The Metabolism of Sulphonamides

3. THE DETECTION OF 3-HYDROXYSULPHANILAMIDE IN THE URINE OF HOSPITAL PATIENTS TREATED WITH SULPHANILAMIDE

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3-Hydroxysulphanilamide has been isolated in a pure crystalline condition from the urine of rabbits which had been heavily dosed with sulphanilamide (Williams, 1946). In the present paper it will be shown that 3-hydroxysulphanilamide is also present as a conjugate in the urines of hospital patients treated with sulphanilamide.

The isolation in a pure state of small amounts of 3-hydroxysulphanilamide from urine is a difficult task uncertain of success. Therefore, a colour reaction has been developed for its qualitative detection in urine. This colour reaction appears to be fairly specific under the conditions used here and depends upon the oxidation of 3-hydroxysulphanilamide by dilute ferric chloride to a compound which can be extracted with ether. The ethereal solution has an intense red colour which shows an absorption band ($\lambda_{\max}$, 530 m μ .) in the visible region of the spectrum.

METHODS

The colour reaction for 3-hydroxysulphanilamide

It is well known that phenols react with ferric salts to give highly coloured compounds. Wesp & Brode (1934) have suggested, from spectroscopic and other data, that the colour produced is probably due to complex co-ordinated ions of the type $\text{Fe}(\text{OR})_6^{3+}$, where OR is ionized phenol.

Fig. 1. Absorption spectra of the red ethereal solutions obtained by the action of aqueous ferric chloride in the presence of ether on, I, synthetic 3-hydroxysulphanilamide; II, *p*-aminophenol hydrochloride; III, extracts of human sulphanilamide urine; and IV, extracts of rabbit sulphanilamide urine.

When a few drops of 1 or 2% FeCl_3 are added to an aqueous solution of 3-hydroxysulphanilamide, a purplish red colour is obtained which rapidly turns red, then brown, finally

giving a brownish cloudy solution. These changes are complete in about a minute after mixing. If, however, the 3-hydroxysulphanilamide solution is first shaken with about 2 vol. of ether and then FeCl_3 added and the mixture shaken, the ether layer becomes coloured an intense red (containing some blue) which is comparatively stable; the aqueous layer, however, undergoes the same colour changes as in the absence of ether. The spectral absorption, in the visible region, of this coloured ethereal solution is recorded in Fig. 1, which also gives the result using *p*-aminophenol hydrochloride instead of 3-hydroxysulphanilamide.

Other considerations, however, indicated that the colour produced in ether from 3-hydroxysulphanilamide and FeCl_3 was probably not that of an iron complex of the phenol, for it was observed that the same colour could be obtained when FeCl_3 was replaced by the oxidizing agents, potassium permanganate, faintly acid sodium dichromate and chloramine T. On oxidation 3-hydroxysulphanilamide should give rise to a quinone imine thus:

Quinone imines, however, are not highly coloured compounds (see Sidgwick, 1942), and it seemed unlikely that the colour could be due to such a compound. The usual way of preparing quinone imines is to oxidize the aminophenol in ethereal solution with silver oxide. A saturated ethereal solution of 3-hydroxysulphanilamide (sparingly soluble in ether) was therefore shaken with Ag_2O at room temperature. After a few minutes the solution had a reddish tinge, and after half an hour the solution had become an intense red (containing some blue) showing an absorption band in the visible with $\lambda_{\max}$ at 532 m μ . (see Fig. 2). In the ultra-violet the solution showed two bands with maxima at 261.5 and 298 m μ . due to 3-hydroxysulphanilamide. The absorption spectrum of 3-hydroxysulphanilamide freshly dissolved in water shows two bands, $\lambda_{\max}$ 259 and 292 m μ . (see Fig. 3). On evaporation of the red ethereal solution to dryness a small amount of a brownish residue was obtained which, when dissolved in water, showed absorption maxima at 257, 291 and 440 m μ ., the last one being of low intensity (Fig. 2). The first two are due to 3-hydroxysulphanilamide. The third one was found present in the absorption spectrum of an aqueous solution of 3-hydroxysulphanilamide which had been allowed to stand for a week at room temperature in the dark ($\lambda_{\max}$, 259, 292 and 442 m μ .; see Fig. 3). The solution at this stage was brownish yellow in colour. It appears probable that in aqueous solution 3-hydroxysulphanilamide is partly transformed into a quinone imine which then

