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Preparation and biological activity of some complexes of trypanocidal phenanthridinium compounds

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The preparation and *in vivo* testing is described of complexes of some phenanthridinium trypanocides with polysaccharides and polymeric materials possessing strong anionic groups. The laminarin sulphate complex of Prothidium bromide [2-amino-7-(2-amino-6-methyl-4-pyrimidylamino)-9-*p*-aminophenylphenanthridium 10,1′-dimethobromide] shows enhanced prophylactic activity in mice challenged with *Trypanosoma congolense* (F.N.). When tested in cattle in East Africa the complex produced less local reaction at the site of injection but prophylactic activity was similar to that obtained with the equivalent dose of Prothidium bromide itself. Possible reasons for these results are discussed.

A NUMBER of new phenanthridinium trypanocides have been produced in recent years. One of the main disadvantages of these has been the excessive tissue reactions at the site of injection, resulting in large swellings which sometimes ruptured (Robson & Cawdry, 1958; Stephen, 1958; Smith, 1959; Smith & Brown, 1960; Cawdry & Knight, 1961).

Several attempts have been made to prolong the period of protection afforded by prophylactic trypanocides, and to reduce the local and systemic toxicities of both prophylactic and curative agents. These have usually taken the form of depot preparations from which the active substance is gradually released. Examples are the relatively insoluble chloride of quinapyramine (Davy, 1950), insoluble complexes of trypanocides with suramin (Stephen, 1958; Stephen, 1960; Stephen & Williamson, 1958; Stephen & Grey, 1960), and incorporation of the active substance in an oil or grease base (Cawdry & Knight, 1961). The soluble complex of dimidium bromide with thymus nucleic acid has been used to reduce systemic toxicity (Seaman & Woodbine, 1955).

Phenanthridinium compounds of value in the treatment of bovine trypanosomiasis include homidium bromide (B. Vet. C.) and Prothidium bromide; the latter also possesses considerable prophylactic activity. Our attention was directed particularly to these compounds and to a lesser extent to other phenanthridinium compounds, all of which possess quaternary nitrogen groups. Antibacterial quaternary ammonium compounds react with many polysaccharides to produce insoluble products (Groves, 1958). Accordingly, an attempt was made to prepare complexes of phenanthridinium compounds which would delay the release of the active component from an intramuscular or subcutaneous injection site. We hoped thereby to reduce the intensity of the local reaction, and to confer prolonged prophylactic activity.

Experimental

MATERIALS

Acacia, agar, carboxyvinyl polymer (Carbopol 934), isometamidium

From the Research Department, Boots Pure Drug Company Limited, Station Street, Nottingham.

[7-(*m*-amidinophenyldiazoamino)-2-amino-10-ethyl-9-phenylphenanthridinium chloride hydrochloride], sodium alginate, sodium carboxymethylcellulose (Edifas B), stearic acid, sterculia and tragacanth were all pharmaceutical materials of commercial origin. Prothidium bromide [2-amino-7-(2-amino-6-methyl-4-pyrimidylamino)-9-*p*-aminophenylphenanthridinium 10,1′-dimethobromide]; homidium bromide B. Vet. C.; R.D. 2787 [2-amino-7-(2-amino-6-methylpyrimidyl-4-amino)-9-*p*-nitrophenylphenanthridinium 10,1′-dimethochloride, $4H_2O$ or $6H_2O$]; R.D. 2902 [2-amino-7-(2-amino-6-methylpyrimidyl-4-amino)-9-phenylphenanthridinium 10-ethomethanesulphonate-1′-methomethane sulphonate], (Watkins & Woolfe, 1956) and laminarin sulphate LM 111 (degraded material as used by Adams, Heathcote & Walker, 1962; Black & Dewar, 1954) were all available in the laboratory.

Degraded carrageen. Commercial carrageen extract proved unsuitable for complexing. A 5·0% w/w dispersion of commercial carrageen extract in 0·01N sulphuric acid was autoclaved at 10 lb in.^{2} for 30 min. When cool the product was clarified by centrifugation and dialysed against distilled water at 5° for 3 days until free of sulphate ions. The filtered solution was a clear colourless liquid (containing 0·7% solids dried to constant weight at 105°): this produced a copious precipitate with Prothidium bromide solutions.

