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T. A. HENRY AND H. C. BROWN

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THE WELLCOME CHEMICAL RESEARCH LABORATORIES
(The Wellcome Foundation Ltd.)
T. A. HENRY, D.SC., *Director*
6, King Street, Snow Hill
LONDON, E.C. 1

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BY

T. A. HENRY, D.Sc.

Director of the Wellcome Chemical Research Laboratories.

AND

H. C. BROWN, C.I.E., M.B., B.Ch. (Camb.), Major I.M.S. (ret.).

Wellcome Bureau of Scientific Research.

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In a previous paper (Brown, 1922) it was shown that *in vitro* conessine, the principal alkaloid of *Holarrhena (Wrightia) antidysenterica* bark (Pyman, 1919) was as toxic to free-living amœbæ as emetine.

It has now been found that the toxicity of both alkaloids towards protozoa is greatly increased in presence of alkali. This is only one out of a number of recent laboratory and clinical observations indicating that the action of alkaloids on protozoa is enhanced in an alkaline medium. Thus Acton (1922) found that quinine is more toxic to Paramæcium in presence of alkali, and, in the same year, Iriye found that the action of emetine on amœbæ was intensified under like conditions. This year, Sinton has made practical use of these observations, and finds that quinine gives better results in malaria when administered with alkalis than when given alone.

The data recorded in the present paper are derived solely from experiments *in vitro*, but as Sellards and Leiva (1923) have pointed out: "In chemotherapeutic work on amœbic dysentery tests for toxicity of a drug for Limax amæbæ are not without value for purposes of obtaining general orientation. Obviously the results cannot be applied to *Entamœba histolytica* any more than the effects of experiments on lower animals can be applied directly to man. It is also perfectly clear that the

effects *in vitro* do not necessarily imply a corresponding action in the animal body."

The increased toxicity of the three alkaloids—quinine, emetine, and conessine—towards protozoa, when applied in an alkaline medium, is so marked that we venture to suggest that it should not be lost sight of in the treatment of intractable cases of dysentery, and it seems possible that if means can be found of taking advantage of this method of increasing the action of these drugs, a step forward may be achieved in dealing with this disease.

Technique.

The quinine and emetine used consisted of the purest alkaloids obtainable commercially; the conessine was part of that prepared by Professor Pyman from Holarrhena bark. The protozoa were obtained from hay infusions. In Acton's work on Paramæcium, cultures isolated from single cells were used, but in the present investigations it was considered more satisfactory to avoid the variations in susceptibility in individual protozoa of the same species, and to study the effect of the reagents on large numbers of organisms of varying susceptibility.

The work of Woodruff (1912) is useful in dealing with the origin and sequence of the protozoan fauna in hay infusions.

During the earlier experiments it was found, when studying the susceptibility of *Colpoda cucullus* to the action of acids, that the character of the infusion played an important part in the resistance of this organism to the action of acids. This is clearly shown in the following table:—

					15 minutes.
Original culture	+	tap-water	+	N/50 HCl.	All dead.
Subculture	+	,,	+	N/50 HCl.	,,
Original culture	+	,,	+	N/60 HCl.	Active.
Subculture	+	,,	+	N/60 HCl.	All dead.
Original culture	+	,,	+	N/70 HCl.	Active.
Subculture	+	,,	+	N/70 HCl.	Sluggishly motile.
Original culture	+	,,	+	N/80 HCl.	Active.
Subculture	+	,,	+	N/80 HCl.	,,
Original culture	+	,,	+	N/90 HCl.	,,
Subculture	+	,,	+	N/90 HCl.	,,

