RESEARCH PAPERS

THE IDENTIFICATION OF THE CLINICALLY-IMPORTANT SULPHONAMIDES

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Received March 23, 1949

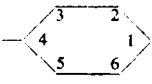
Since the discovery in 1935 of the chemotherapeutic value of the red azo-dye prontosil rubrum, and the subsequent proof that its activity *in vivo* is due to its conversion into sulphanilamide, an immense number of sulphonamide derivatives has been synthesised. The vast majority of these derivatives has found no place in medicine, and in this country some dozen only are in current clinical use.

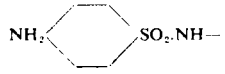
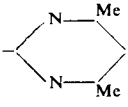
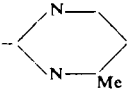
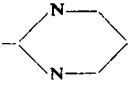
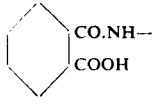
Owing to their relatively low toxicities, the sulphonamide drugs are unlikely suicidal agents, but during their therapeutic use toxic symptoms of varying severity not infrequently develop, necessitating clinical and chemical supervision. Excretion of the drugs occurs essentially *via* the urine, in which they are found either unchanged, in acetylated form, or to a lesser degree as the sulphates or glycuronates of derived phenols, the relative proportions of each form varying with the nature of the original substituent radical (Williams[1]). Thus urine, or blood, is the usual material examined in the biochemical control of sulphonamide therapy, but the toxicologist may be further concerned with the isolation and identification of these drugs when occurring in viscera and medicinal preparations. From an analytical standpoint, a serious difficulty arises from the fact that no reactions specific for the typical sulphonamide linkage have been described. Quantitative determinations of the sulphonamide content of urine and blood are usually based upon such reactions of the free primary amino group as diazotisation (Fuller[2] Bratton, Marshall, Babbitt and Hendrickson[3]), production of the yellow Schiff's bases with *p*-dimethylaminobenzaldehyde (Werner[4]), or the indophenol reaction (Lapière[5-12]). Whilst such reactions have some merit of simplicity, it is evident that interference would result from the presence of other aryl primary amino compounds (Pons and Abel[13]), and clearly the method is ineffective with sulphonamides in which the primary amino group is substituted.

Methods for the qualitative identification of individual sulphonamides figure widely in the literature, and range from the synoptic schemes of Burkham[14], Deniges[15], Pesez[16], and Hoffmann and Wilkens[17,18], to the colour reactions of Chavez[19], Sample[20], and other workers and the crystal reactions suggested by Beck[21], Dodson and Todd[22], and Lapière[5-12]. The present authors have repeatedly investigated the various methods hitherto described, but nevertheless have felt the need for a simple technique for identification of the drugs in the micro-quantities in which they may be encountered in toxicological analyses.

The number of sulphonamides is so large, whilst those of clinical importance are so relatively few, that any attempt at systematic identifi-

TABLE I

SULPHONAMIDE DRUGS (R—  $SO_2 \cdot NR'R''$*

	R	R'	R''	M pt. (°C)	
Sulphanilamide B.P.	NH_2-	$-H$	$-H$	165	
Proseptasine	$C_6H_5 \cdot CH_2 \cdot NH-$	$-H$	$-H$	175	
Sulphacetamide B.P. ...	NH_2-	$-H$	$-CO \cdot CH_3$	183	
Sulphaguanidine B.P.... ...	NH_2-	$-H$		191	
Sulphapyridine B.P.C. ...	NH_2-	$-H$		191	
Uleron		$-CH_3$	$-CH_3$	194	
Succinylsulphathiazole B.P.	$CH_2 \cdot CO \cdot NH-$ $CH_2 \cdot COOH$	$-H$		195	(185)
Sulphadimidine	NH_2-	$-H$		199	(176)
Sulphathiazole B.P.	NH_2-	$-H$		201	
Sulphamerazine	NH_2-	$-H$		236	
Sulphadiazine B.P.	NH_2-	$-H$		255	d.
Phthalysulphathiazole ...		$-H$		260	d.
Soluseptasine	$Ph.CH.CH_2.CH.NH-$ $SO_3Na \quad SO_3Na$	$-H$	$-H$		—
Solupyridine	$Ph.CH.CH_2.CH.NH-$ $SO_3Na \quad SO_3Na$	$-H$			—
Soluthiazole	$Ph.CH.CH_2.CH.NH-$ $SO_3Na \quad SO_3Na$	$-H$			—

