J. Physiol. (1956) 134, 484–496

THE EFFECTS OF 5-HYDROXYTRYPTAMINE ON HYPOTONIC HAEMOLYSIS, AND ON THE POTASSIUM LOSS FROM ERYTHROCYTES DURING COLD STORAGE

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(*Received* 12 *July* 1956)

The many pharmacological effects of 5-hydroxytryptamine (5-HT) seem unlikely to depend on as many underlying physico-chemical actions. The working hypothesis that is being tested is that some of these effects may depend on a smaller number of physico-chemical actions on cell membranes, possibly expressible in terms of permeability to water and water-soluble substances. This was suggested by a similar hypothesis about the actions of indole-3-acetic acid in plants (Veldstra & Booij, 1949). If substantiated, it might help to solve the problem of the physiological function of 5-hydroxytryptamine. Experiments on beetroot cells, the plasma membranes of which are not wholly unlike those of animal cells, showed that 5-hydroxytryptamine and indole-3-acetic acid in concentrations of about 100 μg/ml. allowed the red pigment to escape from the vacuoles much more rapidly than from untreated cells (Pickles & Sutcliffe, 1955). The active uptake of sodium from the medium, and to a smaller extent the passive loss of potassium, were reversibly inhibited, without significant effect on the rate of oxygen consumption.

In the present experiments human erythrocytes, either during cold storage or during hypotonic haemolysis, have been chosen as test objects. It is not supposed that hydroxytryptamine necessarily has any physiological effect on erythrocytes (though Stacey (1956) has recently shown that they absorb hydroxytryptamine against a concentration gradient); but the systems are comparatively simple and well known, and the erythrocyte membrane is not highly specialized. The rates of release of haemoglobin and of potassium from the cells under various conditions have been measured. It is concluded that allowing the cells to absorb hydroxytryptamine tends to diminish their potassium loss and usually accelerates their hypotonic haemolysis; and that the second effect may possibly result from the first.

METHODS

Human venous blood was routinely drawn into a siliconed syringe containing 0·3 ml. heparin solution (5000 u./ml.) to 10 ml. blood, and thereafter handled at room temperature in siliconed glassware. It was first oxygenated with room air, and the erythrocytes were washed three times and finally resuspended in a phosphate-buffered NaCl solution consisting of NaCl 8·51 g, Na_2HPO_4 1·65 g, NaH_2PO_4 0·52 g, and glass-distilled water to 1 l., adjusted if necessary to pH 7·2. The pH of this buffered solution was not detectably altered by the addition of 5-HT in the concentrations used. Dilutions of this solution in glass-distilled water were used for the hypotonic haemolysis.

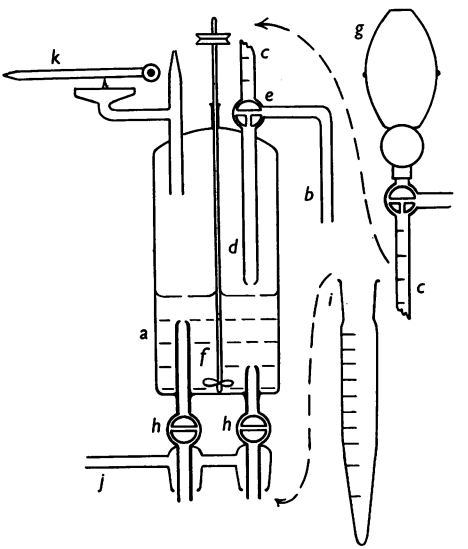

Fig. 1. The mixing and sampling apparatus used in the experiments on hypotonic haemolysis. For description see text.

