I

THE INCLUSION OF MONOHAPTENIC SUBSTANCES IN IMMUNE AGGREGATES

II

HETEROLIGATING ANTIBODY IN ANTI-ARSANILIC ACID SERA

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Kenneth Delford Johnson

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ABSTRACT

I

Methods have been developed for the preparation and purification of iodinated haptens of the arsanilic acid series suitable for critical experiments on hapten binding in immune aggregates. Additional data are given on the preparation and properties of the solid immune adsorbents described by Lerman.

The inclusion of homologous monohaptenic substances in the aggregates formed between these solid adsorbents and homologous antibody has been studied under a variety of experimental conditions. The existence of such hapten binding has been clearly demonstrated, as has the inclusion of hapten in the immune precipitates formed between anti-RBG antibody and both azoprotein and polyhaptenic simple antigens. This demonstration has been interpreted as additional evidence in support of the theory of the bivalence of antibody.

II

The Pauling theory of antibody synthesis predicts that among the antibody molecules synthesized in spatial contact with the stimulating antigen, there should be some molecules having the two binding sites directed against different antigenic determinants on the surface of the antigen. The existence of such heteroligating antibody molecules has not previously been demonstrated. Data presented here are interpreted as evidence for the existence of heteroligating antibody in the anti-RBG sera studied. These data are derived from experiments on (1) the loss of anti-bovine globulin activity on adsorbing anti-RBG sera with R-adsorbent, (2) the recovery of such activity in the eluates from the adsorbent, and (3) the inclusion of arsanilic acid haptens in precipitates formed between such sera and a native bovine globulin precipitating antigen.

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I

THE INCLUSION OF MONOHAPTENIC SUBSTANCES IN IMMUNE AGGREGATES

INTRODUCTION

The relative resistance to reinfection that commonly follows recovery from many types of infectious diseases was recognized long before there was any appreciation of the mechanism of the phenomenon. That this immunity, while primarily directed against the infectious agent stimulating its development, is not completely specific, was recognized by Jenner, who observed that the relatively mild infection of cowpox confers upon its victims an immunity to smallpox. The attempts at the elucidation of the means by which the organism, through such acquired immunity, can resist the invasion of the pathogen, or neutralize the toxic products of its metabolism, have through the intervening years led to our present knowledge of immunochemistry.

The development of the immunity referred to above is accompanied by the appearance in the blood of the immune organism of substances that have one or more of the properties of agglutinating the infecting bacterium (1), facilitating its phagocytosis by the leucocytes of the blood (2), precipitating soluble proteins of the pathogen (3), neutralizing toxins (4), and binding certain fractions of the normal serum proteins in the presence of

these substrates (5). It was soon found that the development of these responses could be stimulated not only by clinical infections, but by the injection of dead bacilli (6), or soluble toxic principles of high molecular weight from bacilli (7), higher plants (8) or animals (9). Immunology lost its exclusive concern with resistance to clinical infections when it was found that the body exhibited a similar response to the injection of innocuous materials such as soluble proteins or cells from a foreign species (10).

The new substances appearing in the blood plasma in response to these varied foreign materials have been called antibodies (11). These have been identified further as agglutinins, precipitins, opsonins, lysins, antitoxins, etc. as their presence has been detected or measured by the ability of the blood plasma or serum to agglutinate cells, precipitate soluble substances, facilitate the phagocytosis of particulate matter, lyse cells, or neutralize toxins. Today there is general acceptance of the theory that all these phenomena are manifestations of the activity of the same kind of antibody, and that the phenomenon observed depends upon the nature of the substrate and the experimental conditions (11).

Substances which, when injected into the animal, give rise to the production of such antibodies are called antigens. Antigens are substances of large molecular

size and at one time it was thought that all antigens were protein in nature (12), but it is now recognized that carbohydrate and lipid substances (13) may be antigenic. Campbell (14) has summarized the criteria that he believes are essential to antigenicity.

Smaller molecules, chemically similar to the antigens stimulating antibody production, may themselves be incapable of eliciting this physiological response, but may manifest their affinities for the antibody in vitro (15). These are called haptens, or "partial", or "incomplete" antigens. They are further classified as "precipitating haptens" or "inhibiting haptens" according to their abilities to form insoluble or only soluble complexes with the antibody (16).

Early attempts to establish a chemical basis for the specificity of antigen-antibody reactions by chemical alteration of the antigen were unfruitful. Obermayer and Pick (17) had shown that rather drastic chemical treatment of an antigen might fail to alter its ability to stimulate formation of antibodies active against the native antigen, although other types of chemical degradation led to complete loss of antigen character. The researches of Landsteiner and his collaborators developed the first and most powerful methods of studying the relationship between chemical structure and immunological specificity. They showed that coupling of acyl groupings to native

proteins formed antigens that stimulated the production of antibodies directed not only against the native antigen, but against the coupled acyl group as well. This was shown by their ability to react with other proteins coupled to the same or chemically similar acyl groups (18). The introduction by these same workers of proteins coupled with diazonium compounds (19) as synthetic antigens, and their demonstration that the antibodies produced in response to their injections could react in vitro with simple diazo dyes prepared from the same diazonium compounds (20) vastly extended the range of chemical groupings that could be studied and freed the researchers from dependence on proteins of indeterminate chemical structure as test antigens.

