depleted of its normal potassium content, small increases occurred on incubation with the two substrates.

6. It is suggested that glucose (or lactate or pyruvate) is required as a source of energy, whilst

L-glutamate serves another function in potassium transport.

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Studies on Suramin

8. THE ACTION OF THE DRUG ON ENZYMES AND SOME OTHER PROTEINS. GENERAL CONSIDERATIONS

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Suramin has a very powerful trypanocidal action *in vivo*, and furthermore, it has a prolonged prophylactic action; e.g. one injection of 1 or 2 g. may protect an individual against sleeping sickness for several weeks or even months. This long protection can almost certainly be accounted for by the long retention of the drug in the blood plasma and tissues, as a result of its firm combination with the body proteins (see Bournsnel & Wormall, 1939; Dewey & Wormall, 1946). The precise mode of action of suramin on trypanosomes or on the body defence mechanisms is, however, not known.

We have studied the action of suramin on proteins as a preliminary to an investigation of the direct action of the drug on trypanosomes and more particularly on the enzymes of the trypanosome. Some evidence is already available that suramin inhibits certain enzymes. Relatively high concentrations ($M/140$) inhibit trypsin (Beilinson, 1929), $M/6000$ suramin is toxic to fumarase but not to urease (Quastel, 1931), and it strongly inhibits hyaluronidase (Beiler & Martin, 1948). According to Stuber & Lang (1926) it retards blood glucolysis, but no evidence of this action was found by Tcherniakofsky & Nattan-Larrier (1937). In spite of its

capacity to combine firmly with all the proteins we have tested, suramin is not a general enzyme poison.

This and the following paper describe investigations made with the following objects: (i) A study of the action of suramin on some enzymes taking part in carbohydrate metabolism. (ii) A qualitative and quantitative study of the combination of the drug with proteins and some enzymes, to determine as far as possible the nature of the protein groups involved and the number of molecules of the drug required to inactivate suramin-sensitive enzymes. (iii) Investigations on the relationship between concentrations required to inhibit some enzymes and the amounts of the drug present in the blood of injected animals. Preliminary accounts of a few of these experiments have been briefly recorded (Town, Wills & Wormall, 1949*a*, *b*; Town & Wormall, 1949; Wills & Wormall, 1949).

EXPERIMENTAL

Suramin determinations. These were made as described previously (Dangerfield, Gaunt & Wormall, 1938; Bournsnel, Dangerfield & Wormall, 1939). Where the amount of drug present was too small to permit the use of the Tintometer disks, e.g. in some of the enzyme studies, standards were made from acid-hydrolysed suramin suitably diluted with

3N-HCl. The suramin values for plasma and suramin-protein complexes have been corrected for small blank values (equivalent to suramin values of about 1 mg./100 ml. of rabbit plasma and 0.1–0.2 mg./g. of protein).

Enzyme activity determinations. Full details of the methods used are given in the following paper (Wills & Wormall, 1950).

Total nitrogen determinations. These were made by the micro-Kjeldahl method, using the distillation apparatus described by Markham (1942). The NH_3 was distilled into boric acid containing a modified Tashiri indicator and titrated with 0.0143 N-HCl (cf. Kabat & Mayer, 1948). Where the amount of material available was small, an 'Aglar' micrometer syringe (Burroughs Wellcome and Co.) was used for the titration. Suramin gives the theoretical value (5.88% N) by this technique.

Suramin injections. A solution of the drug in 0.9% (w/v) NaCl was injected into the marginal vein of one ear of a rabbit and all blood samples were taken from the other ear. Except in special circumstances the dose given was, on a body-weight basis, within the range used therapeutically for man (i.e. 1–2 g./70 kg. man, or 14–28 mg./kg.).

RESULTS

Plasma suramin contents following the intravenous injection of the drug into rabbits

Relatively little detailed information is available about the plasma suramin concentrations of man and other animals following the intravenous injection of the drug. Of necessity the observations on man have often involved a few isolated determinations on sleeping sickness patients receiving one or a series of injections, and the experiments with rabbits and other small animals have often involved the injection of much larger doses than are used therapeutically for man. We decided, therefore, to determine changes of plasma suramin concentration of rabbits over a period of several weeks after the injection of amounts of suramin equivalent on a body-weight basis to the 1–2 g. doses given at each injection to men suffering from sleeping sickness.

In general, a comparison of our results with rabbits and those of others on man (cf. Hawking, 1940) during the first few days after the suramin injection show that there is a general similarity between the two animal species with regard to the initial retention of injected suramin. The 'normal' dose of suramin given to man varies very appreciably. Many authorities recommend an initial dose of 0.5 g., followed by doses of 1 g. a few days later and subsequently at weekly intervals up to a maximum total of about 10 g. Others advocate doses of 1.5–2.0 g., particularly where a single prophylactic injection is being given. In our experiments we decided to confine ourselves to single injections and give doses of 14 and 28 mg./kg. (equivalent to 1 and 2 g., respectively, for a 70 kg. man).

