utes. Immediately prior to test milking on days 16 and 20 an experimental group of 15 rats was injected with 1.25 ml of 25% sodium citrate to cause convulsions (tetany) due to the decrease in ionized plasma calcium. The milk yield of these rats was greatly reduced in comparison with their own previous production and in comparison with the milk yield of the control group on the same day. It is suggested that the contractility of the mammary gland myoepithelial cells by oxytocin is greatly reduced by the lack of plasma calcium. Thus the decreased milk yield of parathyroidectomized rats is due to a reduction in the synthesis of milk and interference with the removal of the milk secreted.

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Effect of Cysteine on Formation of Streptolysin S by Group A Streptococci.*† (29664)

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It has been shown(1,2,3) that serum albumin, Tween 40 and Triton X-205 induced the formation by resting group A streptococci, of an oxygen-stable hemolysin indistinguishable from the serum or RNA hemolysin (streptolysin S) described by Todd(4) Okamoto(5) and Bernheimer(6). Studies employing chromatographic and electrophoretic techniques have indicated that streptolysin S of group A streptococci is a complex of a nonspecific carrier, such as RNA, albumin or tween, and a specific hemolytic moiety(2). The hemolytic moiety which is synthesized by both growing and resting streptococci is destroyed by papain and chymotrypsin and was postulated to be a protein(3,6). More recently, it was shown that streptolysin S induced by yeast RNA is a complex of an oligonucleotide and a peptide. The latter is composed of 10 amino acids(7,8). For optimal formation of the RNA hemolysin by resting streptococci maltose, Mg^{++} and a fraction of yeast RNA resistant to pancreatic ribonuclease are necessary(9). On the other hand, glucose, Mg^{++} and cysteine are essential for formation of the serum, albumin or tween hemolysins(1,2).

The purpose of the present study was to investigate the effect of sulphydryl compounds on formation of the RNA hemolysin. Also it will be shown that the inhibition of hemolysin formation caused by various amino acids analogues is not effective in the presence of sulphydryl compounds.

Materials and methods. Streptococcal strains and culture media. Most of the experiments described were performed with a group A streptococcus type 3 strain S 84

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which was obtained from the State Serum Institute, Copenhagen, Denmark. In some experiments strains C 203 type 3, kindly supplied by Dr. A. W. Bernheimer, N. Y. University, and type 3 Richard's strain and strain Glossy type 6, obtained from the NCTC London, were also used. The streptococci were cultivated at 37°C in 100 ml portions of Brain Heart Infusion broth (BHI —Difco), and harvested from the stationary phase of growth (Klett scale); the bacterial growth was washed 3 times with a final volume of 150 ml of buffered saline (0.15 M NaCl in 0.025 M phosphate buffer, pH 7.4) and resuspended in 10 ml buffer. 0.2 ml aliquots of this cell suspension in a final volume of 1.0 ml were used to prepare hemolysins from resting streptococci (see below).

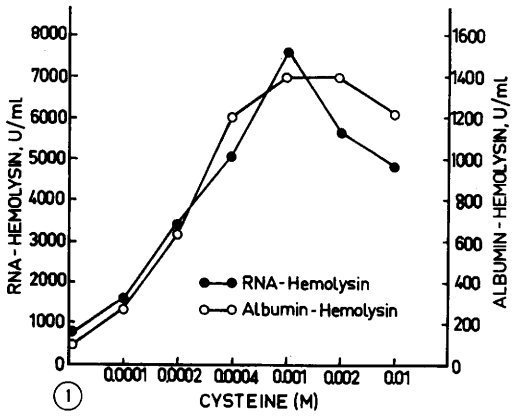
Preparation of hemolysins (a) *RNA—core hemolysin*—RNA-resistant core was prepared from yeast RNA (Mann Laboratories, NYC) by digestion with pancreatic ribonuclease (Worthington, Freehold, N. J.) according to the method described by Tanaka *et al*(10) and Ginsburg and Harris(2). The RNA-resistant core obtained was fractionated on DEAE—Sephadex A-50 columns (Pharmacia, Uppsala, Sweden) by stepwise increase in KCl concentration. Fractions eluted from the column at 0.75 M KCl were used to prepare hemolysin from resting streptococci (2). 0.2 ml of washed streptococci (see above) were incubated for 30 minutes at 37°C with 1 mg/ml of RNA-core fraction to which glucose (or maltose) (0.01 M) and $MgSO_4 7H_2O$ (0.002 M) were added. In other experiments, cysteine free base (0.0001 M-0.01 M) was added to the reaction mixture. Following incubation, the streptococci were removed by centrifugation at 10,000 rpm in a refrigerated centrifuge and the supernatant fluid was assayed for hemolytic activity.

