5. The loss of immunological reactivity at high specific radioactivities or at high levels of chemical substitution with [¹²⁷I]iodine is demonstrated.

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The Electrophoretic Behaviour of some Trypanosomes

BY SHEILA HOLLINGSHEAD* AND B. A. PETHICA†

Department of Chemistry, Manchester College of Science and Technology, Manchester 1,

AND J. F. RYLEY

Imperial Chemical Industries Ltd., Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire

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Evidence has previously been given that certain organic bases show an electrophoretic specificity towards membranes of different cells (Bangham, Glover, Hollingshead & Pethica, 1962). Of particular interest was the effect of the trypanocidal bases Antrycide (quinapyramine), Ethidium (homidium) and Prothidium, and a therapeutically inactive quinapyramine analogue, on the electrophoretic mobility of some neoplastic cells. As trypanosomes are able to circulate freely in the blood during some part of their life-cycle without undergoing phagocytosis, a comparison of their electrophoretic behaviour with that of neoplastic cells may give some indication as to the ability of the latter to metastasize. The present paper describes the effects of these drugs on the electrophoretic mobility of some trypanosomes, and in

addition gives quantitative results on the electrophoretic behaviour of these Protozoa, which have previously been examined only qualitatively (see, for example, Broom, Brown & Hoare, 1936; Grodzensky, 1936; Fischer & Fischl, 1933).

MATERIALS AND METHODS

Trypanosome suspensions. Culture forms of *Trypanosoma rhodesiense* were grown as described by Ryley (1962), and harvested after 5 or 6 days. A rabbit was inoculated intravenously with *T. rhodesiense* from a mouse. Instead of the acute infection seen in rats or mice, a relapsing type of infection resulted which lasted for 3 months. At each relapse, mice were infected from the rabbit, and the variant strains passaged rapidly in mice; one such variant strain has been designated R₂. Blood-stream forms of *T. rhodesiense*, *T. vivax* and *T. equinum* were isolated 3 or 4 days after inoculation from the blood of infected rats by differential centrifuging as described by Ryley (1956), and the blood-stream forms of *T. congolense* and *T. lewisi* were isolated in a similar manner 4–6 days after inoculation.

* Present address: Department of Pharmacy, University of Manchester, Manchester 13.

† Present address: Basic Research Division, Unilever Research Laboratories, Port Sunlight, Cheshire.

Isolated trypanosomes were washed twice and suspended in a solution of 14.5 mM-NaCl and 0.3 mM- NaHCO_3 in 5% (w/v) glucose, pH 7.0 (referred to below as 'glucose-saline'). The isolation procedures were carried out with solutions cooled to 5°. No attempts were made to ensure complete freedom from erythrocytes. Where electrophoretic experiments were performed in 'iso-osmotic saline' (0.145 M-NaCl), the trypanosomes were first washed twice in 0.145 M-NaCl containing 1% (w/v) of glucose, and then suspended in iso-osmotic saline.

Erythrocyte suspensions. Erythrocytes from non-infected rats were washed three times with 100 vol. of 0.145 M-NaCl and once with glucose-saline; after each washing the cells were centrifuged at 1500g for 3 min.

Electrophoretic experiments. Electrophoretic mobilities were determined as described by Bangham *et al.* (1962). For comparison with previous results, mostly obtained at 25° in iso-osmotic saline, it should be noted that the viscosity of glucose-saline at 5° is 1.73 centipoises as against 1.01 centipoises at 25°. Drug solutions were prepared with glucose-saline, and uranyl solutions in glucose-saline without bicarbonate. The quinapyramine analogue used was 1-allyl-4-allylamino-6-(2-amino-1,6-dimethylpyrimidin-4-ylamino)quinaldine 1,1'-di-iodide.

