Mechanism of Intrinsic Factor Action in the Gastrectomized Rat.* (22907)

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It has recently been demonstrated that the gastrectomized rat is unable to absorb vit. B_{12}(1-4). This defect can be abolished by simultaneous administration of rat gastric juice(3,4) or of preparations containing the glandular portion of the rat stomach(2,4). The disturbance in vit. B_{12} absorption in gastrectomized rats therefore is similar to that which exists in patients with pernicious anemia(5,6,7) or in patients in whom a total gastrectomy has been performed(8). However, although human gastric juice or preparations of hog stomach with intrinsic factor activity are capable of enhancing the absorption of Co^{60}–B_{12} in pernicious anemia and in patients following total gastrectomy, these substances are not effective in the gastrectomized rat(1-4).

A number of theories have been proposed to explain the action of intrinsic factor. It has been suggested that it binds vit. B_{12}(9), thereby either "activating" it(10) or making it unavailable to micro-organisms(11,12) or parasites(13,14) present in the intestine. According to a different hypothesis(15) the intrinsic factor acts primarily on the intestinal wall and in some way specifically facilitates absorption of vit. B_{12}. The purpose of our experiments was to determine whether the more significant relation was between intrinsic factor and vit. B_{12} or between intrinsic factor and the intestinal wall.

Methods. Two different technics were used in securing data about the effects of rat intrinsic factor upon Co^{60}–B_{12} absorption in the gastrectomized rat. The first method employed totally gastrectomized rats; and the experimental procedures, which involved determinations of radioactivity of the feces following a single administration of a test dose of Co^{60}–B_{12}, were essentially similar to those previously reported(4). The second method involved experiments designed to determine the radioactivity present in the wall of isolated loops of small intestine created at a second operation in rats that had previously been subjected to total gastrectomy. In these experiments, a test dose of Co^{60}–B_{12} was perfused in the presence of intrinsic factor or of a control substance at an established rate for a specified time through each of a pair of intestinal loops in the same animal. The loops were then washed briefly, removed and measured for radioactivity. The radioactive Co^{60}–B_{12}‡ employed had a specific activity of 1.08 $\mu c/\mu g$ and was given, unless otherwise specified below, in a test dose of 0.015 μg dissolved in 1 ml of water.

A. *Experiments with gastrectomized rats.* After total gastrectomy, animals were fed a vit. B_{12}-free, low residue diet containing 18% casein as the source of protein. Gastrectomized rats were not used in experiments until at least 10 days after operation and only if they appeared to be in good health. At least 5 days elapsed between successive administrations of Co^{60}–B_{12} in the same gastrectomized animal. During the experiments the animals were kept in individual metabolism cages permitting total collection of stools without coprophagy, during a 4-day period following administration of the test dose of Co^{60}–B_{12}, simultaneously with or without substances to be tested for intrinsic factor activity. These were prepared in liquid form and were injected from different syringes in measured amounts by means of a small rubber catheter about 3 mm in diameter, that had been cautiously inserted through the esophagus for a distance of 8-10 cm. The animals had no

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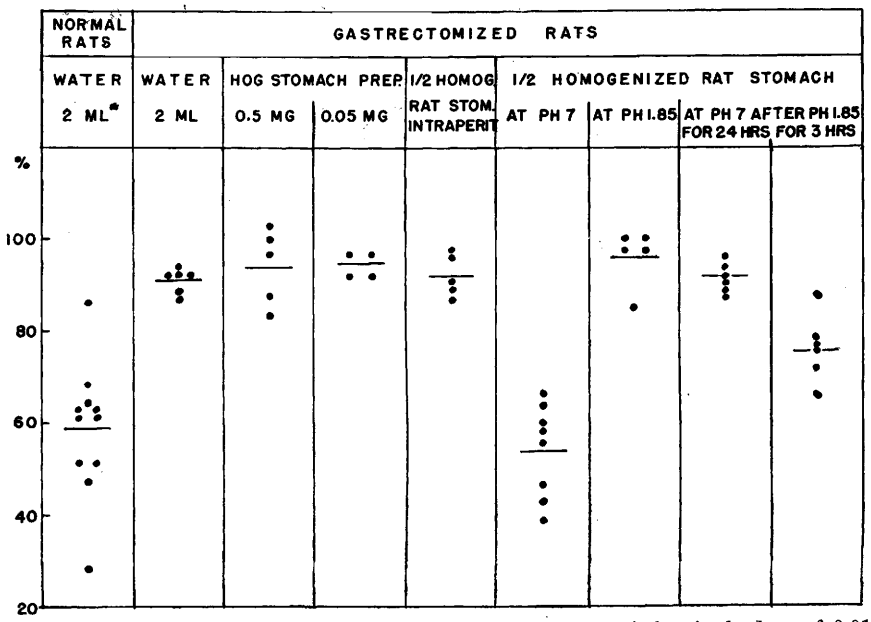