Three rabbits (group 1; nos. 780, 632 and 779, weighing 2.5, 2.9 and 2.45 kg., respectively) each received an intra-

venous injection of 28 mg. of suramin/kg. Samples of blood (6–14 ml.) were taken at intervals, treated with K oxalate and immediately centrifuged and the plasma separated. Suramin and total N determinations were made on suitable portions of these plasmas. A second group of rabbits (nos. 468, 630 and 790, weighing 3.3, 2.4 and 2.45 kg., respectively) each received an injection of 14 mg. of suramin/kg.

The results of these experiments are given in Figs. 1 and 2. They confirm and extend our previous observations (Dangerfield *et al.* 1938; Bournsnel *et al.* 1939). A month after the injection of 28 mg. of

Fig. 1. Plasma suramin after the intravenous injection of 28 mg. of suramin/kg. (averages for three rabbits).

Fig. 2. Plasma suramin after the intravenous injection of 14 mg. of suramin/kg. (averages for three rabbits).

suramin/kg. the rabbits had a plasma suramin concentration of 1–2 mg./100 ml., and with a single injection of 14 mg./kg., the plasma suramin concentration one month later was usually 0.5–1.0 mg./100 ml.

The average values for each group of rabbits gave smooth curves, but there were occasionally appreciable differences between the responses of individual rabbits in any one group, as was observed in investigations on the retention of suramin in the kidney and other tissues (Bournsnel & Wormall, 1939). Hawking (1940) has found that in man also there is a tendency

for marked variations to occur in the capacity to retain suramin in the blood. Rarely, however, was the plasma suramin concentration very much below the appropriate average value given in Figs. 1 and 2.

A few typical results for the individual rabbits of group 1 are shown in Table 1. These, and our other (unpublished) results, show that rabbits injected intravenously with 28 mg. of suramin/kg. invariably

the injected drug in the blood samples taken during the first 24 hr., and the plasma would almost certainly have contained appreciably greater amounts of suramin than those recorded in Figs. 1 and 2 if the animals had not been bled frequently; it is not easy, however, to make any simple correction for this loss. The plasma protein level fell by 12–18 % during the period 1–20 hr. after the injection, but it cannot be assumed that the suramin level is affected to the same extent.

Table 1. *Plasma suramin contents of rabbits injected with 28 mg. of suramin/kg.*

Time after injection	Plasma suramin (mg./100 ml.)		
	Rabbit no. 780	Rabbit no. 632	Rabbit no. 779
5 min.	71	68	68
20 min.	53	54	49
1 hr.	31	36	30
3 hr.	19.4	22.1	18.2
26 hr.	5.4	11.2	5.5
14 days	2.3	3.6	1.9
7 weeks	0.7	1.1	0.7

have a plasma suramin content of at least 70 mg./100 ml. for 3 or 4 min. after the injection, and at least 30 mg./100 ml. at the end of 1 hr. These amounts correspond, respectively, to concentrations of M/2000 and M/5000 suramin (cf. Fig. 1), and this is appreciably above the minimum concentration required to inactivate completely certain enzymes. Furthermore, it should be remembered that, in the experiments involving frequent bleeding of the rabbits, the withdrawal of blood results in the removal of a significant amount of suramin. The rabbits in group 1, for example, lost about 15 % of

The state of the suramin in the blood of injected rabbits

When suramin is injected into the blood stream, most of the injected drug rapidly becomes attached to the plasma proteins, but it is probable that a significant fraction remains free, or at least is only loosely attached to the proteins. We have, therefore, measured, over a wide range of plasma suramin concentrations, the amount of firmly bound suramin in the plasma of rabbits injected with the drug. Suramin which is firmly bound to protein is not removed by methanol, in which suramin is very soluble, and in these experiments we have precipitated and washed the plasma suramin-protein complexes with methanol, to remove as far as possible the loosely attached suramin.

Some of the plasma samples collected from the rabbits of group 1 referred to in the previous section were treated with methanol (6 vol.) and the mixtures centrifuged as soon as possible. Each precipitate was washed twice with methanol (6 vol. each time) and then dried *in vacuo* over CaCl_2 . A suramin determination was made on the whole of each

Table 2. *Methanol-precipitated drug-protein complexes from the plasma of rabbits injected with suramin (28 mg./kg.)*

(The experimental details are given in the text, above. Control experiments with normal rabbit plasma showed that the proteins precipitated by, and washed with, methanol gave an average value equivalent to 0.5 mg. of suramin/100 ml. plasma, and the values for the suramin-containing plasmas have been accordingly corrected for this blank.)

Rabbit no.	Time after injection	Whole plasma	Suramin content of	
			Precipitated complex	
			mg. complex precipitated from 100 ml. plasma	As % of total plasma suramin
780	2 min.	74	54	73
	5 min.	71	51	72
	10 min.	62	46	74
	20 min.	53	40	75
	1 hr.	31	22	71
	3 hr.	19.4	16.1	83
	20 hr.	6.9	6.2	90
	72 hr.	4.2	3.8	90
	14 days	2.3	1.9	(82)
779	5 min.	68	50	73
	20 min.	49	35	71
	1 hr.	29.6	20.7	70
	3 hr.	18.2	12.7	84
	20 hr.	7.0	5.9	84
	14 days	1.9	1.8	(94)

sample, and also on precipitates obtained similarly from normal rabbit plasma.

