intestinal loop led to emulsion stabilization and reduced neomycin effects on lipase.

Previous studies from our unit have demonstrated that neomycin administration to humans resulted in apparent impairment of the *in vivo* hydrolysis of a lipid meal (14). Inhibition of lipase activity was suggested by these observations, although it was not known whether the inhibition was a direct or indirect effect of the neomycin. Interpreted in the light of present data, it is possible that the decreased lipolysis observed *in vivo* can be accounted for on the basis of induced emulsion instability. The known bile-salt precipitating properties of neomycin may contribute to its emulsion unstabilizing potential *in vivo* (3).

Summary. In separating the various effects of neomycin sulfate which may be exerted in systems designed to measure the activity of lipase, it is apparent that the antibiotic enhances the activity of both porcine and human lipase on an insoluble substrate. Additionally, it has the propensity to unstabilize emulsions. These effects are antagonistic and may account for divergent results previously recorded regarding neomycin–lipase interrelationships. Both the enzyme enchancing and emulsion unstabilizing properties of the antibiotic seem to reside in the presence of its free amino groups.

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On the Effects of Tannic Acid on Erythrocyte Membrane Acetylcholinesterase* (32919)

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The occurrence in human serum of agglutinating factors specific for erythrocytes pretreated with tannic acid (TA) has been recently reported from three different laboratories (1–3). Treatment of red cells with tannic acid has been widely used in passive hemagglutination procedures since Boyden made the observation that TA renders erythrocytes capable of adsorbing protein molecues from solution (4). Although it has been shown that this kind of treatment causes reduction in anion permeability (5), alterations in osmotic resistance (6), agglutination (7), loss of agglutinability by blood-group specific antibodies (8), the mechanism by which TA exerts its action on the red cell is not understood. Whereas Bohlmann (6) has

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postulated that at low concentrations, TA is able to traverse the erythrocyte membrane, anion permeability studies (5) have suggested that it only interacts with the red cell surface; however, no investigation has been made of the effects of TA upon a specific red cell membrane constituent.

Among the few enzymes recognized in the membrane of the human erythrocyte, acetylcholinesterase (ACHE) is one of the most investigated. Previous studies have indicated that this enzyme appears to be located at the outer surface of the red cell membrane (9,10). We have shown that erythrocyte ACHE activity is reduced in newborn children affected with ABO hemolytic disease (11). A similar defect has been detected in patients with paroxysmal nocturnal hemoglobinuria (PNH) (12) and it has been reported that normal human red cells exposed to TA acquired some of the characteristics of PNH erythrocytes (13). This communication gives the results of examining the effects of TA on red cell ACHE.

Materials and Methods. Blood obtained from normal adult individuals was centrifuged at 1500 rpm for 5 min and the plasma and buffy coat were removed by suction. The erythrocytes were washed three times with 20 volumes of ice-cold 0.1 *M* sodium–potassium phosphate buffer, pH 7.0. After the last centrifugation a 50% cell suspension was prepared and used immediately. Hemoglobin-free membranes were prepared by osmotically induced hemolysis and stored at 4°C as a 50% suspension. Separation of erythrocytes into young and aged populations was carried out as previously described (14).

A 0.1% stock solution of TA, Fisher Certified Reagent, was prepared daily in 0.1 *M* phosphate buffer, pH 7.0. When the influence of pH was investigated, stock solutions of TA were prepared in the appropriate buffers. The effects of other phenolic compounds were tested with freshly prepared solutions made in phosphate buffer, pH 7.0. Unless otherwise indicated, to one volume of a 50% cell suspension, eight volumes of a correspondingly diluted TA solution were added and incubated at 37°C. Cells treated with buffer alone were used as controls. At the end of the incubation period, erythrocytes were washed thrice with up to 100 volumes of ice-cold buffer and an approximately 50% suspension was prepared after the last centrifugation. Further details are indicated on Table I and Figs. 1 and 2. The ACHE activity was measured at 412 mμ on replicate 0.1% suspensions in 0.1 *M* phosphate buffer, pH 8.0, using acetylthiocholine as substrate and 5:5′-dithiobis-(2-nitrobenzoic acid) as color reagent (11). Specific activity was expressed as ΔOD/min per mg of hemoglobin, the latter measured at 540 mμ as cyanmethemoglobin. Residual activity was related to controls incubated without TA.

Results. Effect of concentration and pH. Incubation of whole erythrocytes with TA at

TABLE I. Effect of TA Concentration on ACHE Activity.