Antibodies are now recognized to be a specifically altered form of serum protein (21). The properties of antibody protein are dependent both upon the species of the test animal and the nature of the antigenic material. Those produced in rabbits in response to the injection of soluble protein antigens, such as those employed in the present study, have been carefully characterized from a physicochemical standpoint. They have the general properties of a serum globulin. In an electrical field, they migrate with a rate characteristic of the γ globulin fraction of the serum (22). In a gravitational field they sediment at a rate that, when taken in conjunction

with a molecular weight of 160,000 derived from light scattering data (23), leads to calculated axial ratios approximating eight to one (24). Chemical reactivity, elementary and amino acid analyses, and antigenicity in foreign species fail to differentiate antibody protein from the normal γ globulins of the rabbit (25). The only characteristic of the antibody molecule that permits its detection and identification is its serological activity against its antigen and related molecular species.

The manner in which an otherwise normal protein molecule is so altered that it will specifically combine with its homologous antigen has been the subject of speculation and investigation since the "lock and key" hypothesis was proposed by Ehrlich (26). His concept, though formulated without the basis of physicochemical concepts adequate to propound a mechanism for the construction of the "lock" complementary in structure to the "key" of the antigen, has survived the attack of numerous speculative theories, and stands today as the foundation of modern theories of antibody specificity.

Although the work of Landsteiner and his associates established beyond question that a complementariness of surface topography and charge distribution was the basis of serological specificity, it remained for Pauling to propose a mechanism consistent with current knowledge of the structure of proteins, chemical thermodynamics, and

the nature of the attractive and repulsive forces exerted between adjacent molecules. Pauling (27) postulated that the normal globulin molecule consisted of a linear polypeptide chain, folded upon itself into a globular structure in such a manner that the stabilization of the structure through the attraction between adjacent loops of the folded chain, attractive forces due to hydrogen bonding, Coulombic forces and van der Waals forces, lead to maximum thermodynamic stability under the environmental conditions of the globulin synthesis. The amino acid sequence in the chain and the precise manner of chain folding lead to the synthesis of a molecule characteristic of the species. He further proposed that, in the presence of an antigen at the site of protein synthesis, the environment was so altered that among the multiplicity of folded structures that differed but slightly in their thermodynamic stability, that structure most nearly complementary to that of the antigen now became the most stable, and, in the folding of the terminal portions of the polypeptide chain, a structural surface was formed upon the antigen acting as a template, and this surface became the active site on the completed antibody molecule.

Pauling's concepts gave a sound theoretical basis to a mechanism that, though widely accepted, had been poorly understood, and gave impetus to highly productive research designed to test and develop these theories.

The extent to which the surface of the globulin molecule is modified in the synthesis of an antibody has not been precisely determined. However, the researches of Landsteiner and van der Scheer (28) set an upper limit on the size of the haptenic grouping that could serve as a single antigenic determinant. Their results indicate that the active site of an antibody molecule cannot be over 700 $\mathring{A}^2$. As the total area of the globulin molecule is about 25,000 $\mathring{A}^2$, it can be seen that the extent of the structural modification is small. This, together with the reasonable assumption that a concavity in the molecular surface is a less effective antigenic template than a protuberance, adequately explains the lack of alteration of antigenic specificity in the globulin molecule by the structural modifications incident to the formation of the antibody combining site.

The complementary structures of the surfaces of the antigen and the combining site of the antibody permit the molecules closely to approach each other, bringing into play intermolecular attractive forces due to hydrogen bonding, electrostatic bonding, and van der Waals attractions. These are all short range forces, and, in order that bond strengths adequate to resist the disruptive forces of thermal agitation be formed, close conformance in surface topography and favorable distribution of structures capable of forming hydrogen or electro-

static bonds are required. These structural limitations are the basis for the specificity of serological reactions.

Mechanism of the Formation of Immune Aggregates

The combination of antigen and antibody through this interaction between their combining sites is the basic step in all immunological phenomena. The mechanism by which this combination leads to the macroscopically visible phenomena in the test tube of the chemist has been, and remains today, the subject of spirited debate. The fundamental disagreement involves the concept of the valence of the antibody molecule. All workers agree that univalent antibody molecules, i.e., molecules having but a single active combining site, exist. Some, notably the school led by Boyd, (29) hold that all manifestations of immunology, including precipitation and agglutination, are the results of the reactions of such antibodies. Others, following the lead of Marrack, Heidelberger and Pauling, feel that those phenomena characterized by the aggregation of dispersed systems of molecular or particulate antigens, are the result of a linking together of the units of antigen through molecules of antibody, with the formation of a framework or lattice of alternating antigen and antibody, in which chain or lattice each unit, with the exception of terminal links, forms two or more bonds to adjacent units of the complementary type (30, 31, 32).

The evidence for the framework theory of precipitation is both theoretical and experimental. The quantitative aspects of the precipitation reaction can be reconciled with mathematical treatments based on the bivalence theory, as has been done by Kendall (33), Pauling, Campbell and Pressman (34), and Goldberg (35). Direct observation by Topley (36), Hooker and Boyd (37), and Lanni (38) of aggregates formed in mixed systems of particulate antigens and their homologous antibodies, indicate that each such aggregate consists of homologous particles, and that only after these primary aggregates become large is there mechanical entanglement with the production of aggregates composed of non-similar particles.

One of the more convincing direct experimental attacks upon the problem was the so-called "R-X" experiment of Pauling, Pressman and Campbell (39). A test antigen prepared by coupling one phenyl-arsonic acid (R-hapten) and one carboxylic acid (X-hapten) to 1-amino-8-hydroxy-naphthalene-3,6-disulfonic acid failed to yield a precipitate with antisera prepared against either arsanilic acid-azo-proteins or carboxylic acid-azo-proteins, but did give a precipitate with a mixture of the two. These results were interpreted by the authors to demonstrate that an alternation of antibody and antigen molecules in a chain or lattice is required to produce an insoluble aggregate, and that the failure of the test antigen to

produce a precipitate with either of the individual sera was due to that fact that with respect to either type of antibody the dye was monohaptenic, and could form only a terminal unit in an antigen-antibody aggregate. Criticisms that the results are invalid because of the possible formation of effectively polyhaptenic polymeric forms of the dye do not account for the failure of the material to produce precipitates with the individual antisera.