Equal volumes of the culture, tap-water and acid were mixed on a slide and observations made under a $\frac{2}{3}$in. objective. The original culture differed from the subculture in that, in the latter, the organisms were growing in the fluid which had been poured off from the original culture, and was, therefore, weaker in products of decomposition. It might at first be thought that the difference in susceptibility to acid of the organisms in the two cultures might be due to difference in age, but the effect was traced in the following way to the difference in power of the two culture fluids to neutralise acid. Fluid from the original culture and the subculture were placed in two tubes and heated at 56°C. for ten minutes, to kill the Colpoda. Then one volume of the original culture was mixed with one volume of the heated original culture medium and one volume of N/60 HCl; the Colpoda at the end of thirty minutes were all active. On the other hand, when one volume of the original culture was mixed with one volume of the heated subculture medium and one volume of N/60 HCl all the Colpoda were dead at the end of thirty minutes, thus showing that the fluid of the original culture medium was capable of neutralising more acid than that from the subculture. Further, if the Colpoda from the two cultures were taken separately and washed twice in tap-water by means of a centifruge, at slow speed, it was found that the two sets of organisms showed no difference in their susceptibility to acids.

It is, therefore, essential, in studying the behaviour of protozoa with alkaloids in different ranges of acidity and alkalinity, that the organism must either be grown in a medium of constant composition, or that it must be freed from the varying amounts of products of decomposition of the hay in the infusion. This latter procedure can be easily performed without injuring the organisms by washing them by means of a centifruge at slow speed in two changes of tap-water, and although the composition and reaction of tap-water vary in different localities, the variation is probably far less than would occur in using hay infusions, even if these were made by a prescribed quantitative method. An artificial culture medium which would remain of constant composition would be still more difficult to obtain.

Dilution with distilled water instead of tap-water gave a very different result. It was found that if equal volumes of the subculture of Colpoda were mixed with tap-water + varying strengths of HCl from N/50 to

N/90, those with N/50 and N/60 were all dead after fifteen minutes, and those with N/70, N/80 and N/90 alive.

If distilled water were used, even with N/100 HCl, the Colpoda were all dead after fifteen minutes. The same result was obtained when using boiled, cooled and filtered distilled water; when, however, boiled, cooled and filtered tap-water was used, even the N/70, N/80 and N/90 preparations were all dead after fifteen minutes.

Hence it appears that ordinary tap-water is capable of neutralising the acid to an appreciably greater extent than is the case with distilled water, and when the tap-water has been boiled for thirty minutes and then cooled and filtered, this power of neutralisation is largely lost.

During some experiments in which protozoa were first acted upon by weak alkalis and conessine was subsequently added, the mixtures were prepared on a slide and observations were made under a $\frac{2}{3}$in. objective, and it was found that when the alkali and alkaloid were added simultaneously death occurred in three minutes; whereas if the dilute alkali, which in itself was incapable of killing the organism, was allowed to act upon the protozoon for twenty minutes and the alkaloid was then added, instead of death taking place in three minutes or less, many of the organisms were alive and active after two hours. It appeared, at first sight, that the alkali by itself had some sort of protective action upon the organism, but this somewhat curious result proved to have a far simpler explanation, namely, that the dilute alkali, exposed to the air on the slide, had been partially neutralised by atmospheric carbon dioxide.

That this was the case was demonstrated by exposing some of the dilute alkali only on a slide for twenty minutes. When one volume of this solution was mixed with equal volumes of culture and alkaloid solution death no longer took place, although when unexposed alkali was used death occurred in three minutes. It is, therefore, fallacious to make these observations on a slide, since even when the droplet is covered, unless the coverslip is ringed to exclude air, some of the organisms will travel to the edges of the drop, where the alkali becomes partially neutralised, and then they will re-enter the drop and cause discrepancies in the results.

In order to overcome this difficulty, the culture and other reagents

were taken up in a capillary pipette, rapidly mixed in a depression on a porcelain slab, and then transferred to a capillary hard-glass tube, which was sealed at both ends, and it was not until this procedure was adopted that consistent results were obtained.

In the following experiments the protozoon used was a species of Glaucoma, a ciliate somewhat similar to, but smaller than, Paramæcium. The culture of this organism was filtered through coarse muslin and washed twice in tap-water by centrifuging at slow speed. For this culture, and for his advice and help, we are indebted to Dr. C. M. WENYON.

The reagents used were dissolved in distilled water, and equal volumes of the cultures and reagents were used throughout.

