I

HAPTEN INHIBITION STUDIES WITH A SERIES OF LOCAL ANESTHETICS DERIVED FROM PIPERIDINO ALCOHOLS

II

THE INCORPORATION OF C^{14}-CARBOXYL-LABELED LEUCINE BY SERUM PROTEINS

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ABSTRACT

PART I

Rabbit antibodies specific for the 3-(2-methyl-piperidino)propyl-*p*-aminobenzoate group have been prepared and the inhibition of precipitation of these antibodies has been studied. It was found that a series of local anesthetics derived from piperidino alcohols were effective inhibitors of precipitation in this system. Reasonable explanations of the serological properties of these drugs have been given but it has not been found possible to establish any relation between their serological properties and their pharmacological properties.

PART II

C^{14}-carboxyl-labeled leucine has been administered to immunized rabbits. While receiving the labeled amino acid they also received intravenous injections of different antibody solutions. It was found that the normal constituents of the serum incorporated the radioactivity at a very rapid rate but the passively transferred antibodies either did not incorporate this radioactivity to any marked extent or else did so only upon the loss of their specific antibody characteristics.

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PART I

HAPTEN INHIBITION STUDIES WITH A SERIES OF LOCAL ANESTHETICS DERIVED FROM SUBSTITUTED PIPERIDINO ALCOHOLS.

INTRODUCTION

It has been known for many years that if an animal is given a series of injections of one of certain high molecular weight substances called antigens, which are usually protein in nature, there is produced in the serum of the animal certain modified proteins called antibodies which react visibly in vitro with the antigen used for immunization. Many investigators have explored the specificity of the reactions and the properties of the substances involved (1) (2) (3). The introduction by Wu (4) of the methods of quantitative chemical analysis and their extensive application by Heidelberger and Kendall (5) and others made possible a far more precise characterization of the various phenomena, particularly the precipitin reaction in which the soluble antigen forms a specific precipitate when added in suitable concentration to the serum of the immune animal.

There are two different ways of performing a precipitin test. In one, varying amounts of antigen are added to a constant amount of antibody; in the other, the amount of antigen is kept constant and the amount of antibody is varied. A typical precipitin curve obtained with rabbit antibody performed by varying the amount of antigen shows a maximum in the amount of precipitate at some antigen concentration. At lower antigen concentrations there is not enough antigen to precipitate completely the antibody and at higher antigen

concentrations soluble complexes are formed with the excess antigen. If the concentration of antibody is varied an inhibition zone in excess antibody can be demonstrated with horse antisera to proteins but horse antipolysaccharide and all rabbit systems do not normally give this effect. The qualitative features of the reactions of antibodies to simple substances. Interpretation of the precipitin reaction in fundamental chemical terms was hampered by the fact that both reactants (the antigen and antibody) are macromolecular and of undetermined structure and the determination of the relative amounts of antigen and antibody in the precipitate has been difficult. The demonstration by Landsteiner and his collaborators that antibodies can be prepared with specificities directed against groups of known chemical structure - synthetic haptens - and that they react with simple synthetic substances whose molecules contain the homologous or similar haptens (6) (7) (8) was a major advance in this field. Beginning in 1917 Landsteiner and his collaborators prepared artificially conjugated antigens by coupling relatively simple chemical substances to proteins (usually by diazotization), and then by injecting these artificial antigens they prepared antibodies to them. The antisera produced were found to contain antibodies capable of combining with the protein used in manufacturing the azoprotein and also antibodies with the specific power of combining with the attached group of known structure - the haptenic group. In preparing antibodies

against azoproteins they found that the antiserum not only has the power of forming a precipitate with the original azoprotein but also can form a precipitate with a different azoprotein containing the same or a very closely related haptenic group. A summary of the results obtained is given by Landsteiner (1). Of especial interest were the experiments by Landsteiner and Van der Scheer (9), in which they prepared antibodies directed against known peptides. These antibodies were found to be quite specific. Landsteiner and Van der Scheer have also differentiated serologically between *d* and *l*-aminobenzoylphenylaminoacetic acid (10), and in other experiments between dextro, levo, and meso tartaric acid (11).

Studies with these antibodies to simple chemical compounds were extended to include the combination with the simple substances themselves. It was found that for a simple substance to form a specific precipitate with antibody it is necessary that its molecule contain two or more haptens (8) (12). While all such polyhaptenic substances do not necessarily form precipitates, no monohaptenic substances do. However, this statement must be qualified because if a monohaptenic molecule has a structure that favors the formation of polymers in saline solution, as among the larger azo dyes, precipitation with purified antibody is observed to occur - the polymer is effectively polyhaptenic (13). In serum the polymerization equilibrium is shifted toward the monomer, since the concentration of free hapten in solution is greatly

reduced by its reversible combination with serum albumin and precipitation may not occur.

Simpler monohaptenic substances do not form precipitates even with purified antibody (13). It has been demonstrated however that monohaptenic substances actually combine strongly with antibody. Landsteiner showed that the presence of monohaptenic substances specifically inhibits the precipitation of antibody by polyhaptenic azoproteins (7) or simple substances (8). This phenomenon has been applied by Landsteiner and others (1) and by Pauling, Pressman, Campbell, et al. (12) in extensive studies on specificity and its structural interpretation. Marrack and Smith (14) presented direct evidence for combination by equilibrium dialysis experiments. They found that when a hapten was dialysed against its specific antibody the hapten accumulated in the antibody solution. Their results were confirmed by Haurowitz and Breinl (15). Lerman (16) has used the method of equilibrium dialysis in an attempt to estimate the free energy change of hapten combining with antibody and similar experiments have been reported by Eisen and Karush (17). The quantitative theory of the reactions of antibodies to simple substances. Pauling and his coworkers have developed a quantitative theory for the precipitation of antibodies to simple substances and have extended this theory to cover hapten inhibition in these systems. An outline of their theory follows. Pauling et al. (18) considered an idealized antigen-antibody system in which the solution

contains antigen molecules A, antibody molecules B, soluble complex molecules A_2B, and molecules AB in equilibrium with a precipitate AB. They assumed that the two bonds in ABA are equal in strength and hence that the equilibrium constants for the two reactions

$$A + B \rightleftharpoons AB \qquad\qquad A + AB \rightleftharpoons A_2B$$

differ only by the entropy factor 4, being $4K$ and K respectively. K is the equilibrium constant for combination of a single haptenic group of an antigen molecule and a single complementary region of an antibody molecule. They derived the equation

$$AB_{(pp)} = A_{total} - s - \frac{1+2Ks}{2+2Ks}\left\{ A_{total} - B_{total} + \left[s(1+Ks)/K + (A_{total} - B_{total})^2 \right]^{\frac{1}{2}} \right\} \qquad (1)$$

in which $AB_{(pp)}$ is the amount of precipitated compound, with solubility s, and A_{total} and B_{total} are the total amounts (per unit volume) of antigen and antibody in all molecular species, including the precipitate.

The curves for the amount of precipitate, as a function of the amount of antigen, calculated with this equation (using arbitrary values for K and s) have the general shape indicated by the experimental points.

This theory was extended to cover the phenomena of hapten inhibition. They considered a system containing in solution the molecular species H, BH, BH_2 and ABH (H = hapten) as well as A, B, AB, and A_2B and assumed the B-H bond

strength to be such that the equilibrium constants for the reactions

$$B + H \rightleftharpoons BH \qquad BH + H \rightleftharpoons BH_2$$

and $AB + H \rightleftharpoons ABH$

were $2K'$, $K'/2$ and K' respectively. K' is the equilibrium constant for combination of the hapten and the complementary region of the antibody. From these assumptions they derived the expressions

$$AB_{(pp)} = A_{total} - s - \alpha(1 + 2K(s + z)) \quad (2)$$

$$A_{total} - B_{total} = \alpha - \frac{s}{4K\alpha} + \alpha K s - z - \frac{\alpha K z^2}{s} \quad (3)$$

$$z = -\frac{1}{2}\left(\frac{s}{2K\alpha} + s + \frac{1}{K'}\right) \pm \left\{\frac{1}{4}\left(\frac{s}{2K\alpha} + s + \frac{1}{K'}\right)^2 + \frac{s H_{total}}{2K\alpha}\right\}^{\frac{1}{2}} \quad (4)$$

in which H_{total} is the total hapten concentration. The other variables are α, the concentration of the molecular species A in solution, and z, the concentration of the molecular species HB in solution.

By combining these three equations they were able to show that at the equivalence zone where $A_{total} = B_{total}$ the amount of precipitate formed is a linear function of the hapten concentration and a relation of the form

$$-\frac{\alpha H}{\alpha AB_{(pp)}} = \frac{c}{K'} + c'$$

where $C = \frac{(Ks + K^2s^2)^{1/2}}{s(1/2 + Ks + (Ks + K^2s^2)^{1/2})}$

and $C' = \frac{1 + Ks + (Ks + K^2s^2)^{1/2}}{1/2 + Ks + (Ks + K^2s^2)^{1/2}}$

was derived. It was found however that the experimental points showing the dependence of the amount of precipitate on the amount of added hapten do not fall on a straight line except in the region of very low hapten concentration - with larger amounts of hapten the inhibition is less than predicted by the above equation. This deviation from linearity was attributed to the heterogeneity of the antiserum which appeared to contain antibodies of greatly varying combining powers. They extended the theory (19) to cover this by assuming that the heterogeneity of the antiserum was such that it could be described by a probability distribution function which is an error function in the effective free energy of combination of hapten and antibody (in competition with antigen); that is, in the quantity $\ln(K'/K_0')$, where K' is the effective hapten inhibition constant of the particular antibody molecule under consideration and K_0' is an average effective hapten inhibition constant. The normalized distribution function used was

$$\frac{1}{\sqrt{\pi}\sigma} e^{-\{\ln(K'/K_0')\}^2/\sigma^2}$$

and the fractional number of antibody molecules with given value of K' in a differential region was

$$\frac{dN}{N} = \frac{1}{\sqrt{\pi}\sigma} e^{-\{\ln(K'/K_0')\}^2/\sigma^2} d\ln(K'/K_0')$$

They then assumed that for each kind of antibody the amount of precipitate is linear in the amount of hapten, with slope proportional to K' and hence the amount of precipitate formed is given by the equation

$$P = \frac{AB(pp)}{AB(pp)_{H_{total}=0}} = \frac{1}{\sqrt{\pi}\sigma} \int_{-\infty}^{\ln(1/H_{total}K_0')} (1 - K'H_{total}) e^{-\{\ln(K'/K_0')\}^2/\sigma^2} d\ln(K'/K_0')$$

The upper limit of the integral represents the value of K' at which the hapten just inhibits completely the precipitation of the corresponding antibody: no precipitate is formed by antibody with larger values of K'. The integral was evaluated in terms of the Gaussian probability integral $H(x)$. The equation then assumed the form

$$P = \tfrac{1}{2}(1 + H(x_1)) - \tfrac{1}{2} H_{total} K_0 e^{\sigma^2/4}\{1 + H(x_2)\}$$

where $x_1 = \frac{1}{\sigma}\ln\left(\frac{1}{H_{total}K_0'}\right)$ and $x_2 = x_1 - \sigma/2$

This equation was used to plot P as a function of $\log H_{total}$ for various values of σ, the heterogeneity index. The shape of such theoretical curves is dependent only

upon the value chosen for σ ; the effect of changing K_0' is simply to shift the curve along the $\log H_{total}$ axis. From the family of curves plotted the values of σ for experimental data can be obtained by fitting the experimental points to one of the theoretical curves. The positions of two curves along the $\log H_{total}$ axis can be used to evaluate the relative K_0's. In a given antigen-antibody system the positions of the curves for two different haptens can be measured by the amount of hapten giving fifty percent inhibition and the ratio of the two values gives the relative K_0'. The expression $\Delta F = -RT \ln K_0'$ gives the difference in the standard free energies of combination with antibody. Usually a given hapten is selected as having a K_0' of unity and the other hapten inhibition constants are evaluated relative to this standard.

Although Pauling and his coworkers have used the theory outlined to arrive at this method of evaluating the differences in free energies of combination of hapten and antibody they point out that

> "the evaluation and discussion of differences in the standard free energy of combination of different haptens with antibody is not dependent on the assumption of any particular theories of the precipitation of antibody and antigen. We may consider that the precipitation with antigen is used as a method of fixing a standard concentration of free antibody (for example, that which corresponds under the conditions of the experiment to the formation of one-half as much precipitate as is formed in the absence of hapten); the concentration of hapten necessary to reduce, by combination with part of the antibody, the concentration of free antibody to the standard value is determined experimentally and the ratio of

> concentrations for two haptens may then be introduced in the well-known equation relating standard free energy change and equilibrium constant to give a value of the difference in the standard free energy of combination of the two haptens with antibody. The only assumption underlying our treatment of hapten inhibition data is that inhibition of precipitation is the result of combination of the antibody with molecules of hapten." (20).

Steric effects in hapten inhibition experiments. It has been possible to interpret hapten inhibition data in terms of the structure of the haptens and the antibody combining site. The present concept of the antibody site is that it is a cavity on the surface of the protein into which the haptenic group fits. This concept has been used by Pauling and Pressman (20) in considering the effect of steric factors in hapten inhibition. Many of their studies were conducted with azophenylarsonic acids and drawings were made of these groups using accepted bond distances and van der Waals radii. Circumferential contours, representing the antibody cavity in the plane of the benzene ring were drawn to fit the groups closely or with radial dilation of various amounts. Scale drawings of various substituted haptens were then superimposed on these drawings and the extent of dilation needed in order for each hapten to fit was measured. Assuming that an increase in the required dilation corresponds to an increase in steric repulsion they were able to correlate this with a decrease in K_o' for different substituted phenylarsonic acids. The substituents were in positions other than the position of the azo

linkage in the immunizing hapten.

The effects of changed electronic van der Waals interaction in hapten inhibition experiments. If a haptenic group has a substituent in the position of the azo linkage in the immunizing hapten there will be little steric effect to consider. However the reaction of such haptens with antibody should be influenced by the interaction of the substituent with the antibody. Pauling and Pressman (20) have also considered this possibility. London developed for the interaction energy of two groups or atoms the approximate expression

$$W = - \frac{3\alpha_A \alpha_B I_A I_B}{2 r'^6_{AB} (I_A + I_B)}$$

in which α_A and α_B are the electronic polarizabilities of the two groups, I_A and I_B are average energy differences for the normal and excited states, and r_{AB} is the distance between the groups. This expression was rewritten in the form

$$W = - \frac{38000}{r'^6_{AB}} R_A R_B$$

in which R_A and R_B are the mole refractions of the groups in cm^3 ($R = \frac{4\pi N}{3}\alpha$) and r_{AB} is in Ångström units. In rewriting this expression I_A and I_B were taken as being 14 electron volts which is an average for several molecules. The expression was then multiplied by a factor of 0.60 to give a 40% correction for the contribution of van der Waals repulsion. This equation has been applied in the calculation

of hapten inhibition constants for substituted haptens relative to an unsubstituted hapten (containing a hydrogen atom rather than a substituent atom or group). In applying the equation four energy quantities must be considered: the interaction of the substituent with antibody, the interaction of the substituent with water, the interaction of the hydrogen atom with antibody, and the interaction of the hydrogen atom with water. The resultant difference in energy of reaction is

$$\Delta w = - \frac{23000}{r'^{6}_{AB}} (R_A - R_H)(R'_{Ab} - R'_{water})$$

where R'_{Ab} and R'_{water} represent the mole refraction values integrated over the region of material in contact with the substituent group. R_A and R_H are the mole refractions of the substituent and the hydrogen. For the studies that were being considered the region of material in contact with the substituent group was considered to be the volume of eight water molecules and so R'_{water} was taken as $8R_{H_2O} = 29.6 cm^3$ and from data on another protein R'_{Ab} was estimated to be $47.2\ cm^3$. The final expression derived can be written in the form

$$\Delta w = - \frac{400,000}{r'^{6}_{AB}} (R_A - R_H)$$

or

$$\log_{10} K_0' = \frac{400,000\,(R_A - R_H)}{2.303\,RT\,r'^{6}_{AB}}$$

This expression was used to evaluate the relative inhibition constants and gave a rough quantitative agreement with experiment when applied to a series of para substituted benzoic acids, phenylarsonic acids, and

p -phenylazophenylarsonic acids. The marked deviations between calculated and observed effects could be satisfactorily accounted for by hydrogen bonding.

An electrostatic effect in hapten inhibition experiments. An interesting calculation has been made by Pressman et al. (21), who found that two haptens which were identical except that one contained a $N(CH_3)_3^+$ group where the other had a $C(CH_3)_3$ group gave a ratio of 15.5 for their hapten inhibition constants. This corresponds to a difference in standard free energy of combination with antibody of 1.5 kcal. Identifying this free energy difference as Coulomb interaction energy between the $N(CH_3)_3^+$ group and the antibody, they calculated that the positive charge of the hapten must be 7 Ångströms from a negative charge on the antibody.

Some other factors in the precipitin reaction. An experiment of fundamental importance to the theory of the precipitin reaction was the demonstration by Pauling et al. (22) that a simple substance containing two different haptenic groups gave only a slight precipitate with antisera to either of the two groups but when treated with a mixture of the two sera gave a relatively large amount of precipitate. This precipitation could be inhibited by a monohaptenic substance of either type. This experiment is a strong support for the "framework" theory of precipitation, which maintains that precipitation occurs as a result of the

formation of a large specifically formed aggregate of polyvalent antigen and at least divalent antibody.

Experiments of the above nature have supported the present concept of antibodies as modified globulins that have portions of their surface complementary to the antigen causing their production. For a specific reaction to occur between a substance and an antibody the substance must have structural features that enable it to fit the antibody site very closely. The forces responsible for antigen-antibody combination are presumably the forces of electronic van der Waals interaction, hydrogen bonding, coulombic attraction, etc., which are effective over short distances only (23), and hence closeness of fit would be required for combination. This idea of complementary structures was first suggested by Breinl and Haurowitz (24), Alexander (25), and Mudd (26), and has come to be rather generally accepted. There is some intimation of it in the early work of Ehrlich and Bordet. The serological properties of simple substances which have pharmacological properties. It has also long been recognized that chemical structure has a very important influence in drug activity (27), and some attempts have been made to use drugs as haptens in serological investigations. Many factors such as passage through membranes, diffusion, and transfer from aqueous to lipoid phases must influence drug activity but it might be expected that molecular configuration would be an important factor in the reaction of a drug at its site of activity. The type of specificity involved might closely

resemble the specificity of an antibody for a haptenic group and if this were true substances with similar pharmacological activity might be expected to have similar serological activity.