Certain similarities between inorganic colloidal systems and immunological phenomena led early workers to attempt to explain the experimental observations in terms of the surface properties of the primary aggregates of antigen and antibody. These theories, although requiring no specific assumptions regarding the antibody valence, explain agglutination and precipitation as the result of nonspecific forces acting upon the primary aggregates between antibody and antigen, and admit the views that both of these phenomena can be produced with univalent antibody. Bordet (40), noting the similarity between adsorption and serological reactions, stressed the influence of variable combining ratios of antigen and antibody upon the properties of the aggregates. His original theory held that the antibody adsorbed in a thin layer over the surface of the antigen particle rendering it susceptible to agglutination or precipitation by nonspecific aggre-

gation. His views are not in accord with modern knowledge of the shape of the particles in immune aggregates. Later workers, including Boyd (29), have held that the interaction of antigen and antibody, by their close approach with the neutralization of polar groups in the region of the specific combining sites and the shielding of adjacent polar groups, so reduce the water-bonding capacity of the surface that the aggregate is much more susceptible to the nonspecific agglutinating or precipitating influences of electrolytes. Some of the experimental evidence offered in support of these views certainly makes this theory attractive, and, particularly in the case of large antigen molecule of high effective valency, such a mechanism may be operative. However, in view of what is known concerning the relative areas of the combining sites as compared to the total areas of the molecules, and in particular the experiments of Pauling et al (41), in which it was shown that erythrocytes coupled to azo groups could be specifically agglutinated by anti-azo sera under conditions such that less than 0.02% of the cell surface was covered by antibody molecules, it cannot be reasonably held that the nonspecific aggregation mechanism can be generally applicable to all agglutination and precipitation phenomena in immunochemistry. The consistent failure of all attempts to produce specific precipitation between monohaptenic antigens and antibody

is one of the most convincing arguments against the theory of the univalence of precipitating antibody.

The question of the valence of antibodies is one of considerable theoretical significance. Presently available quantitative data do not permit an unequivocal answer to the problem. The alternation, or lattice hypothesis of precipitation or agglutination requires a valency of two or higher for precipitating antibody. The Bordet or occlusion theories do not require any assumptions regarding antibody valence, but are not of themselves direct support of a theory of univalence. A clear-cut solution to the problem would perhaps be of greater value in elucidating the mechanism of antibody synthesis than that of aggregate formation. Lanni and Campbell (42) lucidly discuss the implications of antibody bivalency on the former. They point out the lack of any valid reason presently apparent for any a priori assumption that a bivalent antibody molecule should have both of its combining sites directed against a single determinant grouping. The universally admitted existence of univalent antibody presupposes a degree of independence in the machinery of synthesis for the opposite ends of the molecule, and the consistent failure of previous searches for antibody with two or more specific combining sites directed against dissimilar groupings has been frequently quoted as potent support for the theories of antibody univalence.

Interactions of Monohaptenic Substances with Antibody

The nature of the specific combining site of antibodies has been most effectively studied in sera prepared against antigens chemically coupled to haptenic groupings of known chemical structure. The influence of chemical structures in determining the specificity of the hapten can be investigated by measuring the degree of inhibition to precipitation between the antiserum and its homologous antigen as the structure of the inhibiting hapten is altered through known variants. The phenomena of hapten inhibition have been extensively studied since their discovery by Landsteiner and van der Scheer (20, 34, 43), and an extensive mathematical treatment of the problem has been developed by Pauling, Pressman and Grossberg (44).

Pauling et al investigated the degree of inhibition to the precipitation of polyhaptenic azoarsanilic acid dyes and rabbit anti-(arsanilic acid-azo-sheep serum protein) sera by phenyl arsonic acid and numerous related compounds. They found that all derivatives of phenyl arsonic acid containing a single such grouping failed to yield precipitates with that serum, but that such monohaptenic compounds, when present in systems containing the antiserum and a precipitating antigen, would decrease or entirely prevent the formation of specific precipitate. This phenomenon is accepted as being the result of the competition between the simple hapten and

the precipitating antigen for the active sites of the antibody. The degree to which the simple hapten is able to compete with the precipitating antigen is determined by the relative binding energies between the antibody and the competing molecules. From theoretical considerations, these authors were able to develop equations relating the extent of precipitation observed at varying hapten concentrations to a hapten inhibition constant K'_0, which was characteristic of the hapten and the test system. The value of K'_0 was a measure of the relative strength of the antibody-antigen and antibody-hapten bonding forces, and could be correlated with the known structures of antigen and hapten.

It was shown that structures conducive to the formation of Coulombic bonding, hydrogen bonding, and van der Waals attractions all contributed significantly to the total bonding energies, and that the more closely these structures approximated equivalent forms in the immunizing antigen, the more firmly they were held to the antibody. For structures not involved in Coulombic or hydrogen bonding, surface topography seemed to be the important parameter, as shown by the substantial equivalence of methyl and chlorine substituents on the benzene nucleus. Steric hindrance from a space-occupying group on the hapten but not on the immunizing antigen, by preventing a close approach of the antibody and hapten,

markedly decreased the bonding forces. Contrariwise, a "hole" in the surface topography of the hapten as compared with antigen only slightly decreased the bond strength, since it created no problems of steric hindrance and decreased merely the van der Waals forces in proportion to its fraction of the total area of the binding site.