polymerizes to a brownish substance (cf. Sidgwick, 1942) which is responsible for the band at about 440 $m\mu$. It should be noted that the brown material obtained by evaporation of the red ethereal solution, does not give a red solution on redissolving in ether.

Fig. 2. I, absorption spectrum of the red solution obtained by treating 3-hydroxysulphanilamide in ether with silver oxide. II, absorption spectrum of an aqueous solution of the brown residue obtained on evaporation to dryness of the red solution I.

Fig. 3. Absorption spectrum of 3-hydroxysulphanilamide in water. I, fresh solution, $\lambda_{\max}$ 259 and 292 with $\log \epsilon_{\max}$ 4.03 and 3.82. II, and inset, after standing a week, $\lambda_{\max}$ 258.6, 292 and 442 with $\log \epsilon_{\max}$ 4.02, 3.78 and 1.24.

The above observations lead to the conclusion that the red ethereal solution may be due to the formation of a semiquinone type of compound which is stabilized in some way in ether. It was found that ether could be replaced by

certain other solvents such as ethyl acetate, methyl ethyl ketone, *n*-butanol, amyl alcohol, but not by benzene, toluene, light petroleum, carbon tetrachloride or chloroform. The solvents in the former group are compounds containing 'lone pairs' of electrons, and stabilization of the semiquinone type of compound by co-ordination with the solvent is therefore conceivable.

These conclusions may be summarized as follows:

Both maxima, 530 and 440 $m\mu$, can be used for the characterization of 3-hydroxysulphanilamide.

A similar investigation was carried out on the closely related 4-hydroxymetanilamide (4-hydroxy-3-aminobenzenesulphonamide) which is a metabolite of metanilamide (Dobson & Williams, 1946). Although an aqueous solution of this compound gives with FeCl_3 practically the same colours as does 3-hydroxysulphanilamide (3-hydroxy-4-aminobenzenesulphonamide), it does not give rise to a red colour in ether for the intermediate semiquinone stage is apparently not detectable and the brown stage appears immediately in ether. The brownish polymerization product from this phenol shows a band with a maximum at 418 $m\mu$. This band develops very readily when an ethanolic solution of the phenol is allowed to stand (see Fig. 4). The solution turns brown on standing.

Fig. 4. Absorption spectrum of 4-hydroxymetanilamide in ethanol. I, fresh solution, $\lambda_{\max}$ 249 and 299 with $\log \epsilon_{\max}$ 3.8 and 3.6 respectively. II, and inset, after standing 3 weeks, $\lambda_{\max}$ 249, 300.5 and 418 with $\log \epsilon_{\max}$ 3.81, 3.59 and 1.53 respectively.

The other possible metabolites of sulphanilamide, which give coloured solutions with ferric chloride, are *p*-*N*-acetylhydroxylaminobenzenesulphonamide, 2-hydroxysulphanilamide and *p*-aminophenol. Of these only *p*-aminophenol gives the ferric chloride reaction in ether, and in this case the colour obtained is red (with no blue) showing an absorption maximum at 510 $m\mu$. (see Fig. 1). This colour is much

weaker than the one obtained with 3-hydroxysulphanilamide, and it is clear from Fig. 1 that if *p*-aminophenol were present with 3-hydroxysulphanilamide it could not be detected. However, *p*-aminophenol can be eliminated if one takes into account the brownish polymerization product of oxidized *p*-aminophenol. This product shows a band at 395 $m\mu$. which is easily distinguishable from the 440 $m\mu$. band of the polymer of oxidized 3-hydroxysulphanilamide.

