support to this mechanism. Alternatively, red cell treatments effective in promoting attachment might impart to the erythrocyte surface molecular configurations that mimic those of antigen bound antibody. It should be pointed out that several of the treatments used, namely heat, aldehyde, tannic acid and polylysine, induce conformational changes in proteins, and that macrophages display marked affinity, both *in vivo*(16) and *in vitro* (17) for denatured proteins. That a change in the red cell surface proteins might underlie the described interaction is indicated by the attachment to macrophages of aldehyde treated ovalbumin crystals and gelatin DNA coacervates(18) in saline medium (unpublished results).

Summary. Homologous or heterologous red cells subjected to a number of treatments attached to mouse peritoneal macrophages in a saline, protein free medium in contrast to the lack of attachment of untreated red cells. Effective treatments included heat, glutaraldehyde, tannic acid, periodate, polylysine and colloidal silica. Under the same conditions, the modified erythrocytes showed little or no attachment to polymorphonuclear leucocytes. Trypsin or n-ethylmaleimide treatment of erythrocytes did not promote attachment to either cell type. Attachment of treated red cells was temperature dependent, and was abolished or reduced by preincubation of the macrophages with trypsin.

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Plasma Histaminase Activity in Various Mammalian Species; A Rapid Method of Assay.* (31750)

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While investigating the effects of administered histamine and compound 48/80 on plasma histamine levels in monkeys, we observed that squirrel monkeys (*Saimiri sciureus*) exhibited elevations of plasma histamine shortly after administration whereas rhesus monkeys (*Macaca mulatta*) did not. But both species demonstrated signs of increased circulatory histamine as evidenced by gross observations (erythema, hypopnea, reduced blood pressure).† Apparently, rhesus monkeys are unique among mammalian species including

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† J. H. Peters, G. R. Gordon, S. A. Ferguson, in preparation.

man, since rats(1), cats(1,2), dogs(2) and man(3) exhibit elevated plasma histamine levels after receiving compound 48/80.

In an attempt to explain the basis for these observations in rhesus monkeys, we have examined the capacities of the plasma of various species, including man, to degrade histamine. A primary metabolic degradative pathway of histamine in mammals is oxidation by the enzyme, histaminase(4). The reaction proceeds with the consumption of oxygen and the production of ammonia, hydrogen peroxide, and imidazole acetaldehyde. Numerous parameters have been utilized as bases for quantitating histaminase activity. Oxygen consumption(5,6,7) and ammonia liberation(5) have been measured directly. Hydrogen peroxide production has been determined by a microtitrimetric technique(8). In other reports, disappearance of substrate histamine has been followed by bioassay(7,9, 10) or fluorometrically(11). In general, all these procedures require large samples and long incubation periods due to relatively low sensitivities or slow rates of substrate transformation. Recently, a spectrophotometric micromethod for measurement of plasma histaminase in rabbits was reported(12). The basis of this technique is the measurement of the color produced by oxidation of o-dianisidine with the hydrogen peroxide formed in the oxidation of added histamine by plasma histaminase. Peroxidase is included to catalyze the oxidation by hydrogen peroxide. Activities were reported in terms of absorbancies at 470 mμ. After preliminary evaluation, we modified the procedure as follows:

a) Incubation at 37° instead of at room temperature reduced the time of assay from 4 hours to 1 hour or less;

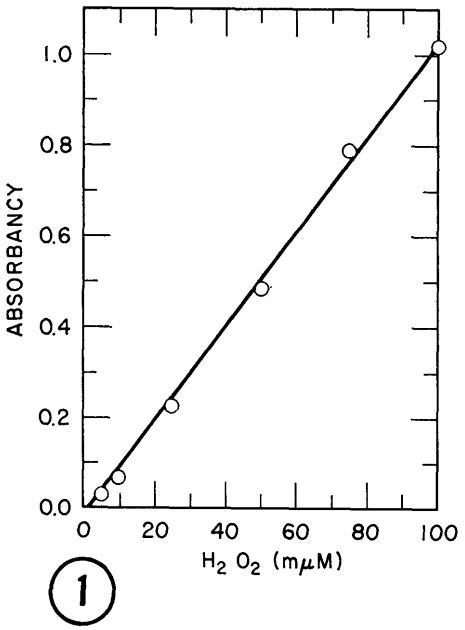
b) The amount of o-dianisidine per sample was increased from 0.05 to 0.25 mg since the original amount did not permit maximum color development;