The direct demonstration of the binding of simple haptens to homologous antisera was accomplished by Marrack and Smith (45) who compared the distribution of arsanilic acid haptens when dialyzed against anti-(arsanilic acid-azo-protein) serum globulins with that observed with antibody-free or normal serum globulins, and found a hapten binding power of the antiserum globulins twelve times that of the control globulins. Haurowitz and Breinl (43) made a direct comparison, in a similar system, of the distribution of the hapten when the antiserum and normal serum were on opposite sides of the semipermeable membrane. Lerman (46), in a more elaborate experiment designed along similar lines, was able to develop equilibrium constants and bond energies consistent with those derived from other experimental approaches.

In view of the extent of the binding energies in the hapten-antibody bonds, it is reasonable to predict that if the alternation or framework hypothesis of

immune precipitates is valid, such a precipitate, formed in the region of antibody excess, and in the presence of simple hapten at concentrations insufficient completely to inhibit the formation of precipitate, would contain the simple hapten attached to the terminal binding sites of the antibodies at the surface of the immune aggregate. Contrariwise, the occlusion mechanism, operating with univalent antibody, would predict the absence of hapten from the precipitate, for those molecules of antibody attached through their single active site to a simple hapten would remain soluble, and would not be included in the precipitate.

The unequivocal demonstration of specifically bound hapten in an immune aggregate is an imposing analytical problem. Among the factors that contribute to the difficulties facing attempts at its solution are the small mass of the hapten in comparison with that of the antibody molecules, the low molecular ratio of hapten to antigen and antibody in the precipitate, the high ratio of free hapten in the system to bound hapten in the aggregate, and the chemical similarity of antigen and hapten. The significance of unambiguous results to the theories of immunochemistry justify elaborate effort to solve the experimental problems, and the availability of present techniques in isotope tracer measurements and suitable sources of isotopic carbon, nitrogen,

sulfur, iodine and other tracer elements gives promise of adequate means to resolve the question of hapten binding in immune precipitates.

Banks, Francis, Mulligan and Wormall (47) have published the only paper dealing directly with this problem. In a carefully planned and executed research, using I^{131} as a tracer, they were unable to demonstrate any hapten binding under the conditions of their experiments. Wormall had earlier shown (48) that immunological specificity of iodinated proteins was largely due to their 3,5-diiodotyrosine groups, as evidenced by the specific haptenic inhibition of precipitation by that compound. Since sensitive and specific methods of measuring both total and isotopic iodine are available, this system seemed to offer the possibilities of accurate and sensitive means for determination of antigen, antibody and hapten in the precipitates.

The immunizing antigens used by these authors were iodinated horse and human serum globulins containing about 14% iodine by weight. The alum precipitated antigen was administered to rabbits by intramuscular injection. The titers of the antisera so prepared are not specifically given, but data from one experiment (their Table 3, line 1) indicate that .33 mg. of antibody nitrogen was precipitated per ml. of anti-(iodinated horse serum globulin)-serum by iodinated

human serum globulin in the absence of any diiodotyrosine. In other experiments, the anti-(iodinated human serum globulin)-serum was tested against iodinated rabbit serum proteins as the precipitating antigen. In this system slightly over 100 μg. of nitrogen per ml. of antiserum was found in the precipitate at the equivalence point. The test antigen was prepared with I^{131} in some experiments, and in other cases with stable iodine.

Diiodotyrosine (abbreviated as DIT by these authors) was prepared by the method of Harrington (49) and purified by multiple precipitation as the free acid from solutions of its salts. The preparation labeled with I^{131} gave 100 counts/μg/minute with the endwindow-counter employed in the study. This compound was also prepared with stable iodine.

A novel form of control to determine the amount of physically held hapten in the moisture of the precipitate was devised by these authors. They added heavy water (D_2O) to the test systems, and determined the excess of deuterium present in the precipitates. They assumed that the deuterium excess found represented a measurement of the physically held water in the precipitates and that a similar fraction of the unprecipitated components of the test system also had been carried through the manipulations by mechanical entrainment.

Total iodine in the washed precipitates was determined by the method of Shahrokh (50) which Banks et al believed was reliable to the closest microgram in the range of ten to one hundred micrograms of total iodine. Iodine 131 was determined with a Geiger counter and thin-window detectors. The actual counting data are not given in the paper, but the authors state that the uncertainty of the counting corresponded to less than 3 micrograms of iodine in the sample counted, an uncertainty that seems very large indeed unless the isotopically labeled materials had decayed to a very small percentage of their original activity by the time the precipitates were counted.

One series of experiments investigated the composition of the precipitates formed from two ml. of rabbit antiserum to iodinated horse serum globulin, four ml. of 0.043% solution of iodinated rabbit serum proteins as precipitating antigen, and varying amounts of 3,5-diiodotyrosine inhibitor. Either the antigen or the hapten was isotopically labeled, and the precipitates obtained after three hours incubation at 37° C and fifteen to eighteen hours at 4° C were analyzed without washing.

Their Table 1 is reproduced below.

