with this site. Similarly, another hallucinogenic substance, LSD, which is a serotonin antagonist at smooth muscle receptors, may also be acting directly on the central nervous system. The receptor sites have a configuration permitting interaction of substances resembling serotonin. Serotonin, like acetylcholine, serves only as a model for specific receptivity. Whether or not the biogenic amines play a role as chemical transmitters in some organisms is irrelevant to the present discussion. The receptor sites themselves, or "locks", are of primary importance; noradrenalin and histamine, as well, serve only as examples of "keys". The number of such sites within the nervous system exhibiting specificity may be very great as evidenced by the variety of pharmacological agents acting upon the nervous system. There may be innumerable endogenous agents, including polypeptides, which may be exerting an action at such sites.

Although the biogenic amines continue to be of immense importance to the chemist in designing new drugs, the biologists have used them, often indiscriminately, in the formulation of untenable theories of chemical transmission and drug action. Considerably more attention must be devoted to understanding the nature of the receptor site; and with the great advances being made in protein chemistry, this problem should be approached.

Summary. The anticholinergic action of a group of glycolic acid esters was compared with their psychotomimetic potency in humans and their efficacy in producing centrally mediated behavioral disturbances in rats and mice. Although all of the effective psychotomimetic agents were potent anticholinergic agents, the agreement between the two frequently did not hold. The findings are in support of the idea that the glycolate esters may, at least in part, be acting directly at synaptic receptor sites and not necessarily by cholinergic blockade.

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Some Chemical and Physical Characteristics of Purified Beta-Inhibitor From Bovine Serum. (32395)

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Following the initial work of Hirst(1) attempts to purify and characterize beta-inhibitor have been the goal of numerous studies (2-15). Although much progress has been achieved in these investigations, there are conflicting reports concerning the nature of beta-inhibitor. The present study was undertaken in the hope of delineating more precisely the physical and chemical properties of beta-inhibitor.

Materials and methods. Virus: The virus used throughout these studies was the Seerey strain of Type A Prime influenza virus as described by Briody *et al*(16).

Titrations: Titrations of hemagglutination (HA) and hemagglutination-inhibition (HI)

were carried out routinely as described by Briody *et al.*

Serum: The serum used as the source of beta-inhibitor was whole bovine serum obtained from Grand Island Biological Co., Grand Island, N. Y.

Isolation and purification: The procedure used was basically that employed by Konno (8) with the substitution of gel filtration and cellulose column chromatography for zymosan treatment. Ultracentrifugation was also employed using discontinuous gradient formation of sucrose or isopycnic gradients of cesium chloride and using the centrifugal technique of Fisher *et al*(17)

Further purification of beta-inhibitor preparations obtained in the above procedure was accomplished by adsorbing inhibitor in equal volumes of concentrated virus preparations (HA titers greater than 1:10,000) in the presence of 0.05 M $CaCl_2$ and centrifuging the complexes formed at 20,000 rpm until supernatant fluid showed no inhibitory activity. Virus-inhibitor complexes collected in the buttons formed in each centrifugation were dissolved in saline containing 0.1 M ethylenediaminetetraacetic acid (sodium salt), and the dissociated virus centrifuged out at 30,000 rpm for 30 minutes. Desorbed inhibitor was then precipitated out of solution with 50% saturated ammonium sulfate and the precipitate dissolved in 0.1 M NaCl containing 0.1% $CaCl_2$ This usually resulted in a 5-10-fold increase in specific activity of the inhibitor.

Electrophoretic analysis: Paper electrophoresis was carried out in a Spinco-Durrum type cell and cellulose acetate electrophoresis was carried out in a Gelman Rapid Electrophoresis Chamber (Gelman Instrument Co., Ann Arbor, Mich.) using the barbitone buffer of Laurell *et al*(18). Proteins were strained with 1% Amido Black in a 1:9 glacial acetic acid-methanol solution. Glycoproteins were stained on paper strips by the method of Köiw and Grönwall(19) and on cellulose acetate by the method of Bodman(20). Both methods employ modified periodic acid-Schiff reactions. Lipids were stained with Sudan Black B(21) and Oil Red O(22). Strips stained for protein were scanned in a Spinco Analytrol recording scanner (Beckman Instruments, Calif.).

