SOME OBSERVATIONS ON THE PHYSICAL AND PHARMACOLOGICAL PROPERTIES OF PICROTOXIN SOLUTIONS

BY P. W. RAMWELL AND JANE E. SHAW*

From the M.R.C. Unit for Research on the Chemical Pathology of Mental Disorders, The Medical School, Birmingham, 15

Received April 5, 1963

A slow and spontaneous decrease in pH was observed with freshly prepared aqueous solutions of commercially available picrotoxin and this change was catalysed by the addition of potassium chloride crystals or by contact with a calomel electrode. The pH at which the solution stabilised was dependent upon the original pH of the solvent and the concentration of the solution. Potassium chloride had little effect upon the pH of freshly prepared saline solutions of picrotoxin. There was no difference in pharmacological activity between freshly prepared picrotoxin solutions, solutions after addition of potassium chloride, or solutions which had been stored.

Since freshly prepared solutions of pure samples of the two recognised constituents of picrotoxin (picrotoxinin and picrotin) were found to be electrometrically stable, the factor responsible for the change of pH may be an impurity; an impurity was detected in commercially prepared samples of picrotoxin by paper chromatography.

Picrotoxinin was the active principle and picrotin was relatively inactive. Slight elevation of the pH markedly reduced the activity of picrotoxin solutions thus supporting an earlier suggestion that for clinical reliability, picrotoxin should be prepared in a buffered solution. No evidence was found to support the belief that Picrotoxin Injection B.P. 1958 should be protected from light.

IN investigations of the properties of a series of neutral substances (pH 6–8) having central actions in low concentrations, a new and interesting response of mice to picrotoxin was observed. In the current literature, we found that although aqueous solutions of picrotoxin were widely described as being "neutral to litmus" (B.P. 1958) the pH was often as low as 4·5. Since there was as much as 2 units difference between the initial pH of freshly prepared solution and the final pH readings, experiments were made to determine whether the pharmacological properties varied with changes in pH. Because there was doubt about whether picrotoxin solutions should be protected from light, the effect of tungsten and ultra-violet light on their activity was examined.

Picrotoxin was first isolated in 1812 by Boullay and it was suggested that it was a mixture or a loosely bound combination of two molecules, picrotin and picrotoxinin. Determination of melting and thaw points of a picrotoxinin-picrotin mixture, thermal analysis and X-ray powder photography of the three substances (Hansen and Jerslev, 1954) have confirmed the observation of Cervello (1911) that picrotoxin consists of 54–55 per cent picrotin ($C_{15}H_{18}O_7$) and 45–46 per cent of picrotoxinin ($C_{15}H_{16}O_6$). Picrotoxin is thought to owe its pharmacological activity

* M.R.C. Scholar.

solely to picrotoxinin, while picrotin is reported to be either "saturated and inert" or to have the same yet weaker action as picrotoxinin (Angelico, 1912; Cervello, 1911).

METHODS

Chemical Procedures

Picrotoxin and its derivatives, and solutions of these substances were always stored at 4° in the dark. Crystalline picrotoxin was prepared from the crude drug by T. & H. Smith Ltd. of Edinburgh. Samples of pure picrotoxinin and picrotin were supplied through the kindness of Dr. J. S. E. Holker of the Department of Organic Chemistry, Liverpool University. All glassware was placed in 3 N hydrochloric acid for 8 hr. before use and then rinsed thoroughly with glass distilled water, in which atmospheric carbon dioxide was reduced by the use of soda lime filters. The pH of solutions was adjusted by the addition of 0·01 N hydrochloric acid or 0·01 N sodium hydroxide; pH was determined with a pH meter using conventional glass and calomel electrodes.

Pharmacological Procedures

All injections were made in a volume of 0·1 ml. into the lateral tail veins of randomly bred albino male mice weighing 20–25 g. The tail veins were dilated by warming the mice for 3 min. at 37°; the room temperature was 22–23°. To reduce the variance, mice were transferred to the laboratory at least 24 hr. before use and the animals were kept in groups of 2–6 per cage (Mackintosh, 1962). After injection, the mice were placed beneath 2 litre beakers for observation.