Component labeled with ^{131}I	DIT added (μg.)	Degree* of precipitation	Composition of Precipitate: Iodine Content: By ^{131}I (μg.)	Composition of Precipitate: Iodine Content: Total I (μg.)	Amount of antigen as percentage of that added
Antigen	0	+++	26.6	29.2	11.3
	1082	++	15.2	16.0	6.4
	2165	+	13.9	14.2	5.9
	4330	±	12.1	12.4	5.1
	8660	tr.	10.6	10.2	4.5
DIT	0	++±	0	30.9	11.6
	1082	++	0**	18.3	6.9
	2165	+	0**	16.9	6.3
	4330	±	0**	13.8	5.2
	8660	tr.	0**	11.8	4.4

*Taken from a parallel set of precipitin tests made with one-twentieth the volumes of solutions used in the quantitative experiment. The readings given are those after three hours at 37°.

**Counts within variation of background and therefore not quantitative, but indicate certainly less than 3 μg. iodine.

The heavy water tracer experiment, designed to determine the extent to which physically dissolved antigen in the water of the precipitate contributed to the small and uncertain values for hapten iodine shown above, was summarized by these workers as follows:

"Samples (6 ml.) of a rabbit antiserum to iodinated horse serum globulin were mixed with NaCl solution containing heavy water (1 ml. containing 0.9% NaCl and 50% w/v D_2O) and ^{131}I-containing 3:5-diiodotyrosine solution (0.25, 0.75 or 2.0 ml. of 0.01M). The antigen (1 ml. of 0.6% iodinated human serum globulin) and sufficient 0.9% NaCl to give a total volume of 10 ml. were then added. The mixtures were kept at 37° for 3 hr. and then at 0 - 4° overnight; the precipitates were separated by centrifuging, and the sides of the tubes were cleaned with filter paper, care being taken to avoid disturbing the precipitate. These unwashed precipitates were then dissolved in water containing a few drops of 0.5 N NaOH and the volume made up to 3.0 ml. Total N and ^{131}I determinations were made on 0.1 and 1.0 ml. samples respectively, and the D_2O content of these solutions, and of the supernatant solutions separated from the precipitates, were also determined.

"The results showed that the amount of combined inhibitor (3:5-diiodotyrosine) in the antigen-antibody complex was very small and probably zero (Table 3). The calculated molecular ratio of combined inhibitor: antibody was sometimes zero, and in no case was it greater than 1:20."

Their Table 3 is reproduced on page 23. In discussing the significance of their results, these authors write:

"The antigen used in our experiments was undoubtedly multivalent, for it contained about sixty 3:5-diiodotyrosine residues per molecule, and furthermore it gave precipitates with antibody:antigen ratios up to over 10:1 in the region of antibody excess in the absence of diiodotyrosine. If the antibody also is

multivalent, and the precipitated complex has a "lattice structure" (cf. Marrack, Spec. Rep. Ser. med Res. Coun., London, no. 230, pp 72, 108, 151.) it seems highly probable that many of the antibody molecules in solution will have some of their valances 'blocked' by combination with diiodotyrosine molecules. Provided the unblocked valances are at least two in number, there seems to be no reason why these antibody-inhibitor complexes should not take part in lattice formation. If the antibody is bivalent and has only one valence blocked, or alternatively, if a few or even all but one of the valences of multivalent antibody are blocked, it seems reasonable to suppose that these antibody-inhibitor complexes should coprecipitate, as do Heidelberger's 'univalent or incomplete' antibodies (Heidelberger and Kendall, J. Exp. Med. 62, 697; Heidelberger, Treffers and Mayer, J. Exp. Med. 71, 271; Kabat and Mayer, Experimental Immunochemistry, p 42. Charles C. Thomas). Thus with a multivalent antibody one might reasonably expect to find the specific inhibitor attached to the precipitates in these 'partially inhibited' systems, as represented diagrammatically for a bivalent antibody in Fig. 2.

"If any inhibitor is attached to the precipitated antibody, one might expect that the amount would be measurable, e. g. more than one molecule of inhibitor per twenty molecules of antibody, for it seems improbable that an insoluble antibody-antigen-inhibitor complex with a molecular ratio of, say, 20:11:1 would become soluble if only one of the antigen molecules were replaced by inhibitor. In most of our experiments with the 'partially inhibited' iodoprotein serological system the amount of inhibitor in the precipitate was significantly less than this. We feel just-

Composition of the unwashed precipitates separating from mixtures of iodinated proteins, their antibodies and 3:5-diiodotyrosine (inhibitor)*

Antigen: Iodinated human serum globulin.
Antiserum: Rabbit antiserum to (iodinated) horse serum globulin.
Inhibitor: 3:5-Diiodotyrosine (DIT) (containing ^{131}I).

		Composition of precipitate						
					DIT			
Antigen added (mμM)	DIT added (mμM)	Antibody (mμM)	Antigen (mμM)	Antibody: antigen (Mol. ratio)	Total (mμM)	Free** (mμM)	'Combined' (mμM)	'Combined' DIT: antibody (Mol. ratio)
7.03	0	12.55	3.85	3.3	0	0	0	------
	440	5.34	2.98	1.8	3.00	2.73	0.27	0.05
	1320	4.70	2.78	1.7	7.89	8.15	-0.26	0
	3520	3.44	2.17	1.6	15.10	15.04	0.06	0.017

** Determined by carrying out the precipitin reaction in a solution containing 5% D_2O, and measuring the D_2O content of the precipitate and supernatant solution.

* Table reproduced from Banks et al (47 a) Table 3, page 375

ified, therefore, in suggesting that our observations support the view that the rabbit antibodies we have studied are univalent, i.e. each molecule has only one combining site for the diiodotyrosine group. On the basis of univalent antibodies, specific inhibition can readily be explained. Antigen and inhibitor compete for antibody, and where the antibody combining site is blocked by inhibitor, this antibody molecule is not able to precipitate, or be precipitated by antigen."