Landsteiner (1) has summarized the earlier data on serological experiments with drugs. The earlier work was undertaken with the hope that non-antigenic poisons after combination with protein might produce antibodies capable of neutralizing their toxic effect. This possibility was tested by Berger and Erlenmeyer (1) with an antiserum to dimethyl-N-pyrrol-phenylarsenic acid, but the results were negative. Stokinger and Heidelberger (28) produced antibodies to thyroglobulin and confirmed the fact that the antithyroglobulin-thyroglobulin precipitation was not inhibited by thyroxine or diiodotyrosine. Hooker and Boyd (29) were unsuccessful in attempts to prepare antimorphine serum but were able to produce antibodies specific for strychnine in rabbits by injections of monoaminostrychnineazohemocyanin. The sera they obtained were very low in antibody and failed to neutralize the lethal effect of strychnine in mice. They also carried out precipitation tests with these antisera and a caseinazostrychnine antigen and found that the precipitation could be inhibited by the presence of the alkaloid and also by some of its amino, nitro, and methoxyl derivatives, but not by the presence of morphine, quinine, tryptophane, or nicotinic acid. Clutton, Harington, and Yuill (30) prepared thyroxyl derivatives of horse serum

globulin and albumin. These substances and thyroglobulin caused the production of antibodies specific to the whole aromatic portion of the thyroxine molecule. Passive immunization with these antisera to thyroxylproteins protected rats against the normal physiological effects of exogenously administered thyroglobulin and thyroxine. The authors thought that the neutralization of thyroxine could be indirect inasmuch as thyroxine may exert its activity after having been converted to thyroglobulin. Butler, Harington, and Yuill (31) studied the immunological properties of aspirin. These workers prepared aspiryl-protein complexes by coupling acetylsalicylazide with horse and rabbit serum globulins. Immunization of rabbits with aspiryl horse serum globulin gave antisera specific to the aspirylamido grouping. Such sera were able partly to protect rats, suffering from pyrexia as the result of injection of yeast, from the normal antipyretic effect of aspirin. They also found that the precipitation reaction between this serum and aspiryl rabbit serum globulin could be inhibited with aspiryl - or salicylglycine but aspirin and salicylic acid were relatively ineffective as inhibitors of precipitation. Wedum (32) investigated the cross reactions of the sulfonamides with antisera produced in rabbits and humans. Cohen and Friedman (33) claim to have succeeded in neutralizing in vitro the physiological effect of small amounts of histamine with the serum of patients treated with histamineazoprotein. However, Coffin and Kabat (34) have reported that the treatment of

guinea pigs with histamine-azoprotein confers no protection against histamine. Allergic reactions to drugs in humans have been studied in many clinical investigations (35).

Perhaps the most extensive series of immunochemical investigations with drug-protein antigens are those of Went, Kesztyüs, and their coworkers (36-46). In their earlier work they too found that immunization with thyroglobulin produced antiserum which was capable of inhibiting the effect of thyroxine in rats and guinea pigs (36). Later they used adrenaline azoprotein to produce what they believed to be a specific adrenaline immune serum in rabbits (37) and found that this antiserum did not affect adrenaline activity in rats and frogs although rats immunized with adrenalylazoprotein did not show the same respiratory effect as normal rats upon the administration of adrenaline (39) (40). Of course, in these experiments that involve the protection of an animal actively immunized with a drug-protein complex a consideration must be given to the possibility of the animal developing a tolerance to the drug by some means not associated with serum antibody formation. This group of workers have also prepared histamine antibodies (41) (42) (43) by using an antigen prepared by diazotizing *p*-aminobenzeneazohistamine to horse or cattle serum. They state that precipitation reactions were not affected by horse or beef protein (42). Inhibition of precipitation of the histamineazoprotein system could be obtained with aniline, *p*-aminobenzeneazohistamine, and histamine, but naphthol or

phenol had no effect. Went and Kesztyüs (44) also have investigated the production of antibodies to novocaine. Novocaine hydrochloride was diazotized and coupled with cattle or horse serum and the novocaine azo cattle serum was used to produce rabbit antibodies. Such antiserums gave precipitation reactions with novocaine azo cattle serum and novocaine azo horse serum but did not react with the proteins of natural cattle serum or natural horse serum. In precipitation inhibition tests, novocaine, larocaine, and anesthesine hindered the precipitation of novocaine azoprotein antibodies whereas orthoform (*m*-amino-*p*-hydroxy-methylbenzoate), aniline, *p*-aminobenzoic acid, *p*- aminobenzenesulfonamide, and cocaine had no effect on the reaction. The authors believed their experiments showed a possible dependence of group specificity on the amino group of the benzene ring in the para position to an esterified carboxyl group. This same group in later reports rediscussed the above results (45) (46) and concluded that their inhibition tests indicated that there was at least a partial specificity to local anesthetics that were derivatives of *p*-aminobenzoic acid. This serum when mixed with procaine (novocaine) inhibited the papulous eruption in an allergic patient.

The studies with local anesthetic drugs are especially interesting as it is known that these compounds are capable of blocking nerve conduction when applied locally to nerve tissue in effective concentrations (47). Although the

problem of nerve conduction is still in a state of controversy (48-54), it appears that acetylcholine plays a very important part in this phenomenon and it is possible that local anesthetics acting on nerve surfaces and at the junctions of nerve with muscle may interfere with the action of acetylcholine. However, in the light of existing knowledge there is no way of connecting the action of these drugs with the chemistry of choline and its metabolic associates.

Object of this research. A large number of benzoates and substituted benzoates have been synthesized and tested for anesthetic action and practically no general rules can be applied for predicting the change of activity resulting from a change in structure (55). Nevertheless, it was thought that the serological investigation of such a series of local anesthetics might yield some interesting data which could possibly be correlated with anesthetic activity. Eli Lilly and Co. prepared a series of compounds derived from substituted piperidino alcohols which have been investigated for toxicity and local anesthetic properties (56). Some of these compounds were made available for the studies discussed in this thesis.

The object of this work was to obtain antibodies specific to one of this series of drugs and to study the inhibition of the precipitation reaction by its analogs. In order to avoid any specific reactions due to the antigenicity of the protein used in the immunization, a different protein was selected to make the antigen used in the precipitation tests.

EXPERIMENTAL

Preparation of antigens. One of the compounds investigated in this series was 3-(2-methylpiperidino)propylbenzoate hydrochloride and this compound was modified by Eli Lilly and Co. to include an amino group in the para position of the benzene ring. The amino group made it possible to diazotize this compound onto proteins. The first attempts to produce antibodies to this drug were made with an antigen prepared by coupling the drug to bovine globulin. In the first preparation 11 g of bovine globulin (Armour and Co. Fractions II and III) were shaken overnight in 150 ml of distilled water; about half of the material dissolved, and the remainder was centrifuged out of the solution. The supernatant was cooled to 0°C. A solution of 0.417 g of 3-(2-methylpiperidino)propyl-*p*-aminobenzoate hydrochloride (L-1) in 8 ml of 0.5 N hydrochloric acid was prepared and cooled to 0°C. An ice-cold solution of 0.096 g of sodium nitrite in a few ml of distilled water was prepared. The ice-cold nitrite solution and L-1 solution were mixed and allowed to stand for 20 minutes in an ice-salt bath. The solution was checked for excess nitrous acid with starch-iodide paper and the excess nitrous acid was removed with a little sulfamic acid. This solution was then slowly added with stirring to the ice-cold protein solution and 1 N sodium hydroxide was periodically added to keep the solution at *p*H 9.0. A large amount of a red precipitate

formed, which would not redissolve. The mixture was dialyzed against water for 48 hours and was then shaken overnight with enough sodium chloride to give a 1% sodium chloride solution. The precipitate which still remained was removed by centrifugation. The original mixture corresponded to about 40 hapten groups per protein molecule, but possibly the material remaining in solution was the protein with the smaller numbers of diazotized groups. The final solution contained only about 1% protein.

In a similar manner the drug L-1 was coupled to rabbit serum albumin and also to human serum albumin. These azoproteins also proved to be relatively insoluble. Further preparations of the L-1-bovine globulin antigen (L-1BG) were made using different ratios of drug to protein but in all cases a large fraction of the material produced was insoluble.

Preparation of antisera and preliminary tests. Over a period of a year a large number of rabbits were injected biweekly with 1 ml portions of the L-1BG antigen but only a few produced measurable quantities of precipitating antibodies. The positive sera were pooled and the pooled material gave about 0.3 mg of precipitate per ml when titrated with the L-1HA (L-1-human serum albumin) antigen. In spite of this low titer a preliminary set of experiments were carried out to see if any inhibition of precipitation could be obtained.

Solutions of the various drugs were made up containing

6 mg of drug per ml in saline solution. One ml of drug solution was placed in each of three tubes and to each tube 1 ml of the pooled serum was added and the mixtures were allowed to stand at room temperature for thirty minutes. To one tube 1 ml of a 1/10 dilution of the L-1HA antigen was added, to another 1 ml of a 3/100 dilution of the L-1HA was added, and to the third 1 ml of a 1/100 dilution of the L-1HA was added. This corresponded to 1.3 mg, 0.39 mg, and 0.13 mg, of L-1HA respectively. These mixtures were allowed to stand at room temperature for two hours and were then stored in a refrigerator for three days. Identical tests were performed with seventeen of the drugs. A control test was performed using 1 ml of saline instead of the drug solution. The precipitates were all washed twice with ice-cold saline, removing the precipitate each time by centrifuging for 30 minutes at 3000 rpm in an International refrigerated centrifuge keeping the temperature near 4°C. The amounts of precipitate in the tubes were measured by nitrogen determinations using the Nessler method. The colorimetric readings were made with a Klett colorimeter. The results are shown in Table 1. As this experiment indicated that inhibition did occur attempts were continued to produce a better antiserum. It is to be noted that no attempt was made to buffer the precipitation tests so this experiment has only qualitative significance.

In an attempt to produce a higher titer antiserum several rabbits were injected with the insoluble L-1BG

material. The material was suspended in saline and sterilized by repeatedly heating it at 56°C. for half hour periods. This was then administered to the rabbits by intraperitoneal injections. Biweekly injections were given over a ten week period but the rabbits failed to show any antibodies specific to the immunizing hapten (L-1). Ten rabbits were given injections of the soluble fraction of the L-1HA antigen. These rabbits also failed to produce significant antibody titers. Finally some fresh sheep serum was obtained and the drug L-1 was diazotized to this whole serum. Enough L-1 was used to give about 40 hapten groups per average protein molecule and the coupling was carried out in the same manner as before. It was noted that the solubility of this material was quite sensitive to pH. In general the antigens were kept at about pH 8 and at this point the L-1SS (L-1-sheep serum) was only partially soluble. However, if the material was adjusted to pH 10 it was practically completely soluble. Some of this material at pH 10 was injected intravenously into some rabbits and the high pH did not seem to affect them adversely. Consequently the antigen was stored at pH 8 and small quantities were taken periodically, adjusted to pH 10, and used for immunization. The rabbits were given biweekly injections of 1 ml each. The results were the most favorable obtained: after ten weeks of injections some of the animals gave serums which yielded a little less than 1 mg of precipitate

per ml when titrated with the L-1HA or the L-1BG antigen. These animals were injected for over a year with the L-1SS material, and continually yielded sera giving from 1/3 to 1 mg of precipitate per ml when titrated with a heterologous antigen. The sera obtained were pooled and the mixture was passed through Seitz filters and stored in sterile bottles. Different pools of this anti L-1SS serum were used for all the later experiments.

Hapten inhibition studies. As sufficient serum was available it was thought that it might be interesting to run parallel experiments with the antibody containing globulin fraction of the serum. A portion of the serum was one-third saturated with ammonium sulfate and the mixture adjusted to pH 7.8 and allowed to stand for one hour at room temperature. The precipitate was centrifuged out and dissolved in saline, the final volume being about half the original volume of serum. The precipitation was carried out twice more and the final solution was dialysed for two days against cold saline. The final solution was diluted with saline to give an antibody concentration similar to that in the original serum.

For these experiments the drugs were dissolved in borate buffer at pH 8.0. Solutions 1.5×10^{-2}, 1.5×10^{-3}, 1.5×10^{-4}, 1.5×10^{-5}, and 1.5×10^{-6} molal in drug were made. The antigen dilution required for maximum precipitation in the absence of any hapten was also made up in the pH 8.0 borate buffer and this was used in the hapten

inhibition tests. All the precipitations were made in the following manner. One ml of the antibody solution was mixed with 1 ml of the drug dilution and the mixture was incubated at 37°C. for half an hour. One ml of the antigen dilution (L-1BG) was then added and the mixture was incubated for another hour at 37°C. The mixture was placed in the refrigerator for 48 hours and the precipitate was treated in the same manner as described previously. In all the experiments using buffer as a diluent the pooled supernatants from the precipitations varied in pH from 7.9 to 8.1. This method of performing the inhibition tests is essentially the same as used in the work of Pauling, Pressman, Campbell et al. (12). The results of the final experiments are given in Table II and Figures I,II, and III. These tests were all carried out in triplicate. Earlier tests performed only in duplicate gave essentially the same results. As can be seen, the whole serum and the globulin fraction gave very similar results.

In a further experiment all the drugs were used in a 1.5×10^{-2} molal solution and also a number of related simple compounds were used as inhibitors. The simple compounds were all used as 1.5×10^{-2} molal solutions in borate buffer at pH 8.0. One ml of these solutions were used in the precipitation tests, which were carried out in the same manner as before. The experimental data are given in Table III. The molecular formula of each drug and simple compound is given in Table IV. The data on the toxicity and anesthetic

properties of the local anesthetics as determined by McElvain and Carney (56) are given in Tables V and VI.

DISCUSSION

Hapten inhibition constants. One of the controlling factors in these experiments was the limited solubility of some of the drugs being investigated. In the first experiment there was some indication that some of the drugs were tending to come out of solution at 4°C. This was especially true of drug L-201, and as this would have a serious effect upon the results it was decided that the most concentrated solution of all the drugs that should be used was 1.5×10^{-2} molal. This limited the data available for the curves shown in Figures I, II, and III, which represent the results obtained from the experiments in which the concentration of hapten was varied. In some cases the amount of precipitate obtained with this maximum drug concentration was still more than half that obtained when no drug was present. However, the curves obtained all show a similar shape, corresponding to a heterogeneity index of five, and by fitting the theoretical curve corresponding to this value to the experimental points the concentrations of the drugs required for fifty-percent inhibition were evaluated. The ratio of the concentration obtained for drug L-1 to the concentration obtained for each of the other drugs was determined and this gave the hapten inhibition constant relative to that of L-1 as unity. For example, from the data of Figure I the concentration of L-1

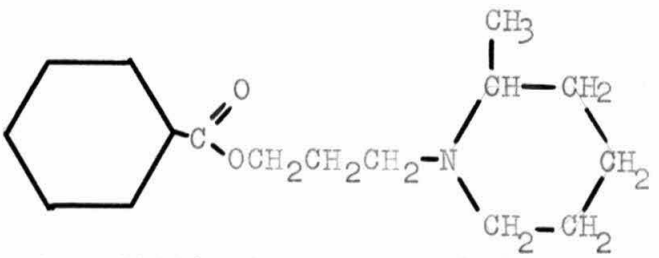
producing 50% inhibition was estimated as $10^{-2.55}$ and the concentration of L-134 producing 50% inhibition was estimated as $10^{-2.05}$. This gives as the ratio of the concentrations $10^{-2.55}/10^{-2.05} = 1/10^{0.50} = 0.32$. This figure, 0.32, is the hapten inhibition constant (K_0') for L-134. In a similar manner the values for the other drugs were obtained. In the second series it was again assumed that the curves are all of the same shape, and the inhibition constants were again evaluated relative to that of L-1.

In the first series the concentrations of the various drugs required for fifty-percent inhibition vary by not more than a factor of 5.3. (The extreme values are $10^{-2.72}$ for L-208 and $10^{-2.00}$ for L-161.) This corresponds to a difference in the free energy of the reactions of the drugs with antibody of $2.303\ RT \log 5.3 = 920$ calories per mole. The drugs are all very similar in their effect as inhibitors of precipitation in the system studied. It is to be noted that the relative inhibition constants vary somewhat in the two series. The serum used in the second series was a pool from the same rabbits but taken after a further series of injections.

As stated earlier, the data obtained with the globulin fraction of the serum were quite similar to those obtained with whole serum (Table II-H), and thus it can be concluded that these drugs interact only slightly with rabbit serum albumin.

The structures of the various drugs used as inhibitors

of precipitation are given in Table IV. The basic structure of the drugs is that of drug L



It is quite difficult to correlate the inhibitory properties of the drugs with their structures, especially considering how little variation in inhibition was shown. However the effects of various modifications of the basic structure are discussed below.

The effect of additional methyl groups on the piperidine ring. It might be expected from the manner in which the drug L-1 was coupled to the carrier protein that substituents on the piperidine ring would be most significant in altering the closeness of fit of the antibody and hapten. Some of the drugs contain additional methyl groups on the piperidine ring and they do decrease the interaction of the drug with antibody.

The two drugs L-135 and L-136, which differ in that L-136 has a methyl substituent in the six position of the piperidine ring, gave as the ratio of their hapten inhibition constants 1.49 in the second series and 1.52 in the first series. If 1.50 is taken as the average of these results a difference in free energy of combination with antibody of 2.303 RT log 1.5 or about 220 calories is obtained. This difference is due presumably to the steric effect of the

second methyl group.