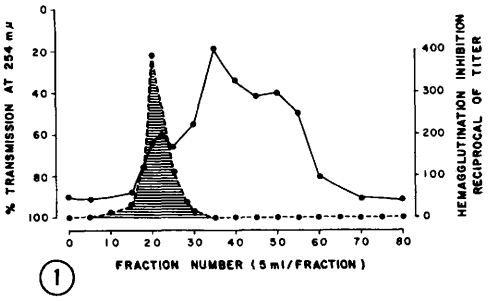
Chemical analysis: Protein was determined by the method of Lowry *et al*(23). Carbohydrate was determined as glucose by the method of Roe(24) using anthrone as the colorimetric reagent.

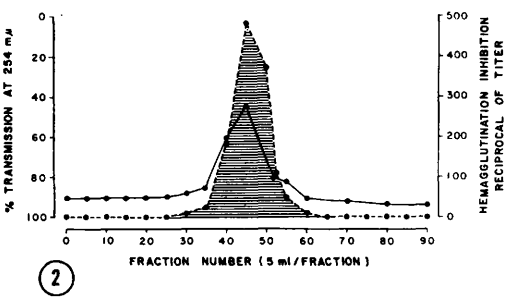
Diethylaminoethyl (DEAE) cellulose chromatography and gel filtration: DEAE cellulose column chromatography was carried out according to procedures as described by Peterson and Sober(25). Gel filtration was carried out using Sephadex G-200 and the procedures recommended by the manufacturer (Pharmacia, Sweden) were generally employed. For rough molecular weight determination the basic technique of Leach and O'Shea(26) was used, substituting tris buffer in place of the citrate buffer recommended by the authors.

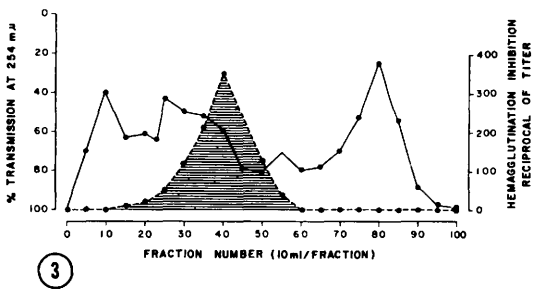
Fixation tests with purified beta-inhibitors: Virus-inhibitor complexes were incubated with either guinea pig or rabbit serum as sources of complement. Incubation time was either 30 or 60 minutes. Routine complement fixation tests were then run to determine if fixation had occurred.

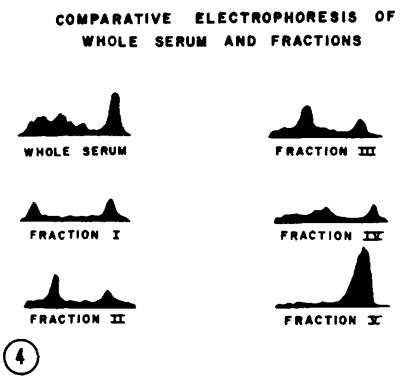
In other tests rabbit albumin-sheep anti-rabbit albumin antiserum was incubated with purified beta-inhibitor alone or in the presence of guinea pig or rabbit complement to determine whether beta-inhibitor is fixed to antigen-antibody complexes *per se* or as a component of complement systems.

Results. Isolation of beta-inhibitor from whole bovine serum by salting out was accomplished by the use of tris-buffered ammonium sulfate solution, pH 7.0, final concentration of sale at 35%. This was followed by precipitation by dialysis against distilled water at pH 7.0 and gave a product whose activity was increased 20-fold over the specific activity of the original serum (based on the ratio of hemagglutination inhibition titer to mg of protein, average activity = 7800/mg protein). Material obtained by this method proved to be very labile, losing as much as 25%-50% of its activity at room temperature over a period of 24 hours. At 4°C it was stable for about a week, at —70°C it was stable indefinitely.









