Biochemical Pharmacology, Vol. 18, pp 2215–2223. Pergamon Press. 1969. Printed in Great Britain

EFFECT OF DRUGS ON THE HEMOLYSIS OF RAT ERYTHROCYTES

H. SHEPPARD, W. H. TSIEN and C. BURGHARDT

Hoffmann-La Roche Inc., Research Division, Department of Pharmacology, Nutley, N.J. 07110, U.S.A.

(*Received* 21 *November* 1968; *accepted* 21 *March* 1969)

Abstract—Twenty-two well known drugs, covering a wide range of pharmacological types, have been examined for their ability to affect the hemolysis of rat erythrocytes induced by hypotonicity, deoxycholate and digitonin. Chlorpromazine, chlorprothixene, phenindamine, desipramine, imipramine and chloroquine enhanced deoxycholate-induced hemolysis and depressed hypotonic hemolysis at low concentrations while stimulating it at high concentrations. Debrisoquin, guanethidine, tripelennamine, leverphanol and cocaine, protected against hypotonic and enhanced deoxycholate-induced hemolysis. Acetylsalicylic acid, indomethacin, chlordiazepoxide, cortisol, cortisone, deoxycorticosterone, hydrochlorothiazide, acetazolamide and prostigmine protected against hypotonic hemolysis without affecting other systems. Digitonin-induced hemolysis was least affected by these drugs. Ethacrynic acid was unique, even among other diuretics, in protecting against deoxycholate- and digitonin-induced hemolysis. Ouabain was also unique in its complete inactivity in the three hemolytic systems. No correlation could be found between the pharmacological and membrane effects.

ANY EVALUATION of drug action, whether of a physiological or biochemical nature, must consider the limiting membrane of a cell or a subcellular organelle as the possible receptor. It was necessary, therefore, to first determine if and how drugs interact with membranes. For the following reasons, the cell chosen for a study of the interaction of drugs and membranes was the rat mature erythrocyte: (1) it is readily available as a free cell suspension; (2) it possesses a plasma membrane but no intracellular membrane; (3) alteration of the membrane to the point of causing hemolysis is readily determined by simply measuring the absorption of the hemoglobin released into the medium.

Some studies have already been carried out on the ability of drugs to interact with the erythrocyte membrane as evidenced by their effects on hypotonic[1, 2] and heat-induced hemolysis.[3,4] While the heating conditions probably result in fragmentation[5] of the membranes, hypotonicity results in colloid osmotic swelling until hemolysis occurs. Other methods of effecting colloid osmotic swelling and hemolysis include the use of agents which directly attack the membrane as deoxycholic acid, a detergent,[7] and digitonin, an agent which forms an insoluble complex with cholesterol. The following data represent the results obtained with a wide variety of substances displaying varied pharmacological activities. The interactions observed in the three hemolytic systems may be viewed as a profile of the drug's ability to interact directly with the red cell membrane or with systems responsible for maintaining the membrane's integrity.

METHODS

Preparation of blood

A male rat was decapitated and the blood drained into a graduated glass centrifuge tube through a plastic funnel coated with 0·2 ml heparin. The whole blood was centrifuged at 900 rpm for 10 min in a model V International centrifuge at room temperature. The plasma and buffy coat were removed with a medicine dropper. The erythrocytes were washed with an equal volume of standard NaCl–phosphate buffer containing 200 mg/100 ml of glucose by inverting the test tube twelve times and again centrifuging at 900 rpm for 10 min. This was repeated twice more with the last centrifugation at 1400 rpm for 10 min. The packed cells were diluted 1 to 4 with the NaCl–phosphate–glucose buffer.

Preparation of the NaCl–phosphate buffer

A stock solution was prepared by dissolving 180 g NaCl, 27·31 g Na_2HPO_4 and 3·74 g $NaH_2PO_4 \cdot H_2O$ in distilled water and bringing to a volume of 2 l. The concentrations in this solution were : NaCl, 1·54 M; Na_2HPO_4, 0·06 M; and NaH_2PO_4, 0·0136 M. Eighty-eight and 34 ml were diluted to 1 l. to give final Na^+ concentrations equal to 0·1575 M and 0·06 M, respectively. The 0·1575 Na^+ solution was isotonic while the other was hypotonic. When glucose was used, 2 g were added and brought to a final concentration of 200 mg/100 ml. These glucose solutions are referred to in the text as the isotonic glucose or hypotonic glucose solutions respectively.