The drugs L-172 and L-161 also differ by a methyl group in the six position of the piperidine ring, and in this case the free energy difference is about 280 calories, although the agreement between the values in the two series is not as good as in the previous example. It may be concluded that the introduction of the methyl group into the six position decreases the free energy of the reaction by about 250 calories.

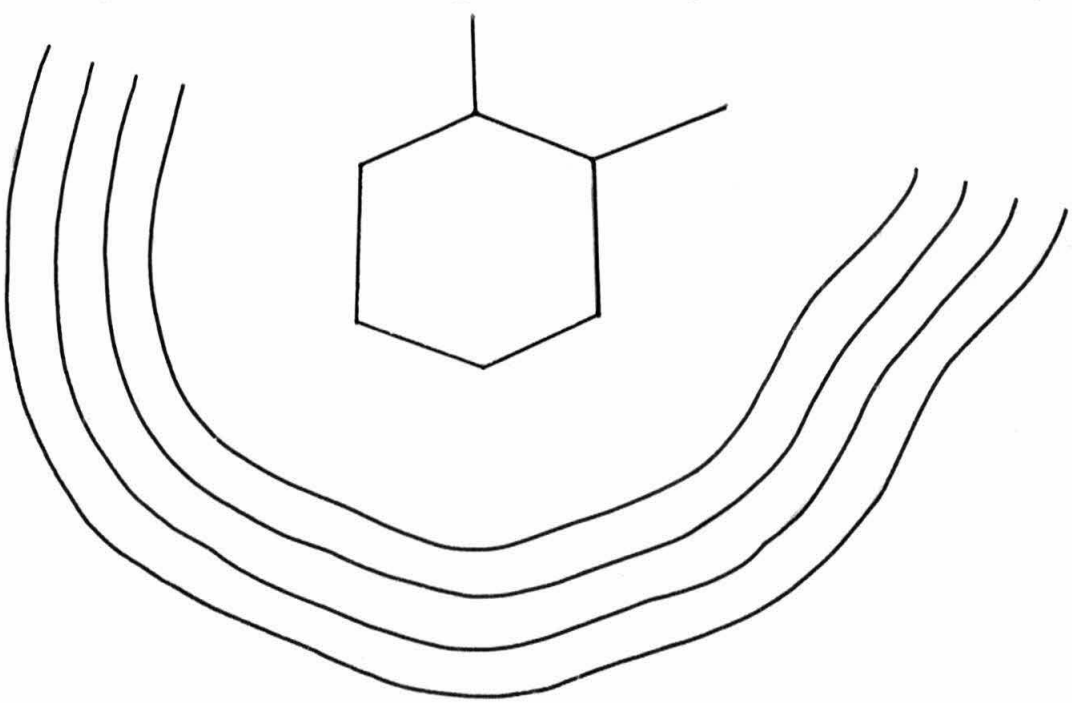
If only the values obtained in the second series are considered it is calculated that the effect of a methyl group in the six position is to decrease the free energy of combination by 160 calories (L-172, L-161), and the effect of a methyl group in the four position is to decrease the free energy by 370 calories (L-172 and L-206, which is the same as L-172 except for a methyl group in the four position of the piperidine ring). The effect of introducing methyl groups in both the four and six positions is to decrease the free energy by about 470 calories (L-172, L-207), which is reasonably close to the sum (530 calories) of the individual effects.

The effect of a methyl group in the three position is uncertain, as in the first series it decreases the free energy by 210 calories and in the second series increases it by about 41 calories (L-172 and L-205).

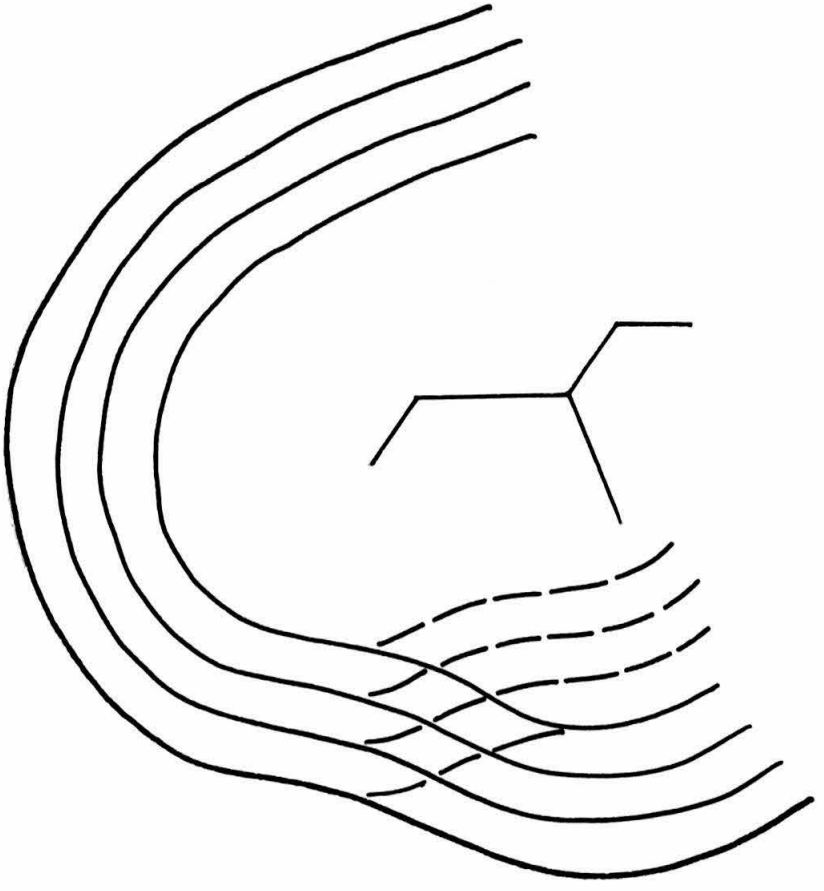
It may be concluded that the methyl group in the four position is the most effective in decreasing the interaction

of drug and antibody. A methyl substituent in the three position has the least effect, probably decreasing the interaction slightly, and a methyl group in the six position has an intermediate effect.

It is interesting to interpret these effects in terms of the radial dilation required of the antibody site in order for the hapten to fit into the site. Drawings were made of the 2-methylpiperidine group with use of the accepted bond distances and van der Waals radii (57), and circumferential contours, representing the required antibody cavity were drawn to fit the group closely or with radial dilation of 0.5 Å., 1.0 Å., and 1.5 Å. The following figure shows the outline of this group in the plane of the four central carbon atoms of the ring. The methyl substituent was taken in the position which places the ring to methyl group bond nearly in this plane in order to give the largest area in this plane.



The second figure shows the outline of the group in a plane perpendicular to the plane of the four central carbon atoms. In this case the methyl substituent was taken in the position which places the ring to methyl group bond almost parallel to this plane in order to give the largest area in this plane. The dashed contours in this figure represent the circumferential contours in a plane behind the drawing. (The six position has no methyl substituent as in the two position). The 'chair' form of the ring was used in these drawings but the 'boat' form could be used without altering the discussion.

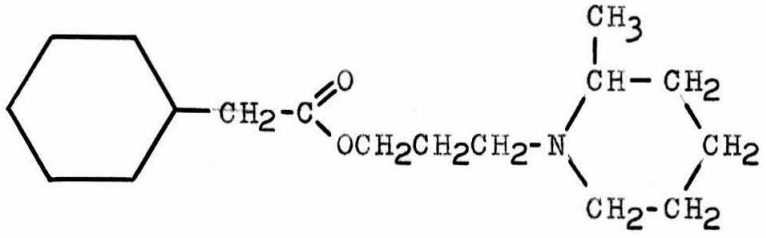


Scale drawings of the other methyl substituted piperidine rings were superimposed on these drawings keeping the nitrogen of the ring in the same position and retaining the same orientation for the ring. The extent of dilation needed for each hapten was determined in both planes. It is obvious from the first figure that a methyl group in the four position would require a dilation of about 1.5 Å. (Approximately equal to the C-C bond distance of 1.54 Å.). Methyl groups in the three and six positions require almost the same dilation, the values being 1.3 Å. and 1.4 Å. respectively. In the second figure it is seen that the methyl group in the four position again requires a dilation of 1.5 Å. if the bond is parallel to the plane of the four central carbon atoms and a dilation of 1.3 Å. if it is in the other position. A methyl group in the three position requires a maximum dilation in this plane of about 1.3 Å. A methyl group in the six position, which is behind the plane of the figure, would require a dilation in this plane of about 1.5 Å.

From these considerations it would be predicted that a methyl group in the four position would have the greatest effect in reducing the interaction with antibody and a methyl group in the six position should have almost as great an effect. The methyl substituent in the three position should have the least effect. Qualitatively the experimental data support this. However the effects of a methyl group in the four position and a methyl group in the six position do not seem to be as close as required and this may be because the methyl group in the six position is closer to the entrance

of the site where dilation may occur more readily. It is perhaps surprising that a methyl group in the three position has such a small effect and this may be a reflection of a general poorness of fit of these antibodies.

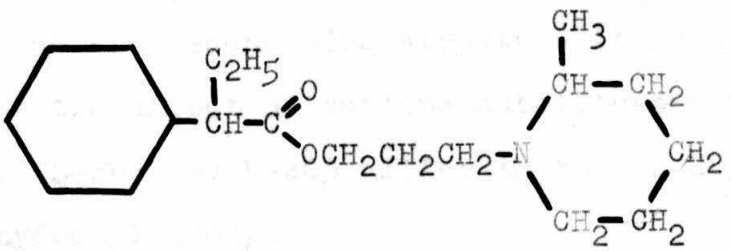
The effect of additional methylene groups. Several of the drugs are modified to include one or more methylene groups adjacent to the carboxyl group. L-134 is an example of this series



These modifications decrease the inhibitory power of the drugs and this can perhaps be explained on the basis of a decreased interaction between the antibody and the second ring structure.

The high heterogeneity index of this series of haptens (5) presumably results from an abnormally great variability of strength of attraction of antibody and azoprotein antigen or hapten. This may be accounted for by the possible variation in configuration of the drugs about the propyl group. Most of the quantitative studies of hapten inhibition have been conducted with haptenic groups of rather rigid structure, whereas in the compounds used in this study a greater variation in configuration is possible; this may explain the relatively poor antigenicity and high heterogeneity in this system. The introduction of further methylene

groups probably increases the variability in structure and consequently decreases the interaction between hapten and antibody. This effect is shown by drugs L-134 (K_0'=0.41), L-137 (K_0'=0.28), L-170 (K_0'=0.45), L-202 (K_0'=0.45), and L-203 (K_0'=0.41) in the second series. In the first series L-170 gave a rather high value (K_0'=0.89) but the others tested were low in this series too. L-171, which contains an ethyl substituted methylene group in this position, does not show the effect to such a marked extent (K_0'= 0.72) and this may be accounted for by interaction of the ethyl group with the antibody molecule. Drug L-171 has the structure



The structure of L-134 is given above.

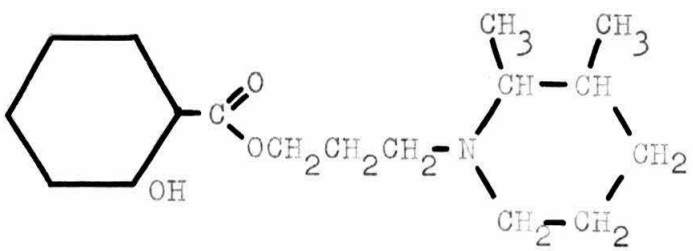
Drug L-171 gave hapten inhibition constants of 0.72 and 0.79 while drug L-134, which is identical with it except for the ethyl substituent, gave inhibition constants of 0.41 and 0.32. It may be concluded that the inhibition constant of L-171 relative to that of L-134 is about 2.1 (1.76/0.37). This K_0' (relative to K_0'=1 for L-134) has a logarithm of 0.31. If the expression

$$\log K_0' = \frac{400{,}000\ (R_A - R_H)}{2.303\ RT\ r_{ab}^6}$$

developed by Pauling and Pressman (20) is applied to this case the value calculated for $\log K_0'$ is about 0.71. In

making this calculation a difference of mole refraction for the ethyl group and the hydrogen of 9.2 cm^3 was used. The distance between the groups (r_{ab}) was taken as the minimum distance of separation, 4.0 Å. As might be expected from the large value of the heterogeneity index, the value calculated is greater than the experimental value. If it is assumed that the separation is somewhat greater than this minimum distance and a value of 4.6 Å. is used a theoretical value in agreement with the experimental one is obtained.

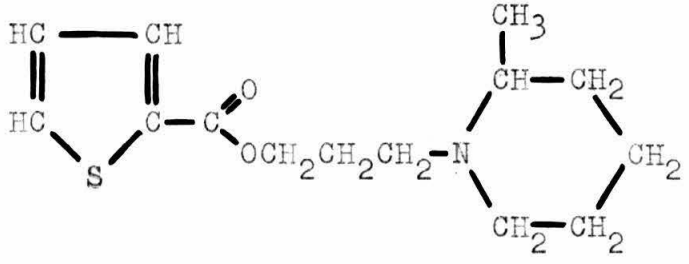
The effect of other modifications in the structure of the drugs. Although some of the drugs contain substituted benzene rings as the second ring structure it is not possible to evaluate the effect of various substituents from the present data. L-204 and L-205 differ in that L-205 contains an ortho hydroxyl group;



This compound gave a K_o' of 0.72, as compared to 0.89 for L-204. L-134 and L-202 differ by a para hydroxyl group in L-202; they have almost the same inhibition constant (0.41 and 0.45 respectively). In the series L-1, L-172, L-173, the values of the inhibition constants are 1.00, 0.67, and 0.81, corresponding to a para amino, an ortho hydroxyl, and an ortho benzoyl substituent respectively. The only marked effect is the low value of 0.14 for L-201, which contains a

para benzoxy group. This is parallel to the difference between L-134 and L-137 in the second series. L-137, which contains a para methoxy group, has a constant of 0.28 as compared with the value 0.41 for L-134. However, in the first series the effect was reversed, the values being 0.50 for L-137 and 0.32 for L-134. L-170, which is similar to L-134 except that the second ring is cyclohexane rather than benzene, gave values of 0.45 in the second series and 0.89 in the first series.

Some of the drugs contain different cyclic structures for the second ring but again not enough different structures are available to investigate their effect. The most notable feature is the high value (1.47 and 1.12) for the inhibition constant of L-208, which has the structure



Inhibition of precipitation by other simple compounds. The effect of certain simple compounds upon the precipitation in the system studied is given in Table III. The simple compounds used are listed at the end of Table IV. Of the simple compounds tested only triethylamine and tri-*n*-butylamine seem to have any significant inhibitory action. Trimethylamine, acetylcholine chloride, choline chloride, and the secondary and primary amines tested caused very little or practically no inhibition of precipitation. Propionic acid

had no effect and benzoic acid had only a small effect at the concentration used.

The above results would seem to indicate that a tertiary nitrogen atom is required for interaction with the antibodies to drug L-1. The failure of trimethylamine to show a significant effect may be due to the methyl groups being not quite large enough. The related structure in the drugs is the propylpiperidine group.

The relation of the hapten inhibition data to the pharmacological data. A comparison of the hapten inhibition activity with the local anesthetic action is given in Table VII; it can be seen that no obvious correlation exists. However, as the inhibition experiments were performed with solutions of equal molality and the pharmacological data were obtained with one percent solutions a further comparison was made with the drugs in groups of like molecular weight. This comparison is given in Table VIII. Again no correlation has been observed between anesthetic action, toxicity, and hapten inhibition properties. It was thought that some slight correlation may have existed between the data of Table I and the local anesthetic action of the drugs, with the poorer anesthetics giving less inhibition of precipitation. However, there is no general overall correlation, and there are exceptions to the trend. Considering the small amounts of precipitate and the manner in which these tests were performed this cannot be regarded as significant.

In conclusion it may be stated that the discussion of

these experiments has provided reasonable explanations of the serological properties of these drugs, but that it has not been found possible to establish any relation between their serological properties and their pharmacological properties.

TABLE I

Hapten Inhibition Experiment with Antiserum to L-1BG Using L-1HA as the Precipitating Antigen.

Antigen Dilution	Protein Precipitate in Milligrams 1:10	3:100	1:100
Saline	0.136	0.288	0.277
Drug L-134	.096	.198	.183
L-137	.072	.172	.167
L-201	.102	.165	.212
L-203	.049	.162	.143
L-136	.072	.156	.186
L-170	.056	.156	.145
L-202	.059	.154	.158
L-204	.038	.121	.135
L-135	.040	.120	.114
L-173	.038	.110	.102
L-208	.019	.102	.116
L-171	.046	.091	.111
L-161	.027	.076	.089
L-205	.016	.057	.070
L-206	.027	.046	.076
L-172	.035	.029	.049
L-1	.035	.032	.027

TABLE II

Hapten Inhibition Experiment with Antiserum to L-1SS Using L-1BG as the Precipitating Antigen.

A. Whole Serum

Drug	Dilution	Klett Readings		Average	Average -Blank	Ratio*
L-171	1.5×10^{-2}	174	171 172	172	96	0.48
	1.5×10^{-3}	211	216 214	214	138	.69
	1.5×10^{-4}	259	262 262	261	185	.92
	1.5×10^{-5}	279	277 279	278	202	1.00
	1.5×10^{-6}	268	275 279	274	198	0.99
L-208	1.5×10^{-2}	157	160 158	158	82	.41
	1.5×10^{-3}	200	206 206	204	128	.64
	1.5×10^{-4}	262	268 271	267	191	.95
	1.5×10^{-5}	276	270 275	274	198	.99
	1.5×10^{-6}	265	275 273	271	195	.97
L-173	1.5×10^{-2}	168	165 166	166	90	.45
	1.5×10^{-3}	212	211 211	211	135	.67
	1.5×10^{-4}	263	262 269	265	189	.94
	1.5×10^{-5}	269	273 271	271	195	.97
	1.5×10^{-6}	269	275 271	272	196	.98
Saline Control		278	278 274	277	201	

*Ratio:- Ratio of amount of precipitate to amount of precipitate obtained in saline control (no inhibitor present)

TABLE II

Hapten Inhibition Experiment with Antiserum to L-1SS Using L-1BG as the Precipitating Antigen.