TA (mg/ml)	Phosphate buffer, pH 7.0		Physiological saline		Phosphate buffer, pH 8.0	
	ΔOD	Activity (%)	ΔOD	Activity (%)	ΔOD	Activity (%)
None	.933	100.0	.937	100.0	.936	100.0
0.020	.660	70.7	.400	42.7	.757	80.9
0.025	.564	60.4	.282	30.1	.726	77.5
0.033	.374	40.1	.138	14.7	.704	75.2
0.050	.255	27.3	.121	12.9	.654	69.9
0.100	.071	7.6	.086	9.2	.525	56.1
0.200	.041	4.4	.077	8.2	.391	41.8

To replicate 0.2 ml of 10% red cell suspensions in indicated diluents, 0.8 ml of TA solution was added. Following incubation at 37°C for 60 min, 10 ml of chilled 0.1 *M* phosphate buffer, pH 8.0, were added. Residual ACHE activity was measured as indicated in the text, expressed as ΔOD/10 min and related to controls incubated without TA.

37°C caused a concentration-dependent loss of ACHE activity. Whereas the effect was relatively discrete in phosphate buffer, pH 8.0, treatment at pH 7.0 or in the presence of saline resulted in a substantial reduction in activity (Table I). At pH 7.0, approximately

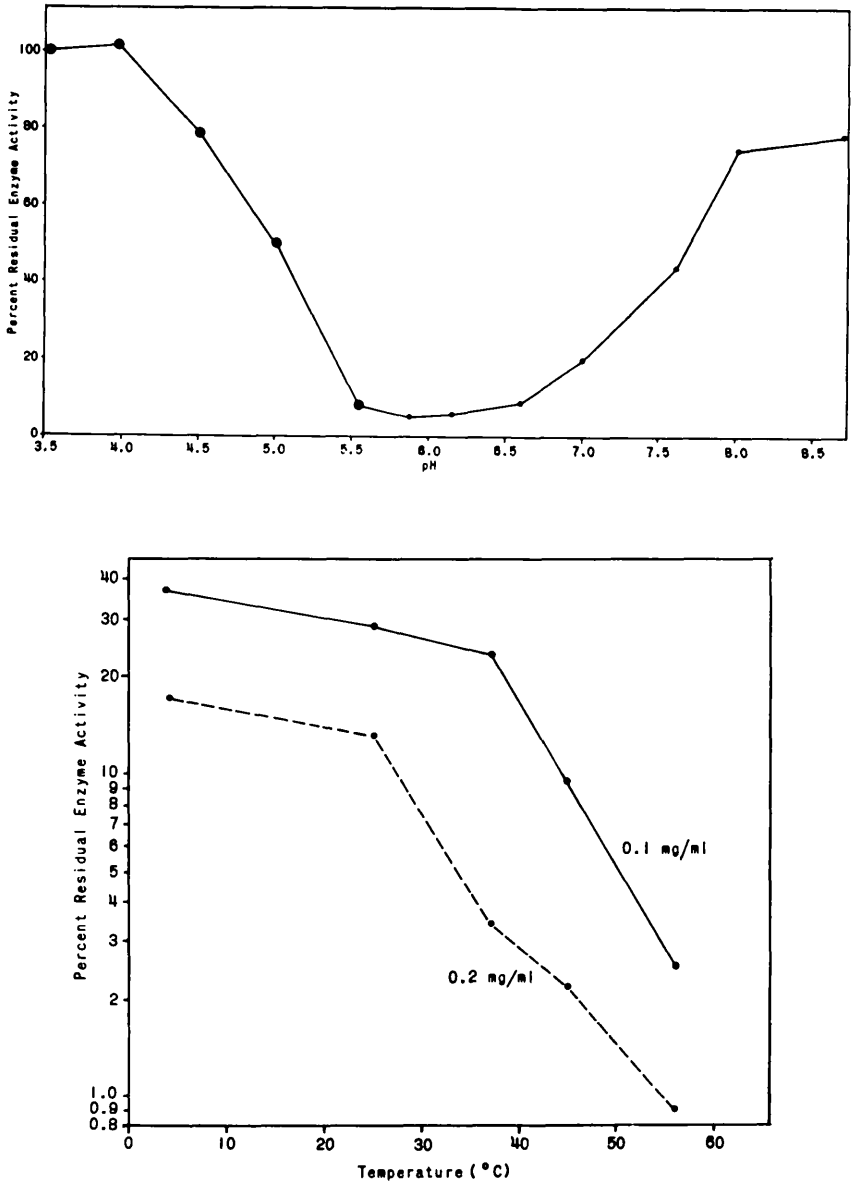
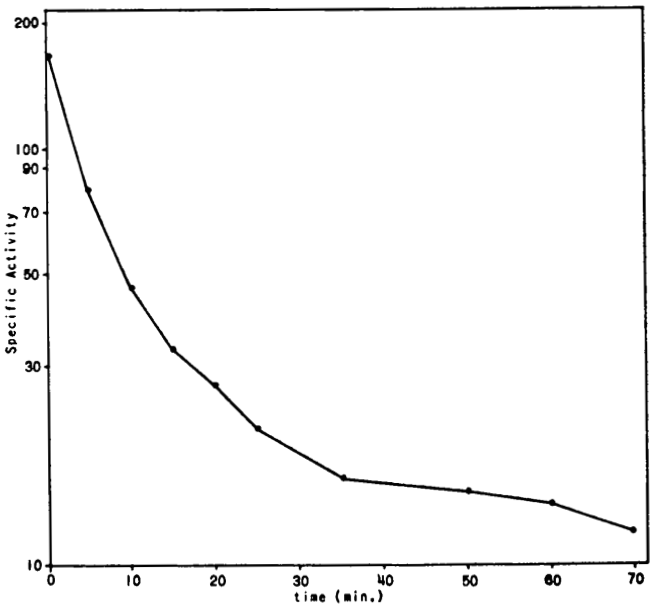


