

SOME OBSERVATIONS ON THE ACTIONS OF STEROIDS  
ON THE METAPLASIA OF THE AMOEBIA,  
*NAEGLERIA GRUBERI*

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INTRODUCTION

Hollande (1942) was the first to show that the form assumed by *Naegleria*, amoeboid or flagellate, was dependent on the salt concentration of the medium. Results of further investigations along these lines confirming and extending Hollande's observations have been published in two earlier papers (Willmer, 1956, 1958). It was shown that solutions of both NaCl and KCl, of increasing concentrations above about 1 mM, caused the amoeba to tend to remain in the amoeboid form, and in concentrations greater than about 50 mM completely suppressed the assumption of the flagellate form. Since the amoeba in these simple salt solutions did not appear to be able to discriminate appreciably between solutions of NaCl or KCl, it was considered possible that the addition of such steroids as deoxycorticosterone and aldosterone might cause the cells to effect some discrimination along these lines since this appears to be their action on the cells of the vertebrate kidney and other tissues. Other steroids also are known to alter the manner in which cells maintain their ionic equilibrium, as, for example, progesterone on the cells of the uterus (Horvath, 1954; Csapo, 1956; Goto & Csapo, 1959) and deoxycorticosterone on blood cells (Jones, 1955). A preliminary account of the successful use of deoxycorticosterone in this way was given at the International Symposium on Cell Movement and Cell Contact in Leiden in 1960 (Willmer, 1961*a*). The present paper amplifies these observations and extends them to the actions of other steroids.

One of the most noticeable differences between *Naegleria* in the amoeboid form and *Naegleria* in the flagellate form is the nature of the cell surface. In the amoeba the surface readily extends into lobose pseudopodia, but in distilled water this property is lost. The cell develops a polarity, and lobose pseudopodia are restricted to the anterior end while filiform pseudopodia may appear for a time at the posterior end. Eventually, in the flagellate, the surface temporarily loses all power to form pseudopodia at all. Since it was considered possible that the steroids might act on the cell surface by entering into the lipid layer (Willmer, 1961*b*), the surface of the amoeba appeared to offer a suitable test object for investigating this possibility, particularly as the steroids can be applied directly to the cell in a simple medium of defined composition.

While the results show that the various steroids do in fact have very different and characteristic actions on the amoeba-flagellate transformation, their interpretation is not immediately obvious.

## METHODS

The *Naegleria* cultures were obtained from the same stock as before and were originally supplied by the Botany School, Cambridge (A 1518). They were maintained, as on previous occasions, on Lemco-agar slopes in test-tubes (Willmer, 1956). The organisms were mostly used on the 4th day after sub-culture, but this varied from the 3rd to the 7th day according to circumstances. On the 4th day the amoebae are large and there are relatively few cysts. After the 4th day the amoebae tend to become smaller and cysts more numerous. There is no evidence that the age of the culture makes any difference to the behaviour of the amoebae either with regard to the time of the first acquisition of flagella or of the occurrence of the maximum number of

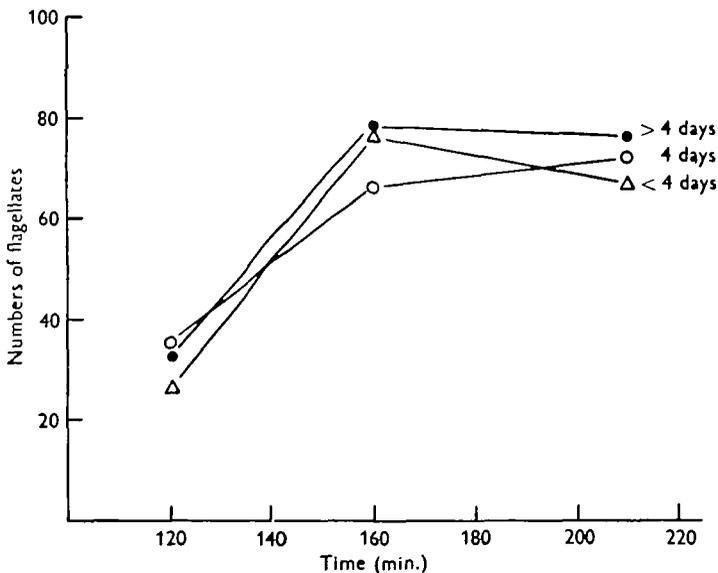


Fig. 1. The effect of the age of the culture of *Naegleria* on the numbers of flagellates present at different times after seeding the buffer solution (B 15 G) with amoebae. Observations made at 21° C.

flagellates (Fig. 1). During subculture, for maintaining the strain, the amoebae were suspended in an M/5000 phosphate buffer (B<sub>2</sub>, see Willmer, 1956) and the usual aseptic precautions were taken.

Since, in the present investigations, it was thought desirable that the amoebae should not be completely lacking any of the main biological ions, a buffer solution (B 15 G) was made up empirically and for convenience as follows:

NaCl	0.015	} per litre of distilled and de-ionized water
KCl	0.015	
CaCl <sub>2</sub> .6H <sub>2</sub> O	0.015	
MgCl <sub>2</sub> .12H <sub>2</sub> O	0.015	
NaHCO <sub>3</sub>	0.015	
Na <sub>2</sub> HPO <sub>4</sub> .12H <sub>2</sub> O	0.015	
NaH <sub>2</sub> PO <sub>4</sub> .2H <sub>2</sub> O	0.003	
Glucose	1.8	

This was convenient to make up, had a pH about 7.2 (B.D.H. Universal Indicator) and though not provoking the change to the flagellate form so actively as distilled water would have done, was yet effective enough in this direction.

In those experiments which were designed to determine whether the amoebae can differentiate between sodium and potassium and whether steroids can alter their behaviour in this direction, the buffer solution was made up without KCl. KCl and NaCl were then dissolved in this buffer at concentrations which when diluted with an equal concentration of the buffer without KCl would yield buffer containing

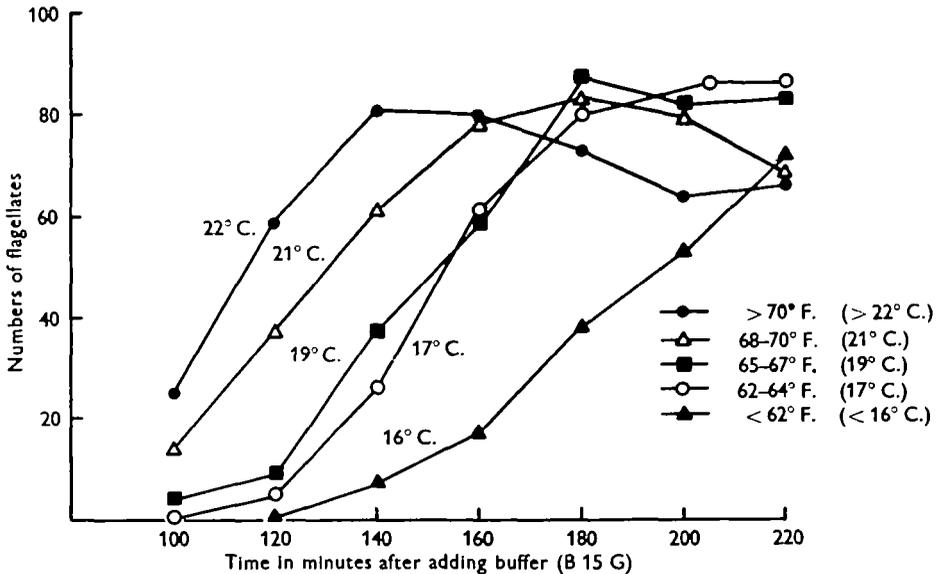


Fig. 2. The effect of temperature on the numbers of flagellates forming in buffer solution (B 15 G).

M/1000 KCl or M/1000 NaCl. In these experiments therefore one buffer solution contained all the original salts (except KCl) plus M/1000 KCl, and the other contained all the original salts (except KCl) plus an additional M/1000 NaCl to compensate for excess osmotic pressure, cation and chloride, introduced by M/1000 KCl.

During the course of the work it became evident that temperature played a much more important role in determining the onset of metaplasia than had been previously realised and much more uniform results have been obtained since the temperature of the room has been kept almost constant at 21° C. Figure 2 shows how by raising the temperature from 16° to 22° C. the duration of the lag phase before flagellates appear decreased from about 120 min. to something less than 90 min., while the maximum number of flagellates appeared at about 140 min. instead of after something more than 220 min. Since these figures are purely relative, there is as yet no information on the total numbers which turn flagellate at different temperatures, though no obvious trend has been observed.