* The drugs are listed under the name given in the British Pharmacopœia or British Pharmaceutical Codex, or if non-official, under a common trade name. The obsolescent azo-compounds prontosil rubrum and prontosil soluble are not included in this survey.

cation in the field as a whole would be unnecessarily cumbersome and would have little practical value. The investigations described in this publication, therefore, have been confined to those sulphonamide drugs commercially available at the present time in this country (Table I).

Residues isolated from medicinal preparations, or from viscera or other biological sources, are examined in a series of separate stages: (1) purification of crude residues, (2) provisional identification as a sulphonamide compound, (3) demonstration of the presence or absence of a free primary amino group, (4) simple crystal tests, (5) colour test indicative of the substituted sulphapyrimidines, (6) final conclusive identification by micromixed melting-point determination. The complete scheme provides a simple and rapid method for the identification of the listed sulphonamides, but in many instances characterisation may be achieved without inclusion of all the stages. It is emphasised that the object of the preliminary stages is essentially the indication of the likely compound, and whilst by the use of control experiments it is frequently possible to obtain a clear identification of an unknown sulphonamide by means of the preliminary tests alone, the ultimate proof should be by micromixed melting-point determination.

Experimental

Isolation of the sulphonamides. Sulphonamide therapy is of so recent introduction that few of the standard toxicological works have any mention of this group of drugs. Bamford[23], in a brief treatment of the sulphonamides, deals largely with methods of determination, and observes " Identification must depend on their isolation—often a difficult process—and examination of physical properties. Generally, however, the history of the case, combined with the results of the non-specific diazotisation and condensation reactions suffice to establish (or, more usually to confirm) the nature of the poison." The difficulties of isolation which this author so rightly mentions are largely attributable to the low solubilities of the sulphonamides in the water-immiscible organic solvents. Literature figures for the solubilities are by no means consistent, but the comparative record of available data (Table II) is useful, particularly when dealing with mixtures of sulphonamides.

In the Stas-Otto process, and also in the tungstic acid method described by Valov[24], sulphonamides are found in the ether extract of the aqueous acidic solution. The solubilities in ether, however, are so low that a small percentage only of the sulphonamide is recovered in this way, but in dealing with mixtures of sulphonamides with other compounds this is a definite advantage, as it frequently affords a ready means of separation. Complete removal from the aqueous acid medium may be effected by addition of half the volume of acetone, followed by thorough extraction with ether. For quantitative determination of total sulphonamides in material from biological sources, hydrolysis of the various derivatives is necessary. Sulphonamides generally, are present partly in the form of N_4-acetyl derivatives, whilst the three disodium cinnamylidene bisul-

phite compounds listed occur (Wien and Hampton[25]) as the parent sulphonamides, their acetyl derivatives, and possibly also as the unchanged bisulphite compounds. Hydrolysis is readily accomplished by refluxing with 2N hydrochloric acid for 20 to 30 minutes.

Purification of crude residues. The low solubilities of the drugs are an undoubted advantage in connection with their purification. As a general method, heating with activated charcoal in acetone solution is found satisfactory; losses due to absorption are negligible, and a clean product, suitable for a preliminary melting point determination, is usually obtained.