FIG. 1. Effect of pH and temperature on ACHE inactivation by TA. (A, top) The 10% membrane suspensions were prepared in each buffer solution. To replicate 0.2 ml of each suspension, 0.8 ml of buffered TA solution (0.05 mg/ml) were added. After incubation at 37°C for 60 min, 10 ml of ice-cold 0.1 *M* phosphate buffer, pH 8.0, were added. Residual enzyme activity was measured as indicated in text and related to controls incubated with buffer alone. (B, bottom) At 4°C, 10 ml of buffered TA solutions were added to replicate 0.5 ml of a 50% membrane suspension in 0.1 *M* phosphate buffer, pH 7.0. Following incubation for 60 min at the temperatures indicated, membranes were washed thrice with 20 volumes of chilled phosphate buffer, pH 7.0. Residual enzyme activity was related to controls incubated without TA.



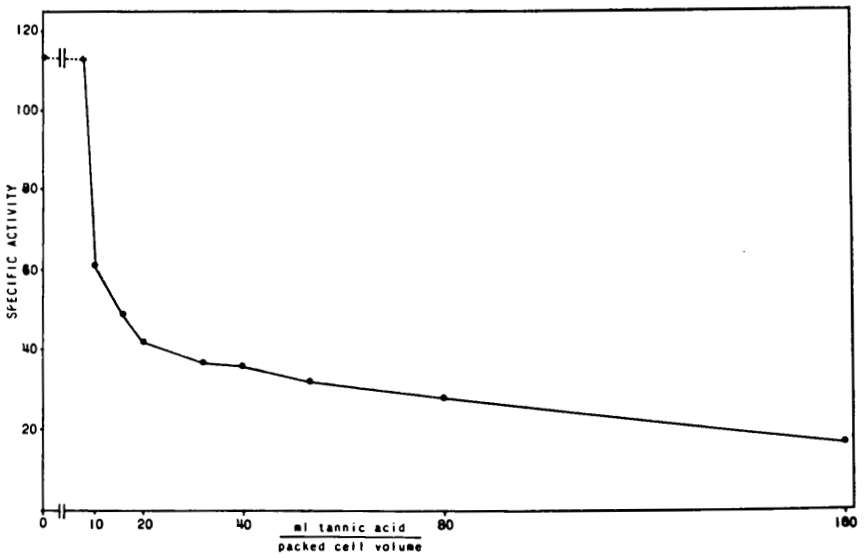


FIG. 2. Effect of time and erythrocyte concentration on ACHE inactivation by TA. (A, top) At 4°C, to replicate 0.5 ml of a 50% cell suspension, 4 ml of TA (0.1 mg/ml) in 0.1 *M* phosphate buffer, pH 7.0 were added, tubes were incubated at 37°C for time indicated and the cells were immediately washed 3 times with 20 volumes of ice-cold phosphate buffer, pH 7.0, and stored as 50% suspensions at 4°C. The ACHE activity was measured simultaneously as indicated in the text. (B, bottom) Eight ml of TA (0.05 mg/ml) in 0.1 *M* phosphate buffer, pH 7.0, were added to replicate tubes containing increasing amounts of a 50% cell suspension (range: 0.1–3 ml). Following incubation at 37°C for 30 min, erythrocytes were washed thrice with chilled phosphate buffer and enzyme activity determined as indicated in text. Abscissa computed with packed cell volume.

75% of the enzyme activity was destroyed by 0.05 mg/ml of TA. Cells exposed to TA at a concentration of 0.05 mg/ml or higher sedimented to the bottom of the tube faster than the controls. Repeated washing of TA-treated erythrocytes did not restore ACHE activity and none was detected in the supernatant liquid following incubation with TA. When red cells were exposed to TA in the presence of 1% bovine serum albumin no reduction of ACHE activity could be detected. However, no restoration of enzyme activity was seen when TA-treated erythrocytes were washed with buffer solutions containing albumin. The successive coating of blood group B, Rh^+ erythrocytes with anti-B, anti-D and antihuman gamma-globulin did not prevent ACHE inactivation by TA. Treatment with the antisera alone had no effect on the enzyme activity. Essentially the same patterns of inactivation were seen with ACHE-deficient erythrocytes from newborn infants with ABO hemolytic disease and with cells separated by density into young and aged populations. The ACHE of hemoglobin-free membrane preparations behaved like that of intact cells.