Apparatus

In the main series of experiments, hypotonic haemolysis took place in the apparatus illustrated in Fig. 1. The main part of the apparatus is made of Pyrex glass, except for the stirrer which is of stainless steel. In use, the mixing chamber *a* is first filled with 29 ml. of hypotonic saline at room temperature (20–23° C) to the level shown. The erythrocyte suspension is drawn up via the intake *b* into the pipette *c*, which is calibrated in terms of the volumes delivered from it. The required volume (usually 0·5 ml.) is then allowed to run down slowly into the delivery tube *d* where it is arrested by turning tap *e*. The stirrer *f* stirs the hypotonic saline rapidly and smoothly; the feathering of the blades is such that the hypotonic saline is driven upwards in the centre of the chamber, its surface remaining approximately flat although it is rotating rapidly. By squeezing the sphygmomanometer bulb *g*, the cell suspension can be quickly ejected from *d* into the hypotonic saline, and haemolysis begins. Four taps *h*, *h* are provided (two only being shown in the diagram), and by turning these in order, at intervals of a second or more, samples of the suspension as it is being lysed are sucked off into the centrifuge tubes *i*, which have been put into position as indicated and partially evacuated via the connexion *j*. Each centrifuge tube normally contains 2 ml. of 3% NaCl solution, which mixes with the hypotonic medium of the suspension and brings it to approximate isotonicity. The unlysed cells and ghosts are separated as rapidly as possible from the supernatant.

The four levels to which the surface of the hypotonic saline is brought as the four taps *h* are opened in succession are illustrated. The pressure changes in the mixing chamber are recorded by means of the tambour *k*, so that the events may be accurately timed. Fig. 2A illustrates the first part of such a record, as first the cell suspension is injected and then the first two samples are withdrawn. Slow-motion cinematography has confirmed that the mixing of the suspension with the hypotonic saline is apparently complete within 0·4 sec (Fig. 2 B–F) and that each withdrawal takes about 0·6 sec. The events are timed from the beginnings of the corresponding deflexions on the record, and are reckoned to the nearest 0·1 sec.

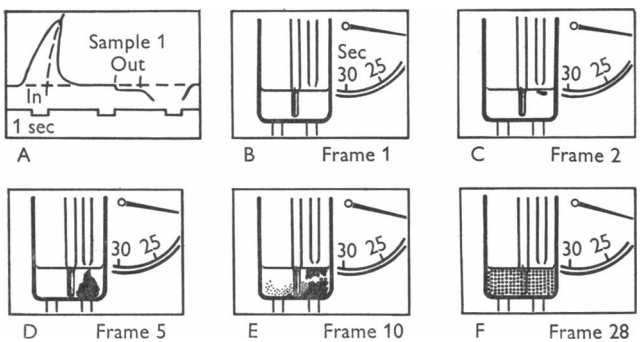

Fig. 2. A, tracing of the first part of a kymograph record made with the mixing and sampling apparatus. B–F, tracings of frames from a cine-film taken at 64 frames/sec, showing the erythrocyte suspension mixing into the saline (in this instance 0·9 % NaCl was used).

Parts omitted for simplicity from the diagram include: two of the four sampling units and further centrifuge tubes, all with ground-glass joints; a tap-funnel for filling the mixing chamber; ground-glass joints to enable the apparatus to be dismantled readily for cleaning; a thermometer in the mixing chamber; kymograph, stirrer-motor and supports.

Estimations

The haemoglobin in the supernatant fluids was estimated colorimetrically by comparison with a series of known dilutions. The unlysed precipitates were completely lysed by being diluted with distilled water, frozen, thawed and further diluted, and their haemoglobin was estimated in the same way. The potassium was determined by flame photometry, by comparison with standards containing approximately the same quantity of potassium and sodium as the unknown. The potassium content of the sodium salts used in making up the solutions was measured and allowed for. From these figures the percentage of the total haemoglobin and potassium that was released from the cells of each specimen by exposure to a measured period of hypotonicity was evaluated. A similar analysis was usually made of a specimen in which isotonic instead of hypotonic saline had been used, as a measure of the potassium and haemoglobin losses from the cells before exposure to the hypotonic medium; these values are quoted as at '0 sec' at the beginning of haemolysis.

Controls

The following facts have been checked by direct experiments which will not be described in full. (1) The normal rate of stirring in the mixing chamber does not itself cause any appreciable haemolysis, although extremely rapid stirring may do so. (2) The withdrawal of samples by suction into the centrifuge tubes likewise itself causes no haemolysis. (3) A very slow liberation of haemoglobin continues for at least 20 min after the hypotonic sample has been mixed into the hypertonic saline, but unless this continued haemolysis is much more rapid during the first 2 min (the least time in which the samples can be centrifuged) no significant error is likely to arise from

this cause. (4) No evidence has been found of any similar slow liberation of potassium after isotonicity has been restored. This question, however, is discussed later.