Wide differences are found in the reported melting points of several sulphonamides. In the case of sulphadimidine, this variation is attributed

TABLE II

SOLUBILITIES OF SULPHONAMIDE DRUGS.*

	Water	2N sulphuric acid	2N sodium hydroxide	Alcohol	Ether	Chloroform	Acetone	Light Petroleum b.pt. 60° to 80° C.
Sulphanilamide ...	1–125	Soluble	Soluble	1–37	1–600	1–4000	1–5	Insoluble
Sulphaguanidine	1–1000	Soluble	Insoluble	1–200	Insoluble	Insoluble	1–300	Insoluble
Sulphapyridine	1–3500	Soluble	Soluble	1–340	1–2200	1–1500	1–65	Insoluble
Sulphadiazine ...	1–13,000	Soluble	Soluble	1–1100	Insoluble	Insoluble	1–170	Insoluble
Sulphamerazine	1–6250	Soluble	Soluble	1–400	Insoluble	1–2000	1–60	Insoluble
Sulphadimidine ...	1–5000	Soluble	Soluble	1–200	1–5000	1–600	1–20	Insoluble
Sulphacetamide	1–150	Soluble	Soluble	1–15	1–600	1–1200	1–7	Insoluble
Sulphathiazole ...	1–2000	Soluble	Soluble	1–200	1–2400	1–375	1–23	Insoluble
Succinylsulphathiazole ...	1–4800	Slightly soluble	Soluble	1–100	Insoluble	Insoluble	1–110	Insoluble
Phthalylsulphathiazole	1–7500	Slightly soluble	Soluble	1–500	Insoluble	Insoluble	1–250	Insoluble
Proseptasine ...	1–32,000	Slightly soluble	Slightly soluble	1–145	1–320	1–1500	1–10	Insoluble
Uleron	1–50,000	Slightly soluble	Soluble	1–250	Insoluble	1–1000	1–20	Insoluble

* Solubilities in water are given numerically throughout. Organic solvent solubilities lower than 1 in 5000 are indicated as Insoluble.

(Northey[26]) to the occurrence of unstable hydrates. A similar explanation may hold for other sulphonamides, and the figures in parenthesis in Table I may represent the melting points of these hydrates. It is further likely that this may also be the explanation of the wide differences in solubilities recorded in the literature.

Provisional identification as a sulphonamide. Using macro-quantities, identification by standard analytical methods presents no particular problem, but on a micro-scale the lack of a specific reaction for the sulphonamide grouping is a serious disadvantage. Lapière[27] has investigated the cobalt reaction described by Parri[28] in connection with the identification of barbiturates, and has found that certain sulphonamides, notably sulphathiazole, sulphapyridine and sulphadiazine, also give the characteristic purple-violet colour. Unfortunately, the reaction cannot be regarded as a general test for the presence of sulphonamides.

Provisional characterisation of isolated and purified residues as

sulphonamide compounds has been accomplished by the present authors in the following manner:

(i) Detection of elements by an adaptation of the micro-method described by Bennett, Gould, Swift and Niemann[29]. The procedure is given in detail in Table III.

TABLE III

MICRO-IDENTIFICATION OF ELEMENTS

1 mg. residue + 5 times weight of Zn dust – CaO mixture (1 : 1) heated in 4·5 cm. × 0·5 cm. tube

Red hot tube dropped into 1 ml. water. Boiled; filtered

NH_3 evolved = NITROGEN

Residue

Filtrate Precipitate with $AgNO_3$ + HNO_3 = HALOGENS

(1) Fragment of solid placed in contact with 1 drop reagent (1 g. NaN_3; 1 g. NaI; 1 mg. iodine; 3 ml. water) held in lower end of open melting point capillary tube. Stream of bubbles issuing up tube = SULPHUR

(2) Acidified with 2N HNO_3; filtered

Filtrate

1 drop each ammonium molybdate, benzidine in acetic acid, satd. sodium acetate (Feigl[31]) Blue colour = PHOSPHORUS

(ii) If nitrogen and sulphur only are detected sulphonamides may be present, and the effect of heating in admixture with soda-lime is then investigated according to the scheme in Table IV.