(b) *Albumin hemolysin* was prepared by incubating streptococci with glucose (0.01 M) $MgSO_4 7H_2O$ (0.002 M) and cysteine (0.0001 M-0.01 M) to which horse serum albumin 25 mg/ml (obtained by ammonium sulfate fractionation) was added(1,2). After 20 minutes of incubation at 37°C, the supernatant fluid was assayed for hemolytic activity.

Determination of hemolytic activity. One hemolytic unit (1 HU) is the smallest amount of hemolysin in a 1.0 ml volume that will hemolyze 50% of a 1% human or rabbit RBC suspension after 45 minutes of incubation at 37°C. In most of the experiments phosphate-saline buffer (see above) was used both for dilution of the hemolysin as well as for preparation of RBC suspensions. In some experiments, buffered saline according to Koyama and Egami(7) (0.08 M NaCl in 0.03 M phosphate pH 7.1) was employed. Degree of hemolysis was determined with a Klett photocolorimeter with a 550 Mμ filter as described previously(2).

Inhibition of hemolytic activity. To assure that streptolysin S (RNA, albumin hemolysins) was the only hemolysin tested under the conditions described, streptolysin O activity was inhibited by including cholesterol (100 μg/ml) in all the experiments(6). Also lecithin 10 mg/ml (vegetable, Mann Laboratories, New York) and trypan blue 100 μg/ml known to inhibit RNA and albumin hemolysins(1,6) were employed as controls. In some experiments iodo-acetate 0.01 M was added to the tubes to inhibit any proteinase that might be activated by cysteine(11).

Results. Effect of cysteine on hemolysin formation. It has been shown that cysteine enhanced the formation of the serum and albumin-inducible hemolysins by resting streptococci(1,3). The effect of cysteine and other sulphydryl compounds on formation of the RNA hemolysin was investigated. Fig. 1 shows that cysteine (0.0001 M-0.001 M) markedly increased the formation of both albumin and RNA hemolysin by streptococci harvested from the stationary phase of growth. Concentrations of cysteine higher than 0.001 M were inhibitory. When RNA core hemolysin was prepared on a larger scale (from 1 liter or BHI culture) 10,000-30,000 Hu/ml of hemolysin were obtained compared with 1,000-3,000 Hu/ml formed in the absence of added cysteine. Similar enhancement of hemolysin formation by resting streptococci was obtained by thioglycollic



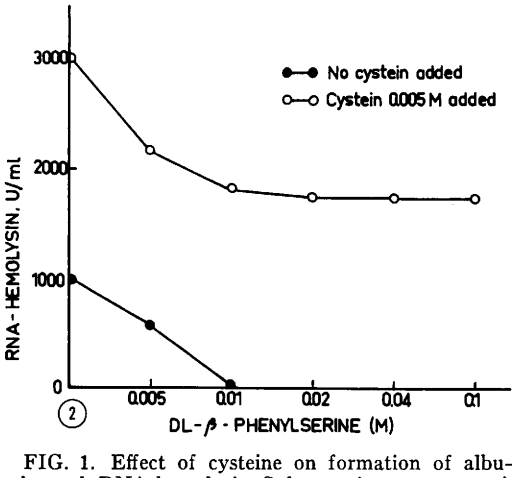


FIG. 1. Effect of cysteine on formation of albumin and RNA-hemolysin S by resting streptococci. (Strain S84) see materials and methods.