In the experiments on cold-storage, the suspensions were stored at about 5° C in stoppered siliconed tubes and the cells were resuspended by gentle shaking once or twice daily.

RESULTS

Hypotonic haemolysis

The general type of result obtained is illustrated in Fig. 3. In this experiment erythrocytes not pre-treated with hydroxytryptamine were lysed in 0·35% saline (i.e. phosphate-buffered saline isotonic with 0·35% NaCl) or in 0·30% saline. The time scale used in Fig. 3B and D is such that the distance from the origin to the point representing time t sec is proportional to $(1-e^{-0.062t})$.

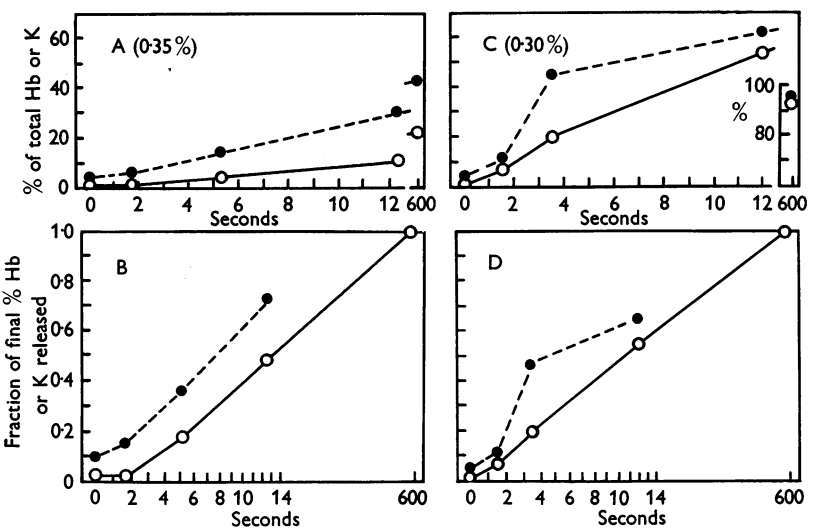

Fig. 3. Results of lysing an erythrocyte suspension, pre-treated with neither hydroxytryptamine nor creatinine, in a buffered saline isotonic with 0·35% NaCl (A, B) or 0·30% NaCl (C, D). Graphs B and D show the same results as A and C, plotted on different scales. ●--●, potassium release; ○—○, haemoglobin release.

This formula is derived from no theoretical considerations, but is found empirically to give approximately straight lines in plotting the percentage haemolysis. Similarly, the scale of haemolysis used in Fig. 3B and D, in which the amount of haemoglobin or potassium liberated by any shorter duration of exposure to hypotonicity is expressed as a fraction of that liberated by 10 min exposure, is again used for its graphical convenience, as the resulting line is not greatly affected by differences in the lysing tonicity within the range used.

The effect of pre-treating the erythrocytes with hydroxytryptamine creatinine sulphate, or with the equivalent creatinine alone, is shown in

Fig. 4. In these twelve consecutive experiments the substances were added to the cell suspensions to give concentrations of 0·1–1·0 mM, and the suspensions were then allowed to stand at room temperature (20–23° C) for 3 hr before the hypotonic haemolysis. The various concentrations used within this range do not give significantly different effects. In Fig. 4A (controls), the points are seen to be distributed roughly around the empirical straight line.