c) The enzymatic reaction was stopped by acidification with HCl. This step also increased the sensitivity of the test by shifting the absorption maximum of the colored product to 540 mμ, a modification utilized in the glucose oxidase method for measuring blood glucose(13). Strong HCl was used for acidification because of precipitation of protein by the H_2SO_4 usually employed; finally,

d) Calibration curves were established by analyzing known concentrations of hydrogen peroxide without plasma since histaminase action is known to produce stoichiometric amounts of hydrogen peroxide from histamine (4). Thereby, activity could be expressed in absolute terms.

Materials and methods. The incubation system for establishing the calibration curve was composed of: 0.8 ml 0.1 M Na_2HPO_4 in 40% aqueous glycerol, pH 7.2; 0.05 ml 0.2 M histamine dihydrochloride (Calbiochem); 0.05 ml 0.04% horseradish peroxidase (Calbiochem) in the above buffer; 0.05 ml 0.5% o-dianisidine (Matheson, Coleman and Bell) in 96% ethanol; and 0.05 ml of dilute H_2O_2 aqueous solutions containing 5, 10, 25, 50, 75 and 100 mμM H_2O_2. These H_2O_2 solutions were prepared immediately before use by dilution of 3.5% H_2O_2 solution (Matheson, Coleman and Bell) with distilled water. The exact molarity of the stock H_2O_2 solution was determined by titration with $KMnO_4$(14). Blanks were samples containing H_2O_2 without added peroxidase. The solutions, in 13 × 75 mm test tubes, were incubated for 1 hour in a water bath at 37 ± 0.5°. After cooling in ice, they were acidified by adding 1 ml ice-cold 9.8 N HCl and allowed to stand at room temperature for 15 minutes. Their absorbancies were then measured at 540 mμ in a spectrophotometer (Zeiss, Model PMQ II) using cells of 1 cm light path. Fig. 1 shows the relationship between absorbancy and concentration of H_2O_2 in the range of 5 to 100 mμM. In these samples, color production before acidification (absorption maximum, 470 mμ) was immediate and was not altered by incubation for 1 hour. Later studies showed that the absorbancies could also be measured in a Klett-Summerson colorimeter equipped with a No. 54 filter using micro tubes.

For assay of plasma (0.05 to 0.10 ml) all the reagents except the H_2O_2 solutions were mixed in an ice bath with sufficient amounts of buffer to yield a final volume of 1.0 ml. Incubation, acidification, and reading of absorbancies were performed as described above. Inherent oxidation by plasma without added



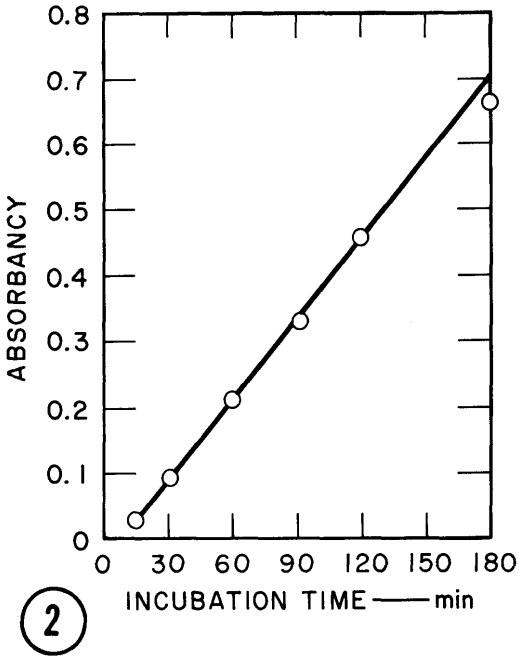


FIG. 1. Calibration curve for estimating histaminase activities in plasma samples.
FIG. 2. Histaminase activity of rabbit plasma vs time of incubation.