Fig. 5. Absorption spectrum of oxidized *p*-aminophenol in ethanol. $\lambda_{\max}$. 234, 302 and 395 with $\log \epsilon_{\max}$. 3.86, 3.38 and 2.51 respectively.

The 395 $m\mu$. band was never encountered in the spectra of any of the urine extracts examined. *p*-Aminophenol (British Drug Houses Ltd.) as taken from the bottle is a black crystalline solid, and its absorption spectrum shows maxima at 234, 302 and 395 $m\mu$. (see Fig. 5). This indicates that ordinary commercial *p*-aminophenol is already in a partly oxidized state, for earlier determinations of the

spectral absorption of *p*-aminophenol only record the first two maxima (cf. Morton & Stubbs, 1940).

The colour reactions of the above-mentioned compounds are summarized in Table 1, which also includes salicylic acid since it was encountered in one of the human urines examined.

RESULTS

The detection of 3-hydroxysulphanilamide in the urine of hospital patients

(1) *Patient P.M.* The urine (3 l.), collected during a course of 26 g. of sulphanilamide, was taken to dryness under reduced pressure. The residue was dissolved in 100 ml. hot water and treated with 50 ml. conc. HCl. The mixture was boiled gently for 2 hr. After cooling the solution was made faintly acid to litmus with 20% (w/v) NaOH and, when cold, filtered. The filtrate was extracted with ether for 5 days in a continuous extractor. The extract was evaporated to a brownish mass of syrupy crystals (7 g.) and as much sulphanilamide as possible was removed by recrystallization from water. 4.3 g. of sulphanilamide, m.p. 162°, were obtained. The filtrate was dried *in vacuo* to a syrupy residue (2.6 g.) which gave colour tests for 3-hydroxysulphanilamide although containing much sulphanilamide. These were separated by chromatography.

The syrupy residue was dissolved in 50 ml. ethanol and 150 ml. dry benzene added. The solution was then chromatographed on an alumina column (6 × 3/4 in.; aluminium oxide, standardized according to Brockmann; Savory and Moore Ltd., London) which was then eluted by ethanol until the eluates gave no diazo test. The eluates yielded 1.8 g. of sulphanilamide. The 3-hydroxysulphanilamide remained on the column forming a yellow band. The latter was separated from the column and exhaustively extracted with hot water. The extract was evaporated under reduced pressure to a clear yellowish glass (0.6 g.) which could not be induced to crystallize. It gave all the qualitative tests

Table 1. *The colour reaction between certain phenols and ferric chloride in water and in ether*

Compound	Colour produced in		$\lambda_{\max}$. of the absorption band of coloured ethereal solution ($m\mu$.)
	Water	Ether	
<chem>Nc1ccc(O)cc1S(=O)(=O)N</chem>	Purplish red → red → brown	Intense red (containing some blue)	530
<chem>Nc1ccc(O)cc1S(=O)(=O)N</chem>	Do.	Pale yellow	—
<chem>Nc1ccc(O)cc1S(=O)(=O)N</chem>	Faint purple-red	None	—
<chem>CC(=O)Nc1ccc(O)cc1S(=O)(=O)N</chem>	Blood-red	None	—
<chem>Nc1ccc(O)cc1O</chem>	Purple-red	Red (no blue)	510
<chem>Oc1ccc(cc1)C(=O)O</chem>	Purple	None	—

for 3-hydroxysulphanilamide (Williams, 1946). For the spectroscopic test, 0.3 g. of the glass was dissolved in 50 ml. water and then 1 ml. of 2% FeCl_3 followed by 50 ml. peroxide-free ether were added. The mixture was shaken and the red ether layer separated and filtered. The absorption spectrum of the ethereal solution showed a band with a maximum at 530 $\text{m}\mu$.