FIG. 2. Effect of cysteine on inhibition of RNA-hemolysin formation by Beta-DL-phenylserine (Strain S84).

acid (0.001 M) and by reduced glutathione (0.005 M) but not by ascorbic acid (0.001-0.01 M). Such enhancement of hemolysin formation by cysteine was obtained with 3 other strains of group A streptococci (C 203 S, Richard's and glossy type 6). To test whether or not the low amounts of hemolysin obtained by us in the absence of cysteine were due to the deficiency of endogenous sulphydryl compounds (cysteine was due to the deficiency of endogenous sulphydryl compounds), cysteine (0.001 M-0.01 M) was added to 100 ml portions of BHI medium prior to inoculation of streptococci. It was found that although the growth of the streptococci was unaffected the yield of hemolysin obtained from such cells was only 50% as compared with controls. Koyama and Egami (7) obtained 4,000-8,000 Hu/ml of RNA hemolysin from streptococci harvested from 250 ml portions of broth containing 0.01 M thioglycollic acid. Such amounts of hemolysin were, however, obtained by RNA mixtures not containing any added sulfhydryl compounds. Since these authors have employed phosphate-saline buffer (0.08 M NaCl in 0.03 M phosphate pH 7.4) of lower salt concentration compared with our buffer (0.15 M NaCl in 0.025 M phosphate), the possibility existed that the high hemolysin titers obtained by them might have been due to the effect of the buffers. It was previously shown that Ehrlich ascites tumor cells exposed to hypotonic solutions are very susceptible to the toxic effect of streptolysin S(12). It was found that a streptolysin S preparation containing 5,000 Hu/ml titrated by the buffers employed by us was equivalent to approximately 20,000 Hu/ml when titrated by the buffers employed by Koyama and Egami(7).

To test whether or not cysteine is essential for hemolysin formation throughout the incubation period (30′) the following experiment was performed. Two ml of washed streptococci were incubated for 15 minutes at 37°C with maltose, Mg^{++} and cysteine. Following incubation, the cells were washed with 100 ml of buffer and resuspended in 2 ml buffer; 0.2 ml of such cell suspensions were used to prepare hemolysin in the presence of different combinations of maltose, Mg^{++} and cysteine. Table I shows that preincubation of streptococci with optimal amounts of maltose, Mg^{++} and cysteine is not sufficient to secure optimal hemolysin formation if these agents are removed by washing prior to addition of RNA. Only the presence of Maltose, Mg^{++} and cysteine throughout the incubation period results in formation of optimal amounts of hemolysin.

Effect of sulphydryl compounds on inhibition of hemolysin formation by amino acids analogues. As shown previously(1,13) DL—phenylserine at 0.005 M markedly inhibited the formation of RNA hemolysin by resting

TABLE I. Effect of Cysteine on Formation of RNA-Hemolysin.

Streptococci* + RNA +			Hemolytic activity, Hu/ml
Maltose	Mg^{++}	Cysteine	
—	—	—	80
+	—	—	1150
—	+	—	150
—	—	+	725
+	+	—	1150
+	—	+	5000
—	+	+	650
+	+	+	5500

* Washed streptococci were incubated for 15′ at 37°C with maltose.

Mg^{++} and cysteine (*Materials and methods*). The cells were then washed with 100 ml of buffer—resuspended and used to prepare RNA hemolysin as shown in reaction mixture column.

streptococci. The reaction mixture employed for formation of hemolysin consisted of RNA, maltose, and Mg^{++} as described by Bernheimer(6) and by Younathan and Barkulis (13). On the other hand, formation of the albumin or Tween hemolysins, in presence of SH-compounds, was unaffected by this amino acid analogue(1). The possibility that the difference in activity of phenylserine in the two systems might have been due to the presence of cysteine was therefore examined.