The experimental procedure was the same as that used on previous occasions. The amoebae were washed from their slopes with buffer solution and centrifuged at a low speed just sufficient to precipitate them in about 3 min. They were then resuspended

in buffer solution and centrifuged again and the process repeated a third time. This usually provided amoebae which were sufficiently free from bacteria for the latter to be unlikely to interfere. In any given experiment the solutions to be tested were used in 0.5 ml. amounts in very carefully cleaned test-tubes. At least four tubes were used for each solution and four tubes contained buffer solution to act as a standard control. As a routine, the test-tubes were washed with ethanol, and then with several changes of distilled water. The tubes were then inoculated, each with a single drop from a uniform suspension of washed amoebae. Counting started about 90–100 min. after inoculation. Uniform drops of about 0.01 ml. (the size depending on the field of view of the microscope) were plated out on to a microscope slide, usually three at a time, and the number of flagellates which appeared in the low-power microscope field during one tour of the circumference of each drop was noted. Although this appears to be a somewhat rough and ready method of recording events it was found in practice to work reasonably well and, in addition, to give the observer a good visual impression of the behaviour of the cells in the various solutions. Counting was generally carried on till at least 240 min. after inoculation, and longer if necessary.

In order to compensate for the varying numbers of amoebae which were present in the original suspension, all figures were expressed as a percentage of the number of flagellates which appeared in the buffer solutions acting as controls. This last number was taken as the average of the maximum numbers appearing in each of the control tubes. Nearly all the data in this paper are therefore given in terms of the percentage  $(100x/y)$  where  $x$  is the number of flagellates in an experimental tube and  $y$  is the maximum number which become flagellate in the control tube. Thus a figure of 20 means that in the particular solution at the particular time the number of flagellates present was only 20% of the mean of the maximum numbers which became flagellate in each of the control tubes. A number 120 means that the experimental tube contained 20% more flagellates than the control tubes did, on the average, at their maximum.

Each experiment was repeated at least four times and, for convenience of plotting the data, readings have in the first instance been grouped into 20-min. periods, so that each point on the graphs represents the mean of at least ten readings and generally very many more. In many cases, the counting period (e.g. 90–240 min. after inoculation) has been subdivided into three, 90–140, 141–180, 181–240, in order to see at what time, if any, the experimental solution was having most effect on the numbers recorded. For reasons which are still not clear, considerable variation in numbers occurred from tube to tube and from experiment to experiment and the standard error  $\Sigma(d^2)/n(n-1)$  was always of the order of  $\pm 4$  or more, so the figures given in the graphs should be interpreted only with this degree of exactitude.

The experimental solutions could well have a different action on the flagellate form from their action on the amoeboid form. This was tested, and found to be so, by allowing the amoebae to become flagellate in the buffer solution, and then adding the experimental substances (in twice their usual concentrations in buffer solution, 0.5 to 0.5 ml.) at about 180 min. after the original inoculation. At this time the number of flagellates was usually near its maximum (see Fig. 1). Counting then started at once, and continued for at least 2 hr. Experience has shown that there is not usually the same lag period between altering the conditions and the resulting change of phase,

When the flagellate changes to the amoeba as when the amoeba changes to the flagellate.

The steroids used were obtained through Messrs George T. Gurr, Ltd., and were Sigma products (U.S.P.). They were made up in ethanol, the highest strength used being determined in some cases by the saturation of this solution. The standard strength of the alcoholic solution was determined by reference to some preliminary observations with deoxycorticosterone in which it was found that a final medium containing  $25 \mu\text{g./ml.}$  had a pronounced effect on the onset of flagellum formation. Other steroids were therefore initially compared with this standard, which meant applying them in solutions of  $7.6 \times 10^{-5} \text{ M}$  concentration. From 0.025 to 0.1 ml. of the strong ethanolic solution of steroid was added to 10 ml. of buffer solution to give the required concentration in the experimental medium, and the control medium received an equivalent amount of ethanol alone. Serial dilutions in buffer solutions were made from the strongest medium when necessary. In one or two cases, to be mentioned later, the steroid was partly thrown out of solution when the alcoholic solution was added to water and the results with the highest concentrations of these steroids are therefore those with a saturated solution of the steroid in water. Sometimes a surface layer separated as a thin film, but in most cases a clear solution was formed.

## RESULTS

### *Amoeba-to-flagellate transformation*

*Na and K.* Nearly all the experiments in this series were designed to see whether the steroids made any difference to the way in which the amoebae reacted to the presence or absence of potassium. The control buffer solutions therefore contained  $\text{M}/1000$  KCl or  $\text{M}/1000$  NaCl, respectively, in addition to the usual mixture minus KCl. These numerous control solutions therefore give a clear answer to the question whether the amoeba discriminates between NaCl and KCl. Figure 3, where each point is the mean of well over one hundred observations, shows clearly that the amoeba does not react differently in the presence or absence of  $\text{M}/1000$  KCl. In some experiments there was some suggestion that the absence of potassium caused more flagellates to appear in the latter part of each experiment but this was probably never significant. Although the distilled water was ion-free, the salts (which were B.D.H. A.R.) may have contained traces of potassium, so these experiments do not show that the amoebae can exist without any potassium in the medium; but  $\text{M}/1000$  solutions of NaCl and KCl, when superimposed on the standard buffer solution without KCl, had precisely similar effects on the amoeba-flagellate transformation, even though in the former the molar ratio of Na/K was almost infinitely large while in the latter it was almost unity (0.65:1). All the solutions contained the same chloride concentration.

*Cholesterol.* It was necessary to ascertain next whether the effect of the steroid hormones was specific, or whether it was a general effect of sterols; so the effects of cholesterol were investigated at  $7.6 \times 10^{-5} \text{ M}$ . They were on a very small scale (Fig. 4) with only a slight reduction in the numbers of flagellates as compared with the controls. It so happened that the reduction was somewhat greater in the KCl-free buffer than in the normal buffer but the difference was not outside the experimental error. It was greatest in the early part of the observation period.

*Deoxycorticosterone* (alcohol) (DOC). The first steroid tested was deoxycorticosterone, in order to see if it caused the amoebae to differentiate between sodium and potassium. In the first experiment, the KCl concentration was M/500 instead of the usual M/1000 and the concentration of DOC was  $7.6 \times 10^{-5}$  M. The results are shown in Fig. 5. It is noticeable that the presence of the extra KCl in the medium caused the numbers

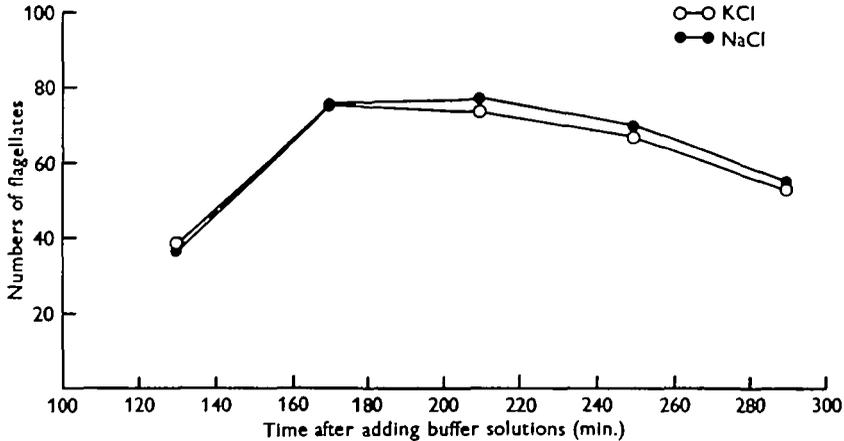


Fig. 3. The effect of M/1000 KCl and M/1000 NaCl on the numbers of flagellates which appear when amoebae are seeded into these solutions in buffer (B 15 G without K).

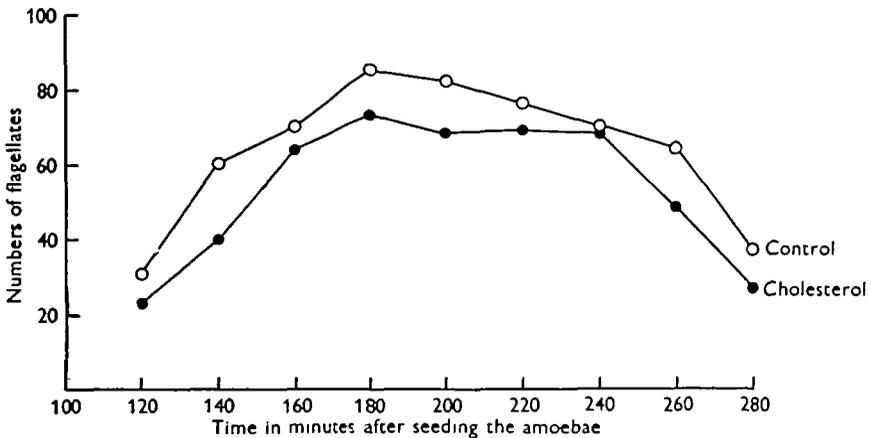


Fig. 4. The effect of  $7.6 \times 10^{-5}$  M cholesterol on the numbers of flagellates formed.

of flagellates to be significantly less in the later part of the experiment than they were in the control solution. The control buffer solution contained no compensating NaCl in this case. On the other hand, in the presence of DOC far fewer cells became flagellate throughout but there was now no noticeable difference between the two buffer solutions used. This could mean that the DOC decreased the ability of the cells to make use of the extra KCl provided. Certainly the action of the DOC in reducing the numbers of flagellates did not depend on the presence of potassium in the medium.