Two responses of mice to picrotoxin may be used as "end-points" (Ramwell and Shaw, 1963). With small doses (7·5 to 25 μg./mouse) the time interval was recorded between the injection and the assumption of a flaccid posture (F.P.) in which the mouse lay full length with its head lowered and stretched out on to the front paws; the hind legs were not properly co-ordinated and tended to be placed awkwardly with respect to the body. The assumption of this posture coincides with the appearance of a short burst of low frequency, high voltage activity in the electroencephalogram. The second "end-point", which was observed with higher dose levels, was the more conventional one, in which the time interval between injection and the first clonic convulsion was measured (convulsion time, C.T.); this response was only observed with doses of picrotoxin greater than 100 μg./mouse. The flaccid posture end-point was always detectable before the first clonic convulsion. There was an inverse linear relationship between the logarithm of the dose and the times elapsing before these two responses.

Exposure of Aqueous Solutions of Picrotoxin to Light

The activity of solutions of picrotoxin of concentrations 25 and 200 mg./100 ml. was tested on the flaccid posture and convulsion time responses, before and after exposure to either tungsten or ultra-violet light

(254 and 366 mμ). The solutions were prepared in distilled water and exposed for varying time intervals to either intense tungsten light in acid-washed pyrex glassware, or to ultra-violet light in silica cells.

Chromatography

For identification of the constituents of picrotoxin single length chromatograms were run in toluene : acetic acid : water (10 : 7 : 3) using Whatman 3 MM paper. After equilibration for 3 or 16 hr. the chromatograms were run for 2·5–3 hr. at 25·5°; 200 μg. of material was applied to each paper. Two colour reactions were employed for identification of the separated substances:

(*a*) Hydroxylamine. The chromatograms were sprayed with hydroxylamine (approximately 5 per cent in 85 per cent ethanol (v/v) and then heated for 3–5 min. at 80°.

(*b*) Silver nitrate. Chromatograms were dipped through a solution of 0·1 N silver nitrate, made just cloudy with 2 N ammonium hydroxide. They were then heated at 45° for approximately 5 min, dipped through 0·5 N sodium hydroxide and left for 10 min. at room temperature.

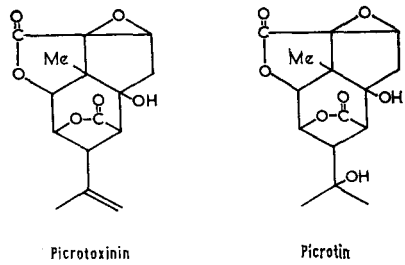
RESULTS

Since the solvent of Picrotoxin Injection U.S.P. 1955 is saline and that of Picrotoxin Injection B.P. 1958 is water, and because aqueous and saline solutions behave differently with respect to pH, the presentation of the results is facilitated if the data obtained using these two solvents is reported separately.

Change in pH of Aqueous and Saline Solutions of Picrotoxin

The pH of aqueous solutions of picrotoxin was found to be proportional to the concentration, and the pH of the water; the pH of the solutions was also modified by contact with calomel electrodes.

Although picrotoxin is widely regarded as a neutral substance, especially in view of the chemical formula (Fig. 1), aqueous solutions were always more acid than the solvent in which they were prepared (Fig. 2). Thus a 3 mg./ml. solution equivalent to Picrotoxin Injection B.P. 1958 attained a final pH, when determined by conventional electrodes, of 5·25 when distilled water of pH 7·15 was used, and with distilled water of pH 4·5 (lower pH limit of "Water of Injection" B.P. 1958) a final pH of 4·44 was recorded.



Picrotoxinin Picrotin

FIG. 1. The composition of picrotoxin (Conroy, 1952).

When measured electrometrically, the pH of freshly prepared solutions rapidly decreased. The change of pH during the 5 min. contact period was rapid, approximately 80 per cent of the change occurring in the first minute (Fig. 3). The slow spontaneous change in pH of freshly prepared solutions was found to be catalysed by the calomel electrode (a crystal of potassium chloride or sodium chloride had an equivalent effect). Whether the fall in pH was spontaneous or catalysed by potassium chloride, the final values were comparable.