Effect of drugs on hemolysis induced by hypotonic glucose, deoxycholate or digitonin

Hypotonic glucose solution. One-tenth ml of the washed blood cell suspension was added to each 5-ml sample of the appropriate drug concentration in the isotonic glucose solution by using a 0·1-ml blow-out pipette. Each point was run in quadruplicate, with the order of pipetting done so that the entire series was repeated four times. In order to express the results in terms of per cent hemolysis, two samples each of isotonic glucose and distilled water–glucose solutions were used. Each sample was mixed on the vortex after the addition of the blood. The samples were incubated for 1 hr at 37°. This is referred to as a preincubation period. After preincubation, the samples were mixed on the vortex to break-up clumps and then centrifuged at 1500 rpm for 10 min. The supernatant was removed by suction. With a 5 ml blow-out pipette, 5 ml of the hypotonic glucose solution containing the appropriate amount of drug was added in the same order as the addition of the blood, and the cells were resuspended with a vortex mixer. The samples were incubated for 30 min at 37°. After incubation, the samples were mixed on the vortex and centrifuged at 1500 rpm for 10 min. The optical density of the supernatant was read at 583 mμ.

Deoxycholate. Preincubations were carried out as described above. After preincubation, 0·15 ml of a 1·0% Na-deoxycholate in isotonic glucose solution was added with a 0·5 ml graduated pipette to each 5-ml sample to give a 0·030% (0·72 mM) deoxycholate solution. Each sample was mixed on the vortex before and after addition of the deoxycholate. The samples were incubated for 30 min at 37°, centrifuged, and the optical density of the supernatant was determined as described above.

Digitonin. The procedure was essentially identical to that of deoxycholate-induced hemolysis, except that 0·2 ml of a 0·01% digitonin solution was added to the preincubated blood samples to yield a final concentration of 0·000384% (3·1 μM).

In each case the hemolytic conditions were chosen such that the per cent hemolysis would fall in the vicinity of the midpoint of the hemolysis curve. A great deal of variation in the extent of hemolysis was encountered and this seemed to reflect differences in the blood samples from day to day.

Materials

The drugs were obtained from a variety of sources: guanethidine, hydrochlorothiazide and tripelennamine from Ciba Pharmaceutical Co., Summit, N.J.; desipramine and imipramine from Geigy Pharmaceuticals, Ardsley, N.Y.; furosemide from Hoechst Pharmaceutical Co., Cincinnati, Ohio; acetazolamide from Lederle Laboratories, Pearl River, N.Y.; cocaine from Mallinckrodt Pharmaceuticals, St. Louis, Mo.; cortisol, cortisone and deoxycorticosterone from Mann Research Laboratories, New York, N.Y.; acetylsalicylic acid, ethacrynic acid and indomethacin from Merck, Sharpe & Dohme, West Point, Pa.; chlorprothixine, chlordiazepoxide, debrisoquin sulfate, levorphanol tartrate, phenindamine tartrate and prostigmine from Hoffmann-La Roche Inc., Nutley, N.J.; chlorpromazine from Smith, Kline & French Laboratories, Philadelphia, Pa.; chloroquine phosphate from Winthrop Laboratories, New York, N.Y.

The glucose (dextrose) and NaCl were Baker Reagent grade, Na_2HPO_4 and $NaH_2PO_4 \cdot H_2O$ were Mallinckrodt analytical reagent. Sodium deoxycholate, M.A. was obtained from Mann Research Laboratories, Inc., and the digitonin was obtained from Hoffmann-La Roche Inc.

RESULTS

The extent of hemolysis produced by different concentrations of NaCl, digitonin and deoxycholate is shown in Fig. 1. The slopes of the deoxycholate and digitonin curves are very similar, while that of the salt is steeper.

It is seen that 200 mg/100 ml of glucose (0·011 M) protects the erythrocytes against hemolysis produced by hypotonic NaCl, probably due to the increased osmolality. In any event, this concentration of sugar was used in all subsequent experiments.