(2) *Patient J.H.* The urine (3 l.) was collected during a course of 24.5 g. of the drug. It was concentrated, hydrolyzed and extracted as in the first case. The weight of material extracted in 7 days was 9.83 g., and this yielded 6.1 g. of sulphanilamide leaving a residue of 2.35 g. for chromatography. The yellow band on the column was prepared and extracted as in the first case, and the spectrogram (Hilger E3 Quartz Spectroscope) of the ether/ FeCl_3 test is recorded in Fig. 1.

(3) *Patient J.K.* The urine (6.32 l.) was collected during a course of 44 g. of sulphanilamide with sodium salicylate. The urine was treated as above, and the material extracted by ether weighed 15.8 g. and contained salicylic acid. Sulphanilamide and some salicylic acid were removed by crystallization from water. The residue (4 g.) for chromatography contained 3-hydroxysulphanilamide, sulphanilamide and salicylic acid. These were separated as follows:

The residue was dissolved in a mixture of 50 ml. ethanol and 50 ml. dry benzene. This solution was passed through an alumina column (35 $\times$ 1 cm.) which was eluted with ethanol until both sulphanilamide and its phenol had been removed. The salicylic acid remaining on the column was extracted with hot water and purified (m.p. and mixed m.p. 153–155°) by recrystallization from acidulated water.

The eluate containing sulphanilamide and the phenol was now taken to dryness and the residue (1.5 g.) dissolved in 25 ml. ethanol and 75 ml. dry benzene. The solution was chromatographed on alumina (column, 20 $\times$ 2 cm.) and the sulphanilamide washed through with ethanol. 3-Hydroxysulphanilamide remained on the column as a yellow band. Part of this band was used for the colour test with positive results. Attempts to isolate the crystalline phenol from the rest of the band failed.

3-Hydroxysulphanilamide in rabbit urine

The colour reaction in this case was carried out on the urine of sulphanilamide-fed rabbits from which pure crystalline 3-hydroxysulphanilamide had been separated by chromatography (see Williams, 1946). The spectral absorption of the urinary product was determined with the following results:

$\lambda_{\text{max.}}$ ($\text{m}\mu$.) $\log \epsilon_{\text{max.}}$	Urinary 3-hydroxysulphanilamide		Synthetic 3-hydroxysulphanilamide	
	259	291	259	292
	4.04	3.81	4.03	3.82

A portion of the alumina column from which the crystalline phenol was isolated was extracted with water as described above for the human urines. The ether/ FeCl_3 test was carried out on this extract. The ether solution had a deep red colour, and its spectral absorption is recorded in Fig. 1. In this case a weak band with $\lambda_{\text{max.}}$ 440 $\text{m}\mu$. was found as well as the band at 530 $\text{m}\mu$. Here evidently the

oxidation had proceeded to some extent beyond the red pigment stage to form some of the brownish material with a band at 440 $\text{m}\mu$.

Detection of 3-hydroxysulphanilamide in guinea-pig urine

Six guinea-pigs were kept for a week on a diet of crushed Lever's cubes containing 1% added sulphanilamide, and cabbage. This diet was only partly eaten. The week's urine (710 ml.) was analyzed for free and total sulphanilamide by the Bratton & Marshall method and was found to contain 1.98 g. total and 1.01 g. free sulphanilamide. The urine was worked up by the procedure used for human urines and very small amounts of 3-hydroxysulphanilamide were detected.

