# *In Vitro* Effect of Some Chemicals on Histaminopexy II: Quantification and Potentiation of Histaminopexy

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**Abstract** □ Optimum conditions for histaminopexic determinations were defined resulting in complete dialysis in 18 hr. at 25°. Complexation of histamine with the dialysis membrane was found to be significant and dependent upon histamine concentration. Due to lack of predictability, individual corrections for this phenomenon were made for each determination. The interaction of gamma globulin was found to follow the law of mass action and was enhanced by quercetin.

**Keyphrases** □ Histaminopexy—*in vitro* determination □ Histamine dihydrochloride, radioactive—dialysis rate □ Membrane binding—labeled histamine □ Protein binding—labeled histamine □ Quercetin, di- and thymoquinone effect—histamine binding

Histaminopexy is the ability of serum proteins to bind and thereby inactivate histamine. This controversial phenomenon, elaborately reviewed by Parrot (1), has been studied in this laboratory and found to be reasonably reproducible when suitable care is taken during the determination. This observation (2), coupled with the finding that quercetin was capable of enhancing the binding of histamine by serum proteins, suggested further study of this interaction which could possibly be the basis of a novel alleviation of symptoms of histamine-related allergies.

## EXPERIMENTAL

A modified dialysis procedure (2) was employed in which a mixture of gamma globulin and an equal volume of aqueous radioactive histamine dihydrochloride was equilibrated against Tyrode's solution. Cellulose dialysis tubing[1] of approximately 25-cm. (10-in.) length for each sample was soaked in Tyrode's solution for a minimum of 4 hr. before use.

Each previously wetted dialysis tube was placed U-shaped into a glass test tube 2.5 × 15.2 cm. (1 × 6 in.) containing Tyrode's solution (10 ml.) such that the open ends of the tube extended beyond the lip. The gamma globulin-radioactive histamine mixture (5 ml.) was introduced into the dialysis tube by use of a syringe fitted with a blunt oral feeding needle. Plastic stoppers were inserted to immobilize the dialysis tubing. All binding studies were conducted in triplicate. A series of confirmatory dialyses was run using Plexiglas cells in which the labeled histamine could be placed on the same side of the dialysis membrane as the serum protein or on the opposite side. When the histamine distribution was similar in both systems, dialysis was considered complete.

During equilibration, the histaminopexic ability of gamma globulin prevented some histamine from dialyzing through the membrane. At the termination of equilibration, the total concentration of histamine inside the tube consisted of histamine–protein complex and free histamine. The external solution contained only free histamine in a concentration equal to that of the internal free histamine.

After dialysis, samples (0.5 ml.) of the external and internal phase were pipeted into separate counting vials filled (15 ml.) with Bray's scintillation fluid. The vials were randomly placed in a scintillator[2] obtaining at least two 5-min. counts for each sample, then "spiked" and recounted.

**Dialysis Rate**—Protein-free solutions of radioactive histamine in Tyrode's buffer were placed in dialysis tubes and were allowed to equilibrate against Tyrode's buffer for 4-, 6-, 8-, and 18-hr. periods at 7° and 25°. The degree of equilibration was determined by measuring radioactivity on both sides of the semipermeable membranes.

**Membrane Binding**—Protein-free dialysis of three concentrations of labeled histamine ($1 \times 10^{-5}$, $6 \times 10^{-6}$, $1 \times 10^{-6}$ *M*) in Tyrode's solution were conducted maintaining a constant length of dialysis tubing in contact with the external solution. The sums of the internal and external histamine concentrations were subtracted from the known concentration of added histamine with the differences assumed to represent the amount of histamine bound by the cellophane dialysis tubes.

**Protein Binding**—The dialysis procedure previously described was followed using several different histamine concentrations ($1 \times 10^{-2}$ to $1 \times 10^{-6}$ *M*). The amount of histamine bound by the gamma globulin (c.p.m. inside − c.p.m. outside/total c.p.m.) was multiplied by the moles of added histamine after correcting for membrane binding to give the moles of histamine bound by the gamma globulin at the various histamine concentrations. This information was related to the number of available binding sites of the gamma globulin and the apparent association constant of the complex (3).

**Chemical Enhancement**—Quercetin, thymoquinone, and dithymoquinone (0.0111% in 20% ethanol) were incubated (1 ml.) at 37° for 1 hr. with 0.1% gamma globulin (10 ml.). To these, aqueous radioactive histamine ($2.3 \times 10^{-4}$ *M*) was added (10 ml.) and incubation was continued for an additional 6 hr. At the end of this period, three aliquots (5 ml.) were inserted into dialyzing tubes and were allowed to equilibrate against Tyrode's solution. Control determinations were conducted without chemicals so that the potentiating effect could be evaluated by comparing the binding of histamine in the presence of absence of chemicals.

## RESULTS AND DISCUSSION

Electrolyte concentration, pH, serum inconsistencies, counting bias, as well as other parameters which influence histaminopexy have been eliminated by the constant use of Tyrode's solution buffered to pH 7.36, gamma globulin as the binding protein, randomization of counting, and standardized experimental conditions.