Typical results of these experiments are given in Table 2. Most of the injected drug, certainly over 70 %, became firmly attached to the plasma proteins during the first 2 or 3 min. after the injection, and during the next 24 hr. there was a slight fall in the percentage of total plasma suramin present in the 'free' state. Even after 3 or 4 days, however, the amount of 'free' suramin was quite significant, and the same may be true after 14 days, but the suramin contents of the plasma and the precipitated complexes were then so small that any difference between the two values has little quantitative significance.

Combination of suramin and egg albumin

With many proteins it is often possible to precipitate a suramin-protein complex merely by adding a neutral solution of the drug to the neutral protein solution. Precipitation only occurs, however, within a certain range of suramin concentration, and with excess of either suramin or protein there is usually no precipitate. We have studied this precipitation with egg albumin in order to determine the best conditions for obtaining the insoluble complexes, and have also estimated the relative molecular amounts of drug and protein in these complexes. The tests were made in quadruplicate, so that two precipitates of each set could be lightly washed with 0.9 % sodium chloride (to remove adhering supernatant liquid) and two with methanol (to remove all the loosely attached suramin).

Exp. 1. Samples (5.0 ml.) of a neutral 9.1 % (w/v) solution of crystalline egg albumin were treated with amounts of suramin ranging from 0.2 to 5.0 mg. dissolved in 0.9 %

NaCl (1 ml.). All the mixtures immediately gave turbidities, and discrete particles gradually separated. The mixtures were kept at 19° for 2 hr., and then centrifuged. The inner walls of two tubes of each set were cleaned with filter paper and the precipitates were washed quickly with 0.9 % NaCl (3 ml.) and then dissolved in saline with the aid of a little $n\text{-Na}_2\text{CO}_3$. The other two precipitates were washed three times with methanol (3 ml. each time) and then dissolved in saline with the aid of a little $n\text{-NaOH}$. The solutions of the washed precipitates were diluted to 5 ml. and suramin and total N determinations were made on samples.

Exp. 2. This was similar to Exp. 1, but a more dilute egg albumin solution and a slightly wider range of suramin concentration were used.

The results of these experiments (Table 3) show that even with a wide range of suramin concentration the composition of the precipitated complexes does not vary very greatly. In Exp. 1, for example, the saline-washed precipitate from the mixture containing 0.4 mg. of suramin had a suramin:egg albumin molecular ratio of 2.3, and 12.5 times this amount of suramin gave a precipitate with a ratio of 3.6. The precipitates washed with methanol contained, as was expected, less suramin than did those lightly washed with saline, and the molecular ratios were lower, possibly because any egg albumin freed from loosely combined suramin would in this case remain in the precipitate. From these and similar experiments it has been found that where these complexes separate from drug-protein mixtures, a large part of the suramin originally present may be found in the precipitated complex. In most of the mixtures referred to in Table 3, for example, over 40 % of the suramin was found in the precipitate and occasionally the amount was over 75 %.

Table 3. *Combination of suramin and egg albumin*

(In Exp. 2 none of the precipitates was washed with methanol. See above for experimental details.)

Exp. no.	Egg albumin concn. in final mixture (g./100 ml.)	Suramin added/6 ml. (mg.)	Suramin content of precipitates washed with		Suramin in precipitate as % of that added		Molecular ratio of suramin/egg albumin	
			0.9 % NaCl	Methanol				
			(a) (g./100 g.)	(b) (g./100 g.)	(a)	(b)	(a)	(b)
1	7.6	5.0	10.6	4.6	12	13	3.6	1.43
		2.0	8.9	4.0	30	29	3.0	1.27
		1.0	9.3	4.3	53	47	3.1	1.33
		0.8	9.0	3.7	61	49	3.0	1.14
		0.6	8.2	4.0	62	68	2.7	1.26
		0.4	7.1	3.1	53	65	2.3	1.02
		0.2	5.2	2.9	45	55	1.7	0.90
2	4.2	17.1	13.4	—	4	—	4.7	—
		8.6	12.7	—	12	—	4.5	—
		2.1	9.5	—	51	—	3.3	—
		1.07	10.2	—	81	—	3.4	—
		0.53	5.6	—	79	—	1.8	—
		0.26	3.6	—	58	—	1.3	—
		0.09	1.3	—	17	—	0.37	—

Other experiments have been carried out in which the pH of the mixtures of suramin and egg albumin was controlled by buffers between pH 6.5 and 8.0.

To the egg albumin solution (6.0 ml. of 8.0% (w/v) crystalline egg albumin) 0.2N-NaOH was added to give the desired pH, followed by 0.2M-phosphate (2 ml.) and water (0.0-18 ml.) to bring all the mixtures to the same volume. Suramin (1.0 ml. of a neutralized 0.03 or 0.10% (w/v) solution) was then added and the mixture was shaken. Precipitates appeared at once in all the tubes, and after the mixtures had stood at about 15° for 2 hr. they were centrifuged. Each precipitate was washed once with 4 ml. of a 0.05M-solution of the appropriate buffer and then dissolved in water (5 ml.) plus 1 drop of 0.5N-NaOH. Suramin and total N determinations were made on these solutions.