RESULTS

Broom *et al.* (1936) described a simple method for determination of charge. This involved the addition of 2 μl . of infected blood to 0.3 ml. of a solution consisting of 1 vol. of 'normal saline' plus 9 vol. of 4% (w/v) glucose. Microscopical examination after 30 min. showed negatively charged trypanosomes to be perfectly free, whereas positively charged flagellates were firmly adherent to one or more erythrocytes. We have applied this test to all the trypanosome strains available to us. The blood-stream forms of *T. lewisi* and *T. cruzi* and the culture form of *T. rhodesiense* mixed with clean rat blood were all unattached under these conditions, but blood-stream forms of *T. equinum*, *T. vivax* and *T. congolense* were completely covered by erythrocytes; blood-stream forms of the parent strain of *T. rhodesiense* usually associated with but a single erythrocyte, but the variant R₂ did not attract erythrocytes.

In preliminary electrophoretic experiments with *T. rhodesiense* (blood and culture forms) and *T. lewisi* (blood form) in iso-osmotic saline at 25°, it was difficult to detect any lateral movement of the trypanosomes under an applied voltage owing to the inherent motility of the flagellates; an electrophoretic mobility was measurable only when the organisms showed a decrease in or a complete loss of motility. As the situation was unsatisfactory for quantitative studies, other experimental conditions were sought. At 5° the motility of *T. rhodesiense* (blood form) was markedly decreased, and hence the electrophoretic measurements and washing procedure were performed at this temperature. As

the electrophoretic mobility in iso-osmotic saline was very small, a medium of low ionic strength (glucose-saline) was used to permit the use of a higher field strength (about 6 v/cm.) in the electrophoretic cell, and hence allow a better estimate of mobility. We observed no differences in electrophoretic mobility between organisms washed in glucose-saline and organisms first washed in iso-osmotic saline with or without 1% (w/v) of glucose, and then suspended in glucose-saline.

Figs. 1 and 2 illustrate the variation with pH of electrophoretic mobility for *T. rhodesiense* (blood and culture forms), *T. lewisi* (blood form) and *T. equinum* (blood form). Table 1 indicates the effect of trypanocidal drugs and UO_2^{2+} ions on the mobility of the same organisms with the exception of *T. equinum*. The mobility at 5° in glucose-saline of rat erythrocytes was 1.35 ± 0.05 (S.E.) $\mu\text{sec./v/cm.}$

Trypanosoma rhodesiense (blood form). The electrophoretic mobility at 5° in glucose-saline

Fig. 1. Variation in electrophoretic mobility of trypanosomes with pH. Solutions were made up with NaCl, HCl or NaOH in 5% (w/v) glucose (I 0.0145) as required. ■, *T. lewisi* (blood form); ▲, *T. rhodesiense* (culture form); ●, *T. rhodesiense* (blood form): electrophoretic mobilities determined immediately after washing (over pH range 4.5–7.5, trypanosomes motile); ○, *T. rhodesiense* (blood form): electrophoretic mobilities determined after standing for about 1 hr. (trypanosomes non-motile).

Fig. 2. Variation in electrophoretic mobility of *T. equinum* (blood form) with pH. The solutions were as described for Fig. 1. Electrophoretic mobilities determined immediately after washing: ●, trypanosomes motile; ▲, trypanosomes non-motile; electrophoretic mobilities determined after standing for about 1 hr.: ○, trypanosomes non-motile.

Table 1. *Electrophoretic mobility of Trypanosoma rhodesiense (blood and culture forms) and Trypanosoma lewisi (blood form) in glucose-saline with added trypanocides and uranyl ions*

The pH of the trypanosome suspensions containing UO_2^{2+} ions at concentrations of 0.1 mM and 1.0 mM was 6.1 and 4.1 respectively; other suspensions were at pH 7.0. The temperature was 5°.