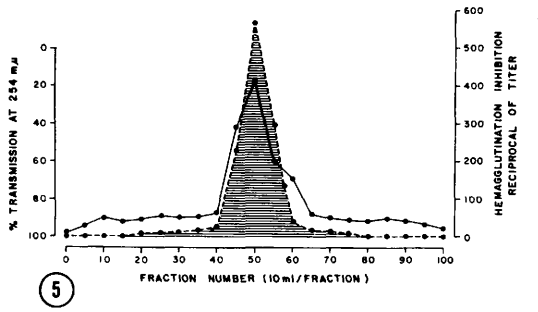


FIG. 1. Gel filtration of whole bovine serum on Sephadex-200 Column eluted with 0.1 M tris (hydroxymethyl) aminoethane (Tris) buffer (pH 7.0). Shaded curve represents distribution of hemmagglutination inhibition activity.

FIG. 2. Gel filtration of beta-inhibitor purified by virus adsorption-desorption. Same conditions as in Fig. 1.

FIG. 3. Chromatography of whole bovine serum on diethylamino ethyl (DEAE) cellulose. Column eluted with 0.1 Tris-phosphate buffer and 0.1 M NaCl in a continuous gradient from pH 7.4 to pH 5.0 Shaded curve represents distribution of hemagglutination inhibition activity.

FIG. 4. Comparative electrophoresis on cellulose acetate of whole bovine serum and of the indicated fractions obtained from experiments represented in Fig. 3. Electrophoretic fraction I = chromatography fractions 0-15 in Fig. 3, fraction II = fractions 20-30, fraction III = fractions 35-50, fraction IV = fractions 53-60 and fractions V = fractions 65-90.

FIG. 5. Chromatography on DEAE cellulose of beta-inhibitor purified by virus adsorption-desorption. Same conditions as in Fig. 3.

Gel filtration experiments on Sephadex G-200 using whole bovine serum consistently gave results indicating that the beta-inhibitor activity was associated with the large molecular fraction of serum (Fig. 1). Activity was always found associated with the ascending arm of the first peak coming off the Sephadex column. This was true over a range of pH 6.0 to pH 8.0. Activity of this fraction was comparable to that obtained by salting out and was relatively more stable, retaining about 70% of its activity for about a week and a half at 4°C.

Gel filtration of beta-inhibitor purified through the virus adsorption-desorption step showed a decided shift to a smaller molecular species (Fig. 2). Molecular weight determination on the purified inhibitor using the technique of Leach and O'Shea(26) indicated a molecule with a weight falling between serum albumin and alcohol dehydrogenase. Calculations indicated that the molecular

weight of beta-inhibitor was approximately 80,000 to 100,000. These calculations were based on experiments using 4 different samples of purified beta-inhibitor, some of which had aged for several weeks at —70°C and this may account for the range in molecular weight.

Chromatography of beta-inhibitor on DEAE columns followed by electrophoretic analysis of fractions obtained in relation to activity indicated that the beta-inhibitor activity migrated with the fast gamma globulin or the slow-moving beta-globulins (Fig. 4). The electrophoretic analysis was the same when done on paper as on cellulose acetate although separation of fractions was more clear cut on cellulose acetate than on paper.

A comparison of Fig. 3 and Fig. 5 shows that the inhibitor purified by virus adsorption-desorption gave a sharp peak and a narrow distribution of activity (Fig. 5) as compared to the activity found in the whole serum (Fig. 3).

Ultracentrifugal studies on the beta-inhibitor gave results that correlated well with the column chromatography and gel filtration. Centrifugation of whole serum in either discontinuous sucrose gradients (5%-40%) or isopycnic gradients of CsCl (density 1.1-1.5) showed activity settling with the heaviest components of serum. However, beta inhibitor which had been purified by virus adsorption-desorption from serum, or active fractions from gel-filtration experiments which had been treated with beta-mercaptoethanol or active fractions from DEAE columns showed beta-inhibitor activity which banded in the region of buoyant density 1.3-1.4 in the CsCl gradient. The result with purified beta-inhibitor in the sucrose gradients was equivocal and not subject to clear interpretation.