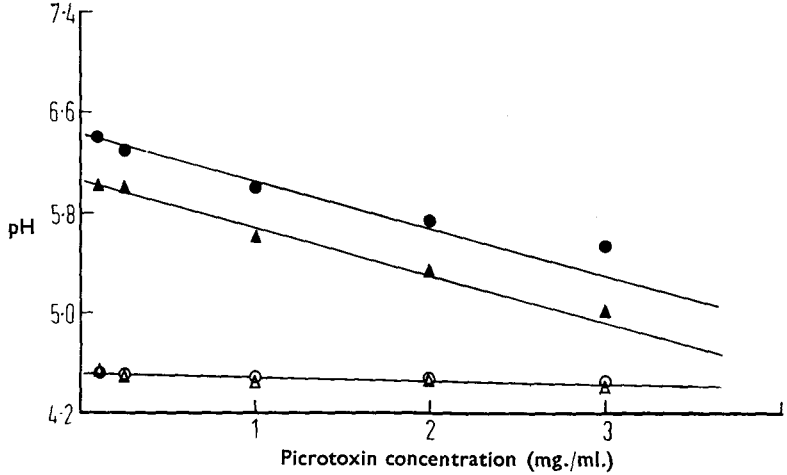


FIG. 2. The pH of aqueous and saline picrotoxin solutions after contact for 5 min. with glass and calomel electrodes.

Aqueous solutions △ Solvent pH 4·5 ▲ Solvent pH 7·3 Saline solutions ○ Solvent pH 4·5 ● Solvent pH 7·3

The pH of solutions in 0·9 per cent saline was also proportional to the concentration of picrotoxin (Fig. 2), and not unexpectedly, the slow spontaneous change in pH which was observed with freshly prepared aqueous solutions, did not occur when the solvent was saline. Generally, the final pH was always lower than the pH of the solvent and the final pH readings were close to those of the equivalent aqueous solutions, i.e. the slight leak of potassium chloride from the calomel electrode or the addition of crystals of sodium chloride had little catalytic activity owing to the saline medium. In consequence, though the initial pH readings of Picrotoxin Injection B.P. 1958 and Picrotoxin Injection U.S.P. 1955 are likely to be different on preparation, providing the pH of the solvents is similar, the solutions may attain the same pH after storage.

Response Times of Mice to Different Solutions of Picrotoxin

Since the pH of aqueous solutions of picrotoxin changed soon after preparation, it was necessary to determine whether there were associated changes in potency. Also, the relative pharmacological activity of aqueous and saline solutions was compared.

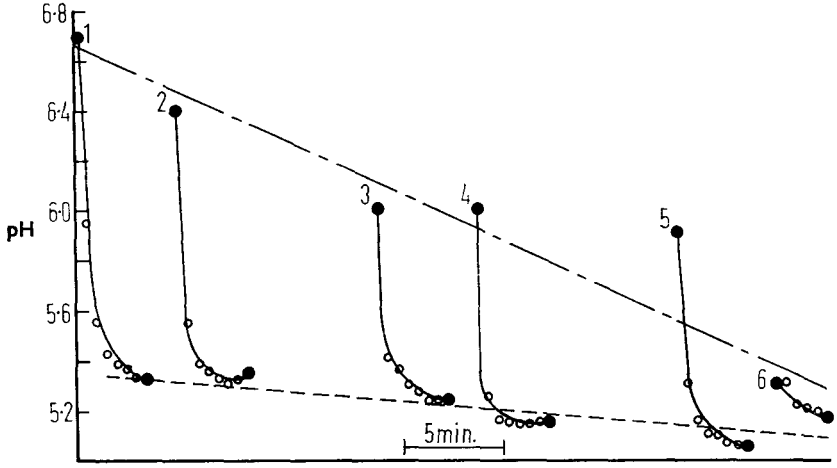


FIG. 3. A 1 mg./ml. solution of picrotoxin was prepared in distilled water (pH 5·72) and divided into 6 aliquots; each aliquot was allowed to stand at room temperature (22°) for a specified time after which the change of pH was measured for 5 min. using glass and calomel electrodes, and then the solution was discarded. The initial pH values of each aliquot (measured within a few sec. of immersion of the electrodes in the solution) decreased almost linearly from 6·7 to 5·5 units over a period of 7 hr. The "final" pH attained is of the same order, whether the fall is allowed to proceed spontaneously or is catalyzed.