Later experiments with the normal buffer solutions with and without K confirmed that the amoeba behaved in the same way in both solutions.

During these experiments it was noticed that there was rather wide variation in the action of the DOC, and partly for this reason it was thought desirable to study the effects of different concentrations on the ability of the amoebae to undergo transformation. The DOC, which was shown (by Dr Short of the Veterinary School, Cambridge) to be a very pure sample, was used in serial dilutions ( $\times 2$ )  $7.6 \times 10^{-6}$  to  $4.8 \times 10^{-6}$  M. The rather surprising results are shown in Fig. 6, from which it is clear that the actual dose is a matter of considerable importance and that in the range of

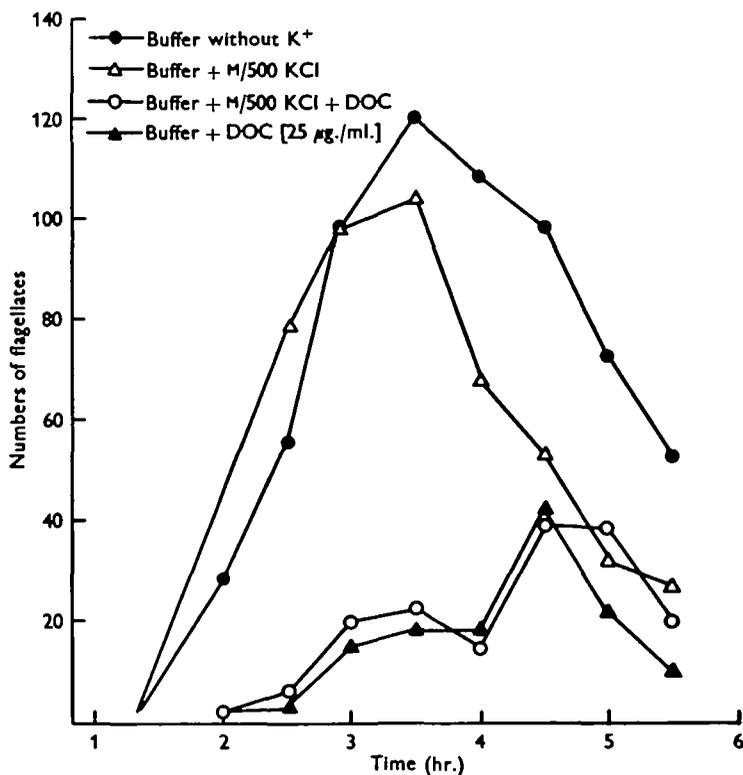


Fig. 5. The effects of  $7.6 \times 10^{-6}$  M deoxycorticosterone and of M/500 KCl on the numbers of flagellates formed.

concentrations from about  $6 \times 10^{-6}$  to  $6 \times 10^{-6}$  M or below, DOC actually encourages the onset of the flagellate phase. This effect is particularly noticeable during the later part of the experimental phase, i.e. after about 200 min. (third period). Indeed in the early stages (first period, from 100–140 min. after adding the DOC) only the concentrations around  $2 \times 10^{-6}$  M produce more flagellates than the controls. On the other hand, the amount of inhibition is much greater in the early stages of the experiment. The suggestion might be made that DOC delays the change to the flagellate form, but also delays the return to the amoeboid form. This, however, is probably not the correct explanation as will be seen when the effects of the steroids applied directly to the flagellate phase are discussed.

*Hydrocortisone and cortisone.* So far as experiments have gone with these corticoids, neither of them has such a definite action as DOC. Cortisone has, so far, only been applied at  $7.6 \times 10^{-5}$  M at which concentration it produced a regular but slight depression in the numbers of flagellates formed. Weak concentrations of hydrocortisone produced some increase in the numbers of flagellates but increasing the concentration to  $1.5 \times 10^{-4}$  M produced no very marked inhibition (Fig. 7).

*Aldosterone.* A single preliminary experiment showed this to be inhibitory to about the same extent as DOC when applied at  $7.6 \times 10^{-5}$  M but there was no trace of an activating effect comparable with that of DOC at lower concentration.

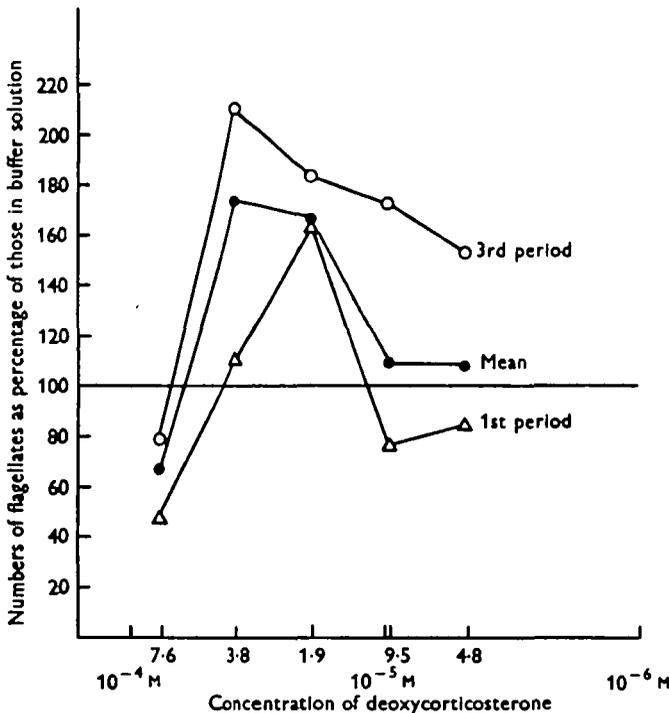


Fig. 6. Dose/response curve for deoxycorticosterone. First period = 100–140 min. after seeding, third period = 180–240 min. after seeding.

*Progesterone.* At  $7.6 \times 10^{-5}$  M progesterone had a very strong inhibitory action on the transformation. The dose/response curve obtained with serial dilutions showed a great similarity to that of DOC though at somewhat different concentration levels (Fig. 8). From complete inhibition of the flagellate form at  $7.5 \times 10^{-6}$  M, almost twice the normal numbers of flagellates appear in the early stages of treatment with  $4.5 \times 10^{-6}$  M. It is interesting to notice that in these experiments, the effects were more noticeable during the first period (100–140 min.) than during the third period (180–240 min.). Inhibitory concentrations were more inhibitory at the beginning (like DOC) but concentrations favouring the flagellate also acted mostly in the first period and differed in this way from similar concentrations of DOC which were most effective in the third period. It would appear from the graph (Fig. 8) that concentrations of progesterone as low as  $10^{-6}$  M (0.3  $\mu$ g./ml.) would probably exert a measurable effect on the amoeba-flagellate

transformation (though they have not actually been tried) and the estimated average concentration of progesterone which may circulate in human blood during pregnancy is 0.142  $\mu\text{g./ml.}$  and may reach 0.268  $\mu\text{g./ml.}$  (Zander, 1955). Thus, allowing for some specialization on the part of the 'target' organs the effective concentrations are not entirely dissimilar.