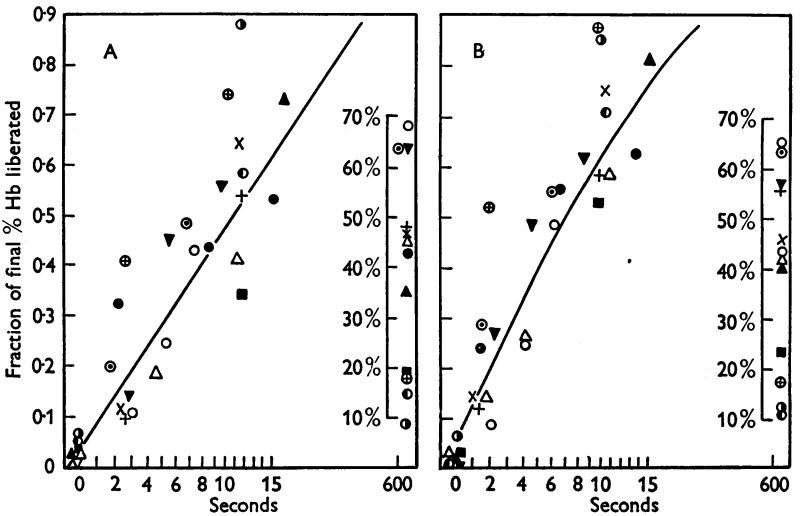

Fig. 4. Haemoglobin release in twelve consecutive experiments in which the erythrocytes were pre-treated with hydroxytryptamine creatinine sulphate (B) or the equivalent creatinine (A). The scales are as in Fig. 3B, D. One type of symbol is used for each experiment; each experiment is represented by 2, 3 or 4 points on each graph. The symbols down the right-hand border of each graph show the percentage of the total haemoglobin in the suspension released by 10 min exposure to the hypotonic medium. The initial concentrations of hydroxytryptamine creatinine sulphate or of the control creatinine in the cell suspension are: ●, ○, 1 mM; ■, ◐, ◑, 0·9 mM; ▼, ▲, △, 0·6 mM; +, ×, 0·4 mM; ⊙, ⊕, 0·1 mM.

The curved line in Fig. 4B represents the time course that would be obtained if any particular degree of haemolysis were reached at a time equal to two-thirds of that indicated by the straight line of Fig. 4A. These lines are included simply to afford a rough indication of the degree of acceleration of haemolysis caused by the hydroxytryptamine. When each point on Fig. 4A is compared with the corresponding point on Fig. 4B, the difference between the two sets of points can be shown by the 't' test to be significant ($P < 0{\cdot}05$). The final degree of haemolysis caused by 10 min exposure to the hypotonic medium is shown by the symbols down the right-hand border of each graph. There is no significant difference between the hydroxytryptamine-pre-treated and the control cells.

Fig. 5 shows a similar record of the potassium liberation. This differs in three ways from the haemoglobin liberation shown in Fig. 4: the percentage

of the intracellular potassium liberated by 10 min exposure to the lysing solution is greater; the fraction of this percentage liberated by any shorter exposure is greater; and the difference between the control and the hydroxytryptamine-pre-treated cells is less.

The relationship between the potassium release and the haemoglobin release in these experiments is shown in Fig. 6. When in any single experiment, such as that shown in Fig. 6A, the percentage of the total intracellular potassium released is plotted against the corresponding percentage haemoglobin release, the resulting points for either the hydroxytryptamine or the control cells lie approximately along a straight line, up to about 30% haemoglobin release.

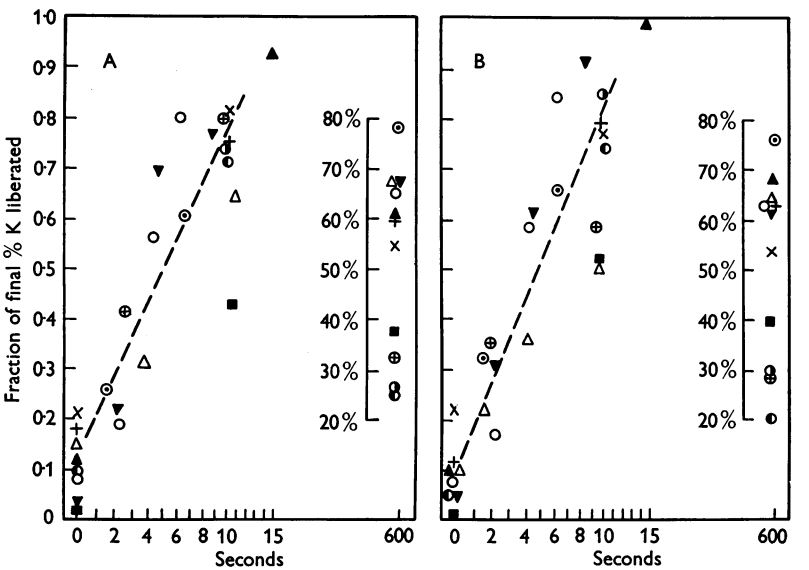

Fig. 5. As Fig. 4, but showing the potassium release in the same experiments. No potassium estimations were made in the experiment shown in Fig. 4 by ●. The broken lines are fitted by eye and have gradients 1·58 and 1·75 times that of the straight line in Fig. 4A.