B. Whole Serum

Drug	Dilution	Klett Readings			Average	Average -Blank	Ratio*
L-134	$1.5x10^{-2}$	192	192	195	193	114	0.58
	$1.5x10^{-3}$	230	-	229	229	150	.77
	$1.5x10^{-4}$	264	265	272	267	188	.96
	$1.5x10^{-5}$	282	273	274	276	197	1.00
	$1.5x10^{-6}$	276	279	281	279	200	1.02
L-170	$1.5x10^{-2}$	168	174	171	171	92	0.47
	$1.5x10^{-3}$	211	215	215	214	135	.69
	$1.5x10^{-4}$	258	271	262	264	185	.94
	$1.5x10^{-5}$	268	272	273	271	192	.98
	$1.5x10^{-6}$	264	272	265	267	188	.96
L-137	$1.5x10^{-2}$	175	176	177	176	97	.50
	$1.5x10^{-3}$	229	233	234	232	153	.78
	$1.5x10^{-4}$	268	266	267	267	188	.96
	$1.5x10^{-5}$	276	274	279	276	197	1.00
	$1.5x10^{-6}$	274	282	274	277	198	1.01
Saline Control		276	274	275	275	196	

*Ratio:- Ratio of amount of precipitate to amount of precipitate obtained in saline control(no inhibitor present)

TABLE II

Hapten Inhibition Experiment with Antiserum to L-1SS Using L-1BG as the Precipitating Antigen.

C. Whole Serum

Drug	Dilution	Klett Readings			Average	Average -Blank	Ratio*
L-135	1.5×10^{-2}	165	162	164	164	87	0.46
	1.5×10^{-3}	209	212	210	210	133	.70
	1.5×10^{-4}	259	255	256	257	180	.94
	1.5×10^{-5}	271	271	266	269	192	1.01
	1.5×10^{-6}	268	273	267	269	192	1.01
L-205	1.5×10^{-2}	181	181	184	182	105	0.55
	1.5×10^{-3}	227	225	223	225	148	.78
	1.5×10^{-4}	260	260	264	261	184	.96
	1.5×10^{-5}	263	265	-	264	187	.98
	1.5×10^{-6}	268	273	273	271	194	1.02
L-1	1.5×10^{-2}	167	165	170	167	90	0.47
	1.5×10^{-3}	197	193	194	195	118	.62
	1.5×10^{-4}	249	246	240	245	168	.88
	1.5×10^{-5}	269	268	261	266	189	.99
	1.5×10^{-6}	271	269	270	270	193	1.01
Saline Control		271	271	262	268	191	

*Ratio:- Ratio of amount of precipitate to amount of precipitate obtained in saline control(no inhibitor present)

TABLE II

Hapten Inhibition Experiment with Antiserum to L-1SS Using L-1BG as the Precipitating Antigen.

D. Whole Serum

Drug	Dilution	Klett Readings		Average	Average -Blank	Ratio*
L-136	1.5×10^{-2}	211	214 212	212	133	0.55
	1.5×10^{-3}	234	260 250	248	169	.70
	1.5×10^{-4}	-	295 -	295	216	.90
	1.5×10^{-5}	313	291 318	307	228	.95
	1.5×10^{-6}	304	316 317	312	233	.97
Saline	Control	316	320 321	319	240	
L-172	1.5×10^{-2}	198	199 203	200	124	.52
	1.5×10^{-3}	259	249 252	253	177	.74
	1.5×10^{-4}	300	312 301	304	228	.95
	1.5×10^{-5}	314	304 308	309	233	.97
	1.5×10^{-6}	310	313 309	311	235	.98
L-161	1.5×10^{-2}	225	227 221	224	148	.61
	1.5×10^{-3}	259	263 260	261	185	.77
	1.5×10^{-4}	302	302 302	302	226	.94
	1.5×10^{-5}	318	317 316	317	241	1.00
	1.5×10^{-6}	320	320 314	318	242	1.00
Saline	Control	322	320 310	317	241	

*Ratio:- Ratio of amount of precipitate to amount of precipitate obtained in saline control (no inhibitor present)

TABLE II

Hapten Inhibition Experiment with Antiserum to L-1SS Using L-1BG as the Precipitating Antigen.

E. Globulin Fraction

Drug	Dilution	Klett Readings			Average	Average -Blank	Ratio*
L-135	$1.5x10^{-2}$	-	136	134	135	60	0.35
	$1.5x10^{-3}$	-	195	201	198	123	.71
	$1.5x10^{-4}$	-	228	236	232	157	.91
	$1.5x10^{-5}$	-	228	246	237	162	.94
	$1.5x10^{-6}$	-	221	245	233	158	.91
L-134	$1.5x10^{-2}$	171	165	-	168	93	.54
	$1.5x10^{-3}$	208	198	-	203	128	.74
	$1.5x10^{-4}$	246	237	-	242	167	.97
	$1.5x10^{-5}$	255	241	-	248	173	1.00
	$1.5x10^{-6}$	258	243	-	251	176	1.01
L-1	$1.5x10^{-2}$	147	144	152	148	73	0.42
	$1.5x10^{-3}$	190	188	192	190	115	.67
	$1.5x10^{-4}$	241	237	237	238	163	.94
	$1.5x10^{-5}$	252	255	252	253	178	1.03
	$1.5x10^{-6}$	248	252	256	252	177	1.02
Saline Control		251	244	249	248	173	

*Ratio:- Ratio of amount of precipitate to amount of precipitate obtained in saline control (no inhibitor present)

TABLE II

Hapten Inhibition Experiment with Antiserum to L-1SS Using L-1BG as the Precipitating Antigen

F. Globulin Fraction

Drug	Dilution	Klett Readings		Average	Average -Blank	Ratio*
L-136	1.5×10^{-2}	148	146 148	147	70	0.43
	1.5×10^{-3}	202	207 183	197	120	.73
	1.5×10^{-4}	245	251 229	242	165	1.01
	1.5×10^{-5}	250	252 229	244	167	1.02
	1.5×10^{-6}	251	251 240	247	170	1.04
L-172	1.5×10^{-2}	153	153 148	151	74	0.45
	1.5×10^{-3}	218	205 205	209	132	.81
	1.5×10^{-4}	237	236 245	239	162	.99
	1.5×10^{-5}	249	246 250	248	171	1.04
	1.5×10^{-6}	235	249 243	242	165	1.01
L-170	1.5×10^{-2}	139	139 140	139	62	0.38
	1.5×10^{-3}	165	167 168	167	90	.55
	1.5×10^{-4}	223	214 222	220	143	.87
	1.5×10^{-5}	245	240 241	242	165	1.01
	1.5×10^{-6}	239	245 228	237	160	0.98
Saline Control		235	247 241	241	164	

*Ratio:- Ratio of amount of precipitate to amount of precipitate obtained in saline control (no inhibitor present)

TABLE II

Hapten Inhibition Experiment with Antiserum to L-1SS Using L-1BG as the Precipitating Antigen.

G. Globulin Fraction

Drug	Dilution	Klett	Readings		Average	Average -Blank	Ratio*
L-137	$1.5x10^{-2}$	-	158	159	158	82	0.46
	$1.5x10^{-3}$	215	211	221	216	140	.78
	$1.5x10^{-4}$	247	248	252	249	173	.97
	$1.5x10^{-5}$	251	-	257	254	178	1.00
	$1.5x10^{-6}$	256	254	260	257	181	1.01
L-161	$1.5x10^{-2}$	157	156	163	159	83	0.46
	$1.5x10^{-3}$	205	203	208	205	129	.72
	$1.5x10^{-4}$	241	234	245	240	164	.92
	$1.5x10^{-5}$	-	251	249	250	174	.97
	$1.5x10^{-6}$	254	242	241	246	170	.95
L-208	$1.5x10^{-2}$	132	-	133	133	57	.33
	$1.5x10^{-3}$	198	196	198	197	121	.68
	$1.5x10^{-4}$	245	-	251	248	172	.96
	$1.5x10^{-5}$	254	265	262	260	184	1.03
	$1.5x10^{-6}$	255	262	261	259	183	1.02
Saline Control		255	255	254	255	179	

*Ratio:- Ratio of amount of precipitate to amount of precipitate obtained in saline control (no inhibitor present)

TABLE II - H

Hapten Inhibition Experiment with Antiserum to L-1SS Using L-1BG as the Precipitating Antigen

Summary of Tests with Most Concentrated Drug Solution

Drug	Whole Serum Ratio	Globulin Fraction Ratio
L-208	0.41	0.33
L-173	.45	
L-135	.46	.35
L-170	.47	.38
L-171	.48	
L-137	.50	.46
L-172	.52	.45
L-136	.55	.43
L-205	.55	
L-134	.58	.54
L-161	.61	.46
L-1	.47	.42

TABLE III

Hapten Inhibition Experiment with Antiserum to L-1SS Using L-1BG as the Precipitating Antigen.

Drug	Klett Readings			Average	Average-Blank	Ratio*
L-208	131	133	135	133	54	0.24
L-204	137	136	135	136	57	.26
L-173	139	140	137	139	60	.27
L-171	141	141	140	141	62	.28
L-205	140	141	140	140	61	.28
L-135	144	151	137	144	65	.29
L-172	144	144	143	144	65	.29
L-161	147	150	153	150	71	.32
L-136	152	151	151	151	72	.33
L-170	156	153	150	153	74	.33
L-202	152	154	152	153	74	.33
L-134	155	154	154	154	75	.34
L-203	-	154	153	154	75	.34
L-206	159	161	157	159	80	.36
L-137	164	163	163	163	84	.38
L-207	172	176	175	174	95	.43
L-201	179	177	177	178	99	.45
L-1	135	135	132	134	55	.25
Saline Control	305 301	293 306	296 297	300	221	

*Ratio:- Ratio of amount of precipitate to amount of precipitate obtained in saline control(no inhibitor present)

TABLE III

Hapten Inhibition Experiment with Antiserum to L-1SS Using L-1BG as the Precipitating Antigen.

Simple Compound	Klett Readings			Average	Average-Blank	Ratio*
1-a	220	218	218	219	139	0.63
1-b	219	216	219	218	138	.63
3	228	236	230	231	151	.69
6	273	262	277	271	191	.87
5	272	272	276	273	193	.88
12	275	277	268	273	193	.88
13	270	275	274	273	193	.88
9	286	278	274	279	199	.90
7-a	281	278	284	281	201	.91
7-b	285	284	278	282	202	.92
8	292	287	285	288	208	.95
4	292	297	283	291	211	.96
11	293	297	295	295	215	.98
2	298	297	301	299	219	1.0
Saline Control	299 298	298 300	305 300	300	220	

*Ratio:- Ratio of amount of precipitate to amount of precipitate obtained in saline control(no inhibitor present)

TABLE IV

Substituted Piperidines Tested for Local Anesthetic Action

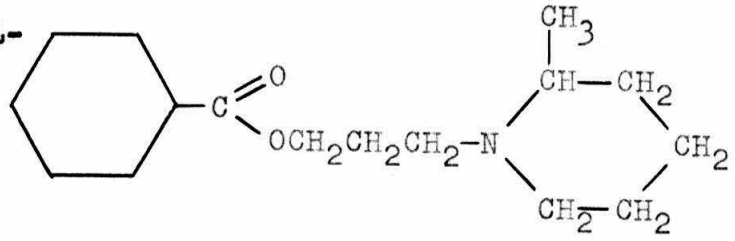
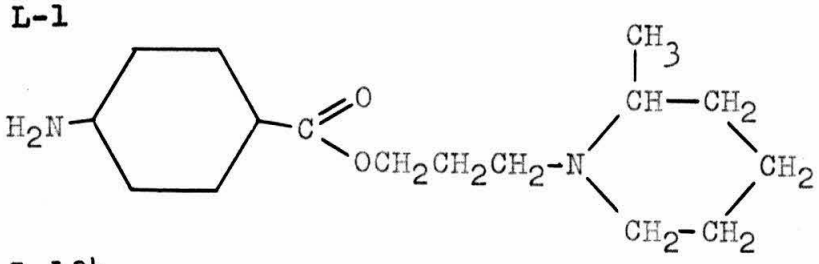
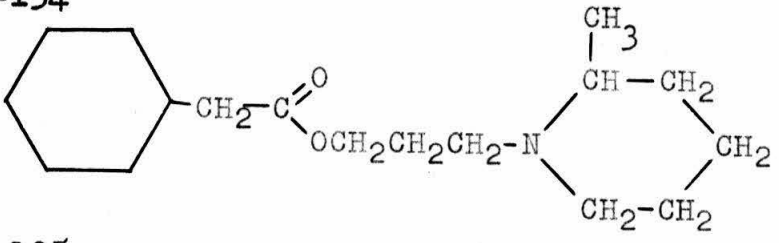
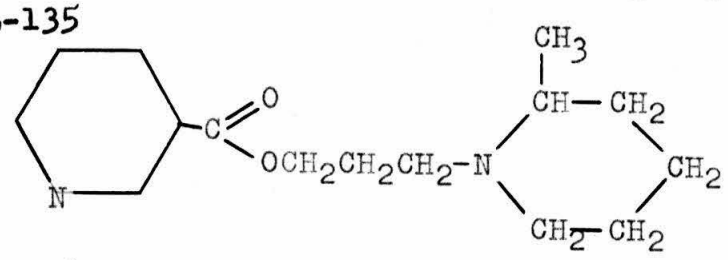
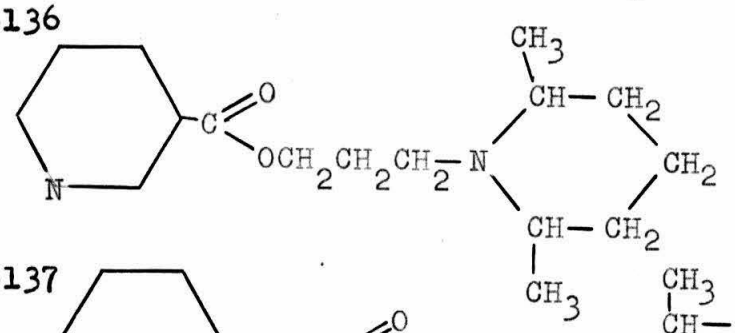
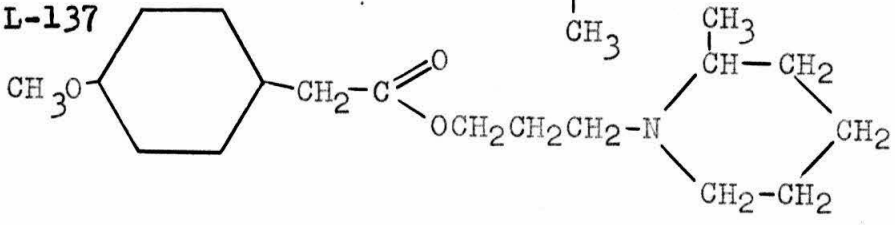
Free Base	Molecular Weight of Hydrochloride
L- 	297.8
L-1 	312.8
L-134 	311.9
L-135 	298.8
L-136 	312.8
L-137 	341.9

TABLE IV

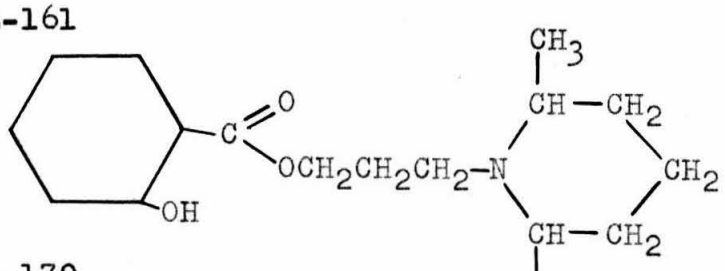
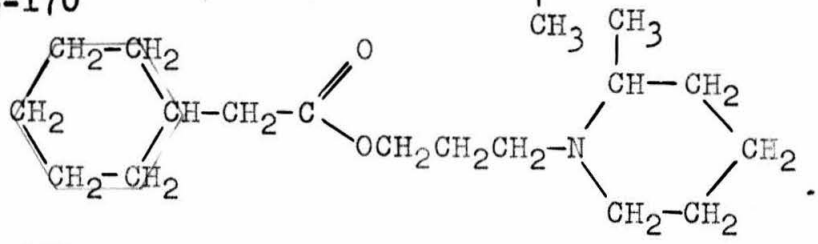
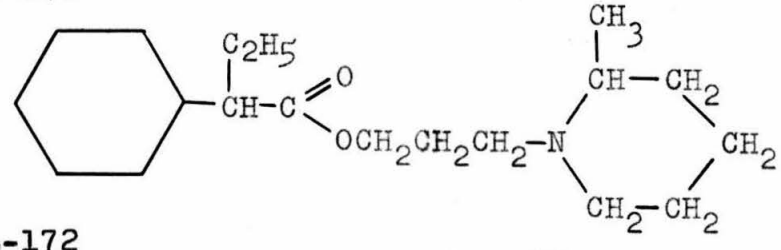
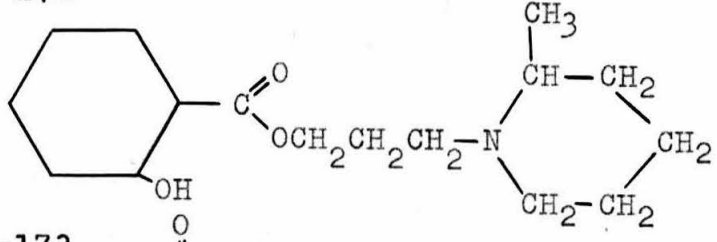
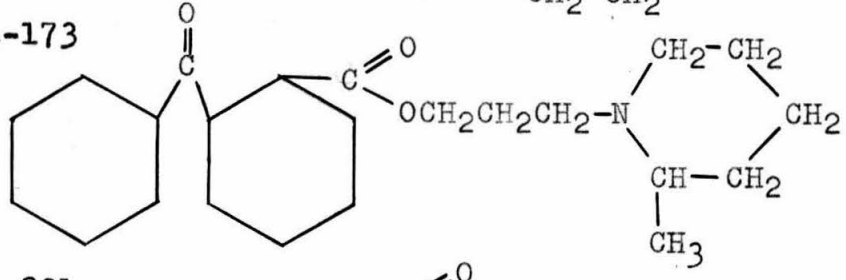
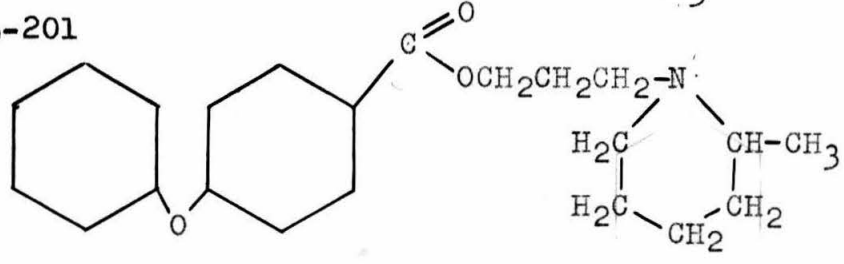
Free Base	Molecular Weight of Hydrochloride
L-161 	327.9
L-170 	317.9
L-171 	339.9
L-172 	313.8
L-173 	401.9
L-201 	389.9

