RESEARCH PAPERS

HYDROLYTIC CHANGES IN SOLUTIONS OF STILBAMIDINE

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Henry[1], working in the Sudan, suggested that solutions of 4:4′ diamidinostilbene dihydrochloride (stilbamidine dihydrochloride), underwent a number of changes, including hydrolysis of the amidine groups to the corresponding mono- and di-amides, when kept in diffuse daylight. He[2] isolated a substance shown by analysis to be 4-carbamyl-4′-amidinostilbene hydrochloride. From kinetic considerations he deduced that a dimer of stilbamidine was also formed. The same author[3] gave further experimental details when this investigation had been completed. Fulton[4] found by chemical methods that, in this country, 1:2:3:4-tetra-(4′-amidinophenyl)-*cyclo*butane was the only product formed on exposure of stilbamidine solutions to light. On account of our interest in this subject Drs. Henry and Kirk in 1947 kindly sent us some bottles of the old solutions of stilbamidine dihydrochloride prepared in the Sudan in 1941 and 1942, in which we have confirmed the presence of 4-carbamyl-4′-amidinostilbene and have also obtained 4:4′-dicarbamylstilbene in pure form. The same substances have now been obtained by us from stilbamidine solutions kept at 37°C. for long periods or on autoclaving the solutions for a few hours. Oastler and Fidler[5] described cerebral lesions in dogs following intravenous administration of stilbamidine solutions which had been autoclaved for a short period. Sen Gupta[6] suggested that the drug had been affected by this treatment. From toxicity experiments with mice we have not obtained any evidence in support of the latter view.

Experimental

Six bottles of 1 per cent. solution of stilbamidine dihydrochloride of approximately 100 ml. volume were received by us from the Sudan in September, 1947, with the information that they had been prepared in 1941-42 and kept in the dark or in diffused daylight. The contents (see Table I) were yellowish in colour and large crystals as well as some microcrystalline material were present, the latter being sometimes very adherent to glass. The two substances were readily separated by their different solubilities in water, in which the larger crystals dissolved. The soluble material after several recrystallisations were shown by analysis to have the composition of 4-carbamyl-4′-amidinostilbene monohydrochloride with two molecules of water of crystallisation. (Found: in solid dried at 90°C.; C, 63·33; H, 5·30; N, 13·85, 14·1; Cl, 21·1; loss at 90°C. 10·45, 10·64 per cent. $C_{16}H_{16}ON_3Cl$, requires C, 63·66; H, 5·34; N, 13·93; Cl, 11·76; H_2O, in hydrated material 10·66 per cent.) The substance

crystallised in thin laths from water, in which it is less soluble than stilbamidine dihydrochloride and had no m.pt. up to 320°C. Its aqueous solution contained Cl′ ions and rapidly decolorised aqueous bromine or

TABLE I

PRODUCTS PRESENT IN OLD SOLUTIONS OF STILBAMIDINE FROM THE SUDAN

Number of sample	*p*H of solution	Total solid	Water-insoluble portion
		mg.	mg.
1	6·8	590	200
2	6·4	505	57
3	6·2	460	30
4	6·4	525	33
5	6·2	395	40
6	6·4	180	37
TOTAL		2655	397

permanganate solutions. When mixed with ammonium nitrate in excess a yellowish somewhat insoluble nitrate was formed which crystallised in fine rods from water m.pt. around 290°C. as given by Henry[2]. The aqueous solutions of 4-carbamyl-4′-amidinostilbene hydrochloride and those of stilbamidine dihydrochloride showed a similar blue fluorescence and their absorption spectra were identical. The microcrystalline material, which proved to be the diamide, was insoluble in common organic solvents but soluble to a limited extent in acetic acid and was somewhat more soluble in ethylene glycol, from which plates and fine rods were respectively obtained with no m.pt. up to 320°C. (Found: N, 10·7 per cent. $C_{16}H_{14}O_2N_2$ requires N, 10·53 per cent.)