The effect of pH on the TA mediated inactivation of ACHE was further investigated over a wider range. Since erythrocytes are unstable at low hydrogen ion concentrations, cell membrane preparations were used in these experiments. As can be seen in Fig. 1A, 90% of the enzyme activity was destroyed by 0.05 mg/ml of TA between pH 5.5 and 6.5. By contrast, TA had little effect below pH 4.5 and above pH 8.0.

Effect of temperature. When ACHE inactivation by TA was determined as a function of temperature, two slopes were obtained. With 0.1 mg/ml of TA a relatively slow rate of inactivation was noted between 4°C and 37°C and a second, very rapid rate between 37°C and 56°C. With 0.2 mg/ml of TA the faster rate was observed between 25°C and 56°C. (Fig. 1B).

Effect of time and cell concentration. Figure 2A shows that ACHE inactivation was time-dependent. The initial rapid loss of enzyme activity, followed by a slower rate indicated saturation of the red cell membrane by TA, suggesting that inactivation was also dependent on the concentration of erythrocytes. When this possibility was tested it was found that the reduction in ACHE activity was inversely proportional to the amount of cells present when the concentration and volume of TA were kept constant (Fig. 2B).

Specificity of TA. No reduction of ACHE activity was seen following incubation of intact erythrocytes for 60 min at 37°C and pH 7.0 with up to 0.2 mg/ml of the following phenolic compounds: gallic acid, caffeic acid, ferulic acid, shikimic acid, vanillic acid and chlorogenic acid. The tanning derivatives, D-catechin and ellagic acid were equally ineffective. Inulin, a vegetable polysaccharide which also renders erythrocytes capable of adsorbing proteins from solution (4), at a concentration of 10 mg/ml did not inactivate ACHE.

Discussion. The foregoing experimental results have demonstrated that treatment of human erythrocytes with TA under conditions commonly used in sensitization procedures causes inactivation of the cell membrane ACHE. The failure to restore enzyme activity by repeated washing of the TA-treated cells with buffer or with albumin-containing solutions indicated that this effect was irreversible, thereby resembling the characteristics observed in tanning processes in which the effect of TA seems to involve both electrovalent and coordinate forces (7). As in the ion permeability studies of Edelberg (5), the action of TA upon ACHE was neutralized by the presence of albumin in the incubation mixture. However, enzyme activity was not protected by coating erythrocytes with blood-group specific antibodies prior to their exposure to TA.

In experiments to determine the factors which influence the adsorption of proteins to tanned red cells, it was found that the amount of human gamma globulin attached was dependent on the concentration of TA used (15). In the present study we found that ACHE inactivation was not only dependent on TA concentration, but also on pH, temperature, and time of incubation. The influence of hydrogen ion concentration was most pronounced between pH 5.5 and 6.5, indicating that the state of ionization of TA (16) plays

an important role in the inactivation of ACHE. Although it has been reported that the cell–TA complex tends to dissociate with increasing temperatures (5), we observed a very rapid rate of enzyme inactivation at temperatures above 37°C.

An inhibitory effect of TA on the reaction of several mammalian enzymes has been reported (17, 18) and by varying the concentration of TA and of the respective substrates it has been concluded that it was competitive in nature (18). Similar results have also been found with a tomato pectinesterase (19). In these studies as in ours, no significant effect was noted with the other phenolic compounds tested. Although TA has been used as a tool in the study of many properties of the red cell during the past 100 years (20), the work reported here represents the first instance in which an irreversible effect on a specific constituent of the human erythrocyte membrane could be demonstrated.

Summary. Treatment of human erythrocytes with tannic acid caused irreversible inactivation of the surface located acetylcholinesterase (ACHE). This effect was dependent on concentration, time, temperature, and pH. The ACHE inactivation was prevented by albumin but not by coating the cells with blood-group specific antibodies. The enzyme was not affected by the other phenolic compounds tested.

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Characterization of Physalaemin-Evoked Rat Saliva and Failure of Autonomic Blocking Agents to Modify Composition* (32920)

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It has recently been shown that physalaemin, a polypeptide extracted from the skin of a South American amphibian, can elicit secretion from salivary glands (1). Furthermore, although in flow rate and general consistency, the saliva resembles that usually evoked by parasympathomimetic stimulation, physalaemin-induced secretion is not blocked by cholinergic blocking agents (2); nor is it prevented when adrenergic blocking agents are administered (2, 3), or when the innerva-

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