Sample 1 tested 0 hr after preparation.
" 2 " 1 " " "
" 3 " 3 " " "
" 4 " 4 " " "
" 5 " 6 " " "
" 6 " 7 " " "

The response times of mice to freshly prepared solutions and to solutions at their final or "acid" pH value were determined. The pH measurements were always made on an aliquot of the original solutions. No significant difference ($P \leqq 0{\cdot}01$) in pharmacological activity could be detected between "acid" and freshly prepared solutions of picrotoxin when both response times were measured; neither was any significant difference found between the activities of freshly prepared aqueous and saline solutions (0·25 mg./ml.) (Table I).

TABLE I

SUMMARY OF RESULTS OF RESPONSES OF MICE TO FRESHLY PREPARED AND "ACID" AQUEOUS PICROTOXIN SOLUTIONS, AND ALSO TO FRESHLY PREPARED SALINE SOLUTIONS

	Concentration mg./ml.	pH	Mean F.P.* (sec.) ± s.e.	Mean C.T.† (sec.) ± s.e.
Freshly prepared solution	0·25 aqueous	6·8	86 ± 5·16	0
	2·00 aqueous	6·4	36 ± 1·38	61 ± 3·24
	0·25 saline	6·6	86 ± 4·32	0
"Acid form" solution	0·25 aqueous	5·1	89 ± 1·50	0
	2·00 aqueous	5·95	32 ± 0·96	61 ± 2·76

* Flaacid posture. † Convulsion time.

Picrotoxin solutions stored in non-acid washed glassware tended to become alkaline. Dilute solutions (0·25 mg./ml.) of picrotoxin in water, when adjusted to pH 9·5 with 0·01 N sodium hydroxide, were 66 per cent as active as the unadjusted solutions. Full activity was restored by neutralising the solution. However, 0·25 mg./ml. picrotoxin solutions of pH 10·5 were pharmacologically inactive and furthermore no activity was detectable after neutralisation (Table II).

TABLE II

SUMMARY OF RESULTS OF RESPONSES OF MICE TO "NEUTRAL", "ALKALINE" AND "NEUTRALISED" PICROTOXIN SOLUTIONS

		pH	Mean F.P. (sec.) ± s.e.
0·25 mg./ml.	controls	6·0	100 ± 3·76
,, ,,	alkaline	9·5	168 ± 10·77
,, ,,	"neutralised"	6·0	107 ± 6·68
0·25 mg./ml.	alkaline	10·5	0
,, ,,	"neutralised"	6·0	0

Response Times of Mice to Solutions of Picrotoxinin and Picrotin

Picrotoxinin solutions (0·25 mg./ml. and 1.00 mg./ml.) were prepared in distilled water of pH 7·3 and the response times of mice to these solutions were tested. There was no significant difference between the activity of these solutions and solutions of picrotoxin of twice the concentration (Table III). Solutions of picrotin (2·0 mg./ml.) caused neither convulsions, nor the assumption of a flaccid posture, but a decrease in motor activity associated with slight ataxia was observed.

TABLE III

RESPONSES OF MICE TO AQUEOUS SOLUTIONS OF PICROTOXIN, PICROTOXININ AND PICROTIN

	Conc. mg./ml.	Mean F.P. (sec.) ± s.e.	Mean C.T. (sec.) ± s.e.
Picrotoxin	0·25	86 ± 3·16	0
	2·00	36 ± 1·38	61 ± 3·24
Picrotoxinin	0·125	87 ± 3·00	0
	1·00	30 ± 2·20	59 ± 3·50
Picrotin	2·00	0	0

Stability of Aqueous Solutions of Picrotoxin to Light

Bryan and Marshall (1948) considered that the unreliability of picrotoxin in the treatment of barbiturate overdosage "might be due to inconstant loss of analeptic activity during preparation and storage of picrotoxin solutions". The British Pharmacopoeia (1958) recommends that Picrotoxin Injection should be stored protected from light, but after exposing 0·25 and 2·0 mg./ml. solutions of picrotoxin to tungsten light for 24 and 96 hr., or ultra-violet light for 24 and 48 hr., we could detect no significant decrease in potency when using the flaccid posture and convulsion time tests (Table IV).