EXPERIMENTAL RESULTS.

Susceptibility to Alkali.— 15 minutes.

Glaucoma	+	distilled water	+	N/50	NaOH.	All dead.
,,	+	,,	+	N/100	,,	Alive.
,,	+	,,	+	N/150	,,	Alive.

Susceptibility to Acid.—

Glaucoma	+	distilled water	+	N/50	HCl.	All dead.
,,	+	,,	+	N/100	,,	All dead.
,,	+	,,	+	N/150	,,	Alive.

It might be thought that distilled water alone would injure the organism, but if one volume of washed Glaucoma was mixed with two volumes of distilled water and sealed in a hard-glass capillary tube, the organism was found to be alive and active twenty-four hours later.

The following table shows the effect of exposing washed Glaucoma to the action of emetine hydrochloride, conessine hydrochloride, and quinine dihydrochloride in presence of (1) distilled water, (2) dilute acids, and (3) alkalis.

TABLE A.

(+ = one or more organisms alive at the end of fifteen minutes.)
(0 = all organisms dead at the end of fifteen minutes.)

	Strength of Alkaloidal Solution.				
	M/10.	M/100	M/1000	M/10,000	M/100,000
Emetine hydrochloride.					
1. Distilled water	+	+	+	+	+
2. N/200 HCl*	+	+	+	+	+
3. N/200 NaHO*	0	+	+	+	+
Conessine hydrochloride.					
1. Distilled water	+	+	+	+	+
2. N/200 HCl*	+	+	+	+	+
3. N/200 NaHO*	0	0	0	0	+
Quinine dihydrochloride.					
1. Distilled water	0	+	+	+	+
2. N/200 HCl*	0	0	+	+	+
3. N/200 NaHO	0	+	0	+	+

* Owing to dilution with the culture medium, the actual concentration of acid or alkali in the preparation was N/600.

The readings were taken after fifteen minutes' interaction at room temperature, and the observations were made with a 1in. objective with dark field illumination.

The most noticeable of these results is the enormous increase in toxicity of conessine hydrochloride to this organism in presence of N/200 sodium hydroxide solution. The toxicity of emetine hydrochloride is somewhat increased by the alkali, as originally pointed out by IRIYE, but not nearly to so marked an extent as that of conessine hydrochloride.

For convenience in making comparable solutions the alkaloidal preparations were made by dissolving a molecular weight (M) in grammes of the appropriate alkaloidal salt in one litre of water, and then diluting to the required strength. M/1,000, therefore, means a molecular weight in grammes of the alkaloidal salt in 1,000 litres, and so on for the other expressions, M/100,000, etc.

Another striking point is the apparently paradoxical result of using quinine dihydrochloride with dilute alkali, the M/1,000 dilution of this salt being more toxic than a M/100 dilution.

This point was investigated by making careful comparison of the action of solutions of (*a*) quinine dihydrochloride and (*b*) quinine monohydrochloride in presence of (1) distilled water and (2) N/200 sodium hydroxide. The results are shown below :—

TABLE B.

	(*a*) Quinine dihydrochloride.		(*b*) Quinine monohydrochloride.	
Strength of solution of alkaloidal salt.	1.—Distilled water.	2.—N/200 NaHO.*	1.—Distilled water.	2.—N/200 NaHO.*
M/10	0	0	—	—
M/20	0	0	0	0
M/40	0	+	0	0
M/60	+	+	0	0
M/80	+	+	0	0
M/100	+	+	0	0
M/120	+	+	0	0
M/140	+	+	0	0
M/160	+	+	0	0
M/180	+	+	0	0
M/200	+	0	0	0
M/400	+	0	+	0
M/600	+	0	+	0
M/800	+	0	+	0
M/1000	+	0	+	0
M/1200	+	+	+	0
M/1400	+	+	+	0
M/2000	+	+	+	0
M/3000	+	+	+	+

* The actual concentration of the alkali in the preparation was N/600 owing to dilution with culture medium. A control test showed in the alkalinised medium without alkaloid, Glaucoma alive and active after two hours.