The solutions fluoresced blue except number 6, which had a greenish tinge. Spectrophotometric analysis showed that samples 1 and 2 contained only saturated material, formed from the parent substance by the action of light, while sample 3 contained only the original material. In samples 4, 5 and 6 both substances were present. Deposits had never been observed by us in this country from solutions of stilbamidine dihydrochloride kept for more than a year at laboratory temperatures, which did not exceed 20°C., in light or dark. A series of solutions was therefore subjected, in stoppered bottles, to different conditions of light and temperature and observed over a period of 6 months; the initial *p*H of the solutions was approximately 6·8 and did not alter appreciably during the experiment. The results obtained are shown in Table II.

The product was practically all monoamide, with only negligible traces of diamide, and formed crystals in some cases 2 cm. long. Temperature is apparently of importance in the reaction as no amides were formed at that of the laboratory. It also seems as if light exerted some influence since solutions kept in complete darkness yielded relatively very small amounts of amide. Good yields of both amides were obtained by autoclaving stilbamidine dihydrochloride solutions; this proved a rapid and convenient method of obtaining both products as shown in Table III.

It appears from the data recorded that hydrolysis of the amidino groups in stilbamidine is readily accomplished by heating solutions of the dihydrochloride under pressure. The amides are much less soluble

TABLE II

RESULTS OF KEEPING STILBAMIDINE SOLUTIONS AT 37° C. FOR A PERIOD OF 6 MONTHS

Number of sample	Solutions of dihydrochloride	Conditions of keeping	First appearance of deposit	Percentage yield
1	100 ml. of 1 per cent.	At 37° C. exposed to electric light for short intervals	8 weeks	31
2	50 ml. of 1 „		6 weeks	22
3	100 ml. of 1 „	Complete darkness at 37° C.	—	None
4	100 ml. of 1 „		16 weeks	5
5	100 ml. of 0·2 „		17 weeks	2
6	100 ml. of 1 „		8 weeks	2·5
7	100 ml. of 1 „	Complete darkness at room temperature 5° to 20° C.	—	None
8	100 ml. of 1 „		—	None

than the parent substance and the yields recorded in the table represent the solid obtained on cooling the treated solutions. Because of the much greater solubility of the parent di-isethionate and resulting monoamide salt it is more satisfactory to start with the dihydrochloride. When a

TABLE III

RESULTS OF AUTOCLAVING SOLUTIONS OF STILBAMIDINE UNDER DIFFERENT CONDITIONS

100 ml. of solution		Treatment in autoclave		Percentage yield	
Nature	Strength	Atmospheres	Hours	Monoamide	Diamide
4 : 4′ diamidinostilbene dihydrochloride	1·0 per cent.	½	½	None	None
	1·0 „	1	½	Trace	—
	1·0 „	1½	4	40	7
	0·5 „	1½	4	24	5
	1·0 „	1½	7	52	16
	1·0 „	2	4	45	11
4 : 4′ diamidinostilbene di-isethionate	1·0 „	1½	4	7	Trace
	0·5 „	1½	4	10	Trace
	10·0 „	1½	2	2·5	Trace
4-carbamyl-4′-amidinostilbene ...	1·0 „	1½	2	—	Trace

solution of the monoamide was treated under the above conditions, conversion to the corresponding diamide took place only to a slight extent. It was found by analysis and spectrophotometric measurements that the mono- and di- amides prepared by us in different ways are identical with the products formed in the Sudan. The absorption spectra of these two substances are indistinguishable from that of the parent *trans*-stilbamidine. The values obtained for the latter and the mono-

amide in aqueous solution and of the diamide in acetic acid were as follows:

	λ max	ε max
4:4′-diamidinostilbene dihydrochloride	328 mμ	37,800
4-carbamyl-4′-amidinostilbene hydrochloride ...	328 mμ	38,200
4:4′-dicarbamylstilbene	328 mμ	40,000 (approx.)

The value ε for the diamide is only approximate on account of its extreme insolubility. The fact that its spectrum was observed in acetic acid does not invalidate comparison with the monoamide since the latter's spectrum was unchanged in this solvent. The two groups $>C=O$ and $>C=NH$ thus appear to be chromophorically identical.