FIG. 1. Radioactivity recovered in feces expressed as percentage of the single dose of 0.015 μg Co^{60}-B_{12} administered together with the substances indicated. Each dot represents the result of an experiment on a single rat; bars indicate averages.

* Data from previous observations(4).

access to food for at least 3 hours before and 3 hours after administration of test materials.

A highly purified preparation of hog intrinsic factor, WES 386,§ was administered simultaneously with Co^{60}–B_{12} to determine whether, in the virtual absence of interfering substances present in cruder preparations of hog intrinsic factor(16), the latter would be effective like rat intrinsic factor. Five mg of this preparation were considered to possess the intrinsic factor activity present in 1 oral unit of a pharmaceutical preparation of vit. B_{12} with Intrinsic Factor Concentrate, U.S.P. In a first group of gastrectomized rats, the test dose of Co^{60}–B_{12} alone was administered in 2 ml of water. In a second group it was given with 0.5 mg of WES 386 in 2 ml of water. In a third group the dose of WES 386 was 0.05 mg in 2 ml of water. From Fig. 1 it can be seen that this hog stomach preparation was without detectable effect upon vit. B_{12} absorption at either dosage level.

§ Kindly made available to us by Dr. W. L. Williams of Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

An intraperitoneal injection of ½ a rat stomach homogenized in 5 ml of water was given to each of a group of gastrectomized rats. Simultaneously, each animal was given the test dose of Co^{60}–B_{12} by stomach tube. As shown in Fig. 1, there was no enhancement of absorption of radioactive vit. B_{12} although this readily occurs when these substances are given together by mouth in such amounts(4).

Homogenized rat stomach was administered at pH 1.85 and at neutrality to determine the effect of maintained acidity upon the action of rat intrinsic factor because of the results of previous observations(17) of similar type in pernicious anemia. The buffering effect of the stomach tissue was such that in order to lower the pH to 1.85, it was necessary to add about 4 ml normal HCl to the test dose of ½ of a homogenized rat stomach, about 0.75 g. The suspension was made up to 5 ml with water. In a first group of gastrectomized rats the rat stomach preparation was given at pH 7 simultaneously with the test dose of Co^{60}–B_{12}. In a second group the rat stomach was given at pH 1.85. In a third group, fol-

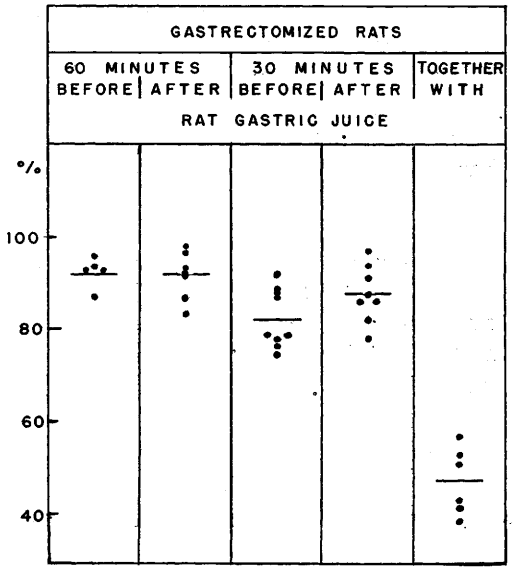

FIG. 2. Radioactivity recovered in feces expressed as percentage of the single dose of 0.015 μg Co^{60}-B_{12} administered together with the substances indicated. Each dot represents the result of an experiment on a single rat; bars indicate averages.

lowing incubation at pH 1.85 for 24 hours at 37.5°C, the rat stomach was given at pH 7 immediately after addition of NaOH. In a fourth group, following incubation at pH 1.85 for 3 hours at 37.5°C, the homogenized rat stomach after similar treatment with NaOH was given at pH 7. The results shown in Fig. 1 indicate that acidification prevented the function of rat intrinsic factor *in vivo*.