**Dialysis Rate**—The variables, time and temperature, were studied to obtain optimum dialysis conditions. The results (Table I) indicated that dialysis was slightly slower at decreased temperature and was essentially complete after 18 hr. at 25°.

Subsequently, 24 hr. and 25° were the conditions maintained for equilibrium. This is in close agreement with previous workers (4, 5). One group (6), using a dialysis period of 4 hr., reported the inability to confirm protein binding.

**Membrane Binding**—The degree of histamine removal from the system through binding to the dialysis membrane was sought due to the possibility of its complete removal. Use of a low concen-

[1] One-inch, 4465-A2, A. H. Thomas Co., Philadelphia, Pa.

[2] Packard.

Table I—*Comparison of Dialysis Rates (% Completion)*

| °C. | Hours | | | | |
|---|---|---|---|---|---|
| | 4 | 6 | 8 | 12 | 18 |
| 7 | 62.0 | 81.0 | 77.5 | 92.5 | — |
| 25 | 72.0 | 81.0 | 95.7 | 95.0 | 99.7 |

Table II—Comparison of Membrane Binding (% Bound)

| °C. | $1 \times 10^{-6}$ | $6 \times 10^{-6}$ | $1 \times 10^{-5}$ |
|---|---|---|---|
| 25 | 18.6 | 18.0 | 16.1 |
| 35 | 18.4 | 15.7 | 13.8 |

tration of histamine which would be essentially protonated under experimental conditions and therefore prone to interact with the negative charge on the cellophane membrane (7) would indicate that a considerable percentage might be bound. Failure to consider this phenomenon would result in erroneously high binding values. Membrane binding was shown to be significant and to be dependent on temperature and histamine concentration (Table II).

The percent binding of histamine decreased with an increase in concentration as expected due to the law of mass action. Since slight deviations in dialysis tube immersion would alter the magnitude of membrane binding, individual corrections were made for this loss of histamine in subsequent studies.

**Protein Binding**—An attempt was made to characterize the reaction of histamine and gamma globulin by defining the number of histamine-binding sites on gamma globulin and the stability of the resulting complex. Since gamma globulin was proposed to be the protein fraction responsible for histaminopexy, it was logical to quantify this interaction.

This determination, based on a reported procedure (3), was estimated graphically (Fig. 1) by relation to the equation derived from the law of mass action:

$$B/A = kn - kB$$

where $B$ = moles of histamine bound per mole of gamma globulin; $A$ = concentration of free histamine; $n$ = number of histamine binding sites of gamma globulin; $k$ = apparent association constant (l./mole).

Theoretically, the number of moles of histamine bound by each mole of gamma globulin ($B$) would increase as larger amounts of histamine were added to the system until a maximum number of moles was bound which would equal the number of binding sites ($n$). In a similar manner, histamine-bound ($B/A$) would increase. According to the above equation, as the amount of histamine bound per mole of gamma globulin ($B$) approached zero, the term $kB$ was eliminated from the equation (*i.e.*, $B/A = kn$). Therefore, the abscissa intercept equaled the number of binding sites ($n$), and the ordinate intercept equaled the apparent association constant multiplied by the number of binding sites ($kn$).

To perform this quantification, it was necessary to assume that binding was not random, that only one class of binding sites was present, and that there was a specificity of histamine for these

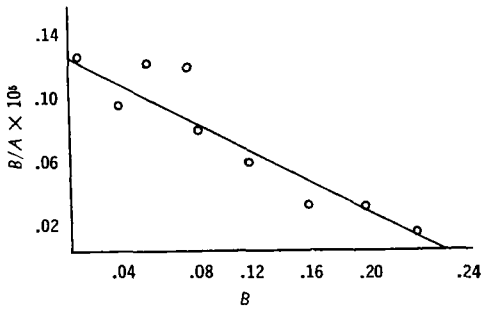


**Figure 1**—*Interaction of gamma globulin and histamine.*

Table III—Chemical Enhancement, Chemical/Control (±*SE*)[a]

| | Quercetin | Thymoquinone | Dithymoquinone |
|---|---|---|---|
| Chemical/Control | 2.8 ± 0.47 | 1.1 ± 0.08 | 1.7 ± 0.10 |
| Samples | 12 | 14 | 16 |

[a] Average binding capacity of gamma globulin = 16.2 ± 1.34%.

sites. Accordingly, results indicated 0.23 histamine-binding sites per mole of gamma globulin, and an apparent association constant of the complex equal to $5.2 \times 10^5$. These figures were obtained by plotting average values of several determinations.

**Chemical Enhancement**—Histaminopexic potentiation by certain compounds was studied with gamma globulin since reproducible histamine binding was apparent at low histamine concentration ($2.3 \times 10^{-4}$ *M*). This initial study was undertaken in an *in vitro* system to confirm previous reports of potentiation (8, 9) with the anticipation of extending this research to structurally related compounds, possibly producing a new classification of antihistamines as discussed in the Introduction.