The drug effects can be seen in Figs. 2–5. Figure 2 has a number of drugs grouped because they have a biphasic effect on osmotic hemolysis. After initially protecting the cells from the hemolytic effects of 0·35% NaCl, higher concentrations of the drugs were found to stimulate hemolysis. In the case of 10^{-3} M, desipramine and imipramine, hemolysis was produced during the preincubation period in isotonic saline. While chlorpromazine at 5×10^{-3} M was reported to cause hemolysis of dog erythrocytes at slightly less than isotonic conditions,[3] in the experiments reported here, no hemolysis was encountered at 10^{-4} M. It was noted that with both chlorpromazine and chlorprothixene the cells became sticky during the preincubation period at 10^{-4} M, and the unusual amount of agitation used to resuspend them did not cause hemolysis. Chloroquine was only weakly biphasic in the hypotonic system.

All of these compounds stimulated deoxycholate-induced hemolysis at concentrations of 10^{-6} M or higher. Digitonin hemolysis was not affected by desipramine or imipramine up to a concentration of 10^{-4} M. At this concentration phenindamine was also inactive, but showed some activity at 10^{-3} M. Chlorpromazine and chlorprothixene both stimulated digitonin-induced hemolysis at 10^{-4} M, while chloroquine was inactive even at 10^{-3} M. Common to all of these drugs except chloroquine was

a central nervous system depression seen at certain doses. However, no relationship exists between their potency as a depressant and their effects in the hemolytic tests. Although chloroquine is not thought of as a nervous depressant, it has been reported to have local anesthetic properties.[6]

In Fig. 3 are some compounds which have been grouped because they protect hypotonic hemolysis while they tend to enhance deoxycholate hemolysis. All were without effects in digitonin hemolysis. Two of these drugs, debrisoquin sulfate and guanethidine, are antihypertensives; tripelennamine is an antihistamine and also, like cocaine, a local anesthetic. Levorphanol is a narcotic analgesic.

The drugs in Fig. 4 are active in protecting against hypotonic hemolysis with no effects in the other systems. Acetylsalicylic acid, indomethacin and cortisol are all anti-inflammatory agents. Though not included, cortisone was indistinguishable from cortisol. The fact that non-anti-inflammatory agents like chlordiazepoxide, deoxycorticosterone and cortisone have profiles similar to that of cortisol makes it difficult to correlate these effects with pharmacological activity.

A number of diuretic agents can be found in Fig. 5, all of which are inhibitors of carbonic anhydrase. Furosemide weakly enhanced hypotonic hemolysis, but had no effect in any of the other systems. Both hydrochlorothiazide and ethacrynic acid protected against osmotic hemolysis. In the deoxycholate-induced hemolysis, ethacrynic acid showed a marked but unique protective effect. No other compound gave this response. In the digitonin-induced hemolysis, ethacrynic acid was again uniquely protective. Acetazolamide, a very potent inhibitor of carbonic anhydrase, was

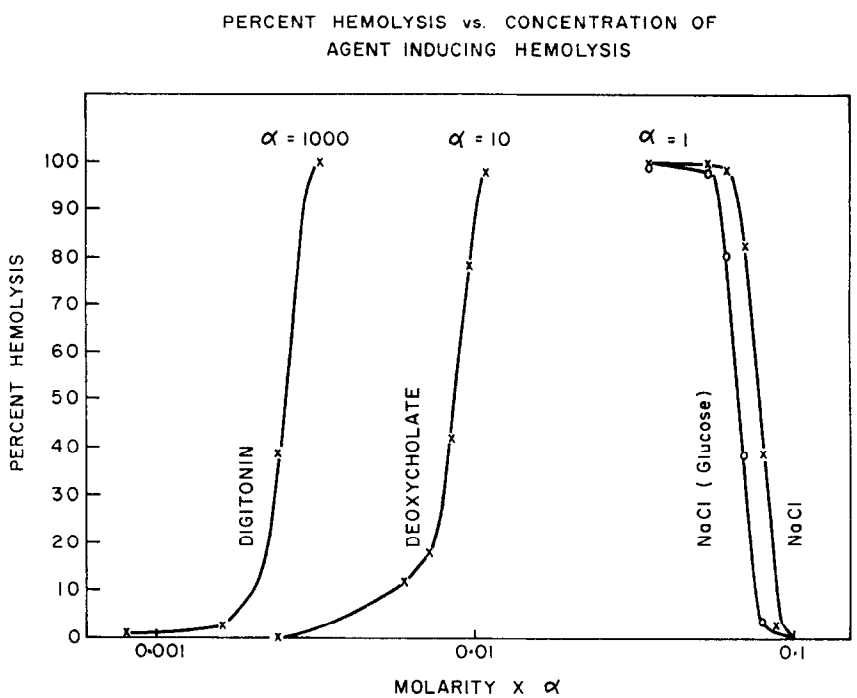

FIG. 1. The degree of hemolysis produced by varying concentrations of digitonin, deoxycholate, NaCl and NaCl + glucose.