TABLE IV

DECOMPOSITION ON HEATING WITH SODA-LIME

0·1 mg. residue heated with equal quantity soda-lime

VOLATILE PRODUCTS
(a) Ammonia—odour; litmus
(b) aniline—odour; furfural/acetic acid

RESIDUE
1 drop saturated $HgCl_2$ added
(a) Sulphide—iodine azide reaction
(b) Sulphite—acidified with HCl, and vapours tested with starch-iodate paper.

The clinically-important sulphonamides listed are all primary amino derivatives, and on fusion with soda-lime are decomposed with the production of ammonia or readily volatile amines, aniline bases and sodium sulphite; sulphathiazoles and the cinnamylidene bisulphite compounds yield, in addition, sodium sulphide. Thus, by the method in Table IV, positive tests for ammonia, aniline and sulphite provide presumptive evidence of the presence of a sulphonamide; sulphide in addition suggests the sulphathiazoles or cinnamylidene bisulphite compounds.

Individual sulphonamides differ considerably in the rate at which the various products are formed, but by using a standard technique the reactions with quantities of the order of 0·1 mg. are equally as satisfactory as those utilising larger amounts of material.

0·1 mg. of the test material intimately mixed with an equal weight

of soda-lime is gently warmed in a small test tube (4·5 cm. x 0·5 cm.). The issuing vapours are tested with a pointed strip of moist red litmus paper introduced just inside the tube. Stronger heat is now applied until an oily distillate accompanied by white fumes approaches to within approximately 0·5 cm. from the open end of the tube. The tube is placed on a white tile, and a pointed strip of filter paper moistened with a 2 per cent. solution of furfural in glacial acetic acid carefully introduced. In the presence of aniline (which may also frequently be detected by odour) a distinct reddish-pink band appears around the tube in the region of the oily distillate. At this stage one drop of saturated aqueous mercuric chloride solution is added to the solid residue in the tube, in order to obviate interference from hydrogen sulphide on subsequent acidification; a fragment of the treated residue is tested for sulphide by the iodine-azide reaction. The contents of the tube are finally heated with dilute hydrochloric acid, and the issuing vapours tested with a pointed strip of moistened filter paper impregnated with starch and potassium iodate, when a blue colour develops in the presence of sulphur dioxide. The results of this test with the listed sulphonamide drugs are summarised in Table V. It should be emphasised that this procedure does

TABLE V

REACTIONS OF SULPHONAMIDES

	Decomposition by soda-lime fusion				Furfural reaction for free NH_2
	Ammonia	Aniline	Sulphite	Sulphide	
Sulphanilamide	+	+	+	−	+
Proseptasine	+	+	+	−	−
Sulphacetamide	+	+	+	−	+
Sulphaguanidine	+	+	+	−	+
Sulphapyridine	+	+	+	−	+
Uleron	+	+	+	−	+
Succinylsulphathiazole	+	+	+	+	−
Sulphadimidine	+	+	+	−	+
Sulphathiazole	+	+	+	+	+
Sulphamerazine	+	+	+	−	+
Sulphadiazine	+	+	+	−	−
Phthalysulphathiazole	+	+	−	+	−
Soluseptazine	−	+	+	+	−
Solupyridine	−	−	+	+	−
Soluthiazole	+	+	+	+	−

not provide unequivocal proof that the material is a sulphonamide, but positive reactions here coupled with indications of unusual insolubility is strong presumptive evidence.

Detection of free NH_2 groups. Aryl primary amino groups may be detected by a variety of reactions, of which diazotisation followed by coupling is perhaps the most usual. The information can however be obtained equally satisfactorily, and much more rapidly, by means of the furfural condensation. 0·1 mg. to 1 mg. of material is placed in a white porcelain dish and treated with 1 drop of 2 per cent. solution of furfural in glacial acetic acid. The liquid is allowed to evaporate spontaneously, during which process a free primary amino group is indicated by the production of an intense red colour, rapidly turning reddish-violet.