TABLE IV

EFFECT OF EXPOSURE OF PICROTOXIN SOLUTIONS TO TUNGSTEN AND ULTRA-VIOLET LIGHT

Dose of picrotoxin μg. injected in 0·1 ml.	F.P. (sec.)		C.T. (sec.)		P
	Controls ± s.e.	Experimental ± s.e.	Controls ± s.e.	Experimental ± s.e.	
	Tungsten light (*a*) 24 hr.				
25	86 ± 2·63	96 ± 5·30	—	—	N.S.
200	—	—	45 ± 1·64	51 ± 7·23	N.S.
	(*b*) 96 hr.				
25	92 ± 3·53	98 ± 6·49	—	—	N.S.
200	—	—	49 ± 4·10	54 ± 3·72	N.S.
	Ultra-violet light (254 and 366 mμ) (*a*) 24 hr.				
25	100 ± 3·76	98 ± 5·33	—	—	N.S.
200	—	—	61 ± 3·56	66 ± 5·14	N.S.
	(*b*) 48 hr.				
25	100 ± 3·76	101 ± 3·39	—	—	N.S.
200	—	—	68 ± 4·42	64 ± 3·64	N.S.

N.S. indicates value for P not less than 0·01. Between 7 and 10 animals per group were used.

Chromatography

Application of 200 μg. of picrotoxin to a single length chromatogram resulted in separation of the compound into four fractions with R_F of 0·84, 0·72, 0·25 and 0·07 respectively. Chromatograms of picrotoxinin and picrotin were also prepared.

The same four spots were identified with both hydroxylamine and silver nitrate colour reactions. When the spots were eluted and tested for pharmacological activity by the convulsion and flaccid mouse response time tests, activity was found in only one of the eluted fractions and this corresponded to picrotoxinin (R_F 0·25).

DISCUSSION

Picrotoxin is a neutral amaroid obtained from *Cocculus indicus*, a climbing shrub indigenous to the East Indies and Malay Archipelago. The drug is present in the seeds of the berries of the plant; also present are the nitrogen-containing bases menispermine and paramenispermine (Blyth and Blyth, 1920). Picrotoxin is extracted from the berries by boiling the powdered fruits with ethanol. The fatty residue is extracted with hot water and picrotoxin is recrystallised from water or ethanol. Picrotoxin has a bitter taste which is discernible at dilutions of 1 in 80,000. Four samples of picrotoxin, including one from the U.S.A., were obtained from different drug firms and all were found to be equipotent; subsequently, the three British samples were found to have originated from the same wholesale source.

Picrotoxinin and picrotin differ by only the elements of one molecule of water (Fig. 1). It is apparent from infra-red spectroscopy that both compounds contain two lactone groups (Conroy, 1952), which are believed to be stable except in the presence of alkali. Until recently, separation of the two compounds was only possible by chemical means involving

bromination. Using samples prepared by this method, we have confirmed that picrotoxinin is active and that picrotin is almost inactive; picrotoxinin was found to be twice as active as the parent substance.

Picrotoxin has been described as having some of the properties of an acid (Blyth and Blyth, 1920) and this statement was of interest in view of both the spontaneous and the catalysed fall in pH observed in freshly prepared solutions of picrotoxin. The reason for the fall in pH was not readily apparent from inspection of the structure of the molecule and, furthermore, solutions of pure picrotoxinin and picrotin did not change their pH values when determined electrometrically with calomel and glass electrodes.

The effect of alkali on picrotoxin solutions was not simple, for solutions which had been adjusted to pH 9·5 with 0·01 N sodium hydroxide were less potent, activity being restored on neutralisation, while the activity of solutions adjusted to 10·5 was permanently lost. The addition of alkali to picrotoxinin leads to the formation of picrotoxic acid which is apparently not pharmacologically active. Slater and Wilson (1951) have pointed out that "reversible" opening of the lactone systems in this series is quite common. Further, Holker (personal communication) has suggested that the process leading to loss of activity involves two stages, the first stage being the hydrolysis of the lactone group formed between the 3-hydroxy and 15-carboxyl groups, which is reversible by acid, and the second stage being the formation of an ether link between carbons 3 and 12, which is stable to acid (Fig. 4). This suggestion provides a basis for the explanation of reversible loss of activity below 9·5 and for the permanent loss of activity of solutions with a pH of 10·5 and above. Bryan and Marshall (1948) studied the reasons for the great variation in the dose of picrotoxin required to counteract barbiturate poisoning, and they noted that elevation of the pH was accompanied by a decrease in the activity of