Such lines invariably have gradients well above 1; that is, for every 1% of the haemoglobin released more than 1% of the intracellular potassium is released. In preparing Fig. 6, such a line has first been drawn through the points representing lysis of the control cells in each one of the twelve experiments shown in Figs. 4 and 5. The gradients of these lines average 2·0 (range 1·3–2·4). Fig. 6B shows that the points representing the potassium and haemoglobin release from the hydroxytryptamine cells lie significantly ($P < 0{\cdot}05$) below the corresponding points for the control cells. That is, although the potassium release is always proportionately greater than the haemoglobin release, the potassium release from the hydroxytryptamine cells is relatively retarded, or the haemoglobin release is relatively accelerated, in comparison with the

control cells. The difference appears to be maintained throughout the whole of the range of lysis plotted; the smaller number of points available above this range show a wider variation and no significant conclusions can be drawn from them.

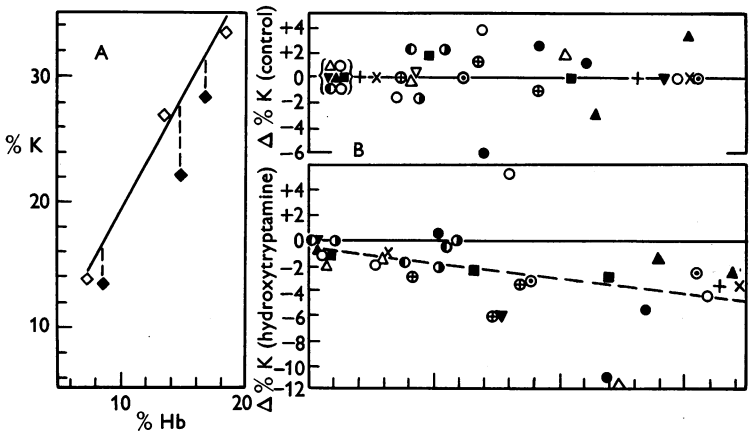

Fig. 6. A: single experiment, showing % K release plotted against % Hb release; solid symbols, hydroxytryptamine cells; open symbols, 'control' cells. The reference line is drawn to give a good fit with the 'control' points. B: results of all twelve experiments shown in Figs. 4 and 5. The deviations of the individual points from the reference lines (Δ % K), drawn for each experiment separately as in A, are plotted against the % Hb release; above, 'control' points; below, 'hydroxytryptamine' points. The broken line shows the calculated regression of the Δ % K on % Hb.

Some additional measurements, the significance of which is discussed later, were made in four experiments. In addition to the normal procedures, separate lots of the erythrocyte suspensions were allowed to take up hydroxytryptamine (with the usual controls) under conditions identical with the usual ones except that during this period the suspending medium was hypotonic (equivalent to NaCl 0·45%) instead of isotonic. This degree of hypotonicity brought the cells to the beginning of haemolysis. They were restored to isotonicity before being put through the usual test.

The results are summarized in Table 1. The general effect of the hypotonic pre-treatment, in comparison with the normal, was to cause a little more haemoglobin and potassium loss during the pre-treatment, to diminish the final (10 min) degree of haemolytic loss of both substances, and to prevent the acceleration of haemolysis by hydroxytryptamine.

Potassium and haemoglobin losses on cold-storage

In nine experiments the cell suspensions were allowed to take up hydroxytryptamine from initial concentrations of 0·1–1 mM and then were cold-stored for periods of 1–5 days, and the potassium losses from the cells were compared

with those from appropriate (creatinine) controls. If the potassium loss from the latter is taken as 100, the mean loss by the hydroxytryptamine-pre-treated cells was $97 \pm 1{\cdot}8$ (S.D. of the individual values). The difference is significant. In all but one instance the very slight loss of haemoglobin that also occurred on cold-storage was smaller from the hydroxytryptamine cells than from the controls. In proportion to the total intracellular content, the haemoglobin loss was considerably smaller than the potassium loss.