Influence of the order of administration of Co^{60}–B_{12} and rat gastric juice when separated by an interval was studied because of the results of previous observations(15) of similar type in pernicious anemia. A first group of gastrectomized rats was given the test dose of Co^{60}–B_{12}, dissolved in 3 ml of water, and this was followed after 60 minutes by 3 ml of rat gastric juice. A second group of rats received the Co^{60}–B_{12} 60 minutes after the rat gastric juice. In a third group, the Co^{60}–B_{12} preceded the rat gastric juice by 30 minutes; and in a fourth group rat gastric juice preceded the Co^{60}–B_{12} by 30 minutes. As may be seen in Fig. 2, there was no definite evidence of difference due to the order of administration of the 2 substances. However, when Co^{60}–B_{12} and rat gastric juice were given simultaneously to a final group of rats the usual effect of rat intrinsic factor was manifest by decreased excretion of radioactivity in the feces.

B. *Experiments with isolated loops of small intestine with intact blood supply.* Gastrectomized rats were fasted 24 hours and then anesthetized by an intraperitoneal injection of Nembutal (pentobarbital sodium). A midline incision was made in the anterior abdominal wall and the ileum was ligated about 10-15 cm from the ileocecal junction. A polyethylene catheter about 3 mm in diameter was then introduced through the wall of the gut about 1 cm above the first ligature and tied in place pointing upward. About 10 cm higher up along the intestine another polyethylene catheter was introduced and tied in place pointing downward. Thus an isolated loop of intestine with intact blood supply was created that could be perfused by test mixtures introduced into the intestine through the upper catheter and flowing out through the lower. A second 10 cm loop of intestine was then created slightly higher up in the intestine than the first and was similarly isolated between 2 catheters. Throughout the operative procedure the intestine was handled with care in order to maintain the circulation as evidenced by arterial pulsation and normal color. The abdominal wall was then closed by sutures, with care to prevent obstruction of the intra-intestinal orifices of the catheters or angulation of the gut by malpositioning of catheters as they emerged through the abdominal incision.

In the performance of experiments, both isolated loops were simultaneously perfused through their respective upper catheters with the 2 different mixtures to be tested at a rate of 1 ml for the first minute and of 0.2 ml/min for the next 15 minutes. After this, the loops were at once rinsed out with 6 ml of saline and then were emptied of the saline solution by introduction of air through the upper catheter. The loops were then taken out of the abdomen, the catheters removed and the mesentery trimmed at its intestinal insertion. Each intestinal segment was then put into a previously weighed tube, containing 3 ml of

TABLE I. Radioactivity of Pairs of Perfused Intestinal Loops Expressed as Counts/Min./g Tissue.

Rat No.	A. Co^{60}-B_{12} perfused with: Rat gastric juice	Heated rat gastric juice
	Upper loop	Lower loop
1	801	83
3	915	227
5	1112	119
	Lower loop	Upper loop
2	922	490
4	641	102
Avg	878	204

Rat No.	B. Co^{60}-B_{12} perfused with: Human gastric juice	Heated human gastric juice
	Upper loop	Lower loop
1	393	211
3	270	233
5	189	301
	Lower loop	Upper loop
2	141	219
4	279	221
Avg	254	237

concentrated H_2SO_4. The tubes were then reweighed to determine weight of the tissue by difference, and its dissolution was effected by vigorous shaking. Finally, the amount of radioactivity present in each tube was determined in a well-type scintillation counter for which the background was about 500 counts/min. The results are expressed in Table I as counts/min/g of tissue. To minimize any differences characteristic of upper or lower loops of intestine the test mixture in one rat was perfused through either the upper or the lower isolated loop and in the next rat the same mixture was perfused through either the lower or the upper loop, respectively. However, in none of the experiments were consistent differences between radioactivity of upper and lower intestinal loops observed.

In one group of rats so prepared *a mixture of 2 ml of neutralized rat gastric juice and 2 ml of 25% human albumin*, to which 0.015 μg of Co^{60}–B_{12} had been added, was perfused through one intestinal loop. Through the other loop were perfused 2 ml of neutralized rat gastric juice which had been heated for 10 minutes at 100°C to destroy its intrinsic factor activity(4) and 2 ml of 25% human albumin to which 0.015 μg of Co^{60}–B_{12} had been added. The results shown in Table I indicate that unheated rat gastric juice was distinctly more active in promoting absorption of vit. B_{12} by the intestinal wall than was heated rat gastric juice. In a second group of such rats the experimental procedure was identical except that *neutralized normal human gastric juice* was employed instead of rat gastric juice as a source of intrinsic factor. The results shown in Table I indicate no consistently greater activity of unheated compared to that of heated human gastric juice in promoting absorption of Co^{60}–B_{12}.