Fig. 2 shows that in the absence of cysteine, phenylserine (0.01 M) completely inhibited hemolysin formation. However in the presence of (0.005 M) of cysteine only approximately 30% inhibition occurred. Even when the concentration of phenylserine was increased to 0.1 M, cysteine (0.005 M) reversed the inhibition to the same extent. Both thioglycollic and thiomalic acids showed a similar effect. Ascorbic acid or ascorbic acid plus cysteine failed to affect the inhibition caused by phenylserine. The effect of cysteine on the inhibition caused by phenylserine varied however in different experiments. In some cases as little as 0.0002 M of cysteine could largely reverse the inhibition caused by 0.01 M of phenylserine. In other experiments no inhibition of hemolysin formation could be obtained by 0.5 M phenylserine. This failure could however be overcome by aging the streptococci for 1-2 hours at 37°C. Under similar conditions, 0.01 M phenylalanine completely reversed the inhibition of hemolysin formation caused by 0.01 M of phenylserine, in confirmation of the findings of Younathan and Barkulis(13) and Sugai and Egami(14). Phenylserine was also found to inhibit the formation of the albumin hemolysin in a system not containing cysteine. The yield of hemolysin was however very small as compared to controls containing cysteine. These results therefore explain the failure of phenylserine to inhibit the formation of the albumin and tween hemolysins produced in the presence of cysteine as published earlier(1). In other experiments, 2 other amino acid analogues, phenylglycine and acetyl-proline at 0.02 M, strongly inhibited hemolysin formation. However, inhibition was also reversed by the presence of cysteine (0.005 M) or thioglycollic acid (0.005 M). Attempts to reverse the inhibition by phenylalanine glycine or proline failed. Moreover, as little as 0.002 M of these amino acids markedly inhibited hemolysin formation. Inhibition of hemolysin formation by tetracyclines and chloroamphenicol was unaffected by cysteine.

Discussion. These data show that cysteine and other sulfhydryl compounds markedly enhance the formation of streptolysin S by 3 strains of group A streptococci harvested from the stationary phase of growth. Thus in the presence of 0.001 M of cysteine 7,500 Hu/ml of hemolysin were usually obtained from strain S 84 compared with 500-800 Hu/ml in the absence of cysteine. When the cultures were scaled up to 1 liter 10,000-30,000 Hu/ml hemolysin were obtained compared with 1,000-3,000 Hu/ml in the absence of added cysteine. Although not all the strains employed yielded such high amounts of hemolysin, the enhancing effect of cysteine was found with all the strains tested. The role of sulfhydryl compounds in the formation of hemolysin is not clear. The fact that no cysteine was found in highly purified preparations of the RNA-hemolysin (8) suggests that the beneficial effect of cysteine is probably not to supply an essential amino acid for synthesis of hemolysin. On the other hand both glutamic acid and serine shown by Koyama and Egami(8) to

be the major components of the hemolysin, markedly enhanced hemolysin formation in the presence of cysteine (unpublished observations).

Bernheimer(6) demonstrated that formation of the RNA-hemolysin by streptococci which grew in the presence of thioglycollic acid, takes place under anaerobic conditions as well as by cell suspensions exposed to air, and that the formation of hemolysin was markedly inhibited by mechanical agitation. This inhibition could be reversed only to a small extent by bubbling nitrogen, but was completely reversed by cysteine(6). Since ascorbic acid failed to enhance hemolysin formation, it appears that anaerobiosis is not a sufficient condition, and that active reduction of sulfhydryl compounds is essential for an unknown metabolic process leading to the formation of the hemolysin. The fact that cysteine is essential for hemolysin formation throughout the incubation period with RNA (Table I), suggests that the streptococci do not possess a pool of endogenous sulfhydryl compounds.

The experiments showing the effect of buffers on the hemolytic titers indicate the importance of tonicity in determination of hemolytic activity. The employment of slightly hypotonic buffers may be important for determination of very small amounts of hemolysin, not detected by the regular hemolysis assays.