PREPARATION OF COMPLEXES

Insoluble complexes. The following method yielded reproducible quantities of insoluble precipitates easy to collect and in a form suitable for subsequent testing.

An excess of 1·0% solution of the phenanthridinium salt in distilled water was added slowly, with stirring, to a 1·0% solution or gel of the complexing substance, also in distilled water. The mixture was stirred at room temperature for 5 min and allowed to stand for 1 hr before collecting the precipitate by centrifugation. The material was resuspended in an equal volume of distilled water, and again centrifuged. The "wet" solid was stored at 5°, assayed by total nitrogen analysis, then resuspended in sterile 2·0% hydroxyethylcellulose (Cellosize QP 15,000). In some instances the precipitated complex was dried in an air oven at 105° or at 60° under vacuum and the solid resuspended in hydroxyethylcellulose solution. Equilibrium dialysis (Graham & Thomas, 1962) gave no evidence of complex formation between any of the phenanthridinium compounds tested and hydroxyethylcellulose. In no instance did the colour of the phenanthridinium solutions change.

Soluble complexes. When saturated aqueous solutions of Prothidium or homidium bromide were added to solutions of agar or pectin no precipitation occurred but the change in colour indicated that a reaction had taken place. This was confirmed by equilibrium dialysis (Graham & Thomas, 1962). Agar complexes of Prothidium and homidium were prepared by the general method described above, evaporated to dryness at 105°, assayed, then redissolved in hot water at the required concentration. Pectin complexes were prepared likewise and precipitated with

an excess of ethanol. For testing purposes the solid was redissolved in water, and the pH adjusted to 3·3 with 0·01M sodium bicarbonate solution.

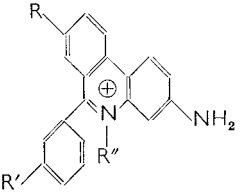
Some properties of the complexes are listed in Table 1.

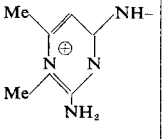
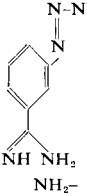
MEASUREMENT OF PROPHYLACTIC ACTIVITY IN MICE

Mice were injected subcutaneously with 1·0 or 0·2 mg/kg of phenanthridinium or with doses of complex containing 1·0 or 0·2 mg/kg of phenanthridinium. The dose was always contained in 0·2 ml of suspension

TABLE 1. PROPERTIES OF THE PHENANTHRIDINIUM COMPLEXES

Phenanthridinium nucleus:



Phenanthridinium	Structure of cation R	R′	R″	Complexing moiety (I = insoluble complex) (S = soluble complex)		Total phenanthridinium content of complex dried to constant weight at 105° (total nitrogen) determination)
Prothidium bromide (cation λ_{max} 466 mμ)		NH_2	Me	Acacia	(I)	35·2
				Agar	(S)	56·5
				Carbopol 934	(I)	55·4
				Carboxymethylcellulose	(I)	57·1
				Degraded carrageen	(I)	44·6
				Heparin	(I)	87·6
				Laminarin sulphate	(I)	53·5
				Pectin	(S)	—
				Sodium alginate	(I)	69·2
				Stearic acid	(I)	68·4
				Sterculia	(I)	62·2
				Tragacanth	(I)	32·9
R.D. 2787 (cation λ_{max} 465 mμ)	As for Prothidium	NO_2	Me	Laminarin sulphate	(I)	65·3
				Degraded carrageen	(I)	57·3
R.D. 2902 (cation λ_{max} 465 mμ)	As for Prothidium	H	Et	Laminarin sulphate	(I)	68·5
				Degraded carrageen	(I)	58·5
Isometamidium (cation λ_{max} 474 mμ)		H	Et	Laminarin sulphate	(I)	65·5
				Degraded carrageen	(I)	53·7
Dimidium (cation λ_{max} 480 mμ)	NH_2–	H	Me	Laminarin sulphate	(I)	81·4
				Degraded carrageen	(I)	65·2
Homidium bromide (cation λ_{max} 482 mμ)	NH_2–	H	Et	Agar	(S)	61·5
				Carbopol 934	(I)	23·6
				Carboxymethylcellulose	(I)	50·2
				Degraded carrageen	(I)	68·6
				Heparin	(I)	97·4
				Laminarin sulphate	(I)	87·1
				Pectin	(S)	20·5
				Sodium alginate	(I)	75·8
				Sterculia	(I)	51·7
				Tragacanth	(I)	38·9