TABLE IV

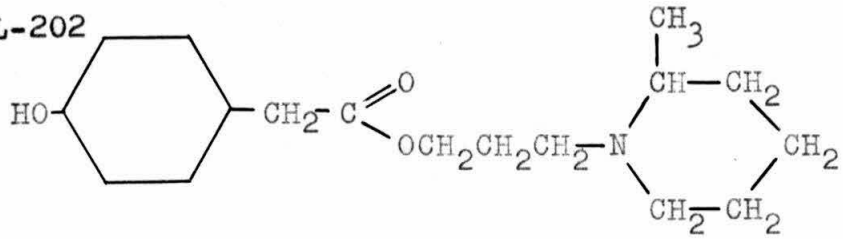
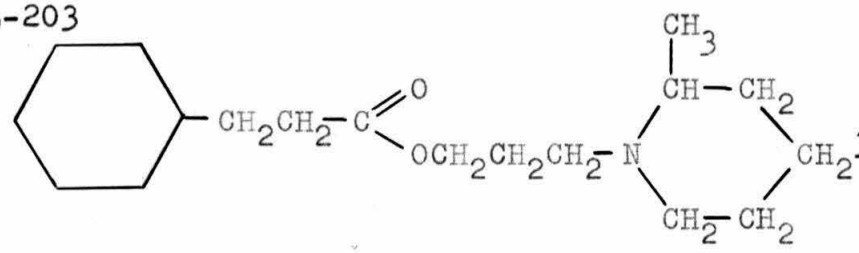
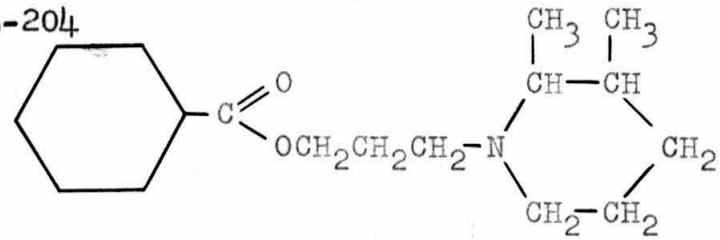
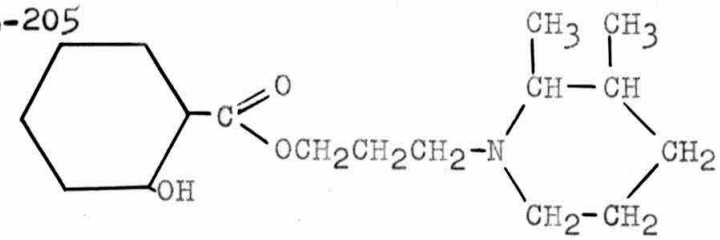
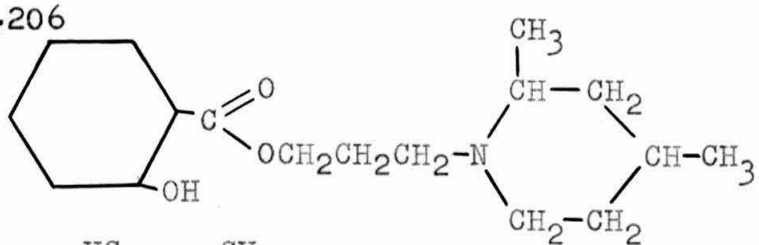
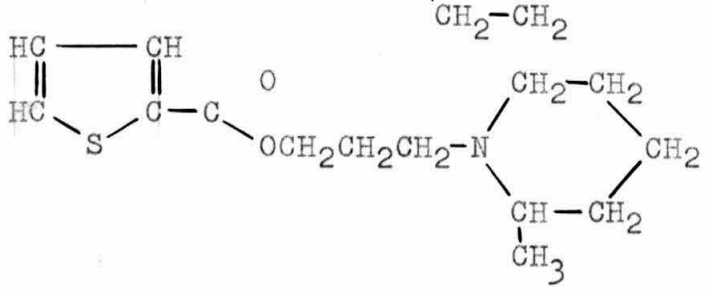
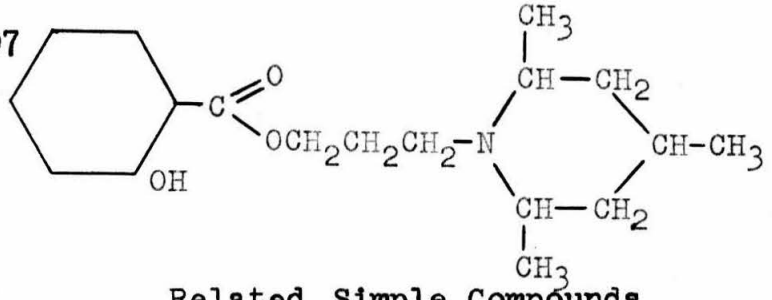
Free Base	Molecular Weight of Hydrochloride
L-202 	327.9
L-203 	325.9
L-204 	311.9
L-205 	327.9
L-206 	327.9
L-208 	303.9

TABLE IV

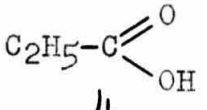
Free Base	Molecular Weight of Hydrochloride
L-207 	341.9

Related Simple Compounds

1 - a Two different
1 - b solutions of

$(C_2H_5)_3N$

2



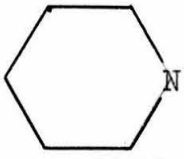
3

$(CH_3CH_2CH_2CH_2)_3N$

4

$CH_3CH_2CH_2OH$

5



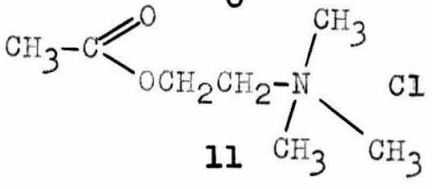
6

$CH_3CH_2CH_2NH_2$

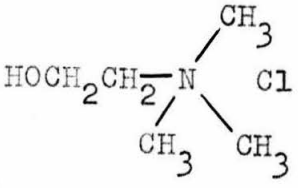
7 - a Two different
7 - b solutions of

$(CH_3)_3N$

8



9



11

$(CH_3)_2NH \cdot HCl$

12

$(C_2H_5)_2NH \cdot HCl$

13

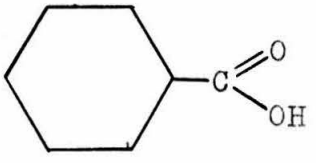


TABLE V

The Toxicities of Variously Substituted Piperidines

Intravenous		Subcutaneous	
Drug	L.D. ± S.E. mg./kg. in mice	Drug	L.D. ± S.E. mg./kg. in mice
L-136	115 ± 6	L-204	1600 ± 69
L-135	100 ± 9	L-202	1550 ±185
L-202	74 ± 4	L-134	800 ± 64
L-203	59 ± 2	L-208	730 ± 43
L-134	52 ± 2	L-203	690 ±117
L-137	42 ± 4	L-	589 ± 65
L-201	35 ± 2	L-207	510 ± 24
L-204	35 ± 2	L-135	500 ± 11
L-161	35 ± 1	L-206	410 ± 48
L-172	34 ± 1	L-161	386 ± 27
L-170	31 ± 4	L-205	345 ± 29
L-207	27 ± 1	L-136	330 ± 39
L-208	26 ± 1	L-171	305 ± 30
L-	22 ± 1	L-170	290 ± 39
L-206	20 ± 1	L-137	235 ± 15
L-171	18 ± 1	L-201	156 ± 17
L-205	18 ± 1	L-173	155 ± 24
L-173	9 ± 1		

TABLE VI

The Local Anesthetic Action of

Variously Substituted Piperidines

Drug	% Solution	T (minutes)	Drug	% Solution	S (minutes)
L-173	0.25	35	L-170	1	50
L-201	0.25	30	L-206	1	45
L-161	1	23	L-161	1	39
L-172	1	21	L-171	1	34
L-206	1	19	L-134	1	30
L-205	1	15	L-202	1	28
L-207	1	15	L-203	1	23
L-	1	10	L-135	1	8
L-208	1	5	L-137	1	7
L-204	1	5	L-136	1	3
L-136	1	0			
L-134	1	0			
L-202	1	0			
L-137	1	0			
L-171	1	0			
L-203	1	0			
L-170	1	0			
L-135	1	0			

T - "duration of topical anesthesia was determined by application of a solution of the anesthetic.....to the rabbit's cornea"

S - "subcutaneous anesthesia was determined on the guinea pig's skin"

TABLE VII

Comparison of Hapten Inhibition with Local Anesthetic Data.

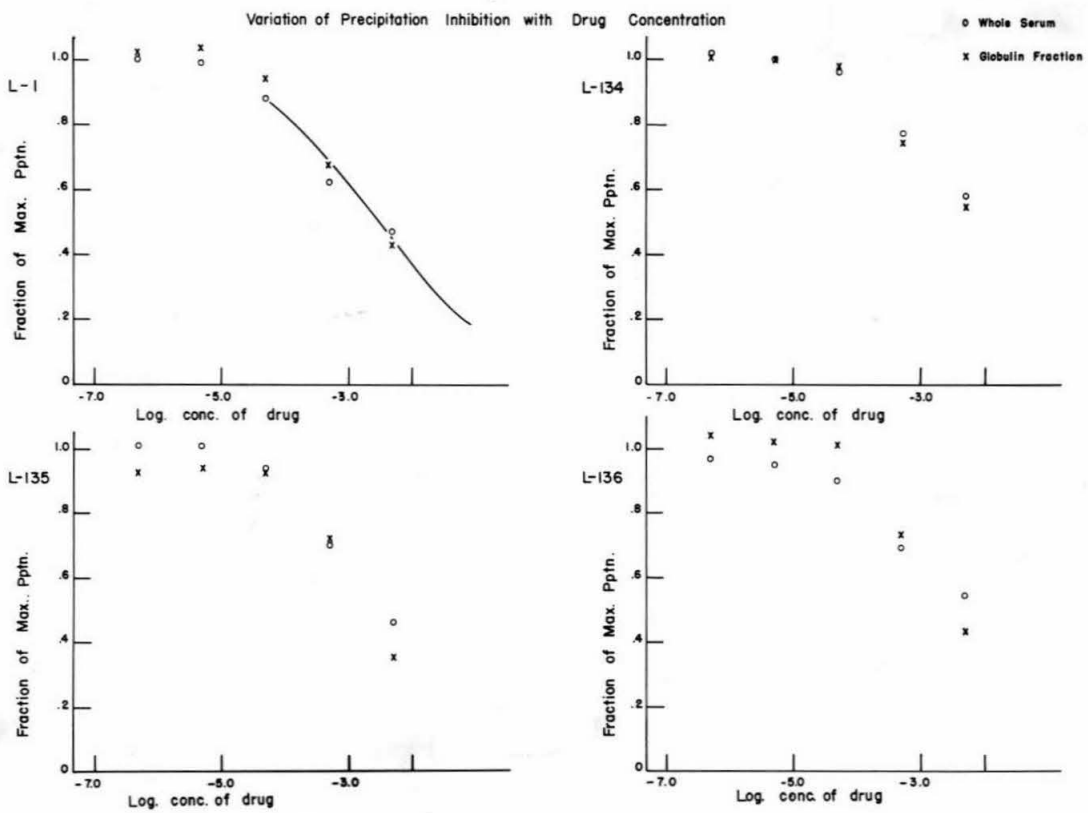
Drug	Hapten Inhibition Constant		Local Anesthetic Action	
	Second Series	First Series	T	S
L-208	1.12	1.47	5	-
L-204	0.89	-	5	-
L-173	.81	1.00	0.25%- 35	-
L-171	.72	0.79	0	34
L-205	.72	.38	15	-
L-135	.67	.85	0	8
L-172	.67	.56	21	-
L-161	.50	.28	23	39
L-136	.45	.56	0	3
L-170	.45	.89	0	50
L-202	.45	-	0	28
L-134	.41	.32	0	30
L-203	.41	-	0	23
L-206	.34	-	19	45
L-137	.28	.50	0	7
L-207	.18	-	15	-
L-201	.14	-	0.25%- 30	-

TABLE VIII

Comparison of Hapten Inhibition and Pharmacological Data.

Drug	Mol.Wt.	Hapten Inhibition Constant		Toxicity		Local Anesthetic Action	
		Second Series	First Series	i.v.	s.c.	T	S
L-208	304	1.12	1.47	26±1	730±43	5	-
L-135	299	0.67	0.85	100±9	500±11	0	8
L-204	312	0.89	-	35±2	1600±69	5	-
L-172	314	.67	.56	34±1	-	21	-
L-136	313	.45	.56	115±6	330±39	0	3
L-134	312	.41	.32	52±2	800±64	0	30
L-205	328	.72	.38	18±1	345±29	15	-
L-161	328	.50	.28	35±1	386±27	23	39
L-202	328	.45	-	74±4	1550±185	0	28
L-206	328	.34	-	20±1	410±48	19	45
L-203	326	.41	-	59±2	690±117	0	23
L-171	340	.72	.79	18±1	305±30	0	34
L-137	342	.28	.50	42±4	235±15	0	7
L-207	342	.18	-	27±1	510±24	15	-
L-173	402	.81	1.00	9±1	155±24	0.25% 35	
L-201	390	.14	-	35±2	156±17	0.25% 30	

Figure 1


Variation of Precipitation Inhibition with Drug Concentration
o Whole Serum
x Globulin Fraction
L-1
L-134
L-135
L-136
Fraction of Max. Pptn.
Log. conc. of drug
1.0
.8
.6
.4
.2
0
-7.0
-5.0
-3.0

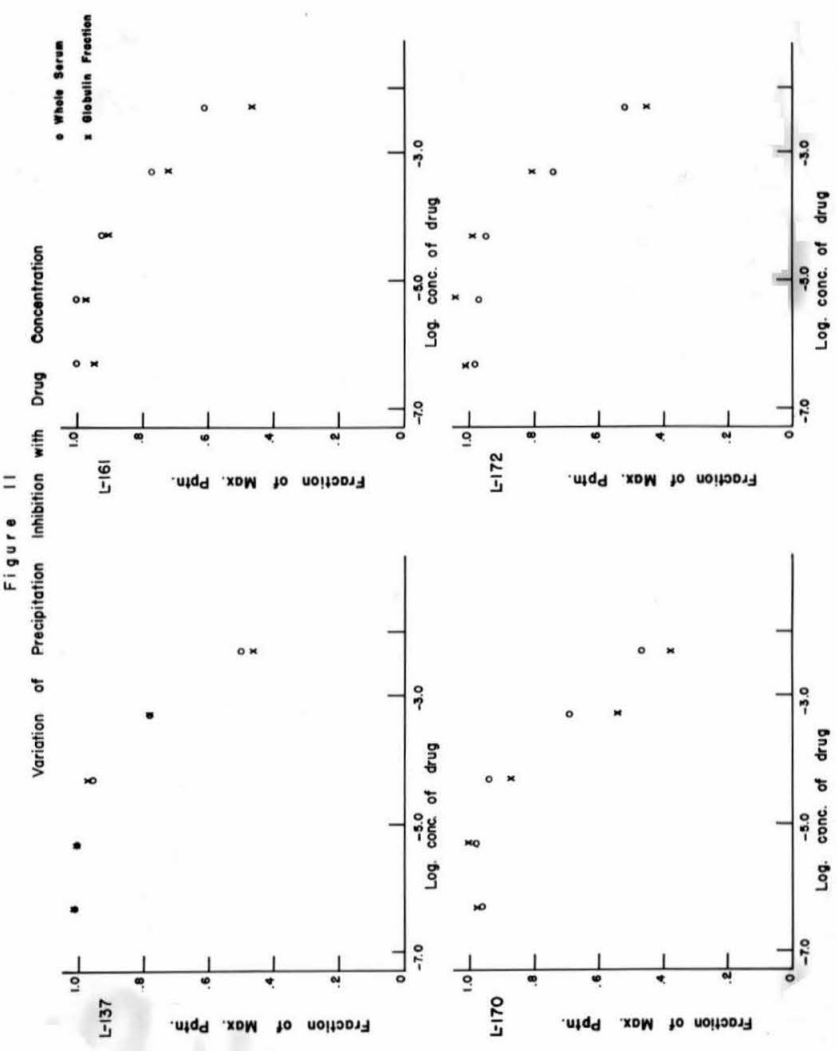
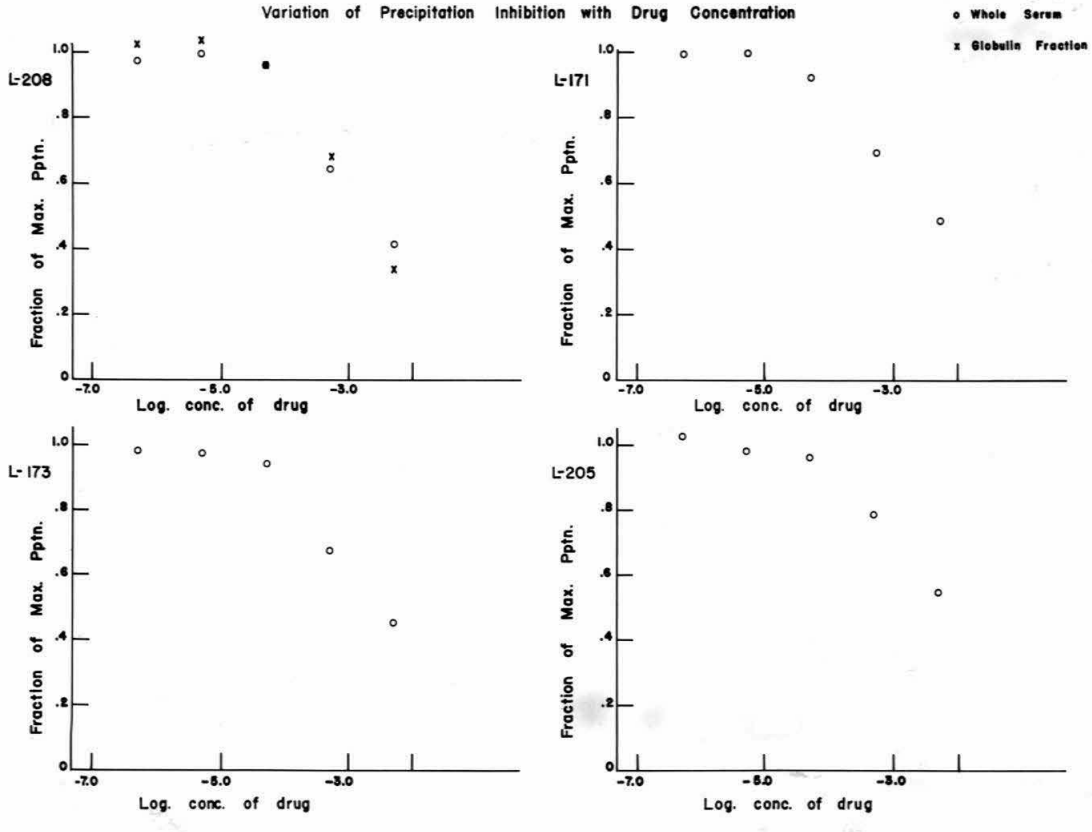