In a final set of experiments the effect of *prior in vitro saturation of the binding capacity of rat gastric juice with labelled or with unlabelled vit. B_{12}*, respectively, was studied by simultaneous perfusion of a pair of isolated intestinal loops. For example, the first mixture was prepared by adding 0.8 μg of Co^{60}–B_{12} to 20 ml of rat gastric juice which was then dialyzed in a cellophane bag for 24 hours against water in the refrigerator. Radioactivity measurements indicated that only 0.22 μg of labelled vit. B_{12} remained within the bag, presumably in a bound state. It was assumed that similar amounts of unlabelled vit. B_{12} remained in an aliquot of the sample of rat gastric juice treated at the same time in the same way after addition of the same amount of unlabelled vit. B_{12}. The contents of the bags were removed and kept frozen until required for use.

When an experiment was run, to 2 ml of dialyzed rat gastric juice containing labelled vit. B_{12} was added an equal amount of unlabelled vit. B_{12} in 2 ml of saline, together with 0.6 ml of saline. Four ml of this mixture, which was prepared immediately before use, were perfused through one intestinal loop, while the other intestinal loop was perfused with a mixture prepared in identical fashion except that the rat gastric juice had been first exposed to unlabelled vit. B_{12} and subsequently, after dialysis, to labelled vit. B_{12}. Because all unbound vit. B_{12} had presumably been lost from the dialysis bag, it was assumed that the vit. B_{12} subsequently added

TABLE II. Radioactivity of Pairs of Perfused Intestinal Loops Expressed as Counts/Min./g Tissue.

Rat No.	Rat gastric juice first saturated with: Unlabelled vit. B_{12}	Labelled vit. B_{12}
	Upper loop	Lower loop
1	108	476
3	468	1368
5	294	587
7	460	1261
8	474	2195
9	862	2209
	Lower loop	Upper loop
2	538	420
4	574	750
6	289	927
Avg	451	1132

could not immediately replace that present in the bound form. Consequently, the experimental design permitted one intestinal loop to be perfused with rat gastric juice in which all or most of the bound vit. B_{12} was radioactive while the other loop was perfused with rat gastric juice in which the same amount of bound vit. B_{12} was non-radioactive. The results are shown in Table II. In the experiment on Rat No. 1 the amount of radioactive vit. B_{12} and of non-radioactive vit. B_{12}, respectively, perfused through each of the two loops was 0.022 μg. In experiments on 8 other rats both these values were 0.031 μg. The data in Table II, with one exception, indicate a greater amount of radioactivity in the wall of the intestinal loop when the rat gastric juice had been initially exposed to labelled vit. B_{12} than when the first exposure had been to unlabelled vit. B_{12}.

Discussion. Human gastric juice(4), hog gastric juice(2) and a commercial hog stomach preparation(1) did not increase the absorption of Co^{60}–B_{12} in the gastrectomized rat. On the other hand, rat stomach preparations(2-4) and rat gastric juice(3,4) were active in this respect. In normal rats with isolated loops of small intestine with intact blood supply during 20-hour periods the absorption of Co^{57}–B_{12} was enhanced by preparations of rat stomach and inhibited by preparations of hog duodenum(18). This apparent species specificity of intrinsic factor was also demonstrated in the present short term experiments with paired isolated loops of small bowel with intact blood supply created in previously gastrectomized, anesthetized rats. As shown in Table I the radioactivity of the wall of the intestinal loop perfused for 16 minutes with Co^{60}–B_{12} and rat gastric juice was consistently greater than that of the paired loop perfused with Co^{60}–B_{12} and rat gastric juice previously heated to 100°C for 10 minutes to destroy intrinsic factor activity(4,19). On the other hand, no consistent difference was observed with heated and unheated human gastric juice. Maintenance of the circulation is seemingly important for the function of intrinsic factor because in experiments(20) with segments of rat intestine removed from the animal, rat gastric juice has been shown to inhibit the uptake of radioactive vit. B_{12} by the tissue.