or solution. The dosed mice were challenged by intraperitoneal inoculation of 20,000 *Trypanosoma congolense* (strain FN) per mouse. The inocula were obtained by harvesting infected blood from mice in which the trypanosome was maintained by serial blood passage. Challenges were normally made at 2, 4 and 8 weeks after the mice had been dosed; but for substances unlikely to be prophylactic the first challenge was made 1 week after the dose. The tail blood of inoculated mice was examined for trypanosomes every 3 days. As it takes a minimum of 4 days for a mouse to die after the first *T. congolense* (FN) appear in its peripheral blood, examination every 3 days means that no mouse could die from trypanosomiasis without trypanosomes being seen.

Results

In some instances complexing the phenanthridinium compounds effected marked changes in the prophylactic properties of the drugs in mice. For the greater part of this work Prothidium was used as it was then the only phenanthridinium with prophylactic properties proven in the field in Africa. Of all the complexes of Prothidium, only that with laminarin sulphate clearly showed enhanced activity in mice. Complexes with heparin and degraded carrageen may have had some slight advantage compared with the drug itself (Table 2). Complexes with acacia, agar,

TABLE 2. THE PROPHYLACTIC TRYPANOCIDAL ACTIVITY OF PHENANTHRIDINE COMPLEXES

Phenanthridinium	Complex	Dose mg/kg†	Number of surviving uninfected mice from groups of 10 challenged at		
			2 weeks	4 weeks	8 weeks
Prothidium ..	Uncomplexed control (results of 5 tests)	0·2	0–5	0–2	0
		1·0	10	7–10	0–3
	Degraded carrageen	0·2	4	2	0
		1·0	9	5	2
	Laminarin sulphate	0·2	10	10	3
		1·0	10	9/9*	10
	Heparin	0·2	4	3	0
		1·0	9	9	5
R.D. 2787.. ..	Uncomplexed control	0·2	0	0	0
		1·0	9	0	0
	Degraded carrageen	0·2	0	0	0
		1·0	9	6	0
	Laminarin sulphate	0·2	0	0	0
		1·0	9	9	9
R.D. 2902.. ..	Uncomplexed control	0·2	0	0	0
		1·0	1	0	0
	Degraded carrageen	0·2	2	0	0
		1·0	0	0	0
	Laminarin sulphate	0·2	0	0	0
		1·0	8	8	2
Isometamidium ..	Uncomplexed control	0·2	2	0	0
		1·0	10	10	0
	Degraded carrageen	0·2	9	6	0
		1·0	9	10	8
	Laminarin sulphate	0·2	4	4	0
		1·0	9	10	8
Dimidium ..	All complexes and uncomplexed control	1·0	No survivors in any group challenged one week after dosing		
Homidium ..	All complexes and uncomplexed control	1·0	No survivors in any group challenged one week after dosing		

* The 10th mouse in this group died from causes other than trypanosomiasis.
† Doses are quoted as their equivalent of prothidium.

Carbopol, carboxymethylcellulose, sodium alginate and sterculia, showed reduced activity, while those with pectin, sodium alginate and tragacanth showed similar activity to the drug itself.

When the Prothidium-laminarin sulphate complex was dried and the solid resuspended just before administration it was less active than the original suspension, although it was still more active than Prothidium, Table 3.

