5$	Ergocristine Ergocristinine $C_{35}H_{39}O_5N_5$
L-leucine	Ergosine Ergosinine $C_{30}H_{37}O_5N_5$	Ergokryptine Ergokryptinine $C_{32}H_{41}O_5N_5$
L-valine	—	Ergocornine Ergocorninine $C_{31}H_{39}O_5N_5$

The relationships of the known water-insoluble alkaloids are summarised in Table III embodying data by Stoll *et al*[13].

The exact mode of linkage of the amino-acids in these alkaloids is not known but Stoll and Hofmann[14] have reported that thermal degradation of ergotamine yielded pyruvyl-phenylalanyl-proline, and Stoll[15] has further reported finding dimethylpyruvyl-valyl-proline, together with lysergic acid amide, in the products from the alkaline hydrolysis of ergocornine.

It is thus clear that the identification of the amino-acids obtained on acid hydrolysis would be of great assistance in the identification of pure alkaloids and, in the absence of major quantities of extraneous amino-acids, might be of help in the examination of cruder preparations. The occurrence of valine in ergocornine and ergocorninine is a specific test for this inter-convertible pair of alkaloids. The presence of phenylalanine would indicate ergotamine or ergocristine and their isomers, further tests such as the identification of the keto-acid and the determination of physical properties being necessary for complete identification of the alkaloids. The finding of leucine would be equally significant for the detection of ergosine and ergokryptine and their isomers.

We have identified the amino-acids in the acid hydrolysates of the alkaloids by the method of paper partition chromatography in the following manner. 10 mg. of the alkaloidal preparation together with 1 to 2 ml. of concentrated hydrochloric acid was heated in a sealed tube at 100°C. for 16 hours. After cooling, the contents of the tube were transferred to an open dish and evaporated to dryness on a steam bath. The dark residue was extracted with 0·2 ml. of distilled water and, without separating the insoluble matter 0·01 ml. of the suspension was placed on the paper. The chromatogram was prepared on Whatman No. 4 paper using *n*-butyl alcohol-acetic acid-water mixture. Although the positions of the amino-acids on the well-dried paper were revealed by their bluish-white fluorescence in filtered ultra-violet light, a more specific test in the presence of fluorescent alkaloids was afforded by the ninhydrin reaction, carried out by spraying the paper with 0·1 per cent. ninhydrin in equal parts of *n*-butyl alcohol and chloroform, drying and then developing by heating in an oven at 100°C. The amino-acids were identified by their R_F values and their ninhydrin colour reactions, leucine and valine yielding reddish purple spots while those due to phenylalanine and proline were grey-blue and yellow respectively. Under the above conditions hydro-

TABLE IV

Alkaloid	Amino-acids, identified chromatographically
Ergotamine	Proline, phenylalanine
Ergotaminine...	Proline, phenylalanine
Ergocristine	Proline, phenylalanine and faint trace of valine
Ergocristinine (ergotinine)	Proline, phenylalanine and faint trace of valine
Ergocornine (ergotoxine)	Proline, valine and trace of phenylalanine

lysis of ergine, the amide of lysergic acid, gave a continuous streak showing a variety of colours, but no major spots reacting with ninhydrin. A similar streak often appeared on chromatograms prepared with hydrolysates of water-insoluble alkaloids but was fainter and caused no difficulty in the identification of the amino-acids.

Using the above technique the results shown in Table IV were obtained with samples of " pure " alkaloids.

From these preliminary experiments it is obvious that not only is paper chromatography a valuable tool for identifying but also for assessing the purity of ergot alkaloids. For example, it is clear that in the cases of the ergocristine and ergocornine examined each alkaloid was contaminated with traces of the other. When assessing the degree of contamination it is necessary to remember that phenylalanine, under the conditions employed, gives less colour than leucine or valine and one of the quantitative applications of paper chromatography should be applied.

Other minor ninhydrin spots have been noted in the chromatograms, but at present no assignment of these to any known constituent of the hydrolysate can be made. Alkaline hydrolysates have not shown the presence of amino-acids but, in general, give two elongated spots reacting with ninhydrin. The substances in these spots have yet to be identified.

Discussion

It was stated early in this paper that available methods of ergot assay lacked specificity and it is felt that the application of paper partition chromatography, now described, has done something to remedy this deficiency.

The need for a specific identification test for ergometrine has long been felt by workers in this field and it is a matter of great importance to the chemical manufacturer who wishes to purchase ergot for the manufacture of ergometrine. By paper chromatography it is a simple matter, even with a small sample of drug, to state whether ergometrine is present and approximately in what quantity. The application to liquid extracts of ergot has also been described.

Of all ergot preparations perhaps injection of ergometrine maleate is the most important. As a result of the present work a much clearer picture of the changes which occur during the manufacture and storage of the injection has been obtained and, in view of the very small quantity of injection required for a test, the chromatographic technique has opened the field for much fuller investigation. It has been seen that conversion of ergometrine to ergometrinine occurs on heat sterilisation of the injection and it may be that sterilisation at room temperature by candle filtration might afford a better preparation. The *p*H of the injection may influence the alkaloidal equilibrium and we have experiments in progress to explore this aspect of the problem. Hydrolysis of the alkaloid in the injection has been confirmed and this factor must be added to the previously accepted causes of deterioration[9]. It is considered unlikely that the hydrolysis of ergometrine in solution can be avoided.

The close relationship of the ergot alkaloids to the polypeptides has been emphasised by the use made of chromatograms, prepared from the

hydrolysates of the alkaloids. In spite of the amount of work so far done on the ergotoxine group of alkaloids it seems doubtful whether complete separation of the individual alkaloids has yet been achieved and, in this connection, it is certain that the chromatograms of the amino-acids will be of great value in testing highly purified specimens for traces of other alkaloids. A long-standing controversy as to whether ergotamine and ergotoxine ever occur together in the same ergot[16] has not been resolved and chromatography may well have something to contribute in this field. Preliminary results with chromatograms, prepared from hydrolysates of single sclerotium of *Claviceps purpurea* have indicated that the amino-acids found are a good indication of the alkaloids present in the drug, about which there is still much to be learnt.

Summary

1. A study has been made of the application of paper partition chromatography in the ergot field.

2. A technique for the separation and identification of ergometrine and ergometrinine, when present in mixtures of total ergot alkaloids, has been described.

3. The method has been extended so that approximately quantitative results may be obtained and, in this way, the ergometrine contents of samples of ergot have been estimated.

4. The changes which take place in injection of ergometrine maleate B.P. 1948, during manufacture and storage have been studied chromatographically. It was found that during heat sterilisation some conversion of ergometrine to ergometrinine occurs. On storage some hydrolysis giving rise to lysergic and *iso*-lysergic acids takes place.

5. By preparing chromatograms from the hydrolysates of water-insoluble ergot alkaloids, identification tests for individual alkaloids have been developed.

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