The results (Table 4) showed that the composition of these complexes which separated from buffered mixtures of egg albumin and suramin varied very little over the range pH 6.5-8.0. They contained

Table 4. *Insoluble egg albumin-suramin complexes formed over the range pH 6.5-8.0*

(The results given below are the averages of duplicate experiments.)

pH	Suramin added to 6 ml. of 8.0% egg albumin (mg.)	Precipitated complex	
		Suramin content (g./g. total N)	Suramin/egg albumin (mol./mol.)*
6.5	0.3	0.55	2.7
7.0	0.3	0.49	2.4
7.5	0.3	0.56	2.8
8.0	0.3	0.50	2.5
6.5	1.0	0.61	3.0
7.0	1.0	0.52	2.6
8.0	1.0	0.67	3.3

* Calculated on the basis of 15.76% N and a mol. wt. of 44,000 for egg albumin.

2.4-3.3 molecules of suramin : molecule of egg albumin, and similar results were obtained with precipitates which separated at pH 7.5 and 8.0 from mixtures of egg albumin and suramin prepared as in the above-described experiment but with 0.75M-sodium bicarbonate-carbonic acid instead of the phosphate buffer. From these results it appears that the combination of egg albumin with two or three molecules of suramin results in the formation of an insoluble complex.

Combination of suramin and urease

Suramin has a strongly inhibitory effect on urease at pH 5 (Wills & Wormall, 1949, 1950), and we decided, therefore, to study the amount of the drug which can be bound by the enzyme.

Crystalline urease was prepared from jack bean meal as described by Sumner & Somers (1947) and dissolved in glass-distilled water to give a neutral solution containing

17.95 mg. N/100 ml. Samples (1 ml.) of this solution were treated with suramin (0.33 mg. dissolved in 0.1 ml. of water) and the mixture kept at 22° for 30 min. with occasional shaking. Acetone (5 ml.) was added, the mixture kept for 3 hr. and centrifuged. The precipitate was washed three times with a mixture of 1 vol. water and 5 vol. acetone (2 ml. each time). The washed precipitate was dissolved in a mixture of 2N-Na₂CO₃ plus a little 0.5N-NaOH and suramin and total N estimated on samples of this solution. The amount of suramin present was too small for matching the final colour against the Tintometer disk and special standards of diluted hydrolysed suramin were used.

The results showed that although only a very small fraction of the added suramin became attached to the urease, there was sufficient in the complex to allow an approximate estimate to be obtained. The result, 0.24 mg. of suramin/100 mg. of complex, corresponds to a suramin : urease molecular ratio of about 0.8 : 1, on the basis of a molecular weight of 483,000 and 16% nitrogen for urease, but this ratio should be regarded as provisional until the experiment can be repeated with a much larger amount of crystalline urease. Furthermore, although tests showed that the urease had been completely inactivated by the added suramin, it is possible that the acetone-precipitated material contained some free enzyme or possibly oxidized or split enzyme. The true suramin/urease molecular ratio might thus be slightly higher than the value obtained here.

Combination of suramin and yeast

Suramin strongly inhibits the glucose-fermenting power of yeast juice, but not that of the intact yeast cells (Wills & Wormall, 1950), and it is suggested that this difference is due to the inability of the drug to pass through the wall of the yeast cell. Support for this view was sought by a study of the capacity of the drug to combine with (a) the intact cells, and (b) the proteins of yeast juice.

Brewer's yeast was washed with water and then with buffer solution (0.2M-phosphate, pH 7.5 or 0.2M-acetate, pH 5.0). Samples (2 ml.) of a suspension of these washed cells in the appropriate buffer solution were treated with 10, 20 or 50 mg. of suramin (in 5 ml. of water) and the mixtures kept at 24° for 1 hr. and centrifuged. Some of the precipitates were washed twice with 0.02M-buffer of the appropriate pH (6 ml. each time), and a suramin determination was made using the whole of the solid. The centrifuge tubes containing the remainder of the precipitates and the deposits were freed, as far as possible, from supernatant solution by the use of strips of filter paper, and the whole solids were used for suramin determinations.

The results of these experiments show (Table 5) that at pH 7.5 little or no suramin passes into the yeast cell. The washed cells contained practically no suramin (0.1-0.4% of that added to the yeast-cell suspension). The possibility that suramin had entered the cell, but had subsequently been removed during the washing of the cells, can be excluded, for

the unwashed cells contained a mere 2.1–3.6 % of the added suramin, an amount which could be accounted for by the presence of a small amount (0.14–0.25 ml.) of supernatant solution in the unwashed cells.