The reasoning expounded above cannot lightly be dismissed, and the experimental observations seem to have been made with considerable care and skill. To challenge the conclusions reached, valid criticisms of the design or execution of the research, or of the interpretation of the results, must be sustained. In which aspects is this paper subject to attack?

The mathematical theories of hapten inhibition, as developed by Pauling et al, permit an estimation of the strength of hapten-antibody bonds in terms of the degree of inhibition observed at given levels of hapten concentration. The limited quantitative data reported for this system permit only qualitative comparison with the arsanilic acid compounds studied by Pauling's group. From their Figure 1 it would appear that a concentration of approximately 87 micrograms of hapten per ml. of final volume was required to reduce the total nitrogen in the precipitate in the optimum proportions region to half that observed in the corresponding region for the

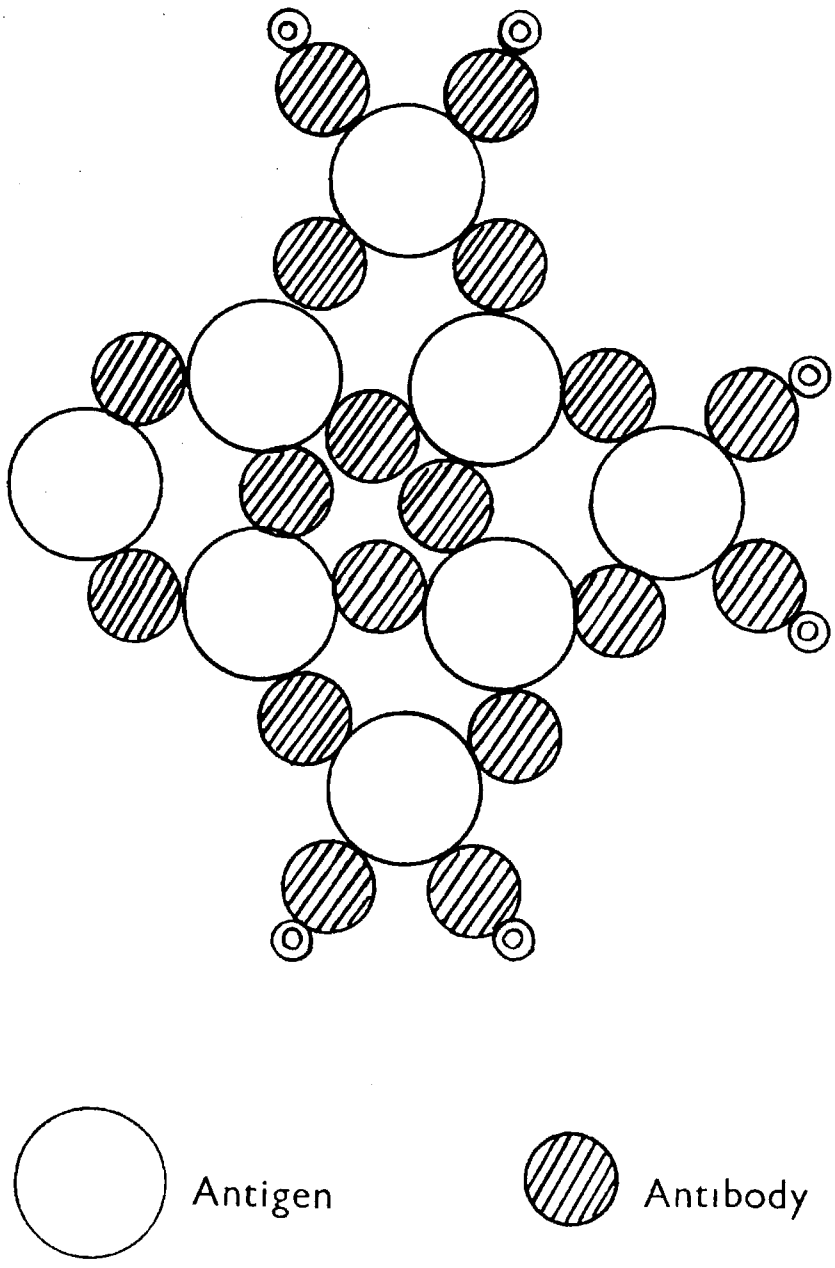

Fig. 2. Hypothetical lattice formed by iodinated protein antigen and bivalent antibody in the presence of the specific inhibitor (3:5-diiodotyrosine).

Reproduced from Banks et al.
Figure No. 1

uninhibited system. This may be compared with values given by Pressman, Brown and Pauling (44) of equivalent degrees of inhibition in their systems at concentrations of haptens ranging from one to three micrograms per milliliter. When an azoprotein, rather than a polyvalent hapten was used as the test antigen, the corresponding values were somewhat higher, but in the case of the better inhibiting haptens, ranged as low as 2.5 micrograms per milliliter. One must conclude then, that 3,5-diiodotyrosine is a relatively poor inhibiting hapten, and that the bonding forces between it and the antibodies produced in response to the injection of iodinated proteins are weak.

Molar ratios of antigen to antibody in the systems employed by Banks et al cannot be calculated with certainty from the data given, but are estimated to be in the range of from 1:1 to 1:2. Since the valence of the antigen is given by these authors as about sixty, the ratio of combining sites of the antigen kind to combining sites of the antibody kind must range from a low of 15:1, if one assumes antibody to be bivalent, to a high of 60:1, on the assumption of the univalence of antibody. With such an effective antigen excess, an excess that would be further increased by the formation of any soluble antibody-inhibitor complexes, one may properly question whether "one might reasonably expect to find

the specific inhibitor attached to the precipitates in these 'partially inhibited' systems".