initially protective against osmotic hemolysis but, like the central nervous depressants, it was biphasic in its action and at 10^{-3} M enhanced hemolysis. However, this drug was inactive in the deoxycholate and digitonin systems.

Prostigmine, an inhibitor of the enzyme, acetylcholinesterase, was protective against hypotonic hemolysis but inactive in the other systems. Ouabain, an inhibitor of the Na–K-activated ATPase, was inactive in all hemolytic systems and was, therefore, not graphed.

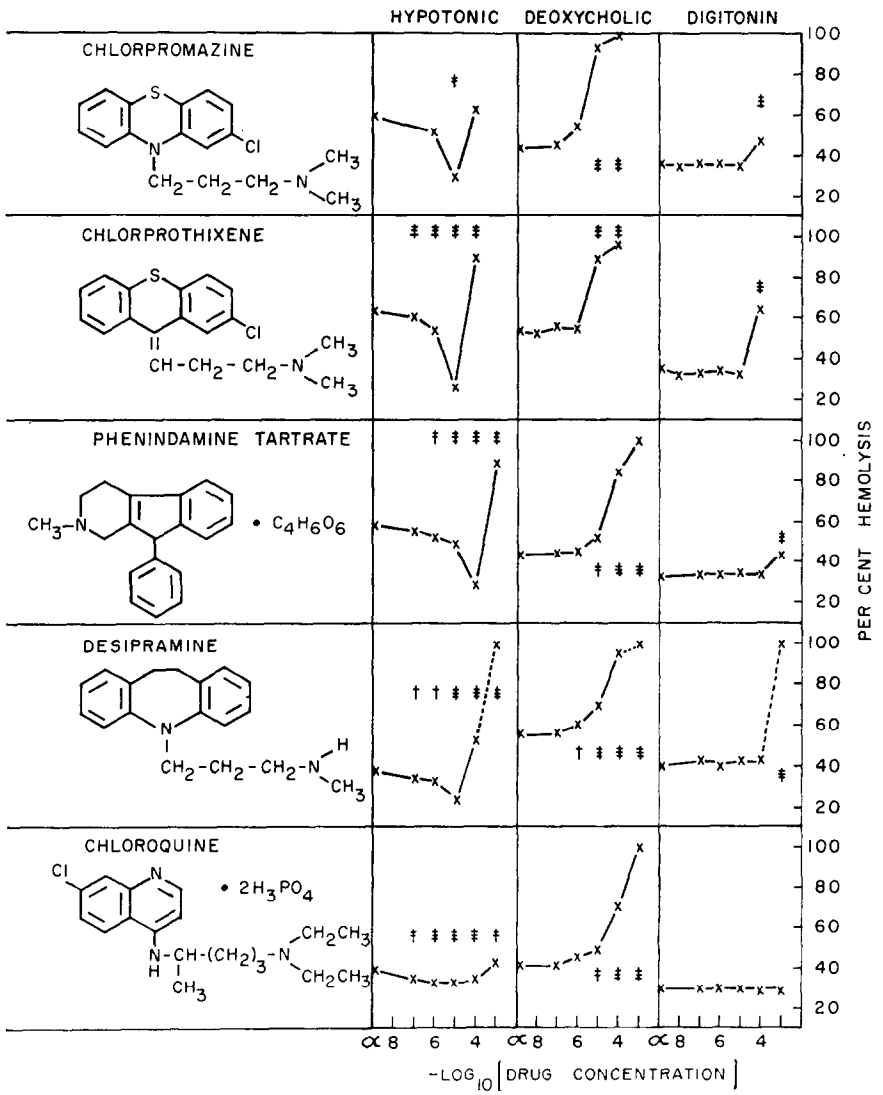

FIG. 2. Hemolytic profiles of drugs which diminish and enhance hypotonic hemolysis. Those points in this and other figures which are significantly different from control are designated according to P values: † is <0·05; ‡ is <0·01; and ‡ is <0·001.