Table 5. *Combination of suramin with yeast cells*

(The values given in this Table refer to amounts of suramin added to, or found in, the cells of 2 ml. of a suspension of yeast cells; the yeast suspension used for the experiment at pH 7.5 contained 2.6 mg. and that used for the tests at pH 5.0, 2.8 mg. of total N/ml. The results given are the averages of duplicates.)

pH	Added	Amount of suramin (mg.)	
		Found in the suramin-treated yeast cells	
		Unwashed cells	Washed* cells
7.5	10	0.36	0.04
	20	0.50	0.04
	50	1.06	0.05
5.0	10	0.75	0.35
	20	0.96	0.37
	50	2.21	0.43

* Washed with dilute buffer solution of the appropriate pH.

At pH 5.0 a small amount of suramin combined firmly with yeast cells, but here also this represented a small fraction only (0.9–3.5 %) of the added suramin. Furthermore, the amount 'fixed' by the cells did not greatly increase with an increase in the suramin concentration of the solution. It would thus appear that the yeast-cell surface has a few protein and possibly other groups which can combine with suramin, and that combination occurs at pH 5 but only to a very slight extent at pH 7.5.

In other experiments complexes were separated from mixtures of yeast juice and suramin by (a) acidification and (b) the addition of methanol at pH 7.4.

Samples (1 ml.) of press juice from brewer's yeast were treated with 2, 5 or 10 mg. of suramin (in 1 ml. of water) and 0.2 M-phosphate buffer, pH 7.4 (0.5 ml.). After the mixtures had stood at 25° for 1 hr., the drug-protein complexes were precipitated from half the mixtures by adding methanol (10 ml.) and these precipitates were washed five times with methanol (6 ml. each time) and dried over CaCl_2 . The complexes in the remaining mixtures were precipitated by adding 2 N-acetic acid, and the precipitates were dissolved at pH 7.4 and reprecipitated a further four times; suramin and total N were estimated on solutions of these acid-precipitated complexes.

The results of this experiment showed (Table 6) that the proteins of yeast juice combine firmly with suramin. Thus at pH 7.4 and when methanol was used to remove all loosely attached suramin, as much as 15 % of the added suramin was present in the complex separated from one mixture. The complexes

precipitated by acid contained, as was expected, much more suramin than did the corresponding complexes precipitated by methanol at pH 7.4, and the amount of drug present in the former was of the same order as those obtained in comparable experiments with serum proteins, egg albumin, etc. The enzymic activity of these precipitated complexes from yeast juice has not yet been studied.

Table 6. *Combination of suramin with the proteins of yeast juice. (a) The acid-precipitated drug-protein complexes, and (b) the complexes precipitated by methanol at pH 7.4*

(The experimental details are given in the text, above; the results recorded are the averages of duplicates and all are in terms of mg./ml. of yeast juice.)

Suramin added (mg.)	Suramin content of the complexes precipitated by methanol (mg.)	Suramin content of the acid-precipitated complexes (mg.)
2	0.31	1.2
5	0.42	2.0
10	0.46	4.1

Inhibition of the succinic oxidase system by suramin-containing rabbit serum and by suramin-protein complexes

After its injection into the blood stream suramin is rapidly 'fixed' by the plasma proteins and we decided to study the inhibiting capacity, towards the suramin-sensitive succinic oxidase system acting at pH 7.4, of (a) the plasma of rabbits injected with the drug, and (b) the separated drug-protein complexes. Since we anticipated that only those plasmas containing much suramin would show inhibitory action

Table 7. *Effect of suramin-containing rabbit serum or plasma on the succinic oxidase system*

(Exp. 1. A rabbit (2.8 kg.) received an intravenous injection of 2.8 ml. of a 5.6 % solution of suramin in 0.9 % NaCl (i.e. 56 mg. of drug/kg.) and blood samples were collected from the ear not used for the injection. The serum was separated at once and tested for inhibitory action on the succinic oxidase system as described elsewhere (Wills & Wormall, 1950). In Exp. 2 a rabbit weighing 2.8 kg. received an injection of 280 mg./kg.; this rabbit died 6 min. later, i.e. shortly after the collection of the first sample of blood.)

	Exp.	Time after injection (min.)	Suramin concn.	Inhibition (%)
Plasma of injected rabbit	1	0	0	0
		2	M/1000	0
	2	4*	M/600	75
Suramin solution (control)	—	—	M/1000	100
	—	—	M/4000	75

* Plasma had to be used on this occasion, for this blood sample would not clot, owing to its high suramin content.

the amount of suramin injected into the rabbits was appreciably higher than the therapeutic dose of 14–28 mg./kg.

The results given in Table 7 show that even a plasma containing a considerable amount of suramin (e.g. m/1000, or 143 mg./100 ml.) may not have a significant immediate inhibitory effect on enzymes or enzyme systems which are strongly inhibited by the free drug.