Addition	Electrophoretic mobility ($\mu\text{sec./v/cm.}$)					
	<i>T. rhodesiense</i> (blood form)		<i>T. rhodesiense</i> (culture form)		<i>T. lewisi</i> (blood form)	
	0.1 mM	1.0 mM	0.1 mM	1.0 mM	0.1 mM	1.0 mM
Quinapyramine	0 to -0.2	0 to -0.2	-0.46	0	-0.61	-0.21
Quinapyramine analogue	0 to -0.2	0 to -0.2	-0.81	-0.71	-0.61	-0.23
Homidium	0 to -0.2	0 to -0.2	-0.89	-0.69	-0.54	-0.47
Prothidium	0 to -0.2	0	+0.34	+0.75	-0.25	+0.56
Uranyl ions	0 to -0.2	+0.90	-0.82	+0.44	-0.41	+0.31
Control	0 to -0.2		-0.91		-0.61	

determined immediately after completion of the washing procedure was zero or had a value in the range -0.1 to -0.2 $\mu\text{sec./v/cm.}$ at pH 7.0. Occasionally a suspension of trypanosomes contained flagellates of two types, one having zero mobility and the other a mobility in the above range. In a single (atypical) suspension examined, the trypanosomes had a very small positive electrophoretic mobility. On standing in glucose-saline at 5° or room temperature the organisms gradually lost their motility and clumped together. The electrophoretic mobility of free motile trypanosomes increased in magnitude as the stock suspension was kept for a period of 1 hr. at room temperature (Fig. 1). Changing of the pH or addition of trypanocidal drugs to the suspension usually resulted in loss of motility accompanied by clumping; the results given in Table 1 and Fig. 1 refer to free organisms. Suspensions of the variant R_2 prepared in glucose-saline showed much less tendency to clump than did the parent strain, but, on the basis of electrophoretic measurements, the two strains were indistinguishable.

Trypanosoma rhodesiense (culture form). This form had an electrophoretic mobility of -0.91 ± 0.02 (S.E.) $\mu\text{sec./v/cm.}$ at pH 7.0 and this varied with pH as indicated in Fig. 1. The mobility at pH 7.0 remained constant over a period of 2 hr. at room temperature, although a gradual loss of motility was observed. From the point of view of loss of motility and clumping, these organisms were less tolerant to pH changes and the presence of trypanocidal drugs than *T. lewisi*, but more tolerant than the blood form of *T. rhodesiense*.

Trypanosoma lewisi (blood form). The electrophoretic mobility was -0.61 ± 0.02 (S.E.) $\mu\text{sec./v/cm.}$ at pH 7.0 and this varied with pH as illustrated in Fig. 1. Mobility and motility at 5° were unaltered when the suspension was kept for 4 hr. in glucose-saline at room temperature. Changes in

pH from pH 2 to pH 10 had little immediate effect on motility, although motility was soon lost in the range pH 2-4; clumping and loss of motility were observed at pH 7.0 in the presence of 1.0 mM trypanocidal drugs. In any given suspension a few individual trypanosomes were attached to erythrocytes, especially at low pH values; occasionally a trypanosome could be observed adhering to two erythrocytes. Two further strains of *T. lewisi* kindly provided by Mr K. N. Brown have been examined. The parent strain P and a derived strain R, claimed to be an antigenic variant, were indistinguishable electrophoretically from each other or from our own strain when examined at pH 7.0.

Trypanosoma equinum (blood form). With some preparations, the entire population was motile at pH 7.0 immediately after washing and had an electrophoretic mobility in the range 0 to +0.4 $\mu\text{sec./v/cm.}$ With other preparations studied, the entire population was non-motile with a mobility close to -0.95 $\mu\text{sec./v/cm.}$ Organisms that were originally motile after washing gradually lost their motility over a period of about 1 hr., did not clump, and showed a change in electrophoretic mobility to -0.95 $\mu\text{sec./v/cm.}$ (Fig. 2).

Trypanosoma congolense and Trypanosoma vivax (blood forms). Blood-stream forms of *T. congolense* and *T. vivax* were extremely difficult to handle, since the use of glucose-saline necessary for electrophoretic measurements caused marked adhesion to erythrocytes and clumping. Such measurements as could be made suggested the trypanosomes had a small negative mobility.

DISCUSSION

As the random orientation of trypanosomes is unaffected by an electric field, there can be no gross localization of charge at either tip of the organism, such as is found with spermatozoa (Bangham, 1961).