The explanation of the apparently anomalous results in column 3 appears to be that the initial toxicity shown in this column is mainly due to the acidity of the quinine dihydrochloride, since it is reduced from M/40 in distilled water to M/20 in presence of the alkali. At M/200 the amount of alkali added is just enough to convert the dihydrochloride into the monohydrochloride, and the renewal of toxicity at this point is due

to this salt. At M/400 the alkali used is enough to combine with all the acid in the dihydrochloride and liberate free quinine: below this point the toxicity is due to free quinine plus free alkali. With quinine monohydrochloride in distilled water (column 4) toxicity stops at M/200, but in presence of alkali (column 5) it goes on to M/2,000. In this case the alkali added is just enough to convert all the monohydrochloride into free base at M/200, and after that the free base is acting in presence of free alkali, but the amount of the latter will, for corresponding points, always be twice as great as in column 3, and this accounts for the toxicity going on to M/2,000 in column 5, while it stops at M/1,000 in column 3.

An interesting point deducible from these results is that quinine base is more toxic to protozoa than quinine monohydrochloride, and both are much more toxic than the dihydrochloride.

The free bases are all so sparingly soluble that saturated solutions in distilled water showed no toxicity towards Glaucoma, but such solutions of emetine (active at M/4,600) and especially conessine (active at M/14,000) became toxic in presence of sodium hydroxide, whilst the quinine solution, though much more concentrated than those of the other two alkaloids, did not kill all the organisms, a few being alive and active at the end of fifteen minutes, though most were dead or only sluggishly motile. The results are summaried below :—

SATURATED AQUEOUS SOLUTIONS OF FREE BASES WITH N/200 NaHO.

Strength.	Quinine.	Emetine.	Conessine.
M/635*	+	—	—
M/4684†	—	0	0
M/9368	—	+	0
M/14052	—	+	0
M/18736	—	+	+
M/23420	—	+	+

* Saturated for quinine.

† Saturated for conessine. Emetine, being more soluble, was made of the same strength.

In view of the striking increase of activity in presence of dilute sodium hydroxide, it became of interest to try the effect of other substances of alkaline reaction. For this purpose equal volumes of diluted normal solutions of the various alkalis and of a saturated

aqueous solution of conessine were used, and the points at which they killed and failed to kill Glaucoma in fifteen minutes determined.

	NaHO.	KHO.	LiOH.	$Ca(OH)_2$.	$NaHCO_3$.	$KHCO_3$.	NH_4OH.	$(NH_4)_2CO_3$.
Kills...	N/500	N/600	N/100	N/20	N/1	N/10	N/200	N/10
Fails to kill...	N/600	N/700	N/200	N/100	N/10	N/100	N/300	N/100

Of the organic bases tried, trimethylamine was itself toxic to Glaucoma at N/100, and did not appear to increase the toxicity of conessine. Pyridine at N/10 is not toxic alone, and appears to have no effect on the toxicity of conessine.

There remains for consideration the question why these alkaloids are more toxic to protozoa in presence of alkalis. It is natural to assume that combination takes place between the alkali and the alkaloid with the production of a more toxic substance; but sufficient is known about the chemistry of these three alkaloids to make it quite certain that such combination does not occur, and toxicity trials on mice of conessine sulphate, with and without the addition of sodium hydroxide, by intravenous and subcutaneous injection, makes it clear that the alkaloid does not become more toxic to mice in presence of alkali, as the following results show:—

INTRAVENOUS INOCULATION.

Mouse, 28 grammes, 0·35 c.c. of 0·1 per cent. conessine sulphate + 0·35 c.c. distilled water.
" 29 " 0·45 c.c. of 0·1 per cent. " " + 0·35 c.c. "
Both died in less than two minutes.
" 22 " 0·3 c.c. of 0·1 per cent. " " + 0·3 c.c. distilled water.
" 22 " 0·3 c.c. of 0·1 per cent. " " + 0·3 c.c. N/100 NaOH.
Both alive and well after forty-eight hours.