Inhibition of urease, and the succinic oxidase system by acid-hydrolysed suramin

There is no evidence that suramin is metabolized in the animal body or in the trypanosome, but the possibility of some degradation of the molecule is not excluded. A study has, therefore, been made of the inhibitory action, on urease at pH 5.0 and on the succinic oxidase system at pH 7.4, of suramin which has been partially or completely hydrolysed by HCl at 100°. The acid hydrolysis used in the determination of suramin involves heating at 100° for 6 hr. with a

Table 8. *Effect of acid-hydrolysed suramin on succinic dehydrogenase and urease*

(Samples of 4.76% suramin were heated with 1 vol. of 10.4N-HCl for various periods in a boiling-water bath. The mixtures were cooled, neutralized with 6N-NaOH and diluted to the required concentration. Extra NaCl was added to the control mixture to allow for the effect of NaCl in the hydrolysed suramin solutions. The concentrations of hydrolysed suramin used in these tests were, in terms of suramin, m/4000 for succinic dehydrogenase and m/6000 for urease, and the tests were made at pH 7.4 and 5.0 respectively. The inhibitor and enzyme were in contact for 30 min. before addition of the substrate. Full details of the enzyme tests are given elsewhere (Wills & Wormall, 1950).)

Extent of hydrolysis	Inhibition (%)	
	Succinic dehydrogenase	Urease
None	85	68
Partial* (a)	83	46
(b)	62	27
(c)	46	23
Complete†	46	23

* (a), (b) and (c). Hydrolysis with HCl for 20 min., 1 hr. and 3 hr., respectively. Suramin determinations on these solutions indicated that the drug had been hydrolysed to the extent of 16, 44 and 78% respectively.

† Because, for reasons given in the text (below), it was necessary to hydrolyse a concentrated solution of suramin, complete hydrolysis was not obtained in this experiment. The colorimetric determination showed that the diazotizable amines produced in the mixture heated for 6 hr. were equivalent to an 82% hydrolysis of the drug.

concentration of HCl which is at least 5.2N, and when neutralized these hydrolysed solutions contain, unless they are extensively diluted, sufficient NaCl to inhibit many enzymes. For these enzyme experiments we had, therefore, to start with concentrated suramin solutions, and when

these are heated with conc. HCl a precipitate appears after about 1 hr., and does not disappear during the remaining 5 hr. hydrolysis. This probably explains why concentrated suramin solutions are not completely hydrolysed by acid hydrolysis for 6 or even 8 hr. (cf. Dewey & Wormall, 1946).

The results of the enzyme tests showed (Table 8) that acid hydrolysis strongly reduces the capacity of suramin to inhibit urease. However, the capacity of hydrolysed suramin to inhibit succinic dehydrogenase is significantly greater than its effect on urease, and these results are consistent with those we have obtained with 1-naphthylamine-4:6:8-trisulphonic acid and other suramin analogues. Thus if enzyme inhibition is a factor in the trypanocidal action of suramin, it is probably the drug which acts and not the completely hydrolysed product.

Combination of suramin with peptone, nucleic acids, etc.

In one set of experiments suramin (5–15 mg. dissolved in 0.5 ml. of water) was added to 5% peptone solution (2 ml.) and the drug-protein complexes were precipitated by, and washed with, methanol as described in some of the experiments above. Suramin was estimated on the dried precipitates.

The results showed that suramin readily combines with peptones, and complexes containing 13.3–19.2 g. of suramin/100 g. complex were obtained. These values are of the same order as those obtained in comparable experiments with egg albumin and serum proteins. The suramin-peptone complexes, like those with gelatin and unlike those with egg albumin and the serum proteins, are not precipitable by dilute acid.

Other experiments have shown that the addition of suramin to solutions of nucleic acid results in the formation of acid-precipitable complexes containing considerable amounts of suramin. Commercial 'yeast nucleic acid' and 'thymus nucleic acid' (both supplied by British Drug Houses) were used, and the complexes were precipitated by adding hydrochloric acid to give maximum precipitation. These precipitates were dissolved at pH 7.0 and reprecipitated twice. In one set of experiments up to 25% of the added suramin was found in the complexes with yeast nucleic acid and up to 80% in the thymus nucleic acid-suramin complexes, and the complexes contained, respectively, 0.39–0.65 and 1.2–2.4 g. of suramin/g. of non-suramin nitrogen. It is our intention to study further this combination of suramin and the nucleic acids.

DISCUSSION

Our investigations described here and elsewhere have shown that suramin combines with certain enzymes just as readily as it does with other proteins. As a result of this combination many enzymes are in-

activated, but some are not; indeed at pH 7-7.5 few enzymes are inhibited very strongly by suramin.

The concentration of suramin in the blood of rabbits shortly after the injection of a normal dose of the drug is considerably above the minimum amount required completely to inhibit certain enzymes in tests *in vitro*. The plasma suramin concentration several days after the injection is still above the level required to give 50% inhibition of the enzymes which are most sensitive to the drug. There is also no doubt that a similar amount of suramin is present in the blood of injected men, for Hawking (1940) found that the average plasma concentration of patients injected with 1 g. of suramin was about 3 mg. after 2 days and 0.6 mg./100 ml. after 9 days; these amounts correspond to concentrations of M/50,000 and M/250,000 respectively. Even these very low concentrations will affect some enzymes, e.g. urease and hexokinase, if the enzyme is relatively pure and if there is no appreciable amount of other protein in the reaction mixture.