Figure 11
Variation of Precipitation Inhibition with Drug Concentration
o Whole Serum
x Globulin Fraction
L-161
L-137
L-172
L-170
Fraction of Max. Pptn.
Log. conc. of drug
1.0
.8
.6
.4
.2
0
-7.0
-5.0
-3.0

Figure III

Variation of Precipitation Inhibition with Drug Concentration



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PART II

THE INCORPORATION OF C^{14}-CARBOXYL-LABELED LEUCINE BY SERUM PROTEINS

INTRODUCTION

Quite recently many workers have attempted to study the reactions of antigens and antibodies with the aid of isotopic tracer methods and it is probable that these methods will be used extensively in the future. However, most of the studies made to date have been carried out by labeling the material to be studied with an element or group not normally present in the molecule itself. Although labeling the normal constituents of a protein is a time consuming and laborious process as compared to other methods of labeling it is certainly the most satisfactory method for the carrying out of certain experiments in which unchanged native molecular structures are required.

The greatest number of investigations have been carried out by iodinating the protein material with I^{131}. Butement (1) was one of the first workers to investigate the possibilities of labeling with radioactive iodine and he found that iodination had little or no effect upon agglutinating antibodies for proteus vulgaris. He is perhaps the only worker to have considered the effect of the distribution of the iodine atoms on the different protein molecules. Pressman and his coworkers have used I^{131} to carry out studies on the "zone of localization" of antibodies (2-14). They prepared antibodies directed against specific animal tissues (usually rat or mouse kidney, lung, or liver) and these antibodies were then labeled with radioactive iodine

and injected into normal rats or mice. The localization of these antibodies was then followed. Warren and Dixon have studied anaphylactic shock in guinea pigs with I^{131}-labeled bovine gamma globulin (15) (16). Eisen and Keston (17) have studied the precipitation reaction of bovine serum albumin and anti-bovine serum albumin using this label. Haurowitz and his coworkers (18)(19)(20) have studied the distribution of I^{131} in rabbits following the administration of iodine-labeled antigens and similar work has been carried out by Dixon, Bukantz and their coworkers (21)(22)(23)(24)(25) including studies on rabbits exposed to X-radiation (21). Knox and Endicott (26) have measured the rate of disappearance of iodine-labeled bovine serum albumin from the blood stream of rabbits. Pressman and Sternberg (27) have studied the rate of iodination of antibodies with I^{131} and later studied the protection of the specific antibody site by haptens during iodination (28). Banks et al. (29) have also used I^{131} to label antigens and antibodies and have conducted hapten inhibition tests with labeled antigens, antibodies, and haptens (30). Masouredis et al. (31) have carried out precipitation studies with human serum albumin labeled with iodine and Gitlen et al. (32) have used this isotope to estimate the half life of protein antigens. Latta (33) has also measured the blood and tissue concentrations of I^{131} after the administration of I^{131}-labeled antigen.

S^{35} has been used also as a label for antigens and antibodies. Boursnell et al. (34) used 4,4'-dichlorodiethyl-sulfone to tag proteins and Pressman et al. have also used

S^{35} for a label in their localization studies by diazotizing aminobenzenesulfonic acid onto the antibodies (35).

Banks et al. (36) used P^{32} in the phosphorylation of proteins and investigated the fate of these proteins when injected into rabbits. A number of workers are continuing this type of work and many of the results are appearing in the Journal of Immunology.

Fewer workers have attempted to isotopically label the normal constituents of the antigen or antibody molecule but this has been done successfully. The earliest studies with labeled antibodies were those of Schoenheimer, Heidelberger, and their coworkers (37)(38)(39). They fed N^{15}-labeled amino acids to rabbits and followed the incorporation of the heavy nitrogen into the serum proteins. Libby and his coworkers (40)(41) have determined the fate of tobacco mosaic virus in mice by following P^{32} activity which was incorporated into the virus from the plant on which it grew. Boursnell et al. (34) and their coworkers have carried out investigations on the antigen-antibody reaction of the lipovitellin-antilipovitellin system by using lipovitellin isolated from the eggs of hens receiving P^{32}-labeled phosphate ion. This group studied the antigen-antibody precipitation reaction of the system but because of the complexity of the antigen used and the reaction of the antibody with phospholipids present they concluded that this was a poor system to study quantitatively (29)(36)(42)(43)(44). They also studied the fate of P^{32} containing proteins in vivo (36)(44). Kooyman

and Campbell (45) were the first to use a C^{14}-labeled amino acid in this type of work and they carried out experiments similar to those of Schoenheimer and his coworkers. Keston and Dreyfus (46) have studied the incorporation of C^{14}-tagged glycine into antibody proteins in vitro in some experiments undertaken to critically evaluate the work on the in vitro synthesis of proteins by tissue slices and homogenates. Similar experiments have been performed by Ranney and London (47). A report on the work discussed in this thesis using C^{14}-labeled leucine has also been given (48).

As stated, Schoenheimer, Heidelberger and their coworkers fed N^{15}-labeled amino acids to rabbits and followed the incorporation of the heavy nitrogen into the serum proteins. Their conclusions were that antibody proteins, like other proteins, take up the heavy nitrogen at a rapid rate during the process of formation. They found that this occurs even when the concentration of circulating antibody is diminishing, indicating that new antibody is being formed though the rate of degradation is greater than the rate of synthesis. The authors also concluded that injected antibody globulin (passively transferred antibody) either did not incorporate the isotope or else did so with the loss of its specific antibody properties, since rabbit antibody injected into a normal rabbit receiving labeled glycine showed only a very small uptake of heavy nitrogen and this seemed to be a non-specific effect.

When C^{14} became available in sufficient quantities

Kooyman and Campbell (45) decided to repeat the work of Schoenheimer et al. with this very easily handled tracer. C^{14} offers the advantage of being detectable in very low concentrations in a relatively simple manner. In some preliminary experiments Kooyman and Campbell prepared C^{14}-labeled leucine and administered this to rabbits by intraperitoneal injections. The rabbits used had been immunized and while receiving the labeled amino acid they also received intravenous injections of different antibody solutions. Radioactivity was found in passively transferred rabbit antibody but none was found in passively transferred horse antibody. Kooyman and Campbell thought it was very probable that an exchange took place with the passive homologous antibody. Since there was some question regarding the quantitative significance of their data, which contradicted the earlier conclusions of Schoenheimer and Heidelberger, the following experiments were performed. They are essentially a repetition of the earlier work by Kooyman and Campbell but were performed under more precise quantitative conditions.

EXPERIMENTAL

The synthesis of carboxyl-labeled leucine. Leucine was chosen for these initial experiments because it occurs in large amounts in serum proteins and also its synthesis from C^{14}-labeled barium carbonate is not too difficult. The activity incorporated into the leucine had to be somewhat arbitrary but considering the earlier work it was decided

to prepare leucine with an activity of about 10,000 counts per minute per mg in the counter used. This corresponds to $10^4 \times 10^3$ or 10^7 counts per minute per gram. Allowing a factor of 20 for the efficiency of the counter the material should have a specific activity of

$$\frac{2 \times 10^8}{3.7 \times 10^7 \times 60} = 9 \times 10^{-2}$$

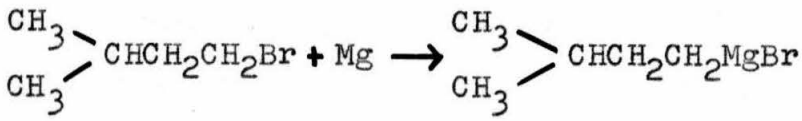
millicuries per gram. As yields of about 30% were expected it was decided that the synthesis would be started from about 0.4 millicuries of barium carbonate and this should yield about 1.5 grams of *dl*-leucine of suitable activity.

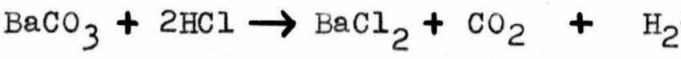
A number of methods for the preparation of leucine have been described, the most important of these being I. Isocaproic acid → bromoisocaproic acid → leucine, II. Strecker synthesis starting from isovaleraldehyde, and III. Saponification followed by decarboxylation and hydrolysis of isobutyl aminoacyl malonic ester. Kooyman (49) had worked out a synthesis of C^{14}-carboxyl-labeled leucine following I and so it was decided to attempt to repeat this synthesis. Although this method is relatively simple it has the disadvantage of starting with a relatively impure material (isoamyl bromide) which yields isomeric compounds in the final product. Since this work was undertaken Borsook et al. (50) have published a synthesis of C^{14}-labeled leucine using the Strecker synthesis and as they obtain the isovaleraldehyde in high purity their synthesis has the

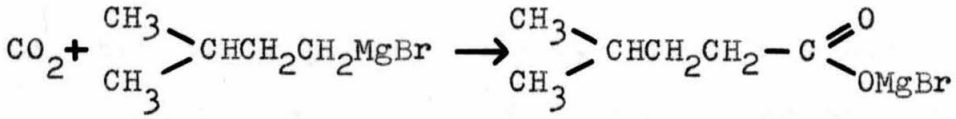
advantage of yielding a purer product.

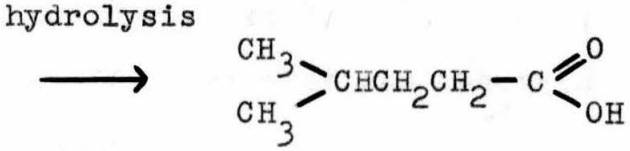
Kooyman (49) had adapted the method of Cheronis and Spitzmueller (51) to convert the bromoisocaproic acid to leucine and doing this reported a high overall yield. However, preliminary trials with inactive material failed to reproduce these high yields and modifications of his procedures were used.

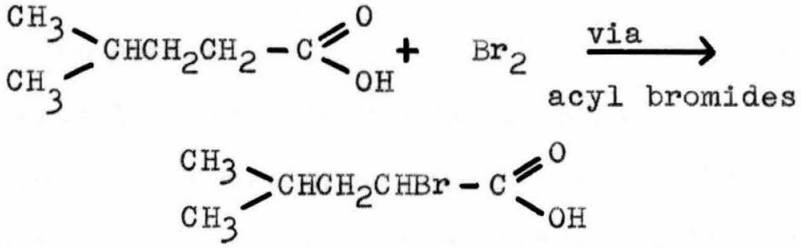
The synthesis was carried out according to the following series of reactions-

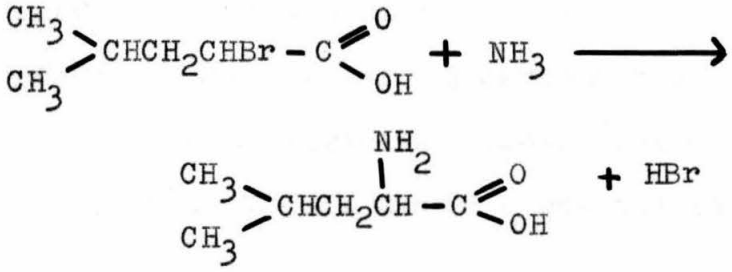
a) 







b) 

c) 

Eastman (white label) isoamyl bromide was distilled several times collecting the fraction coming over at 117° - 119° C. and a solution of isoamyl magnesium bromide was prepared from this. One hundred millimoles (2.4 g) of clean magnesium ribbon was placed in a round bottom flask having a condenser with a drying tube on it. A solution of one hundred millimoles (15.1 g) of the isoamyl bromide in 45 ml of anhydrous ether was slowly added while stirring vigorously. There was no difficulty in starting the reaction especially if a fairly concentrated solution of the isoamyl bromide was added initially. After the reaction had slowed to the point of nearly stopping the reaction mixture was heated in a water bath at 37° C. for a period of about 15 minutes. Unreacted magnesium was removed by filtration under pressure through a sintered glass filter into a flask attached to the carbon dioxide apparatus. The 100 millimoles of isoamyl bromide corresponded to an excess of 150% over what was required to react with the carbon dioxide produced.

The apparatus for the carbonation of the Grignard solution is shown in Figure I. Separating funnel A contained 6 N hydrochloric acid and emptied into tube B which contained the sample of radioactive barium carbonate. The capacity of B was about 3 ml. The three necked flask C contained inactive barium carbonate which furnished carbon dioxide to wash the labeled carbon dioxide through the apparatus. Drying tower D was filled with anhydrous

calcium chloride and the washing tower E contained concentrated sulfuric acid. Stopcock F was used in order to be able to evacuate the apparatus. The washing of the carbon dioxide through the concentrated sulfuric acid seemed to be necessary. G was another stopcock which served to cut off the apparatus when evacuated from the flask H to which the Grignard reagent was transferred. Before a run was started the whole apparatus was evacuated and stopcocks F and G were then closed.

The Grignard reagent was filtered into flask H which was immediately immersed in a dry ice-methyl cellosolve bath. Flask H was then evacuated until the ether solution began to boil and then stopcock I was closed. A few drops of the hydrochloric acid were slowly admitted to tube B containing the 0.4 millicuries of barium carbonate. This corresponded to about 40 mg of the 3% tagged material supplied by the Atomic Energy Commission. After this had reacted more hydrochloric acid was slowly admitted and allowed to overflow into the flask C which contained 7.86 grams of pure inactive barium carbonate. In general a few glass beads were also placed in flask C as they seemed to aid in the mixing of the acid and the solid material. The pressure was allowed to slowly increase to about 70 cm. and then stopcock G was slowly opened. Flask H was gently shaken while the gas was being absorbed. The fall in pressure virtually ceased in about 4-5 minutes at which time stopcock G was closed and F opened. Flask H was

disconnected and 15 grams of crushed ice was added to it. Thirty five ml of 6 N sulfuric acid was added slowly with shaking and the mixture was allowed to warm up to room temperature. The contents of the flask were rinsed into an apparatus for continuous extraction with ether.

The aqueous layer was extracted for four hours and the ether extract was dried over anhydrous sodium sulfate to remove traces of magnesium salts. The isocaproic acid was separated from the non-acidic reaction products by extraction with 4 N sodium hydroxide, using three portions of 12, 4, and 4 ml respectively. This aqueous extract was washed once with 5 ml of ether, acidified and extracted with ether. The solution was dried over anhydrous sodium sulfate and then concentrated under reduced pressure.

The bromination of the small amount of isocaproic acid had proved rather difficult and it was found advantageous to distill the crude isocaproic acid in a small vacuum still at this point and then a small amount of dried stockroom isocaproic acid was used to rinse the tagged material into a one hundred ml round bottom flask for the bromination procedure. Four-tenths of a gram of red phosphorus was added to the acid and a condenser carrying a small separatory funnel and a gas outlet tube was fitted to the round bottomed flask. Twelve grams of bromine which had been dried over concentrated sulfuric acid were added through the condenser in the course of four hours while keeping the temperature of the flask at 50 - 60° C. The flask

was then kept at 70 - 75° C. for another three hours and it was finally heated on a boiling water bath for one hour. The mixture was allowed to stand overnight at room temperature and on the following day 15 grams of crushed ice, 15 ml of chloroform, and 5 ml of a 1% solution of sodium metabisulfite were added. The mixture was stirred for fifteen minutes and the brominated acids were extracted with chloroform after making sure the aqueous phase was quite acid by adding a little sulfuric acid. (This method of bromination is essentially the same as described by Hickenbottom (52).) The chloroform solution was concentrated in vacuo and the brominated acids were distilled in vacuo, the main fraction boiling from 88 - 90° C. at 2 mm. pressure. A little unreacted isocaproic acid was collected with the distillate. The tube containing the distillate was transferred to a pyrex tube 45 cm. in length and 28 mm. in outside diameter. This avoided the loss of any material during the transfer. The large pyrex tube was a standard combustion tube which was capable of withstanding the 10 atm. pressure produced in the treatment with liquid ammonia.