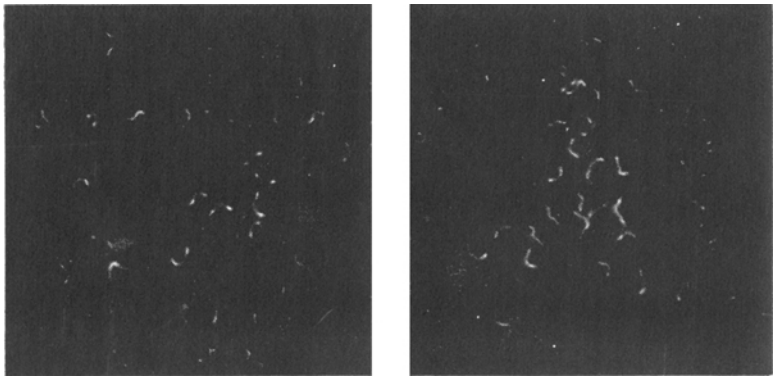
Various methods have been reported for the estimation of stilbamidine, for example fluorescence (Henry and Grindley[7]); colour reaction with glyoxal (Devine[8]), (Fuller[9]); spectrophotometric (Fulton and Goodwin[10]); fluorophotometric (Saltzman[11]). None of these methods is entirely satisfactory. Wien [12] and Hampton[13], using the fluorimetric and colorimetric methods to estimate excretion of stilbamidine in the urine of laboratory animals, found that the values obtained by the latter method were much higher than those obtained by measurement of fluorescence. In view of these results and the possession of similar optical properties by the monoamide and parent substance it seems unlikely that the former is a metabolic product of stilbamidine. Their toxicities as well as those of solutions of stilbamidine autoclaved at 5lb. pressure for 20 minutes, as used by Oastler and Fidler[5], have been compared in mice. The results are shown in Table IV.

TABLE IV

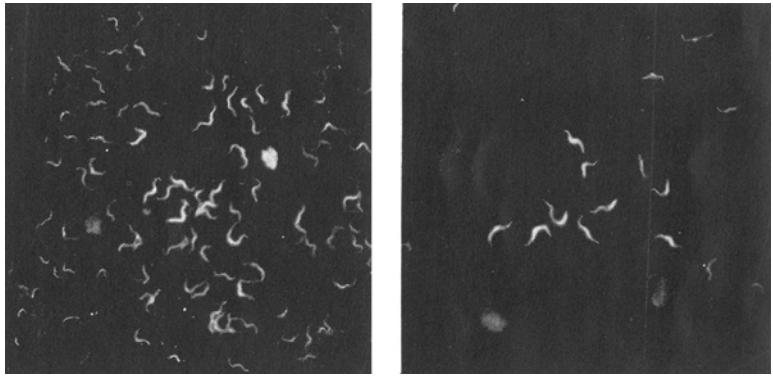
TOXICITY OF VARIOUS SUBSTANCES FOR MICE
D—Died in less than 1 hour after injection
P—Died within a few days of injection
S—Survived observation period of 1 week

Drug	Nature of solution	Effect of doses (mg./20 g. mouse intraperitoneally)		
		2·0	1·0	0·5
4 : 4′ diamidinostilbene dihydrochloride	Fresh	6D, 2P, 2S / 10	2P / 18S / 20	5S/5
	Autoclaved at 5 lb. pressure for 20 minutes	5D/5	1P / 15S / 16	5S/5
	Autoclaved at atmospheric pressure for 20 minutes	5P/5	2P / 18S / 20	5S/5
4-carbamyl-4′-amidinostilbene	Fresh	6D/6	9D / 3S / 12	6S/6

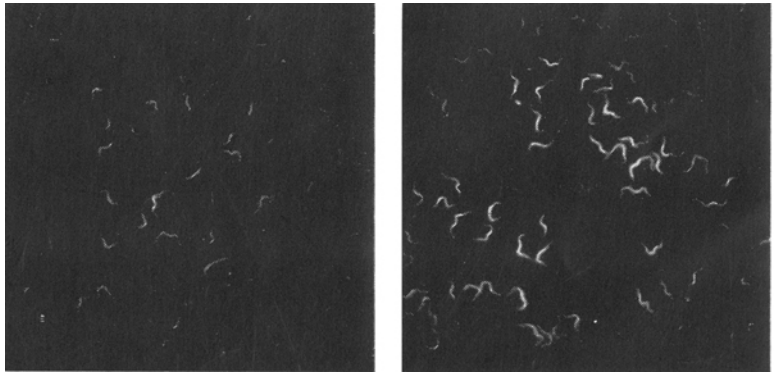
They indicate that 4-carbamyl-4′-amidinostilbene is more toxic than stilbamidine for mice and also that autoclaving of the latter solutions even



Figs. 1 and 4. Appearance under ultra-violet illumination of *T. congolense* and *T. rhodesiense* respectively. Exposed *in vivo* to 4 : 4′ -diamidinostilbene.