histamine was determined in every assay. Activities were estimated by reference to the calibration curve of the net values obtained by substracting the absorbancy of samples without substrate from those containing added histamine. They were expressed as mμM of histamine oxidized/ml of plasma/minute. The lower limit of sensitivity was 0.1 mμM and the reproducibility, ± 0.1 mμM. Human or monkey plasma stored frozen for 4 weeks showed no change in activity. Heating plasma in buffer for 30 minutes at 80° before addition of the other reagents completely abolished the oxidative activity. To test the feasibility of employing longer or shorter incubation times, the relationship between histaminase activity and period of incubation was determined using rabbit plasma. Fig. 2 shows that rabbit plasma histaminase activity increased linearly with incubation times of up to 3 hours. Similar studies with human plasma extended this linear relationship to 5 hours. Although the standard incubation period was 1 hour, these results show that longer or shorter periods can be used for plasma samples of exceptionally high or low activities.

Several experiments were performed using human plasma to define more accurately the activities being measured. Histaminase activities were determined using 0.05-ml aliquots of plasma from 2 subjects and 2-hour incubation periods in the buffer adjusted to pH 6.0, 6.4, 6.8, 7.2, 7.6 and 8.0. Limited inhibition studies were also carried out employing aminoguanidine bicarbonate (Matheson, Coleman and Bell). In addition, the relation of oxidative activity to histamine and putrescine (Calbiochem) concentration was determined employing 0.05-ml aliquots of plasma from 2 volunteers. To compare oxidative activities of human plasma with a commercial hog kidney diamine oxidase preparation (Sigma), we determined the protein content of both types of sample by the biuret method(15). In this last study, oxidative activity against histamine, putrescine, and cadaverine (Calbiochem) was determined. The activities were expressed as mμM of substrate oxidized/mg of protein/hour.

The mice, rats, guinea pigs, rabbits, cats and dogs used in this study were normal healthy adult specimens. Rhesus and squirrel monkeys were housed and fed as described

TABLE I. Plasma Histaminase Activity of Various Species.

Species and sex	No. of subjects	mμM histamine oxidized*/ml of plasma/min, mean ± S.D.
Mouse, Swiss-Webster, ♂	12	<.1
Rat, Sprague-Dawley, ♂	10	<.1
Guinea pig, Hartley, ♂	5	1.3 ± .7
Rabbit, New Zealand white, ♂	13	6.2 ± 3.3
Cat, ♂ and ♀	10	6.1 ± 2.1
Dog, beagle, ♂ and ♀	11	1.4 ± .2
Monkey, rhesus, ♂	8	18.2 ± 6.0
Monkey, squirrel, ♂	6	1.8 ± .8
Man, ♂	6	2.6 ± .3

* Using 10 μM histamine as substrate.

previously.‡ None had received drugs 4 months before blood was taken for histaminase determination. The 6 male human blood donors ranged in age from 27 to 49 years. Mice and rats were bled by heart puncture; all others by venipuncture. Heparinized plasma (10 units/ml of whole blood) was obtained in the usual manner.

Results and discussion. Table I summarizes the plasma histaminase activities found in the various species tested. No activity could be detected in the plasma of mice and rats; plasma from guinea pigs, dogs, squirrel monkeys, and man had relatively low levels of activity, and that of rabbits and cats was somewhat higher. No difference between sexes was noted in the groups of cats and dogs; therefore, these data were combined. Surprisingly high activities were found in plasma from rhesus monkeys. Redeterminations of plasma histaminase levels in 4 squirrel and 4 rhesus monkeys and in 2 human subjects 30 days or more after the initial observations revealed no meaningful change in activity. Therefore, it may be suggested that plasma histaminase activities are stable individual characteristics.