The efficacy of the individual compounds studied was expressed as a ratio of the binding capacity of gamma globulin in the presence of each chemical to the binding capacity in its absence and is summarized in Table III. Although no potentiation was found for thymoquinone and dithymoquinone in earlier work (1), the diminution of the biological effects of histamine by these two compounds is substantiated by *in vivo* data (12).

Although the exact mechanism of this enhancement is unknown, several explanations might be proposed. Since under experimental conditions, the chemical potentiators were allowed to incubate with protein for 1 hr. before adding histamine, it was possible that they might have acted upon the protein in some way so as to expose more binding sites complementary to histamine. This might have occurred by disturbance of the tertiary level of the protein or perhaps by displacement of bound water molecules providing an anydrous binding environment (10).

Another interesting possibility was based on the previously observed conformational adaptability of protein toward substrates (11). If histamine were considered to be the normal substrate for the protein, then one conformation would have been optimum for binding but not necessarily the most thermodynamically stable. The action of chemical potentiators could then have been viewed as either stabilization of the optimum conformation or perturbation of a stable conformation to that optimum for binding. Either action on the protein would have resulted in enhanced binding of histamine.

In independent *in vivo* studies, protection of guinea pigs against a histamine aerosol was afforded by thymoquinone (9, 12) and quercetin (12, 13). This protection was greater than that produced by the classical antihistamine, diphenhydramine, indicating the potential usefulness of these compounds. Although enhanced histaminopexy has been inferred (9) to be the mechanism for this protection, current data are not sufficient to preclude other mechanisms.

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# Two Procedures for Training Differential Responses in Alcohol and Nondrug Conditions

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**Abstract** □ Two simple efficient procedures, automatically programmed in standard operant test chambers, are described for training rats to a high degree of response differentiation solely on the basis of the perceptual or sensory alterations caused by a drug. Rapid learning of differential approach and avoidance responses occurred with a Conflict procedure whereby hungry rats received food reward in one condition (1.2 g./kg. ethyl alcohol for seven animals, saline for seven animals) and shock in the other condition, after every fifth lever press. With a Choice procedure, a separate group of hungry rats learned to press preponderantly the food-rewarded lever during the initial, unreinforced portion of sessions in which they received food reward on an intermittent schedule for presses on one lever in the alcohol condition (left-hand lever for four animals, right-hand lever for four animals) and for presses on the other lever in the saline condition.

**Keyphrases** □ Differential responses—test procedures □ Alcohol, nondrug conditions—perceptual response differentiation □ Choice procedure—test method □ Conflict procedure—test method □ Equipment—differential response testing

The perceptual or sensory alterations caused by drugs enable animals to learn differential responses solely on the basis of whether they are in a drug or nondrug condition (1–10). Such learning may provide part of the explanation for the development of abnormal patterns of behavior under the influence of alcohol or other drugs (11). A technique for training animals to perform differential responses on the basis of their drug or nondrug condition may be used for investigating various problems of pharmacological interest. Problems which have been investigated include tests of similarities or dissimilarities in the perceptual or sensory effects of different compounds (2, 5), measurements of the threshold dose below which the animal cannot differentiate the drug from the nondrug condition (3, 5), and attempts to identify the central or peripheral alteration which is the basis for the differential responses in the drug and nondrug conditions (3, 5, 10).

The ideal training method would be a simple, efficient, automatic procedure which enables rapid learning of a high degree of response differentiation on the basis of a low drug dose compared to the nondrug condition. Most of the methods previously reported (1–6, 8, 10) have required the experimenter to place the animal into the choice situation at the beginning of each brief trial. The locomotor-choice response, measured in these procedures, is generally performed rapidly and hastily, to the detriment of the accuracy of the test. This is especially true of a shock-escape situation (2, 3, 5, 6, 10), in which rather high drug doses have generally been selected.

These disadvantages can be eliminated with the use of a lever-pressing response, in a standard operant test chamber. By means of an intermittent schedule of reinforcements, the test can be extended to require a substantial number of lever presses, thus providing a more deliberate choice by the animal and a wider range of quantitative variant on in the choice response. The prolonged test in an isolated chamber, with automatic programming and recording, provides an efficient procedure and also ensures that the experimenter cannot influence the animal's choice. However, the few operant methods previously reported have not provided simple efficient procedures for rapid learning of a high degree of differentiation of responses. Barry (7) associated food reward in the drug and nondrug conditions with differential environmental illumination, controlled by successive presses on the same lever. This procedure demonstrated that the rats learned a perceptual or sensory discrimination between the drug and nondrug conditions, thus refuting in this situation Overton's suggestion (3, 5) that separate, dissociated habits are learned in the drug and nondrug conditions. However, in this difficult task, the animals failed to achieve a highly consistent discriminative response, even after a large number of training sessions. Harris and Balster (9) reported on a complex procedure with only three rats, trained to choose different levers and at the same time different response rates in the drug and nondrug conditions.