Other proteins strongly protect enzymes against suramin, and the fact that over 70% and sometimes as much as 90% of the drug in the blood of injected animals is firmly bound to the plasma proteins must be considered to militate against any theory of suramin action which postulates the direct inactivation of enzymes in the trypanosome. Furthermore, our *in vitro* tests have shown that plasma containing a very high concentration of suramin has only a small fraction of the inhibitory effect on enzymes shown by an equivalent amount of free suramin.

On the other hand, it is highly probable that the action of the drug on trypanosomes involves the passage of an appreciable amount of the drug into the trypanosome or at least combination of the drug with the organism. The amount of suramin entering the trypanosome will not be easy to measure, for the chemical method we have developed does not permit the determination of less than about 33 μ g., or the detection of less than 5 μ g., of suramin. With this method, however, Hawking (1939) was able to show that a very small but significant amount of the drug was absorbed by living trypanosomes exposed to suramin *in vitro*, though not as much as was absorbed by dead trypanosomes. Issekutz (1933) also found a small amount of suramin in the trypanosomes separated from the blood of infected animals 4-9 hr. after they had been injected with large doses of the drug; the concentration found, 5-10.5 'mg. %' as against 24-42 'mg. %' in the serum at the same time is, however, well above the minimum which we have found to be inhibitory to some enzymes. Thus there is evidence that suramin does enter the trypanosome in amounts which can be detected or measured when there is a relatively large amount of the drug in the external fluid. It will probably be very difficult, however, to obtain

evidence of this penetration or combination of drug and trypanosome when the plasma suramin concentration is low, e.g. several days or weeks after the animal has been given one normal injection of the drug.

It is feasible that suramin may gradually be released *in vivo* from the drug-protein complexes and that it may then enter the trypanosome in amounts which are too small to produce an immediate effect on the enzymes of the trypanosome, but which may be slightly inhibitory over a long period. An amount of suramin which merely gives 25 or 30% inhibition for a protracted period may seriously disturb the metabolic balance in the trypanosome, and it is known that there is a prolonged latent period of contact of suramin and trypanosomes before the trypanocidal effect is manifested (see, for example, Jancsó & Jancsó, 1934). Furthermore, it is feasible that the suramin-protein complex may diffuse into the trypanosome. Thus the fact that most of the plasma suramin is present as a complex with protein does not rule out the possibility that the drug acts by inhibiting some of the essential enzymes of the trypanosome. Experimental support of a more direct nature will be required, however, before such a theory could be justifiably advanced, and with this object in view we are extending our enzyme experiments to the study of the metabolic changes in living trypanosomes. The possibility is also being considered that the drug might act by combining with some nutrient in the medium, e.g. a basic amino-acid or some other base, which is essential for the metabolism of the trypanosome.

If the action of suramin is concerned with the inactivation of trypanosomal enzymes needed for carbohydrate and/or protein metabolism it would appear that this is not a common feature of all specific trypanocides. 'Antrycide', for example, the drug which has more recently been discovered and made available for the treatment of trypanosomiasis in cattle (Curd & Davey, 1949), has no inhibitory action on certain enzymes which are strongly inhibited by suramin (Town *et al.* 1949*b*).

The combination of suramin with enzymes and other proteins appears to be connected with the naphthylamine-trisulphonic acid groups of the drug, but it is also partly dependent on the size of the molecule. It is not known which protein groups are involved, but in all probability they are basic groups. No evidence has yet been obtained, however, that the drug can combine with the basic amino-acids, and furthermore these amino-acids do not protect urease, etc., against the inhibitory action of suramin; amino-acid groupings are, however, often more reactive when present in protein than they are in the free amino-acids, and it is considered likely that one or other of the basic amino-acids is involved in the reaction of proteins with suramin. The sulphydryl

groups of proteins do not appear to be involved (Wilson & Wormald, 1949) and indeed we have found that several sulphydryl enzymes, including xanthine oxidase (which according to Mackworth (1948) is of this type) and cholinesterase, are not significantly inhibited by the drug at pH 7.5 or at about the optimum pH for the enzyme. Furthermore, neither cysteine nor glutathione will protect the sulphydryl enzymes urease and succinic dehydrogenase against the inhibitory action of suramin (Wills & Wormald, 1950). With regard to the free amino groups of proteins, no final conclusion can yet be reached, but our results do not exclude the possibility of their interaction with the sulphonic acid groups of suramin.

Our experiments with many widely differing proteins have shown that the attachment of only a few molecules of suramin to the protein molecule alters profoundly the properties of the protein and produces a complex which is precipitated, in the case of most proteins but not with peptones or gelatin, when the solution is made slightly acid. Furthermore, it is often possible to obtain precipitates of these complexes merely by adding the right amount of suramin to a buffered neutral solution of the protein. In the experiments recorded in Table 4, for example, insoluble complexes of suramin and egg albumin separated at pH 7.0 or 7.5, and these lightly washed complexes contained 2.4–2.8 molecules of suramin/molecule of egg albumin.