PREPARATION OF COMPOUNDS

3-Hydroxysulphanilamide (m.p. 164°). Some improvements were made in the synthesis of Thorpe & Williams (1941). Benzoxazolone was previously prepared in poor yields from salicylamide according to Graebe & Rostovzeff (1902). 50% yields of this compound have been recorded by Scudi & Buhs (1941) using *o*-aminophenol and phosgene. It has now been found that this compound can be prepared in yields of over 75% by simply heating *o*-aminophenol with urea.

o-Aminophenol (50 g.) and urea (50 g.) were heated together on an oil-bath at 180° (bath temp.) until the evolution of ammonia had ceased (c. 1–1.5 hr.). The cooled solid product was recrystallized, and decolorized with charcoal, from a large volume of hot water. The yield of pure benzoxazolone, m.p. 140–141°, was 47 g. (76% of calc.).

Benzoxazolone was converted into 2-oxybenzoxazole-6-sulphonyl chloride according to Scudi & Buhs (1941) and then into the amide. Benzoxazolone sulphonamide was converted into 3-hydroxysulphanilamide by gentle heating with 20% (w/v) NaOH. On cooling and neutralizing with HCl, the phenol crystallized out (cf. Thorpe & Williams, 1941). It was recrystallized from water.

2-Hydroxysulphanilamide (m.p. 154°) was a sample prepared by Thorpe & Williams (1941).

4-Hydroxymetanilamide (m.p. 202°) was prepared essentially according to Kermack, Spragg & Tebrich (1939) (cf. Williams, 1942).

p-N-Acetylhydroxylaminobenzenesulphonamide (*N*⁴-acetyl-*N*⁴-hydroxysulphanilamide), m.p. 223°, was prepared from *p*-hydroxylaminobenzenesulphonamide (1 mol.) and acetic anhydride (1 mol.) according to Burton & Walker (1943).

It was found that if an excess of acetic anhydride were used the product was *p*-O:N-diacylhydroxylaminobenzesulphonamide (*N*⁴-acetyl-*N*⁴-acetoxyulphanilamide). This diacyl compound appears to be new. On recrystallization from water it forms large faintly yellow prisms, m.p. 171°. (Found: C, 44.2; H, 4.55; N, 10.3; S, 11.4. $\text{C}_{10}\text{H}_{12}\text{N}_2\text{O}_8\text{S}$ requires C, 44.1; H, 4.4; N, 10.3; S, 11.8%.)

p-Aminophenol (British Drug Houses Ltd.) was converted into the hydrochloride and used as this salt, thus ensuring that it was largely in the reduced form.

DISCUSSION

With the isolation of crystalline 3-hydroxysulphanilamide from rabbit urine (Williams, 1946) and its detection in human urine by the colour reactions described above, it is felt that a reasonable proof as to the exact chemical nature of the biological oxidation product of sulphanilamide has been provided.

These results have an important bearing upon certain theories of the mode of action of sulphanilamide. Early theories of sulphonamide action, such as those of Mayer & Oechsli (1937) and of Locke, Main & Mellon (1938), postulated that the antibacterial activity of sulphanilamide was due to a metabolic oxidation product, and *p*-hydroxylaminobenzenesulphonamide was considered to be the most likely compound to fill this role. The correctness or otherwise of these theories, however, cannot be proved until the exact chemical nature and the antibacterial properties of the oxidation product have been established. The case against *p*-hydroxylaminobenzenesulphonamide has been considered in an earlier paper (Williams, 1946) and need not be discussed further.

As 3-hydroxysulphanilamide is undoubtedly the oxidation product involved, its antibacterial properties have been tested *in vivo* and *in vitro* (see Appendixes 1 and 2). *In vivo* it has no therapeutic action against haemolytic streptococci in mice, but *in vitro* it shows definite antibacterial activity although of a lesser order than sulphanilamide. Its *in vivo* inactivity may be due to too rapid excretion and/or conjugation to form an inactive glucuronide (Williams, 1943) and an ethereal sulphate. Its slight activity *in vitro* is not unexpected for a number of substituted *o*-aminophenols have been found to be antibacterial *in vitro* (Barber & Haslewood, 1944, 1945; Martin & Rose, 1945). Its *in vitro* activity is, however, different from that of sulphanilamide for it is less affected by the numbers of bacteria than sulphanilamide. A possible explanation for this is that on standing in solution it is partly converted to the quinonoid form which may be more active than the reduced form.