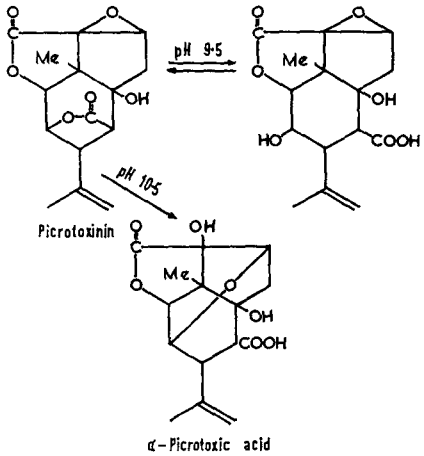


FIG. 4. Suggestion for the formation by alkali of inactive picrotoxic acid from picrotoxin in via an unisolated intermediate.

picrotoxin solutions, e.g. there was anything up to 50 per cent loss of activity between pH 8·5 to 9·0 using a "mouse-awakening" test. They concluded that the rise in pH which led to loss of activity was caused by alkali from the glass container, and to overcome this it was suggested that picrotoxin should be stored in a suitable buffer to maintain the pH below 6·5.

When the pH of freshly prepared picrotoxin solutions decreased, there was no change in potency, such as occurred when similar solutions became alkaline due either to storage in non-acid washed glassware, or the addition of sodium hydroxide. There was no obvious structural basis for the decrease in pH of freshly prepared solutions, and the change in pH was relatively small compared to the concentration used. Consequently it seemed probable that the factor responsible was an impurity. Evidence for this possibility was adduced from paper chromatography of commercially available picrotoxin, as no less than four spots were detected, only one of which possessed pharmacological activity.

A number of analeptics have been widely employed in the treatment of schizophrenia, following the introduction of convulsant therapy by Meduna in 1954. The wide range in the effective convulsant dose of picrotoxin made it less reliable and less extensively used than leptazol, even though the latter possessed the disadvantage of inducing a characteristic terror. The clinical unreliability of picrotoxin was also evident in its use in the treatment of barbiturate poisoning. Since the activity of picrotoxin solutions was markedly reduced by a slight elevation of the pH, this phenomenon may explain the clinical unreliability of the drug. Further, the suggestion that the alkaline nature of some glass containers may be sufficient to cause loss of activity, would appear to be valid, and consequently it is prudent to employ buffered picrotoxin solutions in clinical practice, and also in the laboratory for the accurate determination of biological responses. The presence of alkali may be responsible for the loss of activity previously ascribed to the effect of exposure to light. The spontaneous fall in pH of freshly prepared aqueous solutions did not affect the potency of the active principle picrotoxinin, and it is concluded that the change may be associated with the presence of the impurities that have been demonstrated in commercial samples of picrotoxin.

Acknowledgments. We are grateful to Professor I. E. Bush for his help in the chromatographic separation and detection of the constituents of picrotoxin. Samples of picrotoxin were kindly donated by Abbott Laboratories Ltd., and Kodak Ltd.

REFERENCES

Angelico, T. (1912). *Bioch. Bioph.*, **14**, 827.
Blyth, A. W. and Blyth, M. W. (1920). *Poisons, their Effects and Detection.* 5th ed., p. 475. London: Charles Griffin.
Bryan, G. and Marshall, P. B. (1948). *Quart. J. Pharm.*, **21**, 305–312.
Cervello, C. (1911). *Arch. exp. Path. Pharm.*, **64**, 407–414.
Conroy, H. (1952). *J. Amer. chem. Soc.*, **74**, 491.
Hansen, A. E. J. and Jerslev, B. (1954). *Dansk. Tidskr. Farm.*, **28**, 25–35.
Mackintosh, J. H. (1962). *Nature, Lond.*, **194**, 1304.
Ramwell, P. W. and Shaw, J. E. (1963). In press.