Because relatively crude preparations of hog intrinsic factor may antagonize the absorption of Co^{60}–B_{12} in normal rats(1,21) and human subjects(16), the most highly purified preparation of hog intrinsic factor available to us was tested. As shown in Fig. 1, this preparation, WES 386, in test doses of 0.5 and 0.05 mg, respectively, equivalent to 1/10 and 1/100 of a U.S.P. unit of intrinsic factor activity, and containing less than 0.05 $\mu\mu$g and 0.005 $\mu\mu$g of unlabelled vit. B_{12}, was without effect on the absorption of 0.015 μg of Co^{60}–B_{12} in the gastrectomized rat. Consequently, the species specificity of rat intrinsic factor suggested by others(1) now appears to have been demonstrated and presumably explains the similar inactivity of human gastric juice(4) in the gastrectomized rat. It is of interest, however, that the difference between the activity of human and of hog intrinsic factor in pernicious anemia (22-23) is but slight.

Although several kinds of tissue proteins bind vit. B_{12}(24), extracts of hog stomach mucosa bind relatively large amounts(25) as do highly purified and clinically active preparations of hog intrinsic factor(26,27). Moreover, electrophoretic dispersions of normal human gastric juice exhibit several peaks of binding activity(28) which are reduced by exposure to pH 10 to one component which

alone possesses intrinsic factor activity(29). As shown here rat gastric juice also binds vit. B_{12}. It is therefore possible that binding capacity is a characteristic common to all species of intrinsic factor. According to the results of 9 experiments shown in Table II, with 1 exception, the absorption of Co^{60}–B_{12} was greater when the labelled rather than the unlabelled form of vit. B_{12} was "bound" by the rat gastric juice. This result, which confirms similar observations(30) in pernicious anemia, clearly suggests that binding of vit. B_{12} to intrinsic factor is of importance in its absorption. Recent observations(31) employing rat and hog stomach preparations have likewise emphasized the positive influence of priority of contact with Co^{60}–B_{12} upon its assimilation. However, if vit. B_{12} binding is a property common to the intrinsic factor of different species, it cannot in itself be responsible for the marked specificity of rat gastric intrinsic factor for the rat; and some additional property of rat intrinsic factor must be decisive in determining the ready assimilation of Co^{60}–B_{12} by the gastrectomized rat. Moreover, in view of the observation that crude preparations of hog intrinsic factor enhance the absorption of Co^{60}–B_{12} in pernicious anemia but inhibit it in normal subjects(16) as well as in normal rats(21); and that in chickens the absorption of Co^{60}–B_{12} is inhibited by a diet rich in casein (32), the ability of a substance to bind vit. B_{12} seems by itself actually to oppose the normal process of vit. B_{12} assimilation. Therefore, some other facet of the intrinsic factor molecule must be concerned, and may be conceivably involved in a species-specific relation to the intestinal wall.

Consistent with this supposition is the clinical evidence(15) that the hematopoietic activity of vit. B_{12} is greater in pernicious anemia when the administration of intrinsic factor precedes rather than follows it by an interval of a few hours. In such observations the theoretical opportunity for contact between intrinsic factor and vit. B_{12} should be similar irrespective of the order of their administration. On the other hand, a preparatory effect of intrinsic factor on the intestinal wall would be favored by the prior administration of the intrinsic factor. However, as shown in Fig. 2, when a repetition of such clinical observations was attempted in the gastrectomized rat, even with an interval as short as 30 minutes, no certain differences could be ascribed to the order of administration. Indeed, intrinsic factor activity was detected only with simultaneous administration. This negative result does, however, indicate that in the gastrectomized rat the effect of intrinsic factor is a very transient one. Together with the present experiments with "bound" vit. B_{12}, labelled and unlabelled, it suggests that binding between vit. B_{12} and intrinsic factor is required for its enhancement of the absorption of vit. B_{12}.

In the present experiments with gastrectomized rats it was shown (Fig. 1) that rat stomach preparations given at pH 1.85 were devoid of intrinsic factor activity. However, intrinsic factor activity was present after exposure to pH 1.85 for 3 hours *in vitro,* provided the preparation was neutralized thereafter at the time of administration with Co^{60}–B_{12} to the rat. Because an increase of Co^{60}–B_{12} in the livers of intact rats has been observed to take place within 15 minutes of the administration of Co^{60}–B_{12}(20), 3 hours should have provided ample time for the action of intrinsic factor to become manifest unless acidity was in some way inhibitory to an action of intrinsic factor either within the lumen of or on the surface of the intestine. As the binding capacity of hog stomach mucosal extract for vit. B_{12} is not significantly altered between pH 8 and pH 2(25), and as this also obtains in our experience with rat gastric juice, the inhibitory effect of acidity on the action of intrinsic factor *in vivo* is probably exerted on some other property of intrinsic factor than its binding capacity. Taken together these facts suggest that in addition to binding vit. B_{12} the peculiar activity of intrinsic factor in enhancing the absorption of vit. B_{12} is dependent upon an acid-sensitive relation between intrinsic factor and the intestinal surface.