Beta-inhibitor purified by DEAE cellulose chromatography and viral adsorption-desorption showed a carbohydrate content averaging 4% when determined as glucose by anthrone reaction. The carbohydrate content ranged from 2% to 5% in the 5 samples of purified inhibitor examined. Electropherograms stained for lipid and carbohydrate showed a fairly strong reaction for carbohydrate, with the periodic acid-Schiff reagent, but a very weak stain for lipid using Sudan Black B or Oil Red O. Characteristics of beta-inhibitor purified by DEAE cellulose chromatography and virus adsorption-desorption and treated under a variety of conditions are shown in Tables I and II. These data show the beta-inhibitor to be susceptible to several chelating agents, that calcium ions have a stimulatory effect on the inhibitor, that sulfhydryl reagents are without effect on purified inhibitor.

TABLE I. Effects of Metal Ions and Chelators on Purified Beta-Inhibitor.

Material added to purified beta-inhibitor	Inhibitory activity*
Control—No additions	Taken as 100% active
$+Mg^{++}$ (2.5×10^{-4} M)	100%
$+Ca^{++}$ (2.5×10^{-4} M)	150–200%
$+Fe^{++}, Mn^{++}, Zn^{++}, Cu^{++}$ (2×10^{-4} M)	100%
+EDTA (1×10^{-3} M)	10%
+8-hydroxyquinoline (1×10^{-3} M)	40–45%
+Sodium citrate (1×10^{-3} M)	55–60%

* Control considered as 100% active. Other activities are expressed as per cent of the control activity.

TABLE II. Effects of Sulfhydryl Reagents on Beta Inhibitor.

System	% Stimulation
Beta-mercaptoethanol (%)	
Ultracentrifuged macroglobulin button	+50%
Purified beta-inhibitor	0
Glutathione (1×10^{-3} M)	0
Cysteine (1×10^{-3} M)	0

The following treatments proved to have no effect on purified beta-inhibitor: sodium periodate (5×10^{2} M), receptor destroying enzyme (1:20 final dilution of preparation from Sigma Chemical Co., St. Louis, Mo.), hydrazine sulfate (0.1 M). These were all incubated with the inhibitor for 1 hour at 37°C before testing for activity. Ether extraction (3 extractions at 10:1, at —25°C) and chloroform extraction (3 extractions at 1:1, at 25°C) were also without effect even though electropherograms of beta-inhibitor stained with Sudan Black B indicated some lipid present in the preparations.

Purified beta-inhibitor was destroyed by heat (56°C/30 min), inactivated by incubation with trypsin for 1 hour at 37°C and had a pH optimal range of pH 6.0 to pH 8.0 with maximum activity at pH 7.2. In addition, virus-inhibitor complexes did not fix complement and beta-inhibitor was not fixed by antigen-antibody complexes (rabbit albumin-sheep anti-rabbit albumin antibody), in the presence or absence of complement.

Discussion. The results of this study do not allow an exact characterization of beta-inhibitor, but in conjunction with the results of other workers they do give a better understanding of the nature of this substance. The ability to further purify beta-inhibitor by virus adsorption-desorption has allowed the preparation of a fairly well purified product. The ultracentrifugal and electrophoretic properties obtained in our studies are fundamentally in agreement with those of Krizanova and Sokol(15).

Our results with whole serum would seem to indicate a molecule of large proportions which sediments with macroglobulin fractions and which separates with macroglobulin fractions upon gel filtration. However, the results with beta-inhibitor purified by virus adsorption-desorption indicate that beta-inhibitor is a molecular species much smaller than macroglobulin with a molecular weight in the range 80,000-100,000 and a buoyant density of 1.3-1.4. Such a molecule may complex with macroglobulins in whole serum and this complex may be disrupted by the presence of virus. The union of virus and inhibitor is dependent on calcium ions and the action of the inhibitor may be prevented by depletion of available calcium.