The pH-mobility relationship for *T. lewisi* and the culture form of *T. rhodesiense* (Fig. 1) shows a similar pattern to that observed for lymphocytes, Ehrlich ascites-tumour cells and some phosphatide sols, indicating that the membrane structure may be phosphatide in character (Bangham, Pethica & Seaman, 1958; Bangham *et al.* 1962). Uranyl ions caused a charge-reversal with *T. rhodesiense* (culture form) and *T. lewisi* at a concentration of 1.0 mM (Table 1); a low charge-reversal concentration of UO_2^{2+} ions is characteristic of phosphatide and phosphate surfaces (Krutz, 1949; Bangham *et al.* 1958; Seaman, 1958). The present investigations have been made with a medium of low ionic strength (I 0.0145) at 5°, whereas the comparative studies referred to have used iso-osmotic saline (I 0.145) at 25°. However, the concentration of UO_2^{2+} ions needed to produce charge-reversal with human erythrocytes in glucose-saline at 5° was the same as in iso-osmotic saline at 25° (1.0 mM).

The blood-stream form of *T. rhodesiense* gave an isoelectric point lying between pH 5.8 and pH 7.0 with different suspensions of organisms (Fig. 1); thus, at physiological pH, the trypanosome is capable of circulating in an uncharged state or carrying a very small, usually negative, charge. Uranyl ions do not appear to have any effect on the electrophoretic mobility of the trypanosome; the change in mobility brought about by uranyl solutions is due only to the change in pH of the solutions (Table 1 and Fig. 1), suggesting that the surface of these organisms is not phosphatide in character. Polysaccharide surfaces give low electrophoretic-mobility values that are independent of pH (Douglas & Shaw, 1957). Such a surface on the blood-stream form of *T. rhodesiense* would account for the zero or low mobility observed at pH 7.0, but the pH dependence of the trypanosome mobility suggests the presence of basic and acidic groups in the membrane. From Fig. 1, the pK_a values of the acidic and basic groups are approximately 5.0 and 9.5 respectively, with both types of group ionized in the neutral pH range, suggestive of carboxyl and amino groups. At the moment no clear identification of the surface-charge groups is possible.

The observation that trypanosomes subjected to different washing conditions and unwashed trypanosomes give the same electrophoretic mobility (within experimental error) suggests that the trypanosome surface is not altered during the preparation of the suspension. The loss of some surface component may be responsible for the change with time to a more negative mobility found for suspensions of the blood-stream forms of *T. rhodesiense* (Fig. 1) and *T. equinum* (Fig. 2). A striking change in surface properties accompanies loss of motility and death of *T. equinum*. Though

motile organisms usually carried a small positive charge, the 'non-motile' organisms had a surface that was similar to that of *T. lewisi* and the culture form of *T. rhodesiense* (Fig. 1). There is a general similarity between the blood forms of *T. equinum* and *T. rhodesiense* in the motility-mobility relationships.

Broom *et al.* (1936) noted that the insect and culture forms they examined, and also the blood forms of *T. cruzi* and *T. lewisi*, invariably carried a negative charge, whereas the charge on the blood-stream forms of *T. evansi*, *T. congolense*, *T. brucei* and *T. gambiense* could be either positive or negative, the change in charge of any particular species being associated with a relapse in the particular infection. They claimed that the sign of the charge was correlated with the behaviour of trypanosomes towards erythrocytes in a solution similar to our glucose-saline. Our results with *T. lewisi*, *T. cruzi* and the culture form of *T. rhodesiense* are in agreement with their findings, but, although *T. equinum* seems to carry a small positive charge and associate with erythrocytes in glucose-saline, *T. vivax*, *T. congolense* and the parent strain of *T. rhodesiense* also associated with erythrocytes, but carried if anything a small negative charge. In particular, although the parent strain of *T. rhodesiense* and the antigenic variant R_2 showed the opposite behaviour towards erythrocytes in glucose-saline, they were electrophoretically indistinguishable as were the P strain of *T. lewisi* and the antigenic variant R.