In any case, the high concentration of inhibitor in the system makes the problem of obtaining significance to the small difference between the relatively large figures for total and free inhibitor beyond the limitations of precision claimed by the authors for their results. An analysis of the data shown in their Table 3 is revealing in this regard. In line two, total DIT is given as 3.00 mμ.mol. per ml. antibody solution and free DIT as 2.73. The limits of analytical precision claimed for the iodine analysis are 3 μg. for isotopic iodine, or 1 μg. for chemical assay. Converting these limits to mμ.mols we get 23 mμ.mol. or 7.6 mμ.mol. of iodine, or 11.5 and 3.8 mμ.mols of diiodotyrosine, respectively. Considering that the experiments were carried out with six ml. of antibody solution, and that therefore 18 mμ.mol. of total hapten was found, it is still obvious that there is no valid basis for ascribing any mathematical significance to the failure to establish the presence of even one mμ.mol. of bound hapten, which would be all that one could anticipate were as much as twenty percent of the antibody molecules in the precipitate bonded to the hapten. The experiments with still higher concentrations of inhibitor present an even more unfavorable situation for the

experimenter. The amounts of material available were adequate for the performance of replicate analyses, and it is possible that such were actually performed, but no information is given on the raw experimental data, and however much confidence the authors seem to exhibit in the soundness of their conclusions, the contents of the published results do not justify these conclusions, and taken at face value, would suggest that, a priori, the experiments could neither confirm nor oppose either of the alternative hypotheses against which the results were evaluated.

Any predictions regarding the amount of bound hapten expected to be found in an insoluble serological aggregate must be predicated upon some assumptions regarding the minimum size that such an aggregate must acquire before becoming insoluble. If the antibody be assumed to be bivalent, any complexing of antibody with hapten would terminate the lattice at that point. Substantially all of the hapten-coupled antibody would then occur at the surface of the insoluble aggregate, and the ratio of hapten-coupled to total antibody in the precipitate could not exceed the ratio of the mass of the surface layer of antibody to the total, a ratio which is obviously critically dependent upon the size of the insoluble aggregate. Little experimental evidence is available on this point, but the studies of Lanni (38)

would indicate that specific forces are operative in growth of serological aggregates through a stage that he terms the formation of "seromicrons", which in various systems range from 0.1 to 1 micron in diameter. The smaller limit, a sphere a thousand Ångstroms in diameter, if assumed to have a surface layer of close-packed antibody molecules* standing on their ends, might have most of its contained antibody in such a surface layer. At the larger limit, a seromicron ten thousand Ångstroms in diameter would have only fourteen percent of its total volume in such a surface layer, and if such a layer were uniformly combined with hapten at its outer poles a bound hapten to total antibody ratio of 1:7 would represent an upper limit.

The data of Lerman (46) suggest that at the maximum hapten concentrations at which reasonable amounts of precipitate can be obtained** the proportion of total antibody sites combined with the hapten is 50%. Since the primary assumption, i. e. the surface layer of the seromicron is composed of close-packed antibody molecules, represents a most unrealistic situation, the upper limit set by Banks et al of 1:20 for the bound

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*Campbell and Bulman (14) give the dimensions of rabbit antibody against ovalbumin as 244 Å long by 24 Å diameter.

**In the arsanilic acid system, in albumin-free solutions, 10^{-6} molar hapten will give 50% inhibition of precipitation.

inhibitor:antibody molecular ratio that can be postulated as consistent with their data may in fact represent an implicit admission that the experiment is basically incapable of solving the problem for which it was designed.

The "heavy water" tracer technique for estimating physically held components in the moist precipitate, while ingenious, has not been shown by them to be valid. Unless it can be demonstrated that there is no selective bonding of the deuterium compound by the proteins of the aggregate, the assumption that the fraction of the total heavy water recovered from the precipitate is the same as the fraction of the non-precipitated hapten physically held in the precipitate must remain an assumption only. The huge molecular ratios of total hapten to total antibody in the system operate to minimize any corrections for hapten in the supernatant bound as soluble complexes, and the simple character of the diiodotyrosine largely eliminates any uncertainties regarding possibilities of polymerization in solution or nonspecific adsorbtion on protein surfaces of the precipitate, but the ratio of bound to free water in the aggregate is sufficient to make the value of the distribution ratios between D_2O and H_2O in the bound and free water of definite significance.

An unequivocal solution to the question of hapten binding in immune aggregates would have values far beyond any implications regarding the valence of precipitating antibody. A technique of accepted validity that permitted sensitive and specific detection of bound hapten in serological precipitates would be a powerful research tool for the immunochemist.

A research program designed to test the theories of hapten binding in serological precipitates must meet certain rigid criteria if any significance is to be assigned to the results. Not all of these requirements may be met to the degree that might be desired, but any deficiencies must be explicitly recognized, and the conclusions drawn must be qualified to the extent that these deficiencies dictate.

The most obvious requirement that must be met by the serological systems chosen is homogeneity, particularly with respect to the hapten employed. Since only a very small portion of the total hapten added to the system may be bound to the precipitate, the presence of trace quantities of label-carrying impurities in the hapten solution, may, if they are adsorbed on the precipitate by nonspecific or specific mechanisms unrelated to the prosthetic grouping of the hapten, appear to indicate hapten binding where there may be none, or, in any case, obscure the true value for bound hapten.