The activity of sulphanilamide is unlikely to be

due to an oxidation product for the following reasons:

(a) The amount of oxidation product, i.e. 3-hydroxysulphanilamide, produced is small and is not more than 10% of the dose of sulphanilamide (cf. Shelswell & Williams, 1940).

(b) The antibacterial activity of 3-hydroxysulphanilamide when tested *in vitro* is less than that of sulphanilamide.

(c) When tested *in vivo*, 3-hydroxysulphanilamide shows no therapeutic activity.

It is felt, therefore, that these results dispose of those theories which postulate that the activity of sulphanilamide is due to oxidation products.

It is well known that treatment with sulphanilamide often produces cyanosis, and methaemoglobin and occasionally sulphaemoglobin can be detected in the blood. It is possible that 3-hydroxysulphanilamide is involved here, for it can set up a reversible oxidation-reduction system

which could result in the conversion of haemoglobin into methaemoglobin or into sulphaemoglobin if sulphide is present. *In vitro*, however, 3-hydroxysulphanilamide does not convert haemoglobin to methaemoglobin.

SUMMARY

1. A colour test is described for the detection of 3-hydroxysulphanilamide in urine. The theoretical basis of the test is discussed.

2. By means of this test 3-hydroxysulphanilamide has been detected in the urine of hospital patients treated with sulphanilamide and also in the urine of rabbits and guinea-pigs similarly treated.

3. The absorption spectra of 3-hydroxysulphanilamide, 4-hydroxymetanilamide and *p*-aminophenol have been recorded and discussed in relation to the colour test developed.

4. The bearing of the above results, together with those quoted in the Appendixes to this paper, on certain theories of sulphanilamide action is discussed.

The author wishes to thank Prof. R. A. Morton for assistance with the spectroscopic part of the work, Drs A. R. Martin (Imperial Chemical Industries Ltd.) and M. G. McEntegart for testing 3-hydroxysulphanilamide for antibacterial activity and Dr I. Ansell for the human sulphanilamide urines. The expenses of the work were in part defrayed by a grant from the Medical Research Council.

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Appendix 1. The *in vivo* Antibacterial Activity of 3-Hydroxysulphanilamide

3-Hydroxysulphanilamide was tested *in vivo* for antibacterial activity by Dr A. R. Martin. The following experiment, quoted from Dr Martin's report, shows that 3-hydroxysulphanilamide gives no protection to mice infected with *Streptococcus pyogenes*.

Groups of six mice were infected intraperitoneally with 0.2 ml. of a 1:100,000 dilution of an 18 hr.

culture of *Streptococcus pyogenes* in blood-broth. The drugs used were prepared as dispersions and were given subcutaneously or intraperitoneally. Two doses were given daily, in the morning and in the evening, the first dose being given shortly before infection. Treatment was continued for 3 days where the mice were still alive. The results of one experiment are quoted in Table 2:

Table 2. *Treatment of an experimental streptococcal infection in mice with sulphanilamide and 3-hydroxysulphanilamide*

Treatment	Dose (mg./20 g.)	No. of deaths on		Total deaths	Mean* survival time
		Day of infection	1st day 2nd day		
None (controls)	—	—	4 2	6	0.8
Sulphanilamide, twice daily, subcutaneously	10	—	— 5	5	2.0
	5	—	— 6	6	1.0
	2	—	5 1	6	0.8
3-Hydroxysulphanilamide, twice daily, subcutaneously	10	—	6 —	6	0.8
	5	—	5 1	6	0.8
	2	—	6 —	6	0.8

* Calculated according to Whitby (1938).