Comparison of the results obtained with the blood-stream and culture forms of *T. rhodesiense* shows that the latter corresponds to the form found in the gut of the insect vector. Adaptation to life in the tsetse fly, besides involving a change in environmental conditions (temperature, oxygen pressure, available nutrients) results in a marked change in metabolism (Ryley, 1962), in ultra-structure (Vickerman, 1962), and, from the present studies, it would seem in the nature of the cell surface. From consideration of the changes occurring with *T. equinum* as motility and viability are lost, which seem to involve the loss of surface groupings, it seems possible that such unmasking of inner-surface components takes place with *T. rhodesiense* on introduction into the vector.

The effect of the trypanocides on the culture form of *T. rhodesiense*, the blood-stream form of *T. lewisi* and Ehrlich ascites-tumour cells are similar. The drugs at a concentration of 0.1 mM are effective in decreasing the negative mobility of the tumour cells in the order: Prothidium, quinapyramine, quinapyramine analogue and homodium (Bangham *et al.* 1962). The bases are poorly adsorbed on to the surface of the blood-stream form of *T. rhodesiense*; at a concentration of 1.0 mM,

Prothidium caused gross loss of motility and clumping of all trypanosomes.

Comparison of the findings for trypanosomes with those for other cells previously studied shows that *T. lewisi* is very similar electrophoretically to freely circulating cells such as lymphocytes and tumour cells. On the other hand, the surface properties of the blood-stream form of *T. rhodesiense* are different electrophoretically from any other freely circulating cell so far studied. If the surface of these cells does contain carboxyl groups, the previous hypothesis that all cells carrying surface carboxyl groups will be adhesive to phagocytes will need modification (Bangham & Pethica, 1960). The absence of net charge on the freely-circulating blood-stream form of *T. rhodesiense* is evidence that van der Waals forces play a very minor role in cell adhesion (Pethica, 1961), although the flagellate motility may adversely influence the adhesion process.

SUMMARY

1. Electrophoretic results are presented for *Trypanosoma rhodesiense* (blood and culture forms), and for the blood-stream forms of *T. congolense*, *T. equinum*, *T. lewisi* and *T. vivax*.

2. From a study of the electrophoretic behaviour of trypanosomes at various pH values in the presence and absence of UO_2^{2+} ions, it is suggested that the surface of the culture form of *T. rhodesiense* and of the blood-stream form of *T. lewisi* is phosphatide in character, whereas that of the

blood-stream form of *T. rhodesiense* may be of a polysaccharide or polypeptide nature.

3. Several trypanocidal drugs decreased the negative mobility of the blood form of *T. lewisi* and culture form of *T. rhodesiense* in a manner similar to that found with Ehrlich ascites-tumour cells; they had little effect on the electrophoretic behaviour of the blood form of *T. rhodesiense*.

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Further Studies on the Alkylation of Nucleic Acids and their Constituent Nucleotides

By P. D. LAWLEY AND P. BROOKES

Chester Beatty Research Institute, Institute of Cancer Research: Royal Cancer Hospital, London, S.W. 3

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The principal biological effects of alkylating agents, namely cytotoxicity and mutagenesis, have stimulated interest in their action on nucleic acids, and particularly on DNA. This followed from early work such as that of Herriott (1948), who showed that viruses, particularly those containing DNA, were highly susceptible to inactivation by mustard gas [di-(2-chloroethyl) sulphide], and the demonstration by Auerbach & Robson (1946) that mustard gas was mutagenic.

Specificity of effect among alkylating agents falls mainly under two headings. First, the difunctional agents generally exert a more powerful cytotoxic action than the monofunctional, which may be related to the ability of difunctional agents to cross-link the twin strands of the macromolecular structure of DNA (Brookes & Lawley, 1961a). Secondly, significant differences in the biological action of the simpler monofunctional alkylating agents have been shown, particular attention