The reactions of the sulphonamides are listed for convenience in Table V. It is noteworthy that in the case of the pyrimidine derivatives, the colour is appreciably slower in developing, and may not be apparent until evaporation is complete. Of the listed sulphonamides, positive tests for both sulphide and free $-NH_2$ are given by one compound only, sulphathiazole. Similarly, one compound only, proseptasine, gives negative tests for both sulphide and free $-NH_2$.

Crystal tests. Since the sulphonamides are, in general, soluble both in acid and alkali, acidification of an ammoniacal solution as recommended for the identification of barbiturates (Turfitt[30]) is valueless. It has been found, however, that by a variation in the technique, crystals of the sulphonamides may in most cases be readily obtained. The

TABLE VI

CRYSTAL FORMATION

	Strong solution of ammonia 0·880	Acetic acid vapour
Sulphanilamide ...	Soluble with difficulty	* No peripheral precipitation. Long fine needles radiate from undissolved particles; also occasional hexagonal forms
Proseptasine	Insoluble	—
Sulphacetamide ...	Very readily soluble	—
Sulphaguanidine ...	Insoluble. Slightly soluble hot; long needles on cooling.	—
Sulphapyridine ...	Readily soluble	* Rapid peripheral precipitation. Characteristic 'banded' crystals, with some hexagonal and arborescent needle formations.
Uleron	Readily soluble	* Rapid peripheral precipitation. Minute globules with characteristic 'dumb-bell' shaped crystals. Wrinkled surface skin develops over drop.
Succinylsulphathiazole	Very readily soluble	—
Sulphadimidine ...	Readily soluble	Rapid peripheral precipitation. Minute globules giving large rosettes of brownish needles. Wrinkled surface skin develops over drop.
Sulphathiazole ...	Very readily soluble	* Slow peripheral precipitation. Minute drops coalescing rapidly into large globules, and giving arborescent needle-shaped growths.
Sulphamerazine ...	Readily soluble. On evaporation 'curved' crystals usually obtained	Rapid peripheral precipitation. Three crystalline forms usually obtained: (a) rods, often with bifurcated ends, followed by (b) 'twinned' crystals, and finally (c) aggregates of 'curved' crystals. Any one of these forms is characteristic of the compound.
Sulphadiazine ...	Readily soluble	Fairly rapid peripheral precipitation. Needles individually or in clusters.
Phthalysulphathiazole	Readily soluble	* Slow peripheral precipitation. Thin massed needle rosettes. Wrinkled surface skin forms over drop.
Soluseptasine... ...	Very readily soluble	—
Solupyridine	Very readily soluble	—
Soluthiazole	Very readily soluble	—

* The same crystalline forms are obtained on spontaneous evaporation of the cold ammoniacal solution.

crystalline forms are generally highly distinctive, and it is frequently possible to identify a sulphonamide by means of this test alone.

0·1 mg. of material is finely powdered on a microscope slide and 1 drop of 0·880 ammonia added. The mixture is stirred thoroughly with a fine glass rod and observation made of the ease or difficulty of solution. A drop of glacial acetic acid on the end of a glass rod is then held just above the surface of liquid until a white turbidity appears at the margin of the drop, or, as in the case of sulphanilamide, crystals appear within the drop itself, when the slide is examined microscopically at a magnification of approximately X 50.

A general description of the crystals obtained with the various sulphonamides is given in Table VI, whilst for reference purposes the characteristic forms are illustrated in Figures 1 to 11.

It is regarded as essential that control crystal tests should be made with authentic material, and it is further recommended that after examination of the crystals these should be redissolved by treatment with ammonia vapour or solution, reprecipitated with glacial acetic vapour, and again examined. No loss of material is incurred during this repeated test, but confirmation of the typical crystalline form is obtained.

Vanillin reaction for substituted sulphapyrimidines. When warmed with vanillin and concentrated sulphuric acid the majority of the sulphonamides give a yellowish-green colour; the methylpyrimidine compounds sulphamerazine and sulphadimidine however, give an intense bright red colour. This property has been found useful as a confirmatory test, and has been adapted for micro-quantities.