TABLE 1. The effects of pre-treating the erythrocytes in a hypotonic medium in comparison with the normal, as described in the text. The values below are the means of four experiments. HT +, 0·9 mM hydroxytryptamine creatinine sulphate initially; HT −, equivalent creatinine only. 10 min haemolysis (Hb) or (K) = % of total Hb or intracellular K liberated by 10 min exposure to hypotonicity. 10 sec fraction = fraction of 10 min haemolysis caused by 10 sec exposure to hypotonicity

	Pre-treating solution			
	Hypotonic, HT +	Hypotonic, HT −	Isotonic, HT +	Isotonic, HT −
Hb loss during pre-treatment, %	1·2	1·2	1·0	1·1
K loss during pre-treatment, %	2·9	3·0	2·4	2·7
10 min haemolysis (Hb), %	18	18	22	22
10 min haemolysis (K), %	34	33	40	40
10 sec fraction (Hb)	0·55*	0·53	0·66	0·54
10 sec fraction (K)	0·48	0·48	0·62	0·58

* This mean includes one unusually high value (0·84). When this experiment is omitted the values on this line become 0·46, 0·53, 0·65 and 0·52.

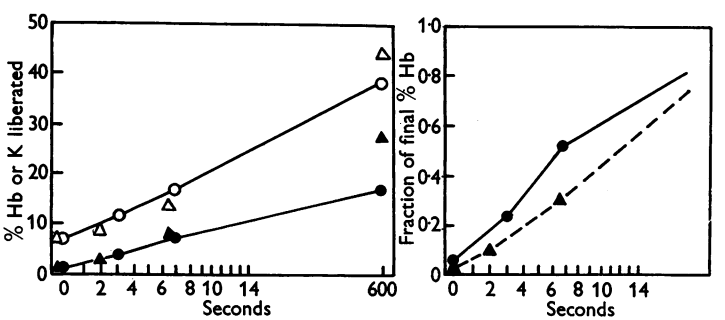

Fig. 7. Hypotonic haemolysis of cells pre-treated with tryptamine 1 mM (▲, △) or of control cells with no addition (●, ○). Open symbols represent K release and solid symbols Hb release. The time scale is as in previous figures.

Effects of other substances

Tryptamine. Tryptamine (1 mM initial concentration) did not proportionately accelerate haemolysis although it tended to increase the fragility of the erythrocytes (Fig. 7). On cold-storage the potassium loss was increased (to 113% of the control in this instance) and the slight haemoglobin loss was not significantly affected.

Lysergic acid diethylamide added to the cell suspension in approximately half the molecular concentration of the hydroxytryptamine appeared to

inhibit the accelerating effect of the latter on hypotonic haemolysis (Table 2). There was no significant effect when the initial concentration of the lysergic acid diethylamide was only 1/133 of that of the hydroxytryptamine.

TABLE 2. Effects of adding lysergic acid diethylamide (LSD+), in the concentrations shown, to the erythrocyte suspension 15 min before the addition of the hydroxytryptamine creatinine sulphate (HT) or the equivalent creatinine only. 'Δ 5-sec fraction' = amount by which the '5-sec fraction' of hydroxytryptamine cells is greater than that of the control cells; the '5-sec fraction' being the percentage haemolysis resulting from 5 sec exposure to the hypotonic medium, divided by the percentage haemolysis after 10 min similar exposure

	Expt. 1	Expt. 2	Expt. 3
Concentration of LSD (μM)	3	55	55
Concentration of HT (μM)	400	100	100
Δ 5-sec fraction (LSD −)	0·13	0·09	0·15
Δ 5-sec fraction (LSD +)	0·11	0·00	0·05

DISCUSSION

Interpretation of the measurements

At first sight it might appear that Figs. 3–7 represented the actual time course of the haemolysis. This is not quite true; the results would represent the precise time course only if the haemolysis stopped immediately and completely in each sample when it was withdrawn into the hypertonic saline. It is known that a slow liberation of haemoglobin continues after the cells have been restored to their normal tonicity, but no evidence has been found of any similar liberation of potassium. Unless the continued loss of haemoglobin is much more rapid in the first two minutes, after the sample has been taken but before the cells can be separated in the centrifuge, it is likely that the graphs representing haemoglobin release genuinely show the time course of the lysis. The question of the potassium loss is more difficult, because it is possible that some cells may become cation permeable without losing their haemoglobin, and remaining so when they are restored to normal tonicity may lose much of their potassium during the first two minutes. Davson & Ponder (1938) showed that 'ghosts' are highly cation-permeable in similar circumstances.