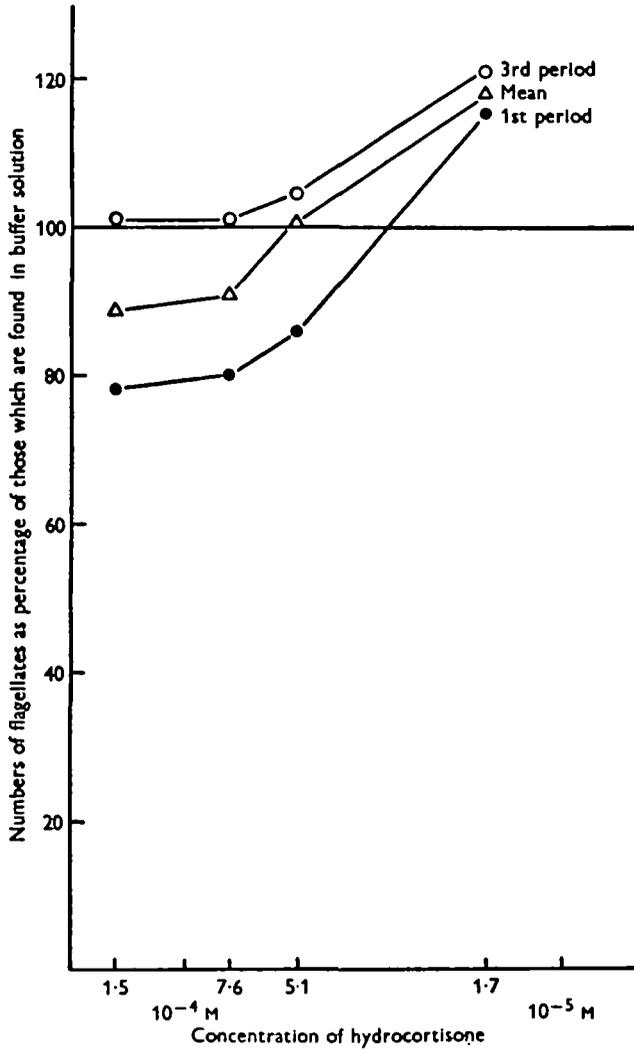


Fig. 7. Dose/response curve for hydrocortisone. First period = 100-140 min. after seeding, third period = 180-240 min. after seeding.

Since progesterone is known to alter the K/Na ratio in certain tissues of the body it was of interest to see whether its action in causing more amoebae to become flagellate depended on the amount of NaCl present in the medium at the same time. Table 1 shows that while there may be some small reduction in its effect at higher concentrations of NaCl substantially more flagellates always appear in the presence of progesterone. The figure for the 500 mM NaCl solutions is somewhat inaccurate owing to the small

numbers of flagellates which are found in this concentration. It may be concluded, however, that the effect of the progesterone is virtually independent of the salt concentration within the limits tested.

Table 1

NaCl (mM)	E/C
500	1.49
125	1.76
0.6 (control buffer)	1.65

$$E/C = \frac{\text{Mean flagellates per tube in presence of progesterone}}{\text{Mean flagellates per tube in absence of progesterone}}$$

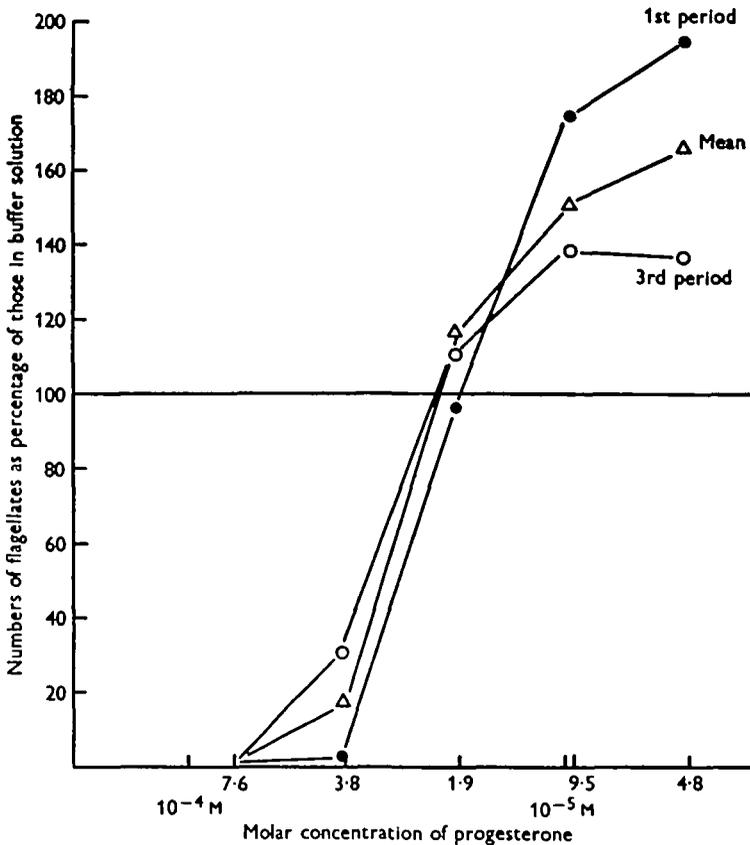


Fig. 8. Dose/response curve for progesterone. First period = 100–140 min., third period = 180–240 min.

*Testosterone.* The characteristic of testosterone throughout numerous experiments has been the variability of the response to it. Whether this depends on some still unknown factor in the amoebae or on some other uncontrolled factor in the experiments has not been determined. Presumably the variability must lie in the amoebae since to other steroids the response has, in general, been much more uniform. It is, however, possible that the state of the steroid and the amount which actually goes into solution

could be important. At the highest concentration the testosterone formed a slight surface film on the buffer solution.

In high concentrations testosterone becomes inhibitory to flagellum formation but at lower concentrations there was generally some favouring of the flagellate form in the latter half of the observation period. The inhibitory effects were always much more noticeable in the early stages of each experiment. Thus at a concentration of  $3.8 \times 10^{-6}$  M only 84 % as many flagellates were counted as in the controls during the period from 100–140 min. after adding testosterone, but during the period from 180–240 min. this had risen to 141 %.

The results with concentrations from  $1.5 \times 10^{-4}$  M downwards are shown in Fig. 9*a*. The results for the third period are somewhat similar to those obtained with progesterone and DOC, but testosterone is far less potent in its actions. During the first period the dose/response curve is of very different shape from those for progesterone and DOC.

*Androsterone.* The dose/response curves for this steroid (Fig. 9*b*) are somewhat similar to those for testosterone though androsterone is much more favourable to the amoeboid form. The mean curve for androsterone compares rather closely with the curve for the first period in testosterone. The curves for androsterone differ increasingly from those for DOC and progesterone. During the first period, all but the weakest concentrations used have strong inhibitory effects on the numbers of flagellates recorded. In the third period, however, there is still a suggestion that intermediate concentrations produce more flagellates than the controls.

*Oestradiol and oestrone.* The dose/response curves for oestradiol (Fig. 10) are somewhat similar to those for androsterone. Except perhaps during the first period and in dilute concentrations, oestradiol is generally inhibitory to the flagellate phase, and the changes in concentration produce comparatively small corresponding changes in effects. During the third period, high concentrations become somewhat less effective in inhibiting the flagellate phase while weaker concentrations, which at first were ineffective or slightly favouring the flagellate, begin to become inhibitory.

In the highest concentration ( $7.6 \times 10^{-5}$  M) the oestradiol was partly in suspension in the buffer solution.

Some preliminary observations with oestrone and with stilboestrol indicated that these substances behaved in a similar manner to oestradiol, but at  $7.6 \times 10^{-5}$  M oestrone was considerably less effective than oestradiol.

Oestrone at  $7.6 \times 10^{-5}$  M was slightly inhibitory to the flagellate form but relatively inactive.

*Androstenedione.* This steroid with ketone groups at each end of the molecule was found to be relatively ineffective at the concentrations at which the other steroids produce their effects (Fig. 11).

*Combinations of steroids.* In the animal body steroids often act in combination. It therefore seemed to be of interest to test whether inhibition produced by one steroid affects the action produced by another steroid. Two combinations have so far been tested, namely progesterone and oestradiol and testosterone and oestradiol.

Concentrations of oestradiol and progesterone, which each produced about 50 % inhibition when given alone, were chosen and their effects separately and in combination are shown in Fig. 12. It is clear that their combined effects are considerably greater

than the effects of either alone. It would be interesting to know how progesterone would act with oestradiol when applied at concentrations which increase the numbers of flagellates.

In a similar experiment oestradiol alone produced only 36% of the number of flagellates in the buffer alone, testosterone 64% and combined they produced 23%. Both steroids were presumably acting independently.