DISCUSSION

It is apparent that no clear-cut correlations can be drawn at this stage of the study which would permit one to predict how the drug would act *in vivo*.

Only one substance, ethacrynic acid, demonstrated any protection against deoxycholate-induced hemolysis. The capacity of this compound to interact with sulfhydryl groups of proteins[7] may be related to its unique effect in the deoxycholate system.

The digitonin-induced hemolysis was most resistant to the modifying effects of drugs. Again, ethacrynic acid was uniquely protective, but only at 10^{-3} M. Chlorpromazine, chlorprothixene and phenindamine on the other hand, enhanced the hemolytic action of digitonin, but only at 10^{-4} and 10^{-3} M.

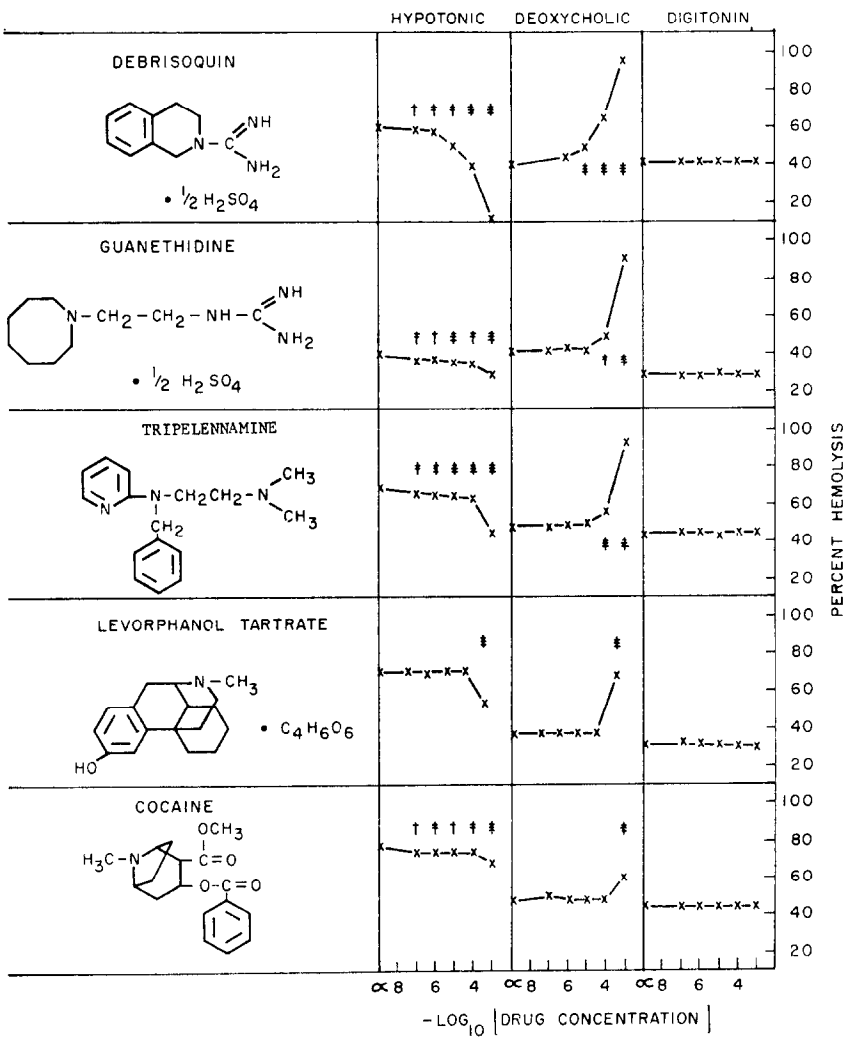

FIG. 3. Hemolytic profiles of drugs which protect against hypotonic hemolysis and enhance deoxycholate hemolysis.