A quantity of vanillin of the order of 0·01 mg. is mixed on a microscope slide with 1 small drop of concentrated sulphuric acid. Into the liquid is dropped approximately 0·01 mg. of the sulphonamide, and the mixture warmed over a micro-flame until fumes are just observable. The slide is placed upon a white tile, and the presence or absence of a red colour arising from the sulphonamide particles is noted.

Final mixed melting-point. The information derived from the foregoing tests is invariably conclusive for any one of the listed sulphonamides, but the conclusion should be checked by a mixed melting-point with a specimen of the indicated compound.

Mixtures of Sulphonamides

With the introduction of proprietary mixtures, e.g. sulphatriad, containing sulphathiazole, sulphadiazine and sulphamerazine, the toxicologist may be required to identify the individual components of such mixtures, either in biological material or during the analysis of actual tablets.

It is clearly essential in such cases to effect a preliminary separation of the components. This may be achieved satisfactorily on the basis of solubility differences (Table II), subsequent identification of each fraction being accomplished by the described methods.

The authors have encountered no insuperable difficulties in the qualitative analysis of mixtures treated in this manner.

Summary

1. A scheme is described for the identification of all the sulphonamide drugs at present available in this country.

2. The six stages of the process are simple and rapid operations, involving no unusual reagents or apparatus:

(i) purification of the crude material by charcoal treatment in acetone solution, followed by melting-point determination.

(ii) preliminary identification as a sulphonamide compound by decomposition with soda-lime.

(iii) detection of free primary amino group by condensation with furfural.

(iv) simple crystal tests based on precipitation from ammonia solution by acetic acid vapour.

(v) vanillin confirmatory test for sulphamerazine and sulphadimidine.

(vi) mixed melting-point determination.

Acknowledgements

The authors wish to express their gratitude to Messrs. May and Baker Ltd. for samples of their proprietary products and for much helpful information throughout the course of this work; to Mr. A. Hay for his valuable assistance with the photomicrographs; and to Dr. H. S. Holden, Director of the Metropolitan Police Laboratory, for his kind permission to publish the results of this investigation.

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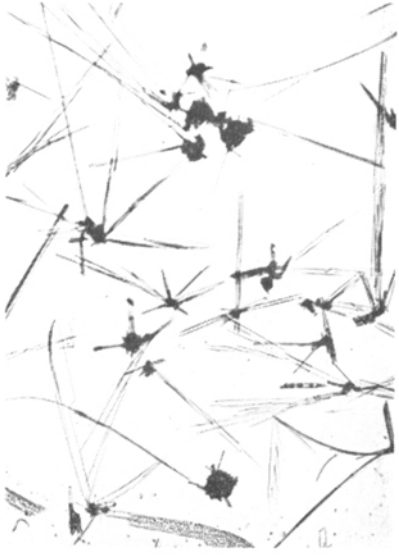

FIG. 1. Sulphanilamide.

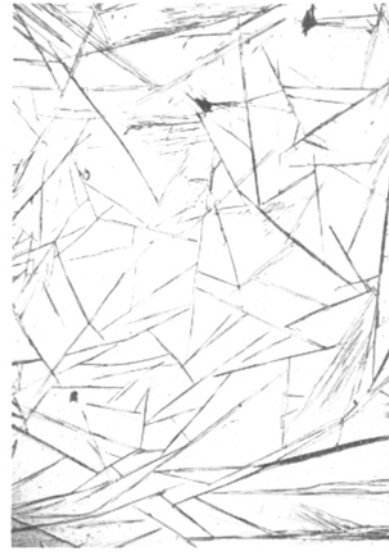

FIG. 2. Sulphaguanidine.

FIG. 3. Sulphapyridine.