Nevertheless, the following considerations suggest that at least part of the potassium loss precedes the haemoglobin loss in time.

(1) It is well known that at certain tonicities erythrocytes lose potassium without losing appreciable quantities of haemoglobin. This fact has been confirmed and the largest amount of this prolytic loss has been estimated as about 4% of the total intracellular potassium. It is unlikely that this prolytic loss would cease in all the erythrocytes as soon as the most fragile of them began to lose haemoglobin. A quantitative analysis of the haemoglobin and potassium losses of cells exposed to various tonicities throughout the whole range has been made; and it fits the assumption that the cells become potassium-leaky when their internal tonicity is greater, by the equivalent

of about 0·02% of NaCl, than the tonicity necessary to make them haemoglobin-leaky. This implies that during rapid hypotonic haemolysis the cells become potassium-leaky before (in time) they become haemoglobin-leaky.

(2) When as in Fig. 8B the potassium released by comparatively long exposure to hypotonicity is plotted against the corresponding haemoglobin release, the resulting line has a gradient not much greater than unity. But if, as in Fig. 8A, a similar graph is drawn showing the effects of short exposure to lower tonicities, the line has a much steeper slope. Brief exposure to low tonicity is relatively more effective in releasing potassium than haemoglobin because some at least of the potassium is released *before* the haemoglobin.

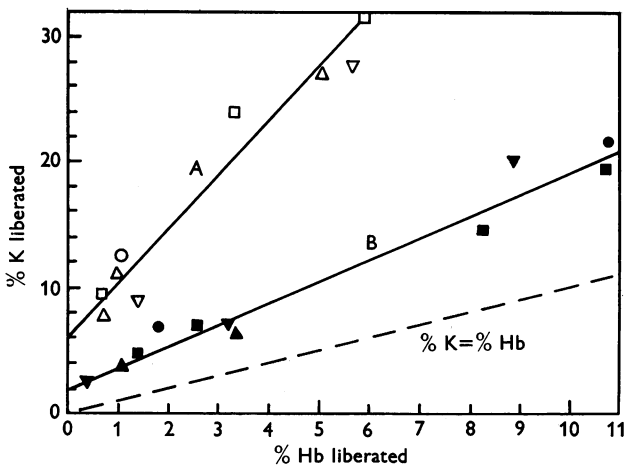

Fig. 8. Comparison of (A) the effects of 10 sec exposure of erythrocytes to very hypotonic solutions with (B) the effects of 20 min exposure to less hypotonic solutions. The solid and open symbols of one type represent points determined in one experiment, and do not refer to the experiments represented by the same symbols in other figures. The regression lines are not calculated.

These two arguments suggest that the results as given approximate to the true time courses of the potassium and haemoglobin losses. But even if they do not, it is unlikely that the general conclusions about the effects of hydroxytryptamine would be invalidated; for it has been found that the difference between the lysis of the hydroxytryptamine-pre-treated cells and the controls tends to decrease rather than to increase if the unavoidable 2 min of delay after the sampling is prolonged. If the delay could be eliminated altogether, it is likely that the apparent acceleration of the lysis by the hydroxytryptamine would be increased rather than decreased.

Significance of the results

A suspension of erythrocytes that have taken up hydroxytryptamine is lysed more rapidly in hypotonic solution than is a control suspension. This

acceleration of haemolysis is shown particularly by the haemoglobin release and to a smaller extent by the potassium release, so that the relation between the haemoglobin loss and the potassium loss is altered. Similarly, hydroxytryptamine-treated cells lose potassium less rapidly on cold-storage, and the slight haemoglobin release is also diminished.

These facts may be qualitatively accounted for as follows. If an erythrocyte in hypotonic solution loses some of its potassium before it begins to lose haemoglobin, then the simple osmotic theory of haemolysis must be modified (there are other well-known reasons for believing this). One would expect the erythrocyte to swell rapidly until it became cation permeable, and then more slowly until it disrupted. If the hydroxytryptamine diminished the permeability of the cell membrane to cations, or at least to potassium, the result would be to continue the initial rapid phase of the swelling in hypotonic solution, with the observed effects on the rates of haemoglobin and potassium release. The diminution in potassium loss on cold-storage needs no separate explanation; and if the small haemoglobin loss on cold-storage results from the cells becoming more cation permeable, then the diminution in this haemoglobin release would follow. A generally disruptive effect of the hydroxytryptamine on the cell membrane, facilitating loss of the haemoglobin, is not excluded; but it seems less likely both from the results of cold-storage and from the fact that under the present experimental conditions no evidence has ever been found that hydroxytryptamine has itself any haemolytic effect. This theory is somewhat similar to that proposed to account for the effects of hydroxytryptamine and other substances on beet cell membranes (Pickles & Sutcliffe, 1955).