It is apparent from the studies reported here that drugs which have the same type of activity in one hemolytic system may behave quite differently in another. A survey of the literature suggests that disparities in the response of hemolytic systems to certain drugs may have resulted from the types of systems used. While Seeman[1] found that procaine, xylocaine and other local anesthetics protected against hypotonic hemolysis, as reported here for cocaine and tripelennamine, others,[3, 4] using heat-induced hemolysis, found these drugs to be inactive. Chloroquine was also found to be inactive in the heat-induced hemolysis,[3, 4] while the activity reported here is in agreement with that found by Inglot and Wolna[2] for hypotonic hemolysis. It is becoming evident that no one hemolytic system will characterize the nature of the interaction of drugs with plasma membranes.

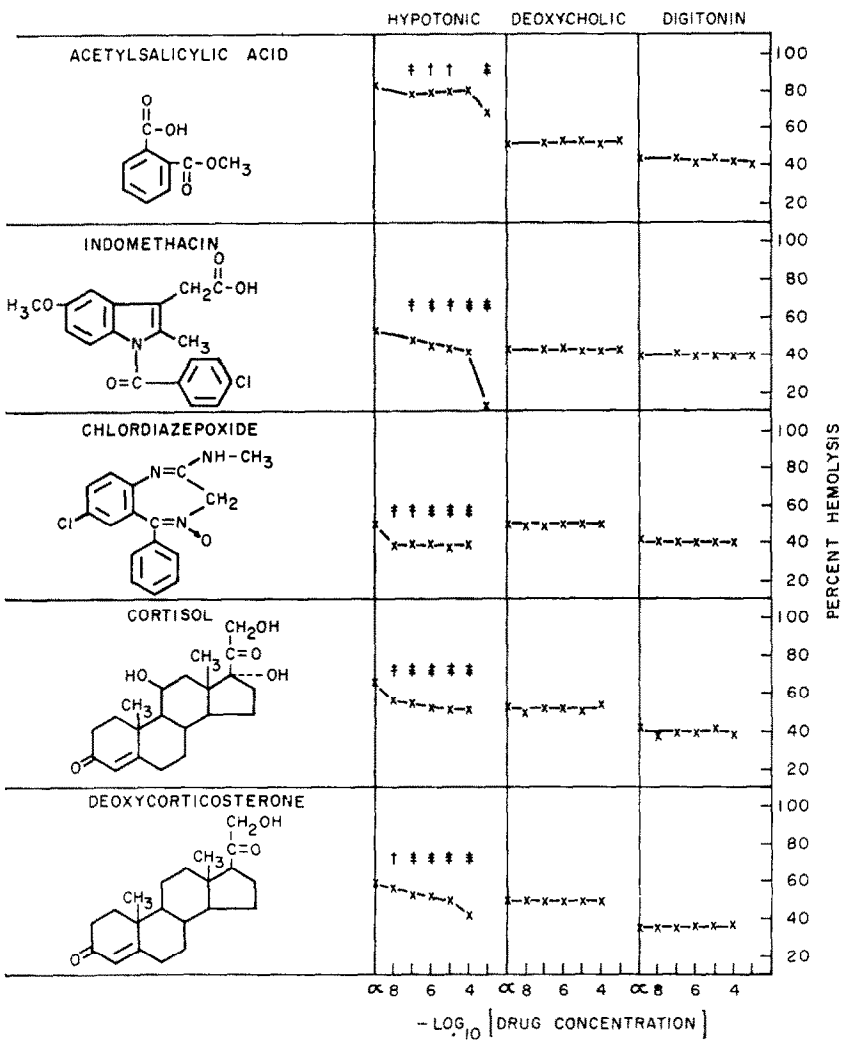

FIG. 4. Hemolytic profiles of drugs which protect against hypotonic hemolysis alone.

At the present moment not enough data are available to permit one to predict what compounds will show activity in any of the systems. A much larger number of compounds would have to be examined. In the deoxycholate system, there is a tendency for weak bases with a higher order of lipid solubility to be active as compared with those which would be less fat-soluble by virtue of their polarity at physiological pH. The observation that the fat-soluble steroids are inactive would point to the need for a weak basic charge. Chlordiazepoxide, however, is a lipid-soluble weak base which is inactive, suggesting that other factors must be involved.