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Appendix 2. The *in vitro* Bacteriostatic Activity of 3-Hydroxysulphanilamide

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Pure synthetic 3-hydroxysulphanilamide was tested for bacteriostatic activity in parallel with sulphanilamide. In the preliminary tests, use was made of 'ditch plates' using 5.0% lysed horse-blood agar to overcome any possible sulphanilamide inhibiting effect of the agar (Harper & Cawston, 1945). Plates were prepared with increasing dilutions of the test substances and were inoculated with streaks, at right angles to the ditch, of a 24 hr. culture of a known sensitive haemolytic streptococcus and of 10-fold dilutions of it—7 streaks being made in all. It was observed that in effective concentrations, 3-hydroxysulphanilamide produced inhibition irrespective of the size of the inoculum whereas

Table 3. *In vitro bacteriostatic activity of 3-hydroxysulphanilamide*

Agar ditch method.

Organism used: β -haemolytic streptococcus.

Inoculum: 1 in 10,000 dilution of a 24 hr. broth culture.

Compound	Concentration in agar ditch (%)	Distance from edge of ditch for which bacterial growth was inhibited (mm.)
3-Hydroxy-sulphanilamide	0.33	15
	0.10	10
	0.03	0 (no inhibition)
Sulphanilamide	0.33	20-30
	0.10	25
	0.03	25

sulphanilamide did not produce inhibition of the undiluted inoculum even in concentrations of 1.0%. The results in Table 3 were obtained with a 1:10,000 dilution of the 24 hr. culture of the test organism as inoculum.

The two compounds were tested for activity against a haemolytic streptococcus in the presence of blood using the slide-cell method (Fleming, 1944) with the following results (see Table 4) after 24 hr. (inoculum approximately 100 bacteria per cell).

Comment

The results of the above observations show, that by the three methods of testing—in agar, in peptone water and in blood—the bacteriostatic concentration effective against a haemolytic streptococcus of 3-hydroxysulphanilamide is 4–10 times that of sulphanilamide when a small number of bacteria is used as inoculum. When *S. aureus* is used as the

Table 4. In vitro bacteriostatic activity of 3-hydroxysulphanilamide

		Concentration, 1 in								
		400	800	1600	3200	6400	12,800	25,600	51,200	124,000
Compound	3-Hydroxysulphanilamide	—	—	+	+	+	+	+	+	+
	Sulphanilamide	—	—	—	—	—	—	—	+	+

+ indicates growth of streptococcus after 24 hr.
— indicates no growth of streptococcus after 24 hr.

Further tests were carried out with serial dilutions of the compound in glucose peptone water against a haemolytic streptococcus and the Oxford *Staphylococcus aureus*. These tests were put up in duplicate with different numbers of bacteria in the inoculum (Table 5).

A *S. aureus* strain which was resistant to penicillin gave results similar to those with Oxford staphylococcus. Strains of *E. coli*, *Ps. pyocyanea* and *B. subtilis* were found to be insensitive to either compound.

test organism in broth, 3-hydroxysulphanilamide was at least as active as sulphanilamide (Table 5).

Apart from this difference in relative activities of the two compounds against haemolytic streptococci and *S. aureus* the range of bacteriostatic activity against different species of bacteria seemed to be the same for the two compounds.

Whereas the activity of 3-hydroxysulphanilamide was generally less than that of sulphanilamide, the size of the inoculum of haemolytic streptococci