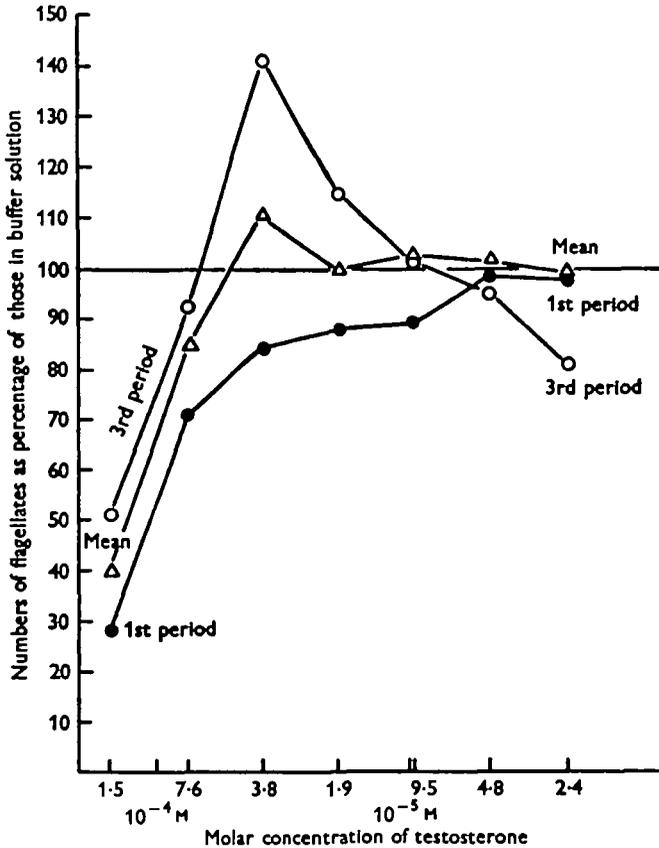


Fig. 9a. For legend see Fig. 9b facing page.

*Vitamin A.* In view of the similarity between the actions of progesterone and vitamin A on certain tissues of the mammalian body, it is of interest that the dose/response curve for vitamin A on *Naegleria* has also some similarity to that for progesterone, though there is probably no encouragement of the flagellate phase at the lower concentrations (Fig. 13). These results, however, were obtained with vitamin A treated in the same way as the steroids and the instability of the vitamin under these conditions could cause errors and the subject needs further investigation when the vitamin is stabilized with proteins.

*Flagellate-to-amoeba transformation*

The results of adding steroids to the amoebae are always somewhat complicated by any action which the steroids may exert on the flagellates themselves. Moreover, since the whole surface of the cell is very different in the two phases, it is quite possible that the actions of the steroids on the two phases of these organisms could be

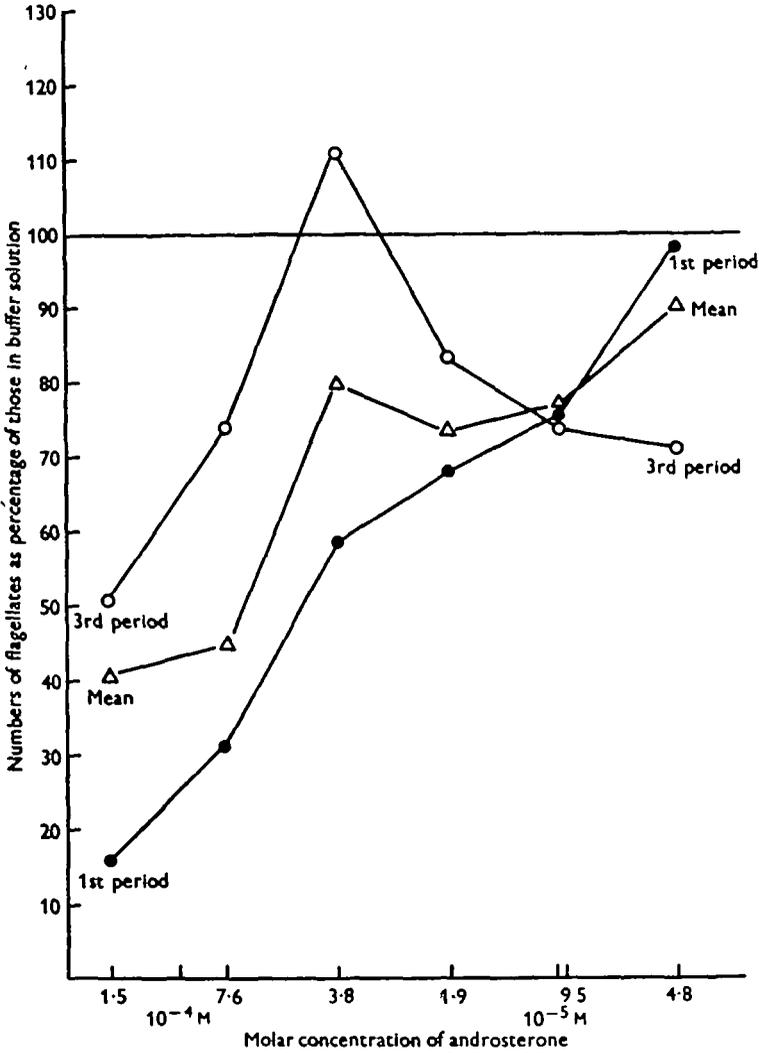


Fig. 9b.

Fig. 9. Dose/response curves for the androgens (a) testosterone and (b) androsterone.

quite different. This has indeed been found to be the case. Similarly, in long-continued experiments, action on the amoebae may complicate the apparent effects of the steroids on the flagellates.

Amoebae were placed in buffer solution till large numbers of flagellates were

produced and then an equal quantity of buffer solution containing twice the required concentration of steroid was added to the experimental tubes, and a similar quantity of buffer to the control tubes. Counting of flagellates started at once. The transformation from flagellate to amoeba can be immediate and very rapid, in this way contrasting with the reverse transformation which requires about  $1\frac{1}{2}$ –2 hr. for its completion.

The results of adding the steroids to flagellates have been expressed as the percentage

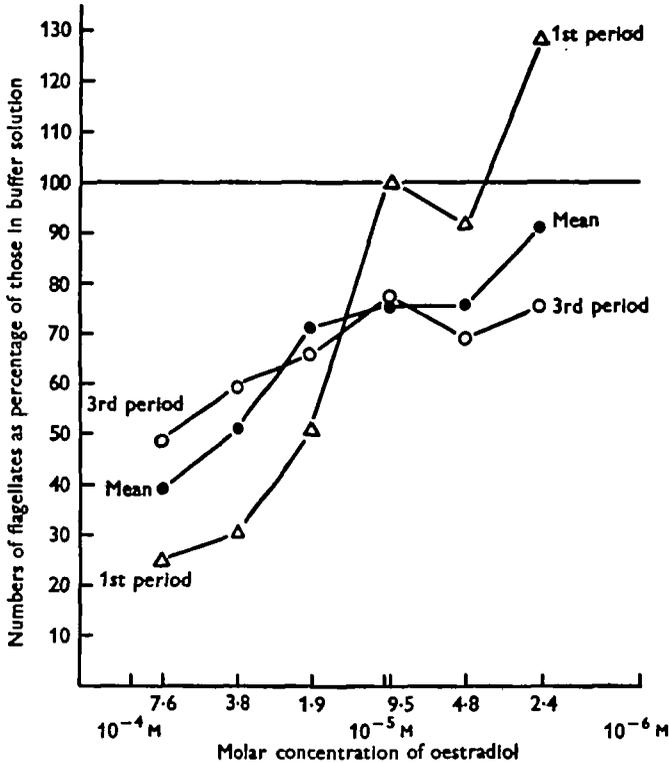


Fig. 10. Dose/response curves for oestradiol.

which the numbers of flagellates present in the steroid-treated tubes form of the numbers in the buffer tubes during corresponding periods after adding the experimental solutions.

*Na and K.* Whereas the amoeboid form showed no preference for sodium or potassium, and equimolar solutions of NaCl and KCl caused the appearance of the same numbers of flagellates, the addition of similar solutions to the flagellates indicated that the KCl solution allowed somewhat fewer flagellates to remain in that condition (Fig. 14). The difference is certainly not great, but is probably just significant and confirms some earlier observations (Willmer, 1961*a*) where buffer solutions containing  $M/100$  NaCl or  $M/100$  KCl had been used in a similar way. It was noticeable also that the variation in numbers of flagellates from tube to tube was always somewhat less in the tubes containing KCl than in those containing NaCl. Reference to Fig. 3, in which the effects of NaCl and KCl on the amoeboid form are set out, shows that at the time when numerous flagellates are present and beginning to turn back to the amoeboid

state the numbers decrease slightly more rapidly when KCl is present. Again the figures are not in themselves significant but they point in the same direction.

It may be concluded therefore that while the amoeba shows no evidence of discrimination between NaCl and KCl the flagellate may have a real preference for KCl.