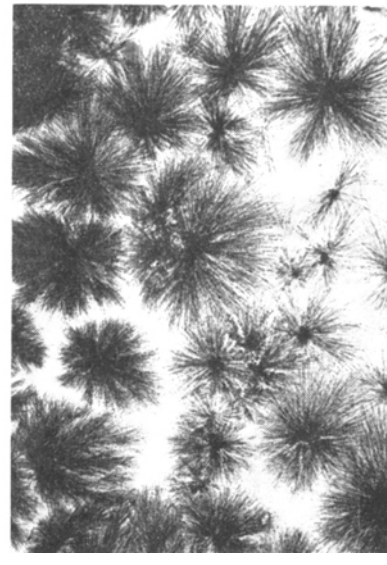

FIG. 4. Sulphadimidine.

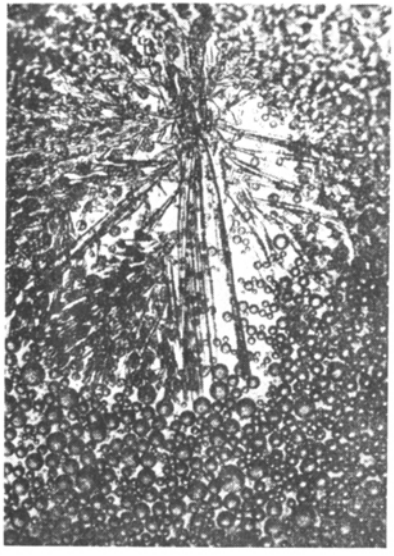

FIG. 5. Sulphathiazole.

FIG. 6. Sulphamerazine (1).

FIG. 7. Sulphamerazine (2).

FIG. 8. Sulphamerazine (3).

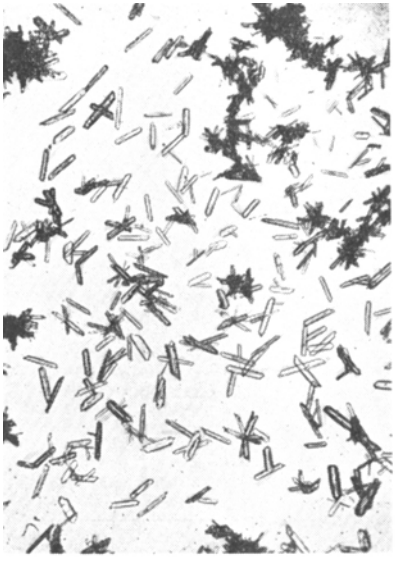

FIG. 9. Sulphadiazine.

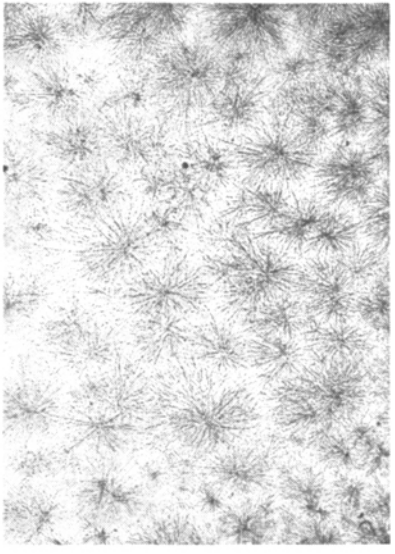

FIG. 10. Phthalysulphathiazole.

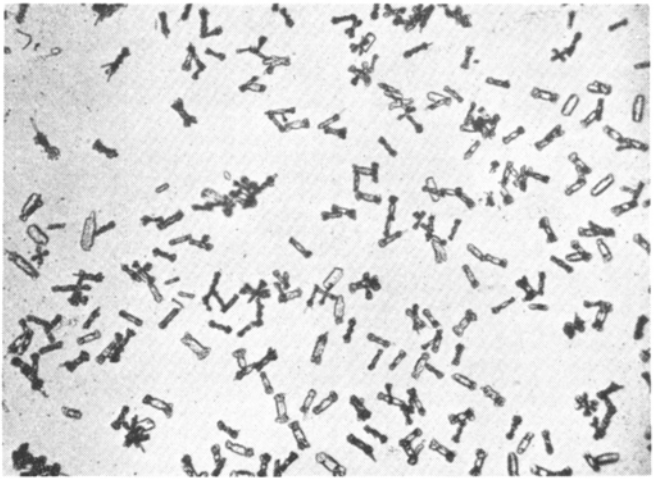

FIG. 11. Uleron.