The fact that inhibition of RNA hemolysin formation by phenylserine is effective only in the absence of added sulfhydryl compounds explains the failure of this analogue to affect the formation of the albumin hemolysin as previously described by us(1). This effect of sulfhydryl compounds is, however, not understood. It is difficult to assume that the effect of cysteine and thioglycollate is due to a competition with phenylserine for incorporation into the hemolysin molecule. Even more surprising is the fact that highly purified preparations of RNA hemolysin do not contain measurable amounts of either cysteine or phenylalanine, which raises the question whether phenylserine is active as a true amino acid antagonist. Since sulfhydryl compounds also interfere with the inhibition of hemolysin formation caused by phenylglycine and acetyl-proline, the role of cysteine is probably not specific. It also appears that a successful inhibition of hemolysin formation by all these analogues, depends on a low level or absence of endogenous sulfhydryl compounds. It is possible therefore, that RNA-hemolysin can be synthesized via 2 pathways of metabolism, one of which is blocked by several amino acid analogues where the alternative pathway is stimulated by SH-compounds. The experimental data, however, do not permit a final conclusion. Further work on the role of SH-compounds in inhibition of hemolysin formation by phenylserine is in progress.

Summary. Cysteine and other sulfhydryl compounds markedly enhance the formation of streptolysin S by 4 strains of group A streptococci. In the presence of cysteine (0.001 M) 10,000-30,000 hemolytic units per ml were obtained from strain S 84 type 3 in comparison with 1,000-3,000 units in the absence of cysteine. The role of cysteine in formation of hemolysin is not clear but it probably acts as a donor of SH compounds essential for an unknown process, rather than as a reducing agent. The inhibition of hemolysin formation by 3 amino acid analogues phenylserine, phenylglycine and acetyl-proline is reversed to a large extent by sulfhydryl compounds. Thus 0.002 M of cysteine reverses to a large extent the inhibition of hemolysin formation caused by as much as 0.1 M of phenylserine.

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Further Studies on Abnormal Serum Proteins in Tumor-Bearing Hosts.* (29665)

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Previous studies on abnormal serum proteins in mice bearing certain transplanted or spontaneous tumors have demonstrated the existence in the host animals of minute amounts of antigenc serum components which were absent in normal control animals of the same strain(1,2). These observations were based on a combination of immunological and biochemical techniques, which led to the production of rabbit antisera against the abnormal serum components. However, the antisera thus obtained reacted also with a number of normal murine serum proteins, and interpretation of the results appeared ambiguous at times.

A new procedure for selective and quantitative removal of certain antibodies from antisera has recently been developed(3). By use of this technique it has now become possible to prepare rabbit antisera *specific* for abnormal components which occur in minute amounts in the serum of tumor-bearing mice; such antisera do not react at all with serum from normal control animals(4).

The sequence of techniques described here may be applied also to other diseases or conditions and may also be employed for detection of abnormal proteins at other sites.

Methods and materials. Mouse serum. Female C3H/He mice with spontaneous mammary adenocarcinoma of one to two cm diameter served as tumor-bearing animals. They were retired breeders from the Jackson Laboratory in Bar Harbor, Maine. Animals with necrotic tumors were rejected. Male littermates were used as normal control animals unless stated otherwise. Mice were bled as described earlier(5), except that no citrate was used and serum was collected.

Murine serum α-globulin fractions. Pooled serum from tumor-bearing mice, in portions of 20 ml, was fractionated by zone electrophoresis on a carrier medium consisting of 3% cornstarch gel with 3% additional potato amylose as described earlier(2,6). An average of 20 protein fractions was obtained from each batch. These fractions were characterized by paper electrophoresis and by plotting the total protein content of each fraction, measured according to Lowry *et al*(7), as a function of the distance of the fraction from the origin. In general, three-fourths of the total α_2-globulin was distributed over 4 to 5 fractions; these also contained variable amounts of α_1- and β-globulins. There was an equal number of fractions encompassing 75% of the α_1-globulin; they contained variable amounts of albumin and α_2-globulin. These α_1- and α_2-globulin fractions partially overlapped each other.

Immunization of rabbits. Young female rabbits weighing 5 to 6 lb received subcutaneous injections of α-globulin (including β-globulin or albumin impurities) in Freund's adjuvant mixture as described by Cohn(8). The initial immunization consisted of one mg of protein per pound of rabbit. Starting 6 weeks after the initial injection, booster in-

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