Summary and conclusions. 1. *In experiments with gastrectomized rats*: (a) The

probable species specificity of rat intrinsic factor was demonstrated by the failure of a highly purified preparation of hog intrinsic factor to increase the absorption of Co^{60}–B_{12}. (b) In consonance with previous observations in pernicious anemia, acidification (pH 1.85) of a mixture of Co^{60}–B_{12} and homogenized rat stomach inhibited the absorption of Co^{60}–B_{12}. (c) In contrast to previous observations in pernicious anemia, separate serial administration of Co^{60}–B_{12} and rat gastric juice failed to show that rat intrinsic factor was more effective in promoting the absorption of Co^{60}–B_{12} when it preceded than when it followed the Co^{60}–B_{12}. Indeed, no certain effect of rat intrinsic factor was detected whether it preceded or followed the Co^{60}–B_{12} by as short an interval as 30 minutes. 2. *In experiments with paired isolated loops of small bowel with intact blood supply created in previously gastrectomized rats*: (a) The radioactivity of the wall of the loop perfused with Co^{60}–B_{12} and rat gastric juice was greater than that of the paired loop perfused with Co^{60}–B_{12} and rat gastric juice previously heated to destroy its intrinsic factor activity. No consistent difference was observed with heated or unheated human gastric juice. (b) The absorption of Co^{60}–B_{12} was greater when labelled rather than unlabelled vit. B_{12} in equal amounts had saturated the binding capacity of rat gastric juice before admixture with an equal amount of the other form of vit. B_{12} just before the intestinal perfusion. 3. *In conclusion,* binding of vit. B_{12} is an important, perhaps indispensable, aspect of the function of intrinsic factor. However, the fact that vit. B_{12} binding is also characteristic of gastric preparations of man and of hog, which are without intrinsic factor activity in the rat, suggests that some property of intrinsic factor in addition to its vit. B_{12} binding capacity is required for intrinsic factor activity. Possibly this property concerns a relation between intrinsic factor and the surface of the intestinal mucosa which is disturbed when experimentally the contents of the alimentary tract are made strongly acid.

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Requirement for Properdin in Hemolysis of Human Erythrocytes Treated with Tannic Acid.* (22908)

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The hemolysis of the abnormal erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (PNH) by normal human serum requires the properdin system, which consists of properdin, the components of complement, and magnesium(1). Normal human erythrocytes treated with tannic acid (TA cells) are also hemolysed by normal serum(2). The present report indicates that the hemolysis of appropriately treated TA cells also requires the properdin system.

Methods. Erythrocytes. Human group O blood was collected in an equal volume of Alsever's solution and stored at 4°C. The blood was stored 4 days before use and discarded after 10 days. The same donor was used throughout, although blood samples from other donors were equally satisfactory. The erythrocytes were washed 3 times in 0.15 M NaCl and suspended to 2% in barbital buffer containing 0.0005 M $MgCl_2$(3,4). *Tanning of cells.* A stock solution of 1% tannic acid in 0.15 M NaCl was stored at 4°C. It was diluted immediately before use in 0.15 M NaCl to concentrations optimal for tanning cells. The final concentration of tannic acid employed was usually 1:15,000. There was some variation from day to day, and concentrations from 1:10,000 to 1:80,000 were tested each day. The activity of the properdin system was demonstrated only for cells exposed to a relatively limited range of concentrations of tannic acid. Concentrations of greater than 1:10,000 caused clumping of erythrocytes, slight spontaneous hemolysis, and marked hemolysis in serum lacking properdin (RP). Concentrations of less than 1:20,000 usually failed to alter the cells. There was little difference in activity whether the tanning was carried out in silicone treated or ordinary glassware. Aliquots of 30 ml of erythrocyte suspension and tannic acid solution of optimal concentration, in separate 150 ml Erlenmeyer flasks, were placed in a 37°C water bath until the contents of each flask reached a temperature of at least 36°C. The tannic acid solution was then poured rapidly into the cell suspension with continuous and vigorous swirling for 5 to 10 seconds. The mixture was immediately transferred to test tubes and centrifuged for 1 minute at approxi-

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