The results of the four special experiments summarized in Table 1 are compatible with the theory. During pre-treatment in the hypotonic medium, the cells swell, and although they are restored to their normal tonicity before the test of hypotonic haemolysis it may be supposed that some effect of the previous strain remains. The cells are seen to have lost more of their potassium during the pre-treatment than have the control cells, and it is possible that during hypotonic haemolysis they become potassium permeable more rapidly than do the controls. Either or both of these effects would account for the slowing and decrease in the extent of haemolysis in the hypotonic solution. The absence of effect of hydroxytryptamine on the hypotonically-pre-treated cells suggests that the mechanism on which the hydroxytryptamine acts, namely the potassium leakage, has already been interfered with.

Possible application of the results to other tissues

The one plant and one animal cell type on which experiments of this general nature have so far been made have been chosen entirely for their practical convenience. It is not supposed that hydroxytryptamine necessarily has any

physiological action on erythrocytes; but the results raise the question—to be answered by further experiment—whether hydroxytryptamine may have the same type of effect on other cells. Broadly, the effect may be described as a *selective alteration in cell-membrane permeability*. One such effect of hydroxytryptamine is well known: the stimulation of plain muscle and nerve by hydroxytryptamine is presumably followed by the same changes in cation permeability as those that follow other modes of stimulation. These changes are primarily increases rather than decreases in cation permeability; but this does not disprove the general hypothesis, since (1) the first effect is on sodium permeability, about which the present experiments give no information; and (2) there is no evidence that the hydroxytryptamine in the mammalian brain acts *physiologically* as a stimulant or transmitter. It is equally plausible (and quite conjectural) that it plays some part, as the adrenocortical hormones may do, in the maintenance of the normal intracellular potassium content.

A possible objection to these conjectures may be that the hydroxytryptamine has been used in concentrations (0·1–1 mM) which are much greater than those in which the substance has its well-known pharmacological effects. Againt this it may be argued that the local concentration in the membrane or other part of the cell is alone of physiological importance, and this remains unknown in all cases; Stacey's findings (1956) suggest that at least the lower concentrations used here are within the physiological capacity of the erythrocytes to absorb hydroxytryptamine against a concentration gradient; and one would not expect to be able to demonstrate a general effect very sensitively on a cell type on which the substance in question has probably no physiological effect. Any value in these experiments lies rather in the general hypothesis that they support.

SUMMARY

1. A mixing and sampling apparatus is described.

2. By means of this, the time courses of the release of both haemoglobin and potassium from erythrocytes during and after hypotonic haemolysis have been studied.

3. The potassium release under these conditions is always proportionately greater than the haemoglobin release. Evidence is given that the former in part precedes the latter.

4. Pre-treating the erythrocytes with hydroxytryptamine accelerates their subsequent hypotonic haemolysis as shown by the haemoglobin release, but diminishes the amount of potassium lost in proportion to the haemoglobin.

5. Hydroxytryptamine-pre-treated erythrocytes lose potassium more slowly on cold-storage than do controls.

6. Tryptamine in similar concentrations does not have either of these effects.

7. Lysergic acid diethylamide inhibits the effect of hydroxytryptamine in accelerating hypotonic haemolysis when it is present in the suspension in approximately the same concentration as the hydroxytryptamine.

8. It is postulated that these effects, together with others described previously, may be explained by a selective alteration in cell-membrane permeability and that this may be relevant to the problem of the physiological functions of hydroxytryptamine.

I wish to thank Dr H. Davson for some helpful comments, and Professor D. H. Smyth for his advice during the preparation of the manuscript. The glass apparatus was made by Mr J. W. Hadfield and the stirrer by Mr C. Stewart and Mr F. Ross, and the experiments were done with the technical assistance of Miss P. Hopkinson.

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