The effects of various chemical and physical agents on the purified inhibitor indicate several features of importance in this molecule. It appears to be a protein, sensitive to trypsin. It may contain a carbohydrate moiety in its structure, but this appears to be of no importance to its activity as an inhibitor in view of its continued activity following periodate treatment. Likewise, any lipid moieties the molecule may carry are of no consequence as far as its inhibitory activity is involved since the activity is resistant both to ether and chloroform extraction.

The effect of sulfhydryl agents on the inhibitor is of some interest. In purified form, the inhibitory activity of the molecule was not affected by these agents and this may be taken as an indication that intact sulfhydryl groups do not play a role in the combination of inhibitor with virus. However, in the ultracentrifugally separated inhibitor (presumably complexed with macroglobulins) the action of beta-mercaptoethanol enhanced the activity. This enhancement of activity may occur by the reduction of disulfide linkages between the inhibitor and the macroglobulins thus releasing inhibitor or it may be the result of a reduction of disulfide linkages in the macroglobulins causing unfolding of macroglobulin structures and a subsequent release or unmasking of the inhibitor.

In addition, while some of the characteristics of beta-inhibitor suggest properties similar to complement (calcium ion requirement, heat lability), its inability to fix to antigen-antibody complexes in the presence or absence of complement and its resistance to hydrazine treatment indicate it is not a component of the complement system. Further, the inhibitor when combined with virus did not fix complement, indicating that it is not a classical antibody which is in keeping with the rough determination of its molecular weight (80,000-100,000) and its electrophoretic character.

It would seem then that beta-inhibitor is a naturally-occurring molecule present in normal bovine serum which can be detected by its ability to combine with influenza virus to prevent hemagglutination by the virus. Its actual role in serum is unknown and the question as to whether it serves as part of the innate defense mechanisms of the host animal is open to speculation.

Summary. In its natural state beta-inhibitor is complexed with the macroglobulin fraction of serum. When purified by adsorption-desorption, beta-inhibitor is a smaller molecular species in the range 80,000-100,000 molecular weight with a buoyant density of 1.3-1.4. The purified inhibitor is inactivated by trypsin, is heat labile and requires calcium ions, but is resistant to ether, chloro-

form and hydrazinie, is unaffected by sodium periodate, receptor destroying enzyme and beta-mercaptoethanol, and cannot be removed by antigen-antibody complexes. Virus can combine with the purified-inhibitor to prevent hemagglutination. Such virus-inhibitor complexes, however, cannot remove complement. From these data, beta-inhibitor apparently is a protein which can complex with macroglobulins. The staining of electropherograms with Sudan Black B and with periodic acid-Schiff reagent indicates the presence of lipid and carbohydrate, but the resistance of beta-inhibitor to ether and chloroform indicates that these substances do not appear to be essential for activity. Enhancement of inhibitory activity by treatment with beta-mercaptoethanol when the inhibitor is complexed with macroglobulin suggests perhaps that disulfide bonds may act to hold the complex together.

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Studies on Immune Suppressive Drugs.* (32396)

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The efficacy of the purinethiols in suppressing the immune response has been well documented(1-3). These agents can be potent inhibitors of humoral antibody formation(4). However, drugs that decrease the host's capacity for circulating antibody formation will decrease resistance to infection. Such drugs must be used with caution in patients who receive homografts. If a dichotomy exists for immunological responsiveness, as has been suggested(5,6), it should be possible to inhibit the homograft response without producing discernible effects on humoral antibody production. Previously we have reported that β-D-arabinosyl-6-mercaptopurine (7) did not inhibit humoral antibody forma-

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Abbreviations used: α-TGdR, α-D-2′-deoxythioguanosine; 6-MP, 6-mercaptopurine; 6-DMHP, 6-(2,2-dimethylhydrazino)-purine; β-D-ara-6MP, β-D-arabinosyl-6-mercaptopurine; β-L-6MPR, β-L-ribosyl-6-mercaptopurine; Me6MPR, methylthioinosine, β-D-ribosyl-6-methylthiopurine.