Dry ammonia gas was condensed into the pyrex tube containing the mixture of bromoisocaproic acid and isocaproic acid making use of a drying tube containing sodium hydroxide pellets to keep the system dry. The tube was maintained at -80° C. and sealed when 25 ml of ammonia had been condensed. The tube was allowed to warm up to room temperature and the contents were mixed. The tube was allowed to stand for

forty-eight hours at room temperature, encased in an iron pipe as a safety measure. The tube was cooled again to -80° C. and opened. The ammonia was allowed to slowly evaporate off. The last traces were removed by evacuating the tube while keeping it at 40° C.

The resulting solid product was repeatedly washed with 95% ethyl alcohol using a total volume of 75 ml. The product was recrystallized from 50% alcohol as described by Marvel (53). The final product melted with decomposition below the prescribed 332° C. but the benzoyl derivative was prepared which melted at the required 141°C. A sample prepared by Dr. Kooyman was analysed and on duplicate determinations gave for the total nitrogen 10.8 and 10.8%, for amino nitrogen by the Van Slyke method 10.7 and 10.6%, for alpha amino nitrogen by the ninhydrin method 10.8 and 11.0%, as compared to the theoretical amount of nitrogen of 10.68%.

The final yield was about 1.5 grams which corresponds to an overall yield on barium carbonate of about 30%. No attempt was made to racemize the mixture as this would have involved the loss of valuable material. The presence of the *d* isomer would not influence the planned experiments as the object was only to obtain a high level of radioactivity in the serum proteins. The solution from the recrystallization yielded a little more product upon concentration and addition of alcohol.

The measurement of the radioactivity. Usually the measurement of C^{14} activity is accomplished by converting the

material to carbon dioxide and either determining the activity of the gas or reacting the gas with barium hydroxide and counting the activity of the barium carbonate (54). However, as all the materials to be counted in this work were protein in nature it was decided to investigate the possibility of counting the material as a protein precipitate. From an absorption standpoint counting as protein material would be advantageous since barium carbonate is only about 6.1% carbon and protein material is at least about 30% carbon. However, it is difficult to obtain smooth reproducible surfaces on the protein precipitates and many procedures had to be tried in order to determine the most satisfactory procedure for mounting the protein material. The most important observation made was that the material had to be relatively free from water in order to avoid caking and cracking of the sample surface. The procedure finally adopted for preparing the samples was the following. The protein material to be counted was suspended in 1% saline and washed three times in a small centrifuge tube. The material was then finely ground in 95% ethyl alcohol and this was followed by three washings in 95% ethyl alcohol and three washings in anhydrous ethyl ether. About five to twenty mg of this material was made into a slurry with a few ml of anhydrous ether and this was transferred to a brass cup which was fitted so as to deposit the residue upon evaporation of the ether on to a weighed aluminum disk. The disk had a circular exposed

surface of one centimeter in radius which resulted in the residue having a surface density of about 1.5 to 6.5 mg per cm^2. The use of some adhesive material on the aluminum disks was tried but in general this was not necessary. Quite often the material would cake and crack on the disk but when this occurred pulverizing the material with a little anhydrous ether and allowing this to evaporate off gave a uniform surface. Care had to be taken to avoid swirling the slurry as this caused uneven deposition of the solid. Self absorption curves were plotted using several different tagged protein materials and these were found to be very similar to the curves for barium carbonate (54)(55)(56). A curve plotted using the antigen-antibody precipitate material from the experiment with rabbit 63 described later is shown in Figure 2. The values of the specific activity of the samples of less than 2.5 mg per cm^2 were used to evaluate the specific activity, n_o, of very thin samples. Self scattering is apparently not too great in these samples. The lower curve in Figure 2 gives the ratio of this specific activity of a very thin sample to n the observed specific activity of a thick sample. As can be seen over this relatively narrow range the curve is practically linear. This curve was used to correct the radioactivities of all the samples measured for self absorption. Sufficient data were available to show that all the protein samples gave similar self absorption effects. The measurements of the radioactivity are given later with the nine-tenths

statistical error (57). The experimental errors introduced are undoubtedly much greater than this.

The source of the radioactivity of the proteins. One question that arose in the counting of the radioactivity was to what extent the C^{14} was still present as leucine. Samples of rabbit serum albumin that had been tagged in the experiments of Dr. Kooyman were analysed for specific leucine activity. This was carried out by digesting a sample of the albumin in the presence of a large excess of inactive pure *l*-leucine. The use of hydrochloric acid seemed to be the most satisfactory for carrying this out. In general samples were digested for 24 hours at 100 - 120° C. and allowed to stand for some time at room temperature. The samples were evaporated to dryness in a desiccator and the residues were dissolved in water and neutralized with a small amount of sodium hydroxide. The solutions were decolorized by boiling for two minutes with norite and filtering. The solutions were again evaporated to dryness in a desiccator. The residues were redissolved in a small amount of water, and ethanol was added. The solutions were allowed to stand overnight in a refrigerator until a small amount of precipitate had formed. The precipitates were filtered out, washed with a mixture of alcohol and ether, and allowed to dry. The specific activity of this material which should be largely leucine only corresponded to 50 - 55% of the total activity of the albumin. No attempt has been made to investigate the distribution of

the radioactivity any further but the determination of where the rest of the activity is would furnish an interesting problem.

All the counting was done with a "Tracerlab" Geiger-Mueller counter using a "64" scaler. The end window of the Geiger tube had a thickness of 1.4 mg per $cm.^2$.

The incorporation of C^{14} activity by the blood proteins of rabbits receiving C^{14}-carboxyl-labeled *dl*-leucine. The primary purpose of these investigations was to determine if passively transferred antibody molecules could incorporate radioactivity given to an animal in the form of a labeled amino acid. To determine this the following experiments were carried out. A healthy male rabbit (Rabbit 63) weighing eight pounds had been immunized against bovine globulin *p*-azophenylarsonate (RBG) receiving the last immunizing injection on the day this experiment started. The rabbit then received three daily intraperitoneal injections (day 0, 1, and 2) of 30 ml of a 1% solution of the labeled leucine. The rabbit thus received an amount of radioactive material equivalent to about 9×10^6 counts per minute in the counter used. The leucine solution had been sterilized by filtration through a Seitz filter. On the third day it received two intravenous injections of a rabbit antiovalbumin solution receiving a total of 27 ml of solution. This solution contained 33 mg of protein per ml and gave 6.1 mg of precipitate per ml when titrated with ovalbumin. On the next day (day 4) the animal received

11 ml of a horse antibody solution which contained 49 mg of protein per ml and gave 12.5 mg of precipitate per ml when titrated with type I pneumococcus polysaccharide and 17.0 mg of precipitate per ml when titrated with type II pneumococcus polysaccharide. On day five 70 ml of blood were taken from the rabbit's ear and this was defibrinated by shaking with glass beads. The rabbit then received 35 ml of sterile saline. On day seven 60 ml of blood were taken from the ear and defibrinated. The defibrinated blood samples were centrifuged and the serum was removed. Small portions of the serum samples were titrated with bovine globulin *p*-azophenylarsonate to determine the optimum ratio. Using this ratio the active antibody was precipitated from 20 ml portions of the sera. The samples were allowed to remain 48 hours in the refrigerator before centrifuging off the precipitate. The precipitates were washed 3 times with cold saline and were then prepared for counting as outlined above. Background was determined before and after each sample was counted and after the counter had been allowed to run continuously for a twelve hour period it was found to be practically constant. The radioactivities measured with the nine-tenths statistical error are given in Tables I and II. These values were corrected to counts per minute per 10 mg of sample making the necessary correction for self absorption and the resulting values were listed.

In the earlier work the question of the effect of

complement being bound by the precipitates was raised. Complement is a naturally occurring substance or group of substances present in serum which is needed in addition to antibody for the bactericidal and hemolytic reactions of immune serum (58). Complement is removed to a large extent by the specific precipitation of antigen and antibody, being part of the precipitate complex. The presence of radioactive complement would have little effect upon the specific activity of the active antibody precipitate but would have a marked effect on precipitates of low specific radioactivity. It was suggested that the radioactivity of the passive antibody precipitates in the experiments of Dr. Kooyman might be due to complement present. Assuring the removal of a material as complex as complement would be very difficult but it is known that complement activity is destroyed by heating the serum at 56° C. for half an hour. The supernatants from the active antibody precipitations which should have little complement activity in any case were therefore heated at 56° C. for half an hour. They were then tested in the standard manner for complement activity (59) and were found to be negative. This still did not completely assure the removal of any material which might be carried down with the precipitates in a more or less nom-specific manner but it at least made it rather unlikely. The solutions were recentrifuged and titrations were run with ovalbumin to determine the optimal ratios for precipitating the passive rabbit antibody and

the precipitations and counting were carried out as for the active antibody. In a similar manner the horse antibodies were precipitated with a mixture of the two polysaccharides in the correct proportions and counted. The results are also shown in Tables I and II.

As these results were contrary to the earlier work it was decided to repeat the experiment with the same animal. The rabbit received a few more injections of the immunizing antigen and then on day twenty four it received an intraperitoneal injection of 30 ml of the 1% tagged leucine solution. Over the next two days it received four intravenous injections of 10 ml of a rabbit antiovalbumin solution. This solution gave 4.5 mg of precipitate per ml when titrated with ovalbumin. On the next day (day 27) 55 ml of blood were taken from the animal and this was treated in the same manner as were the previous samples. The results are given in Tables I and II. As shown the passively transferred rabbit antibody was again found to contain no radioactivity.

For a further check on the above results the experiment was repeated with a different animal and different antigen-antibody systems. A healthy female rabbit (Rabbit 361) weighing about seven pounds received a long series of injections of crystalline bovine serum albumin solution and had a high titer of anti-bovine serum albumin antibodies. On the three days following the last immunizing injection (day 0, 1, and 2) it received 27, 24, and 20 ml of a 1% solution of the labeled leucine. On the next two days

(day 3 and 4) it received four 10 ml intravenous injections of a solution of rabbit anti-bovine globulin *p*-azophenyl-arsonate serum. This material gave 10.9 mg of precipitate per ml when titrated with bovine globulin *p*-azophenylarsonate. The rabbit was then bled on days 5, 6, 13, 20, and 27 taking 40, 65, 40, 30, and 30 ml of blood on the respective days. The bleedings were all from the ear and after each bleeding the animal received an intraperitoneal injection of sterile saline. Between the third and fourth bleedings the immunizing injections were again started. The samples were treated in the same manner as before checking for complement activity after the removal of the active antibody. In all cases the active antibody was precipitated first. The amount of antibody in each sample is given in Table VIII, and the amount of radioactivity in each precipitate is given in Tables V and VI. It is to be noted that the passive antibody in this case did show some radioactivity although not as much as was found in the earlier work. To determine if this radioactivity was merely the result of adsorption the passive antibody precipitates were resuspended in saline and again washed by the procedure previously outlined. The material from the second bleeding showed a real decrease in the amount of radioactivity. A different sample of the passive antibody material from the second bleeding was washed in a more thorough fashion than the other sample and it was found to contain an intermediate amount of radioactivity. The data are shown in Table VI.

It seemed of interest to compare the amount of radioactivity in the antigen-antibody precipitates with that present in the other serum proteins that were available. The fibrin from each bleeding was washed, dried, and pulverized and then rewashed and treated and mounted in the same way as the antigen-antibody precipitates. The fibrin activities are given in Tables IV and VIII and I and V. Samples of the erythrocytes from each bleeding were washed repeatedly with saline and were laked with distilled water. Ninety five percent ethyl alcohol was added to the clear solutions and the precipitates were centrifuged out. The precipitates were treated in the same manner as the other materials and assayed for radioactivity. The amounts of radioactivity found are given in Tables I, V, IV, and VIII. The supernatants from the antibody precipitations were treated with alcohol and the resulting precipitates were likewise treated, mounted, and assayed for radioactivity. The amounts of radioactivity found in this material is also given in Tables I, V, IV, and VIII. The variation in the amount of radioactivity in counts per minute per 10 mg of sample, corrected for self absorption, is shown in the graphs of Figures 3 and 4.

DISCUSSION

The results obtained in these experiments on the incorporation of a C^{14}-labeled amino acid by rabbit serum proteins are essentially what one would predict from the

heavy nitrogen experiments of Schoenheimer et al. (37)(38) and do not show the relatively high radioactivity in the passive antibody material that the results of Kooyman (45) indicated. However, as shown above, in some cases the passive antibody material did contain some C^{14}. This could be removed at least to some extent by extended washing of the precipitate and would thus seem to be non-specific. It is to be noted that the passive horse antibody which the earlier work had claimed to be inactive was just as radioactive or even more radioactive than the rabbit passive antibody again indicating that both effects are non-specific. In general great care must be exercised in evaluating the meaning of radioactivity in precipitates such as obtained in these experiments. A great deal of the radioactivity may be non-specific (46). This may or may not account for the results obtained by Kooyman (45). In some of his experiments the passive antibody was precipitated before precipitating the active antibody and one might expect more non-specific material to be brought down in the first of a series of antigen-antibody precipitations.

It is possible to estimate the half life of the different serum constituents from curves similar to those of Figures 2 and 3 if it is assumed that the radioactivity is not part of a molecule that is reutilized to make more of the same material. However, as large samples of blood were taken at each bleeding the data are not too significant in this regard.

The rate of decrease of activity is what might be expected from similar work on man and the rat (60). It is interesting to note that the red cells are still incorporating activity when the activity of the other serum constituents is decreasing rapidly. This may not only be a reflection of the much longer half life of the cells as compared to the soluble proteins but also a reflection of the utilization of preformed material in the synthesis of the red cell protein.

In conclusion it may be stated that while the normal constituents of a rabbit's serum incorporate radioactivity given in the form of an amino acid at a very rapid rate passively transferred antibodies either do not incorporate this radioactivity to any marked extent or else do so only upon the loss of their specific antibody characteristics.

TABLE I

Rabbit 63 - Immunized Against Bovine Globulin

P-Azophenylarsonate and Injected with C^{14}-Labeled Leucine

Time in days	0	1	2	3	4	5	7	24	25	26	27
Injection of C^{14} leucine (1% solution)	30 ml	30 ml	30 ml					30 ml			
Injection of rabbit anti-ovalbumin				27 ml					20 ml	20 ml	
Injection of horse antibody (SI SII)					11 ml						
Bled						70 ml	60 ml				55 ml

	Radioactivity Found Counts per min. per 10 mg		
Antiovalbumin Precipitate	0	0	0
Horse antibody Precipitate	10	8	-
Anti RBG Precipitate	81	68	32
Red cell protein	14	14	66
Fibrin	144	111	36
Alcohol ppt. from supnt.	117	99	77

TABLE II

Radioactivity of Antibody Proteins from Rabbit 63, Immunized Against Bovine Globulin p-Azophenylarsonate and Injected with C^{14}-Labeled Leucine

Active antibody - Anti-RBG Precipitate

	Weight	Counts/minute	Counts/min./10 mg
Bleed 1	4.3 mg	35.7 ± 2.5	81
Bleed 2	10.8 mg	58.3 ± 2.5	68
Bleed 3	18.6 mg	36.8 ± 2.0	32

Passive homologous antibody - Antiovalbumin Precipitate

	Weight		Counts/min.	Recounted Counts/min.
Bleed 1	2.7 mg	Background	25.5 ± 0.8	27.7 ± 1.5
		Sample	26.1 ± 0.8	28.4 ± 1.5
		Background	25.9 ± 1.0	26.3 ± 1.5
	2.7 mg	Background	24.6 ± 0.9	25.0 ± 0.8
		Sample	24.9 ± 0.8	27.2 ± 0.6
		Background	25.1 ± 0.8	26.6 ± 0.7
Bleed 2	2.4 mg	Background	25.3 ± 0.8	
		Sample	25.4 ± 0.6	
		Background	25.8 ± 0.7	
Bleed 3	6.5 mg	Background	25.1 ± 0.7	
		Sample	25.4 ± 0.7	
		Background	25.5 ± 0.7	

Passive horse antibody - Anti-SI SII Precipitate

	Weight	Counts/minute	Counts/min./10 mg
Bleed 1	9.4 mg	8.0 ± 0.4	10
Bleed 2	2.4 mg	2.0 ± 0.4	8

Errors given are all nine-tenths statistical error.