The magnification of Figures 1-10 is approximately × 40, and of Figure 11, × 50.

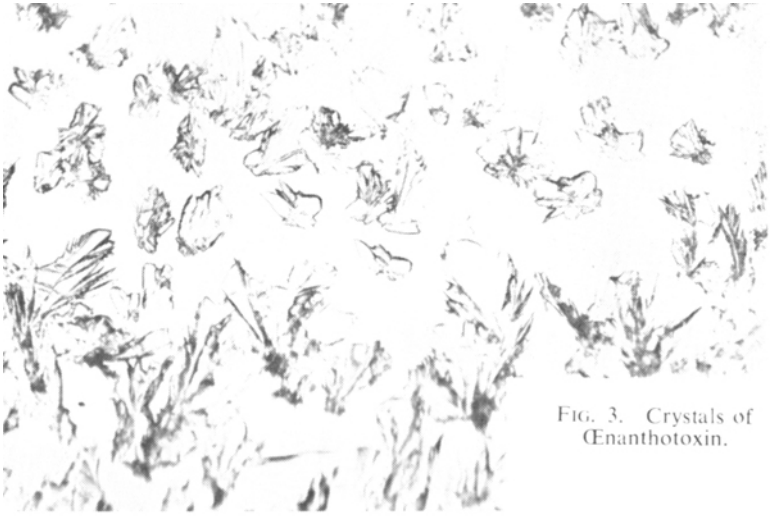

FIG. 3. Crystals of Œnanthotoxin.

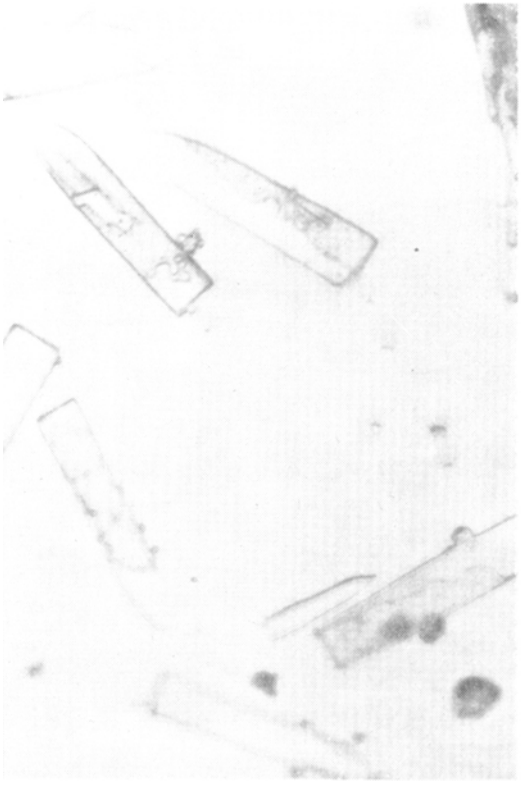

FIG. 4. Œnanthotoxin after recrystallisation from ethyl alcohol.

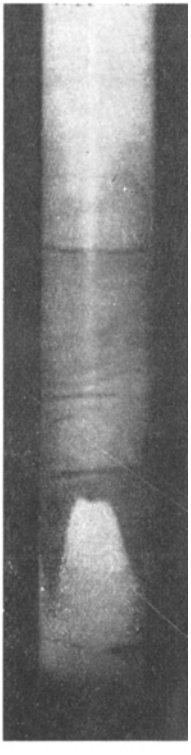

FIG. 1. Chromatogram of toxic principle of *Œnanthe crocata*.