While certain laboratories have attempted to use either the heat-induced[3, 4] or hypotonic hemolysis[6] as tests for anti-inflammatory activity, it is suggested that this

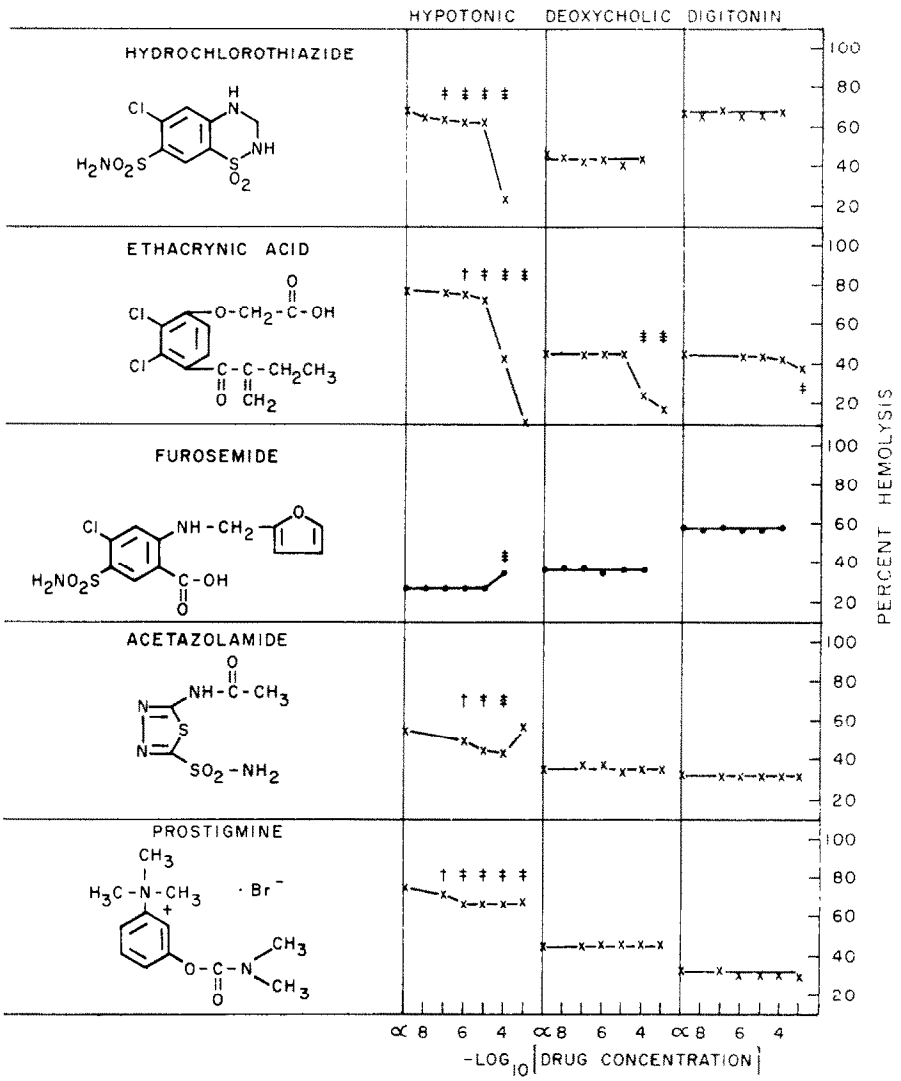

FIG. 5. Hemolytic profiles of diuretic agents and prostigmine.

is too simplistic. It is true that, to date, no anti-inflammatory agent tested has been found to be negative in the system used, but it is also true that many non-anti-inflammatory agents are also positive. It is possible that further testing in other hemolytic systems will eliminate many of the false positives. It is also possible that, by obtaining a profile of responses in several systems, many agents can be crudely classified. It would be naïve to suppose that such an approach *in vitro* would be an adequate primary screen, since it is obvious that a drug's action is also a function of accessibility to its receptor, which in turn depends on its distribution in the body, metabolic alteration, absorption at the tissue site, and excretion. Even assuming that all drugs act by some physico-chemical means at the membrane level, not all membranes are the same.

By examining the effects of drugs on these hemolytic systems and obtaining a better idea of the mechanism by which hemolysis is induced, it should be possible to learn about the structural features necessary for a compound to show activity. In addition, such an approach could help to elicit more about the structure of the erythrocyte membrane and the relationship of this structure to the activity of its constituent enzymes.

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