There appears to be a clear association between those species that respond to compound 48/80 by exhibiting elevations of plasma histamine and relatively low plasma histaminase activities (rats, cats, dogs, squirrel monkeys, and man). Therefore, the inability to detect elevations of plasma histamine in rhesus monkeys receiving compound 48/80 or histamine can be explained by their unusually high capability to oxidize circulatory histamine.§

In attempts to elucidate the characteristics of the plasma histaminase activities measured in these studies, we determined the activities of plasma from 2 human subjects against different concentrations of histamine and putrescine. The optimal concentration of histamine in this system was between 40 and 50 μM (Fig. 3); higher concentrations caused inhibition. Substrate inhibition of histaminase by high concentrations of histamine has been reported previously by others(4,7). This Figure also shows that when the same amounts of putrescine were employed no optimal concentration was observed. Determination of the optimal pH for histaminase action of 2 human plasma samples yielded values of pH 6.4 and 6.8. Kapeller-Adler(4) reported the activity of purified hog kidney histaminase, which exhibited no activity against putrescine or cadaverine, to be optimal at pH 6.8. At this time, our studies do not provide sufficient information to indicate whether plasma contains different enzymes for the oxidation

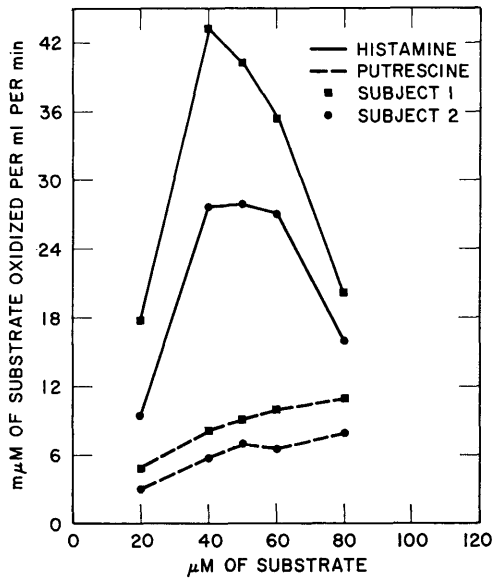


FIG. 3. Effect of substrate concentration on human plasma histaminase activity.

‡ J. H. Peters, G. R. Gordon, S. A. Ferguson, in preparation.

§ J. H. Peters, G. R. Gordon, S. A. Ferguson, in preparation.

TABLE II. Inhibition of Human Plasma Histaminase Activity by Aminoguanidine.

Plasma	Aminoguanidine concentration	Histaminase activity*	% Inhibition
Subject 3	0	8.2	0
	10^{-6} M	7.4	10
	10^{-5} M	1.8	78
" 4	0	17.7	0
	10^{-6} M	16.0	9
	10^{-5} M	3.8	79

* Same units as Table I, using 30 μM of histamine as substrate.

of histamine and putrescine or a single enzyme with markedly different affinities for the two substrates.

Aminoguanidine has been reported to be an active inhibitor of histaminase. Table II shows that, in our system, 10^{-5} M aminoguanidine strongly inhibited the oxidative activity of the two human plasmas tested, while 10^{-6} M was relatively ineffective. Previous *in vitro* tests of aminoguanidine inhibition of histaminase preparations derived from cat kidney(7), hog kidney(4), and rat lung(9) gave qualitatively similar results. Furthermore, Lindell *et al*(16) reported that aminoguanidine was an effective inhibitor of histaminase action in intact human subjects.

Finally, we compared the ability of plasma from 2 human subjects with that of commercial hog kidney diamine oxidase to oxidize histamine, putrescine and cadaverine. Table III shows that the predominant activity of both human plasma samples was on histamine. The commercial preparation had approximately equal but low activity with all three substrates.

Summary. A rapid microcolorimetric method for measurement of plasma histaminase has been described. Its lower limit of sensitivity is 0.1 mμM histamine oxidized/ml of plasma/minute and its reproducibility, $\pm$ 0.1 mμM. In a series of mammalian species surveyed, no activity was detected in the plasma of mice and rats; plasma from guinea pigs, dogs, squirrel monkeys, and man exhibited relatively low activities; plasma activity of rabbits and cats was somewhat higher; plasma from rhesus monkeys was more active than that of any other species. Studies of pH optimum, aminoguanidine and substrate inhibition, and substrate specificity using human plasma indicate that the primary activity measured was histaminase activity.