*Deoxycorticosterone.* As recorded elsewhere (Willmer, 1961*a*), the addition of DOC, at such a concentration as to produce  $7.6 \times 10^{-5}$  M in the experimental tubes,

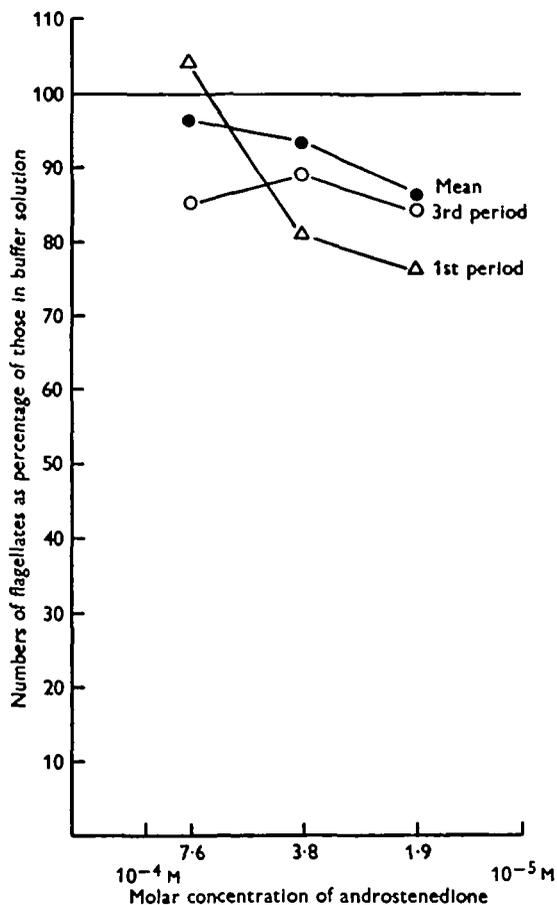


Fig. 11. Dose/response curve for androstenedione.

was found to produce some decrease in the numbers of flagellates as compared with the addition of a corresponding amount of buffer solution. This applied when potassium was omitted from all the solutions, but applied much more noticeably when the buffer solutions (experimental and control) contained M/1000 KCl (Fig. 15). This result was of interest for two reasons. First, the DOC acts, at this concentration, in a similar way on both amoeba and flagellate in that it tends to favour the amoeboid form. Secondly, it accentuates the somewhat doubtful preference of the flagellate for potassium rather than sodium, into something very real.

*Hydrocortisone.* At  $7.6 \times 10^{-5}$  M this steroid produced no obvious difference in the numbers of flagellates when added to the medium containing flagellates.

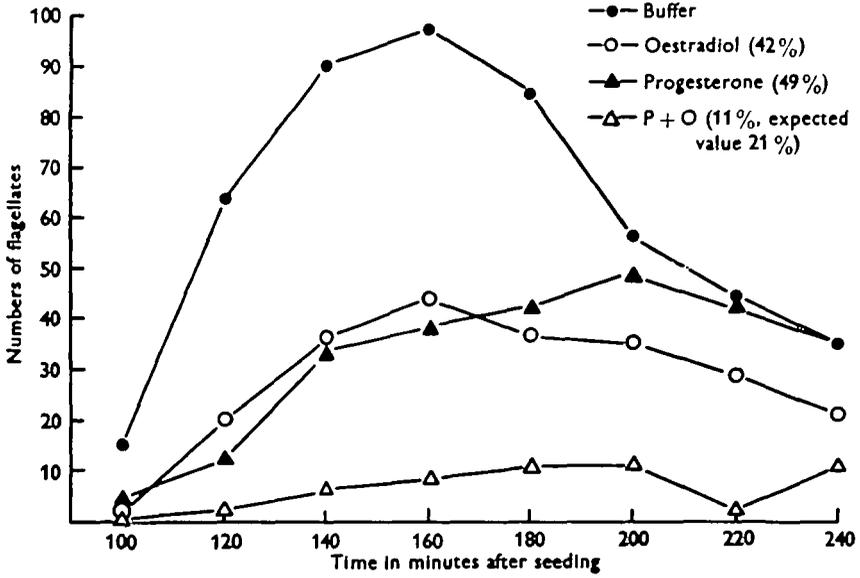


Fig. 12. The combined effects of progesterone ( $3.8 \times 10^{-8}$  M) and oestradiol ( $3.8 \times 10^{-8}$  M) on the numbers of flagellates which appear after seeding the solutions at zero time.

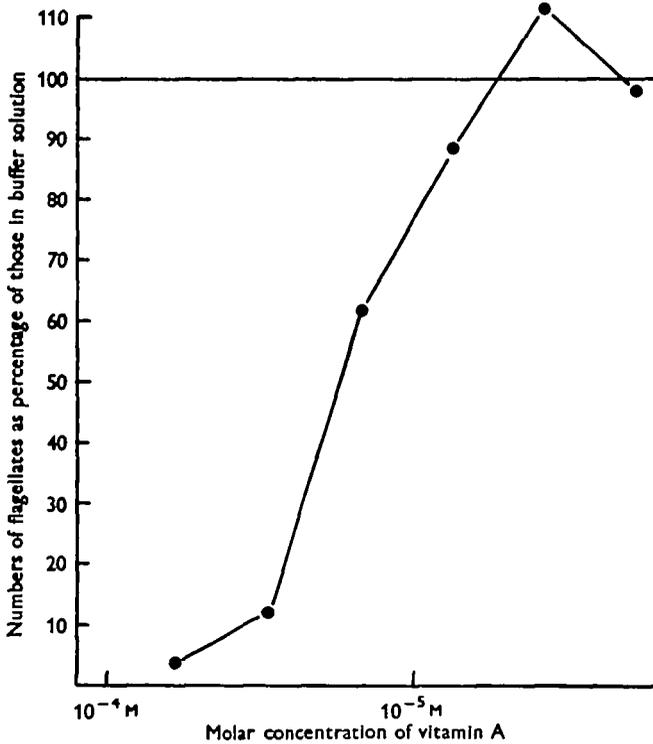


Fig. 13. Dose/response curve for vitamin A.

*Progesterone.* The results obtained when progesterone was added to the flagellates were surprising. It will be remembered (see Fig. 8) that at  $7.6 \times 10^{-5}$  M progesterone completely suppressed the transformation from the amoeboid to the flagellate. When, however, this concentration was applied to the flagellate it was practically without action. There was, paradoxically, some indication of a trivial increase in the numbers of flagellates during the later phase of the experiment in the presence of KCl, but certainly there was no general suppression as might have been expected. On the other hand, when added at one-tenth this concentration (i.e. at  $7.6 \times 10^{-6}$  M, at which concentration

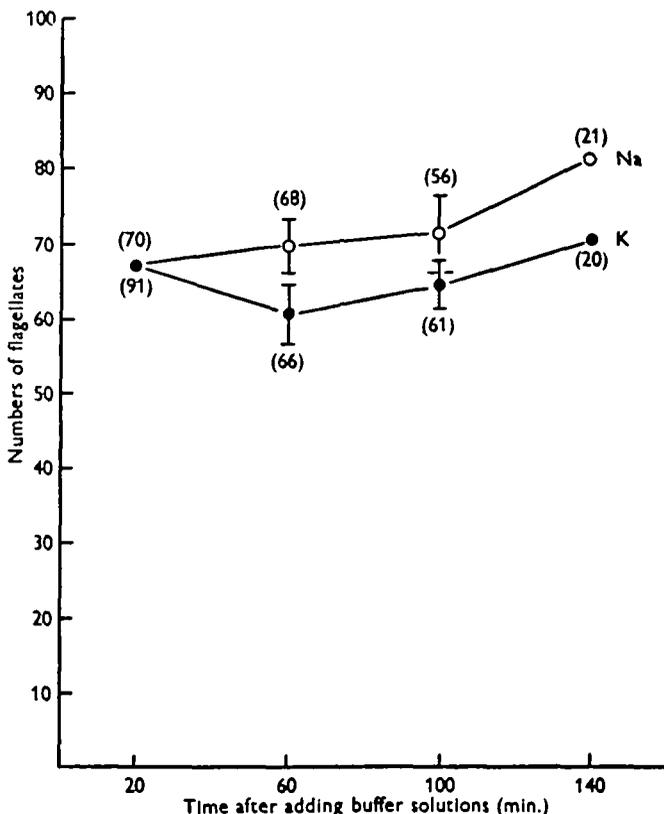


Fig. 14. The effect of adding to *Naegleria* already in the flagellate phase NaCl or KCl, at such concentrations in buffer solution as to produce a final concentration of M/1000. Numbers in brackets indicate the numbers of observations. The standard errors are also indicated.

it activated the change from amoeba to flagellate) it caused a significant decrease in the numbers of flagellates (Fig. 16). Thus there were two paradoxical results (Fig. 17). In strong concentration, progesterone applied to the amoeba suppressed the change to the flagellate but was almost without action on the flagellate. In weaker concentration, progesterone encouraged the change from amoeba to flagellate, but also showed signs of encouraging the reverse action if applied to the flagellate directly. Thus, in the transformation of flagellates into amoebae there was the curious situation that weak concentrations of progesterone were more effective than strong ones. In the present