Table 5. In vitro bacteriostatic activity of 3-hydroxysulphanilamide

		Concentration of drug, 1 in								
		Approx. no. of bacteria	Observation made after hr.	400	800	1600	3200	6400	12,800	25,600
3-Hydroxysulphanilamide	Haemolytic streptococcus	10,000	24	—	—	+	+	+	+	+
		10,000	48	—	—	+	+	+	+	+
		100	24	—	—	—	+	+	+	+
		100	48	—	—	+	+	+	+	+
	<i>S. aureus</i>	10,000	24	—	+	+	+	+	+	+
		10,000	48	—	+	+	+	+	+	+
		100	24	—	—	+	+	+	+	+
		100	48	—	+	+	+	+	+	+
Sulphanilamide	Haemolytic streptococcus	10,000	24	—	—	—	+	+	+	+
		10,000	48	+	+	+	+	+	+	+
		100	24	—	—	—	—	—	+	+
		100	48	—	—	—	—	+	+	+
	<i>S. aureus</i>	10,000	24	+	+	+	+	+	+	+
		10,000	48	+	+	+	+	+	+	+
		100	24	—	—	+	+	+	+	+
		100	48	+	+	+	+	+	+	+

+ indicates bacterial growth. — indicates no bacterial growth.

affected the results with sulphanilamide. While increase in the inoculum within the range tested did not affect the action of 3-hydroxysulphanilamide it

had a marked effect in reducing the action of sulphanilamide both on solid and liquid media (Table 5).

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The Toxicity of Arsenic Solutions for Tissue Slices

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When arsenic enters the blood stream from the lungs, it is rapidly fixed by the red cells. After a short latent period, haemolysis occurs. It has been generally held that no arsenic entering the body can escape reaction with the erythrocytes, and that poisoning of vital organs is thus due entirely to the substances liberated from the red cells on lysis. In recently published work, Levy (1946), however, has shown that the lethal dose of arsenic for mice, as measured by the arsenic absorbed in exposures to the gas causing 50 % mortality, which varies inversely with the gas concentration, can be as little as 0.67 mg./kg. At this value for the median lethal dose, arsenic is much more toxic than arsenites, the most toxic arsenic derivatives likely to be formed in the reaction between the gas and the erythrocytes.

In the same paper (Levy, 1946) experiments were described in which the arsenic content was determined of tissues from rabbits killed immediately after short exposures to arsenic. Although the tissues were freed from blood before the onset of haemolysis, certain organs were found to contain considerable amounts of arsenic. Liver had the highest arsenic content, while kidney and lung also contained comparatively large quantities. It was concluded that this arsenic represented a fraction of the arsenic which had been removed from physical solution in the plasma before it could be fixed by the red blood corpuscles. None of the arsenic fixed in this way is released until haemolysis commences (Thauer, 1934). Since it seemed possible that the effects of unchanged arsenic on vital organs might explain, partly at least, the high toxicity of the gas for the whole animal, it was decided to determine the toxicity of arsenic, in comparison with that of an arsenite, for surviving liver and kidney slices.

EXPERIMENTAL

To determine the toxicity of arsenic for the tissue slices, it was not considered adequate to shake them in suspension in Ringer solution with a gas phase containing the arsenic derivative, since, to compare the effects with those produced by arsenite solutions, assumptions would have been required as to the distribution of arsenic between the gas and liquid phases. A technique was accordingly developed for treating the surviving tissues with arsenic solutions in absence of a gas phase, under conditions resembling as closely as possible those obtaining *in vivo*.

Method

Rat-liver or kidney slices were treated for exactly 20 min. at 37° with the arsenic solution in a closed vessel completely filled with liquid. The solution was composed of a mixture of oxygenated Ringer solution (50–90 % of the total volume) and Ringer solution (10–50 % of the total volume) saturated with arsenic in hydrogen. Other slices from the same animal were treated with either pure Ringer solution or with a solution of arsenite in Ringer solution, both of which contained quantities of oxygen and hydrogen similar to those present in the arsenic vessel. Sufficient oxygen was always present to supply the tissue slices for 20 min. After this treatment, the oxygen consumption of the slices was measured by the Warburg technique (Dixon, 1934). Phosphate Ringer solution (Krebs, 1933) containing glucose (0.2 %) was used in all stages of the experiments.

Procedure

The gas towers. In all, four towers were required for saturating Ringer solutions with various gases. Each contained 200 ml. fluid. If these towers are numbered 1, 2, 3