SUBCUTANEOUS INOCULATION.

Mouse, 19 grammes, 0·3 c.c. of 1 per cent. conessine sulphate + 0·3 c.c. distilled water.
" 19 " 0·3 c.c. of 1 per cent. " " + 0·3 c.c. N/100 NaOH.
Both alive and well after forty-eight hours.

It seems more likely, therefore, that the action is due to the effect of the alkali on the organism itself, but even when equal volumes of N/200 sodium hydroxide and suspensions of protozoa are allowed to interact for one hour and the alkaloid is then added, death does not take place more rapidly than when the alkali and alkaloid are added simultaneously. In

protozoa, stained with neutral red and exposed to sodium hydroxide solution, there is no indication of the entry of the alkali until toxic doses of the latter are used, and even then the red colour (acid) of the food vacuoles is not changed to yellow (alkaline) until just before movement ceases. If, however, washed Glaucoma are mixed with a dilute solution of neutral red together with N/200 NaOH, and a saturated solution of conessine base is then added, the vacuoles, which are at first bright red (acid) turn yellow some minutes before the organism becomes motionless; a similar result is obtained when the conessine is added to the organism and N/200 NaOH is subsequently added. Emetine also acts in the same way. If Glaucoma, stained with neutral red, is killed either by the application of moderate heat, or by oxalic acid or ether, then the food vacuoles remain red (acid) even after the death of the organism; and when a similar preparation is made in presence of the same dilute alkali, and is exposed to moderate heat on a warm stage, it is not until the organism disintegrates, and the food vacuoles are shed into the surrounding alkali, that they immediately become yellow.

Mere physical changes in surface tension, facilitating the penetration of the protozoa by the alkaloid, seem to be excluded by the fact that the action is restricted to free alkalis, and is not shown by salts of the alkali metals with the exception of the carbonates, which, however, only cause a slight increase in the toxicity of the alkaloid.

It seems clear from these results that the free alkaloid exerts a direct toxic effect on the organism, and that it is only after that effect has been produced that the alkali comes into play.

As regards the clinical application of these observations upon emetine-resistant cases of dysentery, taking into consideration the recent work of Sellards and Leiva, where rectal injections of quinine dihydrochloride were apparently successful in effecting a cure in a cat suffering from acute amœbic dysentery, there is evidence for assuming that a more suitable means is at our disposal by increasing the toxicity of the alkaloid towards the protozoon without using a larger dose of alkaloid.

So far all attempts at markedly raising the alkalinity of the fæces by taking alkalis by the mouth have failed; large doses of bicarbonate of soda have been taken for several successive days, also the alkaline mixture advocated by Sinton for malaria has been tried without avail. When,

however, large doses of bicarbonate of soda together with heavy magnesium carbonate and bismuth carbonate were taken, a slight increase in the alkalinity of the fæces did occur. For the suggestion to try this mixture we are indebted to Professor MACLEAN.

Whether the oral administration of alkali will sufficiently raise the alkalinity of the content of the lower gut and the mucosa itself, in which amœbæ are embedded, to markedly increase the toxicity of the alkaloid towards the parasite is difficult to say, and we consider that the rectal administration of the alkaloid in the presence of dilute alkali would enhance the chances of action.

Clinical experiments on these lines are now in progress, and a preliminary report has been given by WILLMORE in his paper which appears in this issue of the TRANSACTIONS p. 26).

The net results of this work, so far as therapeutical possibilities go, seem to be as follows:—

1. The toxicity of quinine, emetine and conessine towards the protozoa used in the experiments is considerably increased in an alkaline medium, and it is possible that if this condition can be realised *in vivo*, the action of these alkaloids in protozoal diseases may be made more certain and efficient. Clinical experiments on these lines have been instituted.

2. These results confirm SCHAEFFER'S observations that quinine dihydrochloride (acid) is less active than quinine monohydrochloride (neutral), and emphasise ACTON'S suggestion that for injection quinine base would probably be more efficient and possibly non-irritant, if a means can be devised for injecting it in solution in this form.

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