TABLE III

Rabbit 63	Injected Material
Labeled leucine	90 ml of a 1% solution. Activity of leucine was 11,000 counts per minute per mg in the counter used
Horse antibody	11 ml of solution. Solution contained 49 mg of protein per ml and gave 12.5 mg/ml of precipitate with S I antigen and 17.0 mg/ml of precipitate with S II antigen
Rabbit antibody	27 ml of solution. Solution contained 33 mg of protein per ml and gave 6.1 mg of precipitate per ml when titrated with ovalbumin
Second Experiment	
Labeled leucine	30 ml of the same 1% solution
Rabbit antibody	40 ml of solution. Solution gave 4.5 mg of precipitate per ml when titrated with ovalbumin

TABLE IV

Radioactivity in Blood Proteins of Rabbit 63

Fibrin:

	Weight	Counts/minute	Counts/minute/10 mg
Bleed 1	16.6 mg	155 ± 3.2	144
Bleed 2	19.9 mg	128 ± 3.1	111
Bleed 3	17.3 mg	39.3 ± 1.5	35.5

Red cell protein:

	Weight	Counts/minute	Counts/minute/10 mg
Bleed 1	21.9 mg	17 ± 0.5	14
Bleed 2	23.7 mg	17.5 ± 0.7	14
Bleed 3	23.3 mg	79.1 ± 1.5	66

Alcohol precipitate from supernatant of antibody precipitations:

	Weight	Counts/minute	Counts/minute/10 mg
Bleed 1	28.3 mg	143 ± 3.5	117
Bleed 2	19.3 mg	113 ± 3.0	99
Bleed 3	21.6 mg	91 ± 3.0	77

Errors given are all nine-tenths statistical error

TABLE V

Rabbit 361 - Immunized Against Bovine Albumin and Injected with C^{14}-Labeled Leucine

Time in days	0	1	2	3	4	5	6	13	20	27
Injection of C^{14} leucine (1% solution)	27 ml	24 ml	20 ml							
Injection of rabbit antibody (anti-RBG)				20 ml	20 ml					
Bled						40 ml	65 ml	40 ml	30 ml	30 ml

Radioactivity Found
Counts per min. per 10 mg

Anti-bovine albumin (active antibody)	36	40	21	16	
Anti-RBG (passive antibody)	2.1	1.5			
Fibrin	56	50	17	9	5.5
Red cell Protein	13	8	33	28	25
Alcohol ppt. from suptnt.	69	63	34	22	13

TABLE VI

Radioactivity of Antibody Proteins from Rabbit 361, Immunized Against Bovine Serum Albumin and Injected with C^{14}-Labeled Leucine

Active antibody - Anti-bovine albumin Precipitate

	Weight	Counts/minute	Counts/min./10 mg
Bleed 1	9.5 mg	29.0 ± 1.4	36
Bleed 2	10.8 mg	34.5 ± 1.4	40
Bleed 3	6.7 mg	13.3 ± 1.0	21
Bleed 4	1.1 mg	1.8 ± 0.4	16 ± 5
Bleed 5	no antibody		

Passive antibody - Anti-RBG Precipitate

	Weight	Counts/minute	Counts/min./10 mg
Bleed 1	18.0 mg	2.8 ± 1.5 2.2 ± 1.0	2
Bleed 2	9.4 mg	3.8 ± 1.0	5
Bleed 3,4,5, no antibody			
	Recounts		
Bleed 1	10.8 mg	1.4 ± 1.0 2.2 ± 0.6	2
Bleed 2	6.2 mg	0.9 ± 1.2	1.5 ± 2
	repeated with fresh material		
Bleed 2	9.6 mg	2.4 ± 1.1	3

Errors given are all nine-tenths statistical error

TABLE VII

Rabbit 361		Injected Material
Labeled leucine		71 ml of a 1% solution. Activity of leucine was 7800 counts per minute per mg in the counter used
Rabbit antibody		40 ml of solution. Solution gave 10.9 mg of precipitate per ml when titrated with RBG

Antibody Concentrations in Serum Samples

Bleed 1	Anti-bovine albumin	3.8 mg of precipitate per ml
	Anti-RBG	1.8 mg of precipitate per ml
Bleed 2	Anti-bovine albumin	1.8 mg of precipitate per ml
	Anti-RBG	1.0 mg of precipitate per ml
Bleed 3	Anti-bovine albumin	2.1 mg of precipitate per ml
	Anti-RBG	negative
Bleed 4	Anti-bovine albumin	1.6 mg of precipitate per ml
	Anti-RBG	negative
Bleed 5	No antibody	

TABLE VIII

Radioactivity in Blood Proteins of Rabbit 361

Fibrin:

	Weight	Counts/minute	Counts/min./10 mg
Bleed 1	17.7 mg	61.5 ± 1.0	56
Bleed 2	21.7 mg	59.5 ± 1.2	50
Bleed 3	23.9 mg	20.4 ± 0.9	17
Bleed 4	20.4 mg	10.3 ± 0.7	9
Bleed 5	20.5 mg	6.4 ± 0.6	5.5

Red cell protein:

	Weight	Counts/minute	Counts/min./10 mg
Bleed 1	16.3 mg	13.6 ± 0.6	13
Bleed 2	18.6 mg	8.8 ± 0.6	8
Bleed 3	18.0 mg	36.9 ± 1.0	33
Bleed 4	15.7 mg	30.2 ± 1.0	28
Bleed 5	22.4 mg	29.8 ± 1.0	25

Alcohol precipitate from supernatant of antibody precipitations:

	Weight	Counts/minute	Counts/min./10 mg
Bleed 1	28.0 mg	84.9 ± 1.3	69
Bleed 2	29.4 mg	77.1 ± 1.3	63
Bleed 3	13.2 mg	32.8 ± 1.0	34
Bleed 4	12.5 mg	20.6 ± 0.6	22
Bleed 5	19.5 mg	14.5 ± 0.4	13

Errors given are all nine-tenths statistical error

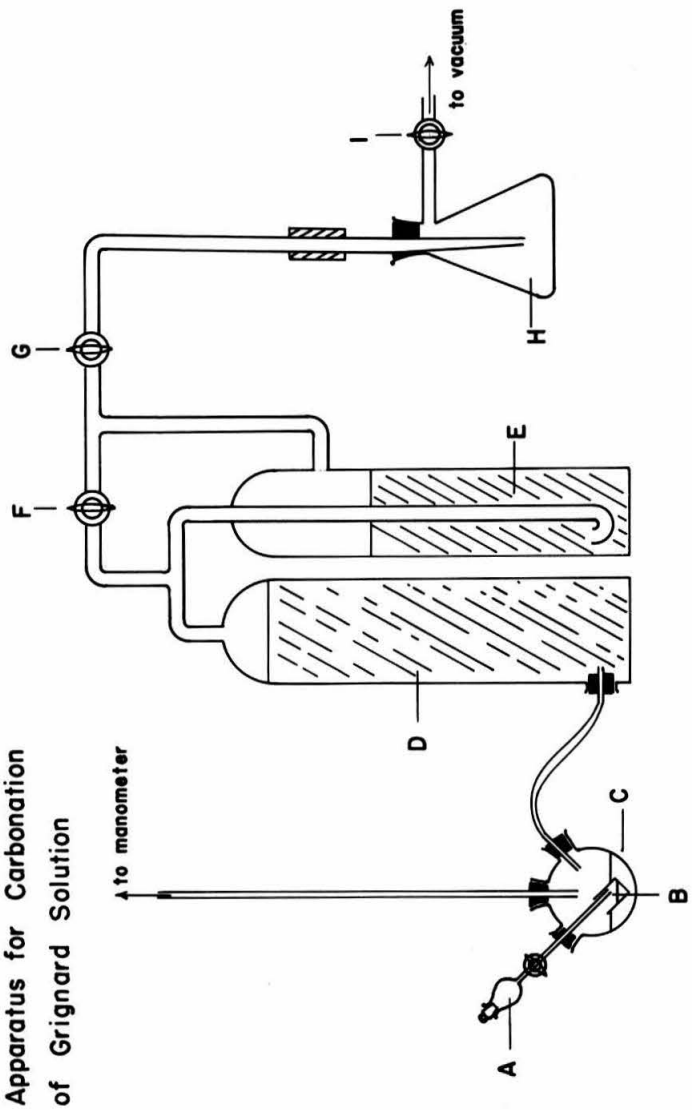


Figure I

Apparatus for Carbonation of Grignard Solution

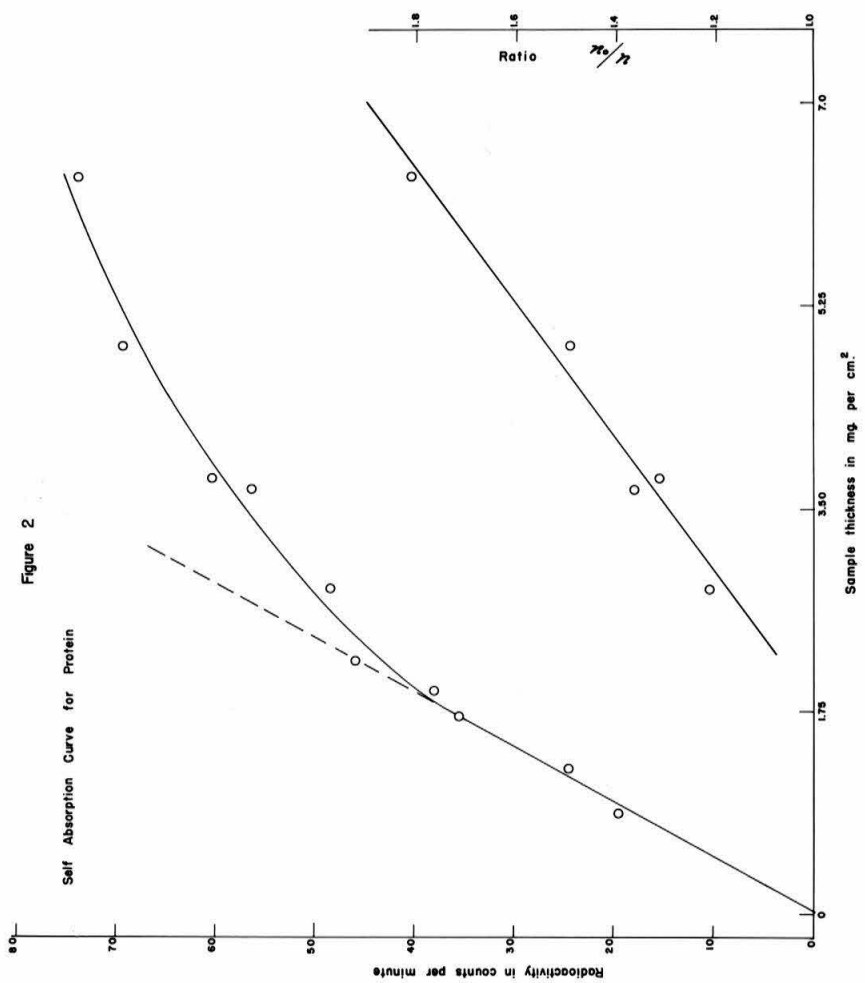


Figure 2
Self Absorption Curve for Protein
Radioactivity in counts per minute
Sample thickness in mg. per cm.²
Ratio
0
1.75
3.50
5.25
7.0
10
20
30
40
50
60
70
80
1.0
1.2
1.4
1.6
1.8

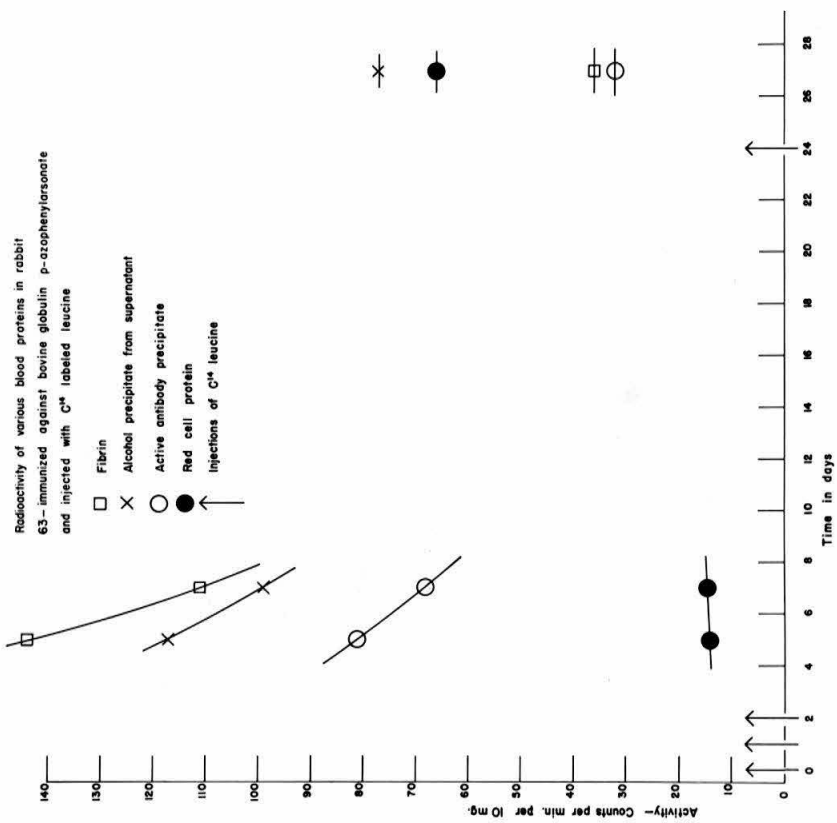


Figure 3
Radioactivity of various blood proteins in rabbit
63- immunized against bovine globulin p-azophenylarsonate
and injected with C¹⁴ labeled leucine
Fibrin
Alcohol precipitate from supernatant
Active antibody precipitate
Red cell protein
Injections of C¹⁴ leucine
Activity- Counts per min. per 10 mg.
Time in days

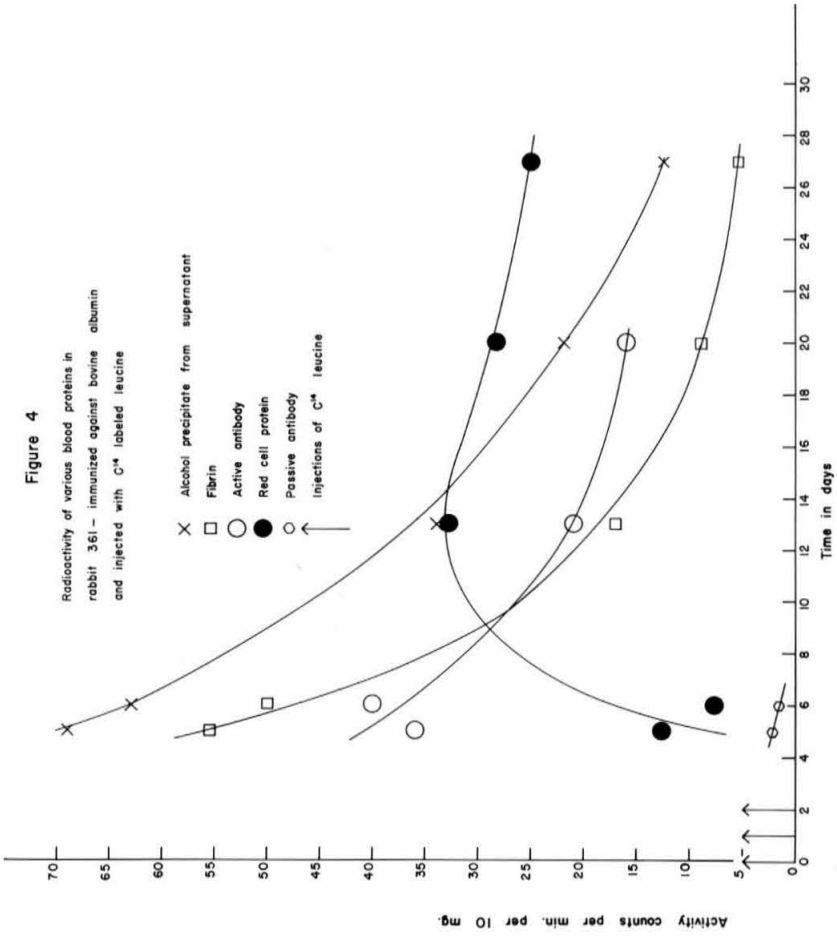


Figure 4
Radioactivity of various blood proteins in rabbit 361 – immunized against bovine albumin and injected with C14 labeled leucine
Alcohol precipitate from supernatant
Fibrin
Active antibody
Red cell protein
Passive antibody
Injections of C14 leucine
Activity counts per min. per 10 mg.
Time in days

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Propositions

1. It is proposed that it is not valid to draw conclusions about the fate of protein antigens from the experiments with I^{131} labeled materials (1) (2). Further evidence is required that the fate of the I^{131} also represents the fate of the antigen. This evidence could be obtained with protein material that contains C^{14} in the normal polypeptide chain and has also been iodinated with I^{131}.

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2. A quantitative examination of the precipitin reaction in a protein-antiprotein system should be carried out with immune serum from animals receiving C^{14}-labeled amino acids. It would be relatively easy to determine antigen-antibody ratios and the influence of numerous factors upon the reaction.

3. Complement from guinea pigs receiving a labeled amino acid should be used in a reinvestigation of the fixation of complement. By mixing different components of labeled and non-labeled complement the relative amounts of each component may be obtained.

4. A number of workers have studied the incorporation of labeled amino acids into proteins in the in vitro synthesis of protein by surviving tissue. It has been proposed that some of these amino acids are not incorporated by the

formation of peptide bonds (3). The difficulty encountered in removing radioactivity from passive antibody precipitates (Part II of this thesis) would tend to support this. The nature of the combination might be investigated by equilibrium dialysis studies.

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5. Equilibrium dialysis of labeled simple substances might be used to estimate the valence of antibodies to simple substances and the ΔF of combination of simple substance and antibody. Some of the difficulties, such as polymerization of dye haptens, encountered in the reported work (4) (5) could be avoided.

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6. The explanation offered for the solubility of horse antitoxin - toxin precipitates in excess antitoxin (6) does not seem reasonable. The explanation is based on the effect of hydrophilic groups on the antibody molecules. The influence of solubility of antibody might be investigated by coupling charged and uncharged groups of similar structures onto the antibody molecules.

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7. It is proposed that the adsorption isotherms of formaldehyde on dried proteins should be obtained. It would be of interest to carry out these measurements with diphtheria toxins

of varying toxicities to see if any relation could be established with the detoxification by formalin.

8. There do not appear to be any data in the literature on the relative oxidation potentials of the sulfoxides. It is proposed that they be investigated as possible agents for the Oppenhauer oxidation (7). It may be that they would extend the range of oxidation potentials available.

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9. It has been proposed that the equilibrium

$$2\ RMgX \rightleftharpoons R_2Mg + MgX_2$$

exists in Grignard solutions and attempts to measure the equilibrium constant have been made by precipitation of the RMgX and MgX_2 with dioxane (8). The validity of this procedure has been questioned because the rate of the reaction is unknown (9) (10). It is proposed that the addition of labeled R_2Mg before the dioxane treatment could be used to determine if the conclusions from the dioxane studies are valid.

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10. It is proposed that some of the reported discontinuities in adsorption isotherms are not real. In particular discontinuities in the adsorption isotherms of carbon dioxide on charcoal have been reported but there appears to be some

discrepancy between the two methods of obtaining the data (11). It is proposed that these isotherms should be obtained by using an apparatus containing a modified McLeod gauge (12). A variable volume apparatus could also be used.

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11. It is proposed that a more satisfactory method for the determination of density gradients in sea water is needed. It may be possible to obtain these gradients from the corresponding variation in the velocity of sound. The necessary measurements might be made with an ultrasonic interferometer (13).

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