Figs. 2 and 5. The same parasites respectively exposed under the same conditions to 4′-carbamyl-4-amidinostilbene.



Figs. 3 and 6. *T. congolense* and *T. rhodesiense* respectively not exposed to drug.

at a pressure of 1 atmosphere has not led to significant increase in toxicity.

Therapeutic tests on mice infected with *T. rhodesiense* and *T. congolense* were carried out with 4-carbamyl-4'amidinostilbene hydrochloride as shown in Table V. It was not possible to test 4:4' dicarbamylstilbene in the same way on account of its insolubility.

TABLE V

RESULTS OF TREATMENT OF TRYPANOSOME INFECTED MICE.
R Blood free from trypanosomes, but relapse occurred.
N Blood never free from trypanosomes.

Mice infected with :	Drug	Effect of doses (mg./20 g. mouse intraperitoneally)	
		0·5	0·25
T. rhodesiense	4-carbamyl-4'-amidino stilbene hydrochloride	1R/15N/16	6N/6
T. congolense		2R/14N/16	1R/5N/6

It is evident that 4-carbamyl-4'-amidinostilbene is inactive therapeutically in the above infections and the life of each mouse was prolonged for only a short period. On the other hand 4:4'-diamidinostilbene is curative at high dilutions in the former infection, but requires a dose approaching the maximum tolerated (1 mg./20 g. mouse) to eradicate *T. congolense* infections. In order to find out the distribution of these drugs in trypanosomes exposed *in vivo* to their action, use was made of their similar fluorescent properties. For this purpose mice heavily infected with *T. rhodesiense* and *T. congolense* were treated with a solution of 0·5 mg. of each drug intraperitoneally and 1 hour later when the trypanosomes were still actively motile, blood smears of treated and untreated animals were made on a quartz slide. The slide was mounted dry without cover glass on the Beck-Barnard ultra-violet microscope and the object was illuminated by means of a quartz dark ground illuminator using the group of lines of the magnesium spark spectrum at 2830Å. Micrographs were taken using a Zeiss 4 mm. apochromat (N.A. 0·95) and a Zeiss No. 2 projection ocular. The length of exposure was 2 minutes in each case. The correction collar on the objective was adjusted to give the best image at a magnification of X 150. The appearances produced are those shown in Figures 1 to 6.

In the case of both trypanosomes exposed to stilbamidine selective absorption of the drug has occurred, as shown by the presence in them of two bright granules. The position of one granule corresponds to that of the blepharoplast, but the nature of the other at the anterior end has not been determined. The remainder of the cytoplasm does not fluoresce more brightly than that of untreated trypanosomes. There is absence of fluorescence in accompanying red cells which are not readily visible. The

inactive 4-carbamyl-4′-amidinostilbene appears to have been generally absorbed throughout the bodies of the trypanosomes as indicated by the increased brightness compared with that of parasites not exposed to the drug.

Summary and Conclusions

The formation of 4-carbamyl-4′-amidinostilbene and 4:4′-dicarbamylstilbene from solutions of stilbamidine had been shown to occur when the latter were maintained for a number of weeks at 37°C., in diffuse light, and to a lesser extent when kept at the same temperature completely in the dark. When the same solutions were maintained at temperatures which varied from 5° to 20°C. the formation of amides did not take place. Henry's observations made in the Sudan have been confirmed. Good yields of the amides were obtained by autoclaving solutions of the parent substance at 1 to 2 atmospheres pressure for several hours. The monoamide was inactive against *T. rhodesiense* or *T. congolense* infections of mice and does not appear to be selectively absorbed by the trypanosomes like the active stilbamidine. The fact that solutions of stilbamidine autoclaved under the conditions employed by Oastler and Fidler undergo no demonstrable change and are not more toxic for mice than similar solutions freshly prepared, suggests that the lesions encountered by these authors in dogs were due to unchanged stilbamidine.

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