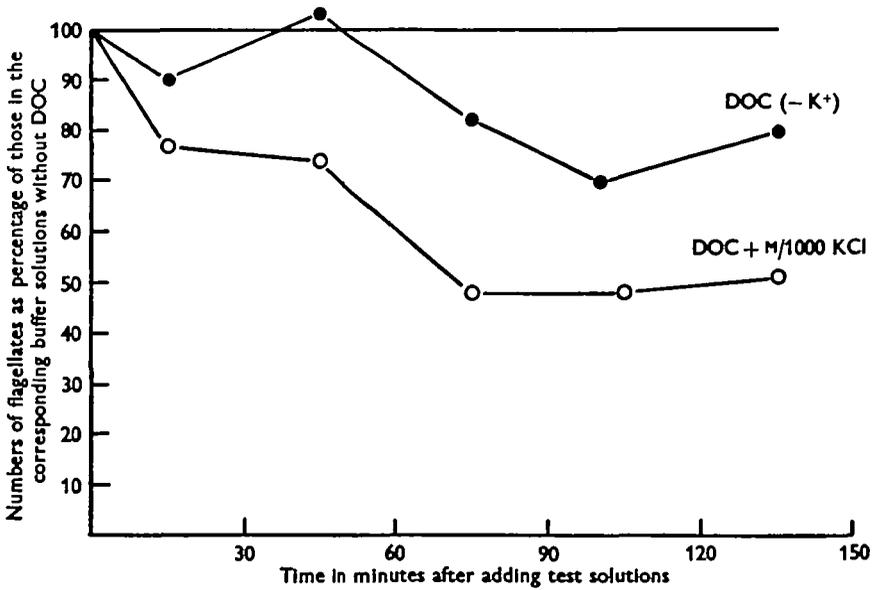


Fig. 15. The effects of adding deoxycorticosterone (to produce an effective concentration of  $7.6 \times 10^{-8}$  M) to the flagellates in the presence of M/1000 KCl and in the absence of KCl.

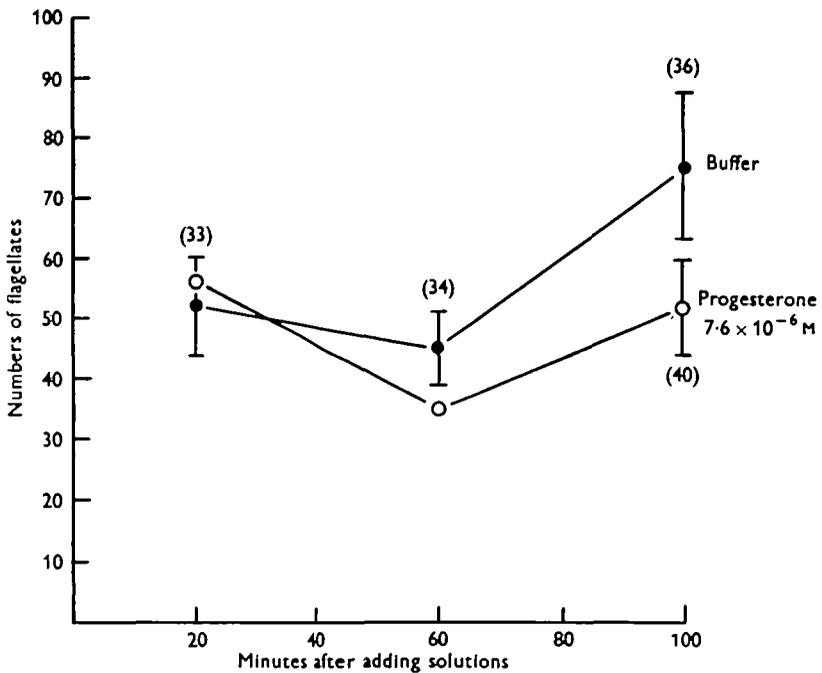


Fig. 16. The effect of adding progesterone (at  $7.6 \times 10^{-6}$  M) to *Naegleria* already in the flagellate phase.

state of ignorance it is difficult to envisage the sort of mechanism which would bring this about. Possibly it could be explained along the following lines. The amoeba and the flagellate have membranes which are oppositely orientated. The amoeba is concerned, let us say, with ejecting cations, the flagellate with collecting them. If the action of the more dilute progesterone is considered as the physiological effect, this could be to accelerate the pump in both cases. The amoeba would then lose cations too quickly under its influence and have to go over to the flagellate form or die. The flagellate, on the other hand, would gain cations more quickly than normal and so return more

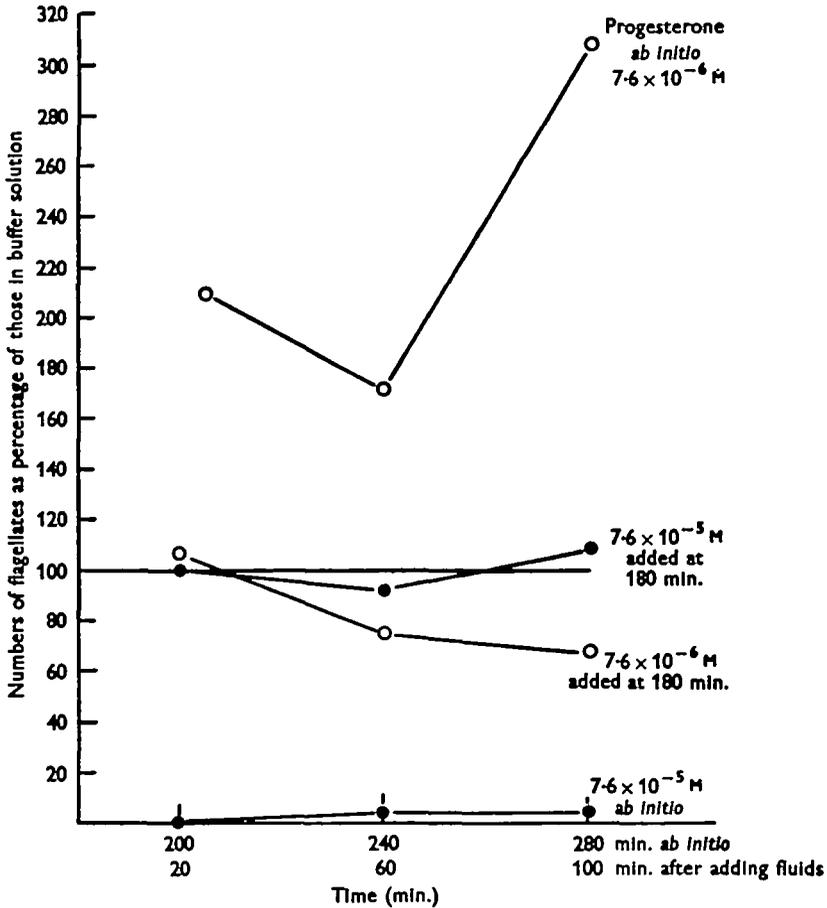


Fig. 17. The effects of progesterone at two different concentrations when applied to *Naegleria* (a) in the amoeboid form (*ab initio*) and (b) in the flagellate form, i.e. 180 min. after first placing the amoebae in buffer solution.

quickly to the amoeboid form. If the strong concentrations blocked the pump or decreased the permeability of the membrane, the amoeba would stay amoeboid and the flagellate would be unable to gain the necessary cations to cause it to change back to the amoeboid form. While it cannot be pretended that this is anything more than a suggestion, it does indicate that an explanation in terms of membrane permeability is not immediately out of court. It also suggests that further work with these amoebae

in simple solutions of different ionic and steroid concentrations is likely to be profitable in elucidating some of the actions of steroids.

*Testosterone.* This steroid, applied to the flagellate at  $7.6 \times 10^{-5}$  M, had rather little action, but showed some indication of encouraging the return to the flagellate form, particularly towards the end of the observation period (Fig. 18). Its action was in this way comparable with that of progesterone. No preference for Na or K was indicated.

*Oestradiol.* This has only been applied to the flagellate form at a concentration of  $3.8 \times 10^{-5}$  M and it caused a definite and almost immediate decrease in the numbers of flagellates though its action was not as potent in this direction as when it was applied to the amoeba (Fig. 18). Again no preference for K or Na was noticed.