Somewhat similar but lower ratios were obtained with crystalline urease, for the complex precipitated by acetone from a mixture of the drug and enzyme had a suramin/urease molecular ratio of 0.8 : 1. On the other hand, inhibition experiments with varying amounts of suramin suggest that two molecules of the drug combine with each active centre of urease (Wills & Wormald, 1950), but the two phenomena, the formation of a sparingly soluble complex and enzyme inhibition, do not necessarily require the same number of molecules of the drug. The amount of suramin in these precipitated drug-enzyme complexes is very small, and the difficulty in obtaining relatively large amounts of crystalline enzymes has prevented us from making these experiments on a larger scale. We hope, however, to obtain more information about these drug-enzyme molecular ratios with the aid of suramin containing ^{35}S .

SUMMARY

1. The suramin content of the plasma of rabbits has been determined at intervals during the first

month after the intravenous injection of either 14 or 28 mg. of suramin/kg., doses which are equivalent to those given to man.

2. The injection of 28 mg./kg. plasma gave an initial concentration of at least $\text{M}/2000$ suramin, and a concentration of over $\text{M}/5000$ was maintained for at least 1 hr. after the injection; these concentrations are sufficient to effect complete inhibition *in vitro* of hexokinase, succinic dehydrogenase, urease and a few other enzymes. Several days later the plasma still contained about $\text{M}/40,000$ suramin, a concentration sufficient to effect marked inhibition of some enzymes *in vitro*.

3. Concentrations reached or maintained after the injection of 14 mg. of suramin/kg. were approximately half those following the injection of 28 mg./kg.

4. About 70–90% of the suramin in the blood of these injected rabbits was firmly combined with the plasma proteins.

5. The plasma proteins, because of their capacity to combine with the drug, strongly protected enzymes against the inhibitory action of suramin, and the suramin-containing plasma had only a relatively slight inhibitory action on the suramin-sensitive enzymes.

6. The possibility that the suramin which is combined with the plasma protein may gradually be released in the blood stream and may then pass into trypanosomes in amounts sufficient to inactivate essential enzymes of the trypanosomes, is discussed.

7. The inability of suramin to inhibit fermentation by whole yeast in contrast to its effect on fermentation by yeast juice is due to the inability of the drug to pass through the wall of the yeast cell.

8. Experiments with egg albumin and urease suggest that the combination of one or two molecules of the drug with each molecule of protein may be sufficient to produce insoluble drug-protein complexes or to inactivate the enzyme.

9. Suramin hydrolysed by acid has little inhibitory effect on urease and only about half that of an equivalent amount of suramin on the succinic oxidase system.

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Studies on Suramin

9. THE ACTION OF THE DRUG ON SOME ENZYMES

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Evidence is available that suramin inhibits a few enzymes (for a review of the literature see Town, Wills, Wilson & Wormall, 1950). For reasons given in the preceding paper (Town *et al.* 1950) we have studied the action of very small amounts of the drug on a considerable number of enzymes under conditions as closely related as possible to the physiological. This paper is concerned mainly with experiments on non-proteolytic enzymes, with a few observations on the milk-clotting enzymes.

The primary object of the experiments described here was to find out whether suramin, in amounts comparable with those which are sufficient to destroy trypanosomes in the blood and tissues of man and other animals, has any inhibitory effect on enzymes likely to be present in the trypanosome. Where marked enzyme inhibition occurs, a study has been made of the mechanism of the inhibition and the conditions under which the drug reacts with the enzyme. In view of the importance of carbohydrate for the nutrition of trypanosomes, special attention has been paid to those enzymes taking part in carbohydrate metabolism, i.e. the enzyme systems concerned with yeast fermentation and muscle metabolism. In this connexion it is of interest to note that Marshall (1948) has recently found that trivalent arsenicals and straight-chain diamidines inhibit

certain enzyme systems concerned with carbohydrate metabolism in *Trypanosoma evansi*, and he has shown that the breakdown of glucose by trypanosomes follows almost exactly the pattern of glucose metabolism by yeast.

EXPERIMENTAL

Materials

Urease. (a) *Soya bean extract.* Soya bean flour (British Drug Houses) (1 g.) was extracted with 95 ml. of water and the mixture centrifuged; the clear solution was mixed with 5 ml. of 0.2 M-acetate buffer (pH 5.0) and centrifuged; the clear supernatant solution was used for the tests. (b) *Crystalline jack bean urease.* This was prepared as described by Sumner & Somers (1947); a solution containing 9 mg. of protein/l. was used.

Succinic oxidase, succinic dehydrogenase and cytochrome oxidase. The preparation of these enzyme systems from pig heart was carried out as described by Straub (1939) as far as the precipitation at pH 4.6. The precipitate was suspended in 5 vol. of 0.1 M-phosphate (pH 7.4) and 1 ml. of the mixture used for each test.

Yeast juice. For different experiments baker's yeast, brewer's top yeast and brewer's bottom yeast were used. After it had been well washed with water, the yeast (100 g.) was mixed with kieselguhr (50 g.) and washed sand (100 g.), and the mixture ground to a dough in a heavy mechanical mortar. The dough was kept at room temperature for