TABLE 3. COMPARISON OF THE ACTIVITIES OF DRIED AND FRESHLY PREPARED PROTHIDIUM-LAMINARIN SULPHATE COMPLEXES

Substance	Equivalent dose of Prothidium in mg/kg sc.	Numbers of surviving, uninfected mice after being challenged 8 weeks after dosing
Prothidium	1 2	0/9 2/9
Prothidium-laminarin sulphate, dried	1 2	2/9 3/9
Prothidium	1 2	0/5 2/5
Prothidium-laminarin sulphate, freshly prepared	1 2	5/5 5/5

Prophylactic phenanthridinium compounds other than Prothidium were complexed only with laminarin sulphate and degraded carrageen. Those compounds possessing prophylactic activity in their own right, all showed enhanced activity in mice after complexing with one or other of these complexing moieties. The activities of isometamidium and R.D. 2787 were enhanced after coupling with either, that of R.D. 2902 only after coupling with laminarin sulphate. Two phenanthridiniums possess mainly curative activity, viz dimidium and homidium, and these were complexed with all of the agents used with Prothidium. In mice, no prophylactic activity was apparent.

The complexes of homidium and Prothidium were compared with the parent compounds for local irritancy by intradermal injection into guinea-pigs. The assessment of differences in reaction proved difficult and inconclusive but the laminarin sulphate complexes appeared to be no more irritant than either homidium or Prothidium bromide alone (R. Bough, personal communication).

Evaluation of the Prothidium-laminarin sulphate complex in cattle in East Africa showed that the reaction at the site of injection had been considerably reduced (R. Fairclough & E. F. Whiteside, personal communications). The standard dose to cattle of Prothidium bromide (2·0 mg/kg) injected into the dewlap can produce a necrotic lesion up to 20 to 30 cm in diameter which may burst. When the same dose of Prothidium bromide was given as the complex the size of the lesion was reduced to between a tenth and a fifth, and did not burst. The period of protection against trypanosomiasis, however, was almost exactly the same as that given by a dose of the bromide equivalent to the dose of complex.

Discussion

The formation of complexes of Prothidium may result in an increase, a decrease or no change in prophylactic activity against *T. congolense* in mice, according to the complexing substance used (Table 3). These changes in activity presumably reflect the ease with which each complex is split under physiological conditions to liberate its trypanocidal moiety into the blood. The complex most active in mice, that with laminarin sulphate, presumably releases phenanthridinium at an optimum rate, which is sufficient to maintain trypanocidal activity in the blood without allowing unduly rapid excretion. When a complex like that with Carbopol is less active than its equivalent of Prothidium, the binding of the two molecules would seem to be so strong, that trypanocidal levels of the drug cannot be maintained in the blood.

Because it apparently had the greatest biological interest, particular attention was paid to the properties of the Prothidium-laminarin sulphate complex. Precipitation of this complex under a wide variety of conditions and concentrations showed that approximately 11 parts of the bromide reacted with 10 parts of laminarin sulphate. The complex was evidently loose since the supernatant washing liquids were invariably slightly coloured. The wet complex dried on a glass plate as translucent reddish brown scales, but there was a reduction in the prophylactic activity in the dried material (Table 3). When the complex was precipitated from dilute solution and concentrated by centrifugation the precipitate varied in consistency from a suspension to a thixotropic gel according to the concentration of solid.

It was disappointing that the increased duration of prophylaxis obtained in mice with the Prothidium bromide-laminarin sulphate complex was not matched by any increase in prophylaxis in cattle in Africa. This may be explained by comparing the situations at the site of injection in mice and cattle. In mice the complex presumably forms a depot at the site of injection, while the bromide alone does not. The latter supposition is supported by observations that no depot has been seen in mice at post-mortem examination after subcutaneous or intramuscular injection of the bromide, and that the period of prophylaxis in mice was the same whether the drug was given by subcutaneous, intramuscular or intravenous injection. In addition, when the site of injection in mice of the Prothidium bromide-laminarin sulphate complex is examined 8 weeks after the injection traces of coloured complex can still be seen. Thus the complex in mice is probably effecting prolonged prophylaxis mainly by liberating the drug slowly from its local depot, in contrast to the bromide, which is apparently bound in tissues generally in the body and slowly released from them.

When Prothidium bromide alone is administered to cattle by subcutaneous or intramuscular injection, the local reaction around the site of injection would appear to have the effect of converting the sealed-off drug into a local depot. If so the injection of cattle with a ready formed depot would be unlikely to confer longer prophylaxis.

The ready formed depot complex in cattle, however, markedly reduces the local tissue reaction, an effect which may be due to a slow release of drug from the complex, thus preventing too sudden an exposure of tissues round the injection site to high concentrations.

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