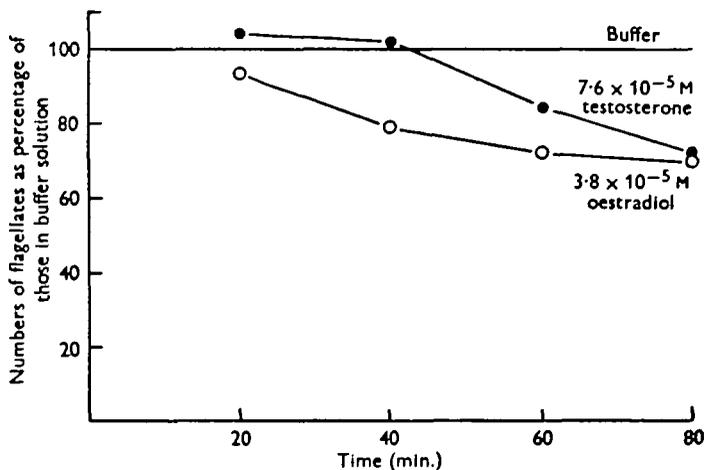


Fig. 18. The effects of adding testosterone and oestradiol, each of  $7.6 \times 10^{-5}$  M, to *Naegleria* in the flagellate form.

#### DISCUSSION

The experiments described in this paper can only be considered as introductory to a very large number of possible investigations which immediately suggest themselves as applicable to a system in which pure steroids can be applied to isolated cells living normally and actively in solutions not far removed from distilled water. The chemistry and stereochemistry of the steroid, the state of the amoebae, the ionic concentration of the environment and a variety of factors of this sort can all be varied at will and thus the material can be regarded as extremely suitable for an investigation of steroid action in general. In fact, this living system and the artificial phospholipid membranes, which have recently been prepared (for example by Mueller, Rudin, Tien & Wescott, 1962), seem to offer excellent opportunities for the investigations of drug and hormone actions at their very roots.

In the present system, there are certain drawbacks which sometimes make interpretation difficult. The amoebae are fed on bacteria and a sort of balance has to be maintained in the cultures between the amoebae and their living food supply. The amoebae therefore may be used at different stages of their nutritional cycles, and although there is no correlation between the age of a culture, i.e. the days since it was

last subcultured, and the numbers of flagellates which it produces under standard experimental conditions, this does not mean that such nutritional factors are not a cause of some of the unaccountable variation which occurs from experiment to experiment. In the actual experiments the bacteria, though inevitably present, are probably not a serious source of error within the usual period of the experiment, though they might become so if the experiment were prolonged much further.

In assessing the numbers of amoebae which become flagellate, the early phases of an experiment in which an experimental solution is added to the amoeba probably are the most reliable provided that the material has acted on the amoeba within that time. When, however, the experiment is continued, the results obtained by the present technique become more difficult to interpret. The numbers of flagellates may then be influenced by the numbers which are returning to the amoeboid form and it is possible that some individuals may cycle periodically between the amoeboid and the flagellate forms. Moreover, once the flagellates are present in the medium, the influence of the experimental treatment on them may have to be added to its influence on the amoeboid cells. To emphasize this point the results given for the various steroids have been partly broken down into those which were obtained in the first 40 min. of the recording period and those obtained in the last 60 min. Sometimes they are very different. This consideration, of course, always complicates the interpretation of the results of adding steroids and the like to the cultures when many of the cells are already in the flagellate form.

Finally, the very great influence of temperature on the amoeba-to-flagellate transformation is a very important factor, but fortunately one which can be reasonably easily controlled. The influence of temperature on the flagellate-to-amoeba transformation has not yet been assessed.

In the present series of experiments the upper limits of concentration of the steroids have in some cases been set by the solubility of the compound in the aqueous medium. This difficulty could presumably be overcome in certain instances by providing a carrier or a solubilizing agent, but this has not been attempted on the grounds that this procedure would vitiate the results with respect to the direct action of the steroid on the organism. The doses which have been used are mostly high by comparison with physiological concentrations in mammalian fluids. This, however, does not detract from their interest, since one cannot regard the amoeba as in any sense a 'target organ' for hormone action, and undoubtedly a great deal of the sensitivity to hormone action in the body depends on the specialization of the target organ itself.

In reviewing some of the positive results achieved in this investigation, a summary of which is given in Table 2, perhaps three points stand out particularly. First, the different action of some of the steroids, according to whether the amoeba is in the amoeboid or the flagellate form, emphasizes the different character of the cell in its two forms. Morphologically, of course, the surfaces of the cells are very different, and it is tempting to look first at the surface properties of the cells in attempting to analyse the action of the steroids. Secondly, the evidence is very suggestive that the flagellate form is much more selective than the amoeboid form in its preference for K rather than Na and experiments are urgently needed now to investigate the actual ionic content of the organism under different controlled conditions. A few pilot experiments by one of us (J. L. P.) and by Dr Dunham at the Zoological Laboratory, Chicago, indicate that the potassium concentration of

the amoebae is probably above that of the medium and regulated at about 11 m-equiv./l. while the Na concentration is more variable and probably more nearly equal to that in the medium. Thirdly, the effects of varying the concentration of the different steroids seems to divide the active steroids into two main categories, exemplified by progesterone and deoxycorticosterone on the one hand, and by androsterone and oestradiol on the other. The action of the first two is both qualitatively and quantitatively dependent on concentration while that of the others is only quantitatively dependent. It is interesting to note that  $\text{NaHCO}_3$  and sodium lactate solutions produce effects on the amoeba

Table 2. *Summary of the actions of the various steroids tested*

Steroids	Dose	Applied to the amoeba	Applied to the flagellate
Cholesterol	$7.6 \times 10^{-6}$	--	.
Deoxycorticosterone	$7.6 \times 10^{-6}$	--	(Particularly in presence of $\text{K}^+$ )
	$3.8 \times 10^{-6}$	+++	.
Hydrocortisone	$1.5 \times 10^{-4}$	-	.
	$7.6 \times 10^{-5}$	+	o
	$1.9 \times 10^{-5}$	-	.
Cortisone	$7.6 \times 10^{-5}$	-	.
	$1.5 \times 10^{-4}$	(---)	.
Aldosterone	$7.6 \times 10^{-4}$	(--)*	.
Progesterone	$7.6 \times 10^{-5}$	-----	o (?+)
	$4.5 \times 10^{-4}$	+++	-
Testosterone	$1.5 \times 10^{-4}$	--	-
	$3.8 \times 10^{-5}$	+	.
Androsterone	$1.5 \times 10^{-4}$	---	.
	$3.8 \times 10^{-5}$	--	.
	$4.8 \times 10^{-6}$	-	.
Oestradiol	$7.6 \times 10^{-5}$	---	-
	$1.9 \times 10^{-5}$	--	.
	$2.4 \times 10^{-6}$	-	.
Androstenedione	$7.6 \times 10^{-5}$	-	.

., Not tested; +, increases flagellates; -, decreases flagellates; o, no action; \*, single experiment only.

(Willmer, 1956) like those produced by DOC and progesterone, though, of course, they only act at much higher concentrations, while NaCl, KCl etc. have dose/response curves similar to those of oestradiol and androsterone. These observations perhaps indicate that the phase of the amoeba is dependent mainly on the ionic balance of the cell and that the steroids are capable of modifying this by acting on the membranes or other mechanisms responsible for maintaining the balance. There is as yet no immediately obvious structural feature of the steroids which is capable of explaining why the particular steroids act as they do on the amoeba.

#### SUMMARY

1. The age of the culture of *Naegleria gruberi*, i.e. the time since the last subculture, is of little importance in determining the numbers of cells which turn flagellate when placed in the extremely dilute buffer solutions used.

2. Between 17° and 21° C. there is a very great decrease in the time which the amoebae require in order to become flagellate when placed in buffer solution.
3. There is no evidence that the amoeba, as such, differentiates between solutions of KCl or NaCl. On the other hand, the flagellate form is somewhat more reactive to KCl than to NaCl, and fewer flagellates are found in KCl solutions.
4. The metaplasia is affected by the presence of steroids in the medium.
5. The more interesting actions of the steroids tested may be summarized as follows. At high concentrations, progesterone and deoxycorticosterone, when applied to the amoeba, prevent the change to the flagellate form. When applied to the flagellate, progesterone has little action, but deoxycorticosterone encourages the return to the amoeboid form, especially in the presence of K<sup>+</sup>. In lower concentrations both steroids favour the change from amoeba to flagellate and also from flagellate to amoeba. Other steroids have characteristic effects.
6. While the dose/response curves indicate qualitatively different effects of concentration of progesterone and deoxycorticosterone they only show quantitatively different effects with oestradiol and androsterone.
7. Progesterone acts on amoebae at concentrations which are comparable with those at which it acts in the human body.
8. When both progesterone and oestradiol are applied together at concentrations which suppress the flagellate form the effects are additive. The same applies to mixtures of testosterone and oestradiol. An activating concentration of progesterone has not yet been tested in combination with an inhibiting dose of oestradiol.

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