TABLE III. Oxidative Activity of Human Plasma and Diamine Oxidase Against Various Substrates.

Preparation	Oxidative activity* when substrate† was Histamine	Putrescine	Cadaverine
Plasma, subject 3	5.3	.8	.7
" " 4	13.6	2.2	1.7
Diamine oxidase, hog kidney, Sigma	1.1	1.6	1.2

* mμM substrate oxidized/mg of protein/hr.
† 30 μM of substrates.

ADDENDUM: In conjunction with later studies involving assays of human, rhesus and squirrel monkey plasma, we measured concurrently the activities when either the monoamine oxidase substrate, tyramine, or histamine served as substrates under the conditions of Table I. For 11 squirrel (8♂, 3♀) and 12 rhesus (8♂, 4♀) monkeys and 10 human (7♂, 3♀) subjects, the mean ($\pm$ S.D.) mμM of substrate oxidized/ml of plasma/min was 0.6 ($\pm$ 0.2), 0.7 ($\pm$ 0.4) and 1.2 ($\pm$ 1.0), respectively, when tyramine was substrate; and 1.8 ($\pm$ 0.6), 14.7 ($\pm$ 2.9) and 1.7 ($\pm$ 0.5) respectively when histamine was substrate.

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Effect of Locally Applied Anti-Inflammatory Substances on Rat Skin Wounds. (31751)

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The underlying property common to all anti-inflammatory substances is the ability to interfere with one or more of the biochemical responses of tissue to injury. Thus, these drugs may variously interfere with: (a) energy production in connective tissue by inhibiting generation of ATP, anaerobic glycolysis and oxidation of reduced DPN(1,2), (b) increased capillary permeability and edema formation by inhibiting formation and release of biogenic amines or bradykinin(3-5), (c) formation of granulation tissue by inhibiting synthesis of collagen and acid mucopolysaccharides(6-17). Tests for anti-inflammatory compounds have been based on each of these aspects of the inflammatory response. However, the metabolic actions of drugs may either be exerted directly on the inflamed tissue or indirectly by disrupting homeostasis. Thus drug-induced anorexia, toxicity, analgesia, counterirritation or activation of the hypothalamo-hypophyseal-adrenal axis may in turn modify the course of an inflammatory response(18-20), making it frequently difficult to distinguish between direct and indirect drug actions *in vivo*.

The responses of the skin to wounding are typical of those observed in any inflammatory process and include increased capillary permeability, edema formation, pain, leukocytic infiltration, fibroplasia and granulation(21, 22). The degree of healing of the wound may be easily measured by the determination of its tensile strength(23). Furthermore, the healing process may be divided into 3 consecutive phases(21) each of which may be studied separately. These are: (a) the lag or substrate phase which is characterized by an "acute inflammation" (1-5 days); (b) the fibroplastic or collagen phase (5-14 days); (c) the maturation of scar phase (15 days).

We have now studied the effects of local application of several anti-inflammatory substances on each of these phases of healing skin wounds in rats. Such studies may provide an insight into the stage in the inflammatory process at which various types of anti-inflammatory substances exert their action.

Materials and methods. Male Wistar rats weighing 170 $\pm$ 10 g were used in these studies. On day 1, the rats were anesthetized with ether, the skin of the dorso-lumbar region was shaved with electrical clippers and a wound approximately 1 inch in length was incised. A weighed amount of the test material was sprinkled along the incised wound and the wound was closed with 3 Michel wound clips. Eight to ten rats from each group were killed and the skin wound tensile strength was measured on day 3, 9 and 15. The tensile strength of the wounds was measured *in situ* according to the method previously described(23).

Briefly, the apparatus used to measure the wound tensile strength consisted of 2 alligator clips, a ball-bearing pulley system, plastic volumetric cylinders and a constant rate of flow water source. After the rat was killed, one clip was attached to one side of the wound and to a stationary post; the other clip was attached to the opposite side of the wound and to the volumetric cylinder *via* a ball-bearing pulley system. A constant flow of water was allowed to fill the cylinder until the wound was disrupted. The volume (ml) and/or weight (g) of water was recorded and