Situations such as those prevailing in the work of Banks et al, in which the maximum anticipated bound hapten is but a few hundredths of one percent of the total hapten added to the system ought to be avoided, but the degree of purity required is far in excess of that which has been deemed adequate by earlier workers in the field of hapten inhibition. The haptens employed by Pauling's group were checked for homogeneity by column chromatography, and were described variously as "essentially pure" "contained only traces of impurities" and "contained five to ten percent of a colored impurity" depending upon the compound and the preparation involved. Anyone at all familiar with the characteristics of commercially prepared dyes of even the highest grade knows that heterogeneity and nonuniformity from lot to lot are substantially universal.

The technical problems involved in preparing haptenic dyes in the requisite purity, and criteria for the establishment of homogeneity are not inconsiderable, but unless they can be solved for the system chosen, no significance can be attached to measured values of hapten binding.

The entire question of selective mechanisms in heterogeneous systems is one that only recently has begun to receive the attention that it deserves. It has almost universally been assumed that the distribu-

tion of "label" in a system is equivalent to the distribution of the labeled molecule. Such an assumption is not necessarily valid. For example, moderate iodination of a protein is said (51) not to affect its native immunological specificity. Such an iodinated protein may be precipitated with antibody formed against native protein. Under conditions of antigen excess, only a small fraction of the total antigen is precipitated. The presumption that the degree of labeling of the antigen in the precipitate is precisely equal to that of the total antigen in the system is then no longer justified. It seems reasonable to believe that among the statistical population of iodinated antigen molecules, those molecules having the lowest degree of iodination would retain in greater degree their native immunological characteristics, and would be selectively concentrated in the precipitate. The iodine content of the precipitate would then fail to give a measure of the precipitated antigen.

The data of Banks et al (their Table 1)* suggest that the reverse phenomenon is occurring in their experiment, with a concentration of label in the precipitate, for the qualitative degree of precipitation decreases from +++ to a trace, while the amount of label in the precipitate decreases by only 60%.

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* Page 20, this thesis.

Definition of the Research Problem

In the determination of hapten binding in homologous systems, the choice of a hapten implies that the chemical nature of the antigenic grouping in the antigen is also precisely known. The use of an azoprotein coupled to the same haptenic group used in the synthesis of the inhibiting dye is the most direct approach to the immunizing antigen. The precipitating antigen can be a polyhaptenic compound of known structure or another protein, antigenically distinct from the immunizing protein, coupled to the same hapten. The use of a serum protein from the same animal as that immunized for the preparation of the antiserum will minimize possible antiserum-protein reactions in the test system. The use of a polyhaptenic dye as the precipitating antigen introduces the possibility of difficulties due to non-specific adsorption of the dye to the protein molecules and to association in solution into polymeric forms of indeterminate size and structure, but does simplify the analysis of the precipitate to determine antibody content.

The third component in the serological system, the antibody is somewhat more difficult to obtain with a high degree of homogeneity. The marked capacity of serum albumin to adsorb small molecules, and particularly simple dyes, makes it necessary to work in, or at least carry out controls with, albumin-free solutions.

The precipitation of the γ globulin of the antiserum with one-third saturated ammonium sulfate furnishes a convenient method to eliminate the albumin and a large portion of the serologically inactive globulins. The low-temperature, low-salt fractionation of Cohn (52) gives substantially similar results. Electrophoresis (53), or electrophoresis-convection (54) are additional techniques dependent upon the physical properties of the antibody molecule, and are unable to make any separation of antibody from serologically inactive proteins with similar physical properties.

The ultimate that can be obtained in homogeneity of antibody preparation is attained through the use of "specific" methods. These methods all involve the separation of the antibody from serologically similar proteins through the formation of an insoluble aggregate between the antibody and a soluble or insoluble antigen. The washed aggregate is then dissociated into its components through a variety of techniques, such as treatment of the aggregate with distilled water, physiological saline at elevated temperatures, hypertonic saline, dilute acids, dilute alkalies, or strong solutions of homologous simple haptens. The literature on the purification of antibodies by specific methods has been reviewed by Campbell (16).

Since serological aggregates are fundamentally a part of a reversible system, it is reasonable to assume that due care must be exercised that the precipitates as analyzed are representative of their equilibrium composition in the system under study. The rate of dissociation of aggregates of large molecules is apparently slow, for exhaustive washing of such precipitates with cold saline removes very little antigen or antibody. It cannot be assumed that this is the case for hapten-antibody complexes, and preliminary studies in this research indicated that well-washed aggregates were indeed free of bound hapten. The only completely satisfactory manner of eliminating the possible effect of dissociation of haptens is to separate the precipitates from as much of the fluid portions of the system as possible, and to correct for nonspecifically held materials by suitable auxiliary measurements. The smaller such corrections can be made, either through effective physical removal of the supernatant from the precipitate, or through the choice of conditions and systems that give significant hapten-antibody interactions at low hapten concentrations, the less will be the uncertainty in the values for bound hapten.

The choice of systems studied should be guided by the availability of analytical techniques adequate in sensitivity and specificity to permit analysis of the precipitates for antibody, antigen and hapten by sub-

stantially direct means, and in no case should the value of hapten in the precipitate be dependent upon the relatively small difference between calculated results of greater magnitude. Before the ready access that we now have to isotopic tracers, this requirement imposed severe restrictions upon such choice. Today the worker is permitted far greater latitude.

No program of research can be considered suitable until adequate controls have been designed. In the ideal case, if two complete serological systems, having a minimum of cross reaction between the two, and for which completely equivalent series of test and inhibiting haptens, differing only in the prosthetic groups, can be prepared, each such system may operate as a control for the other. If this ideal cannot be attained, great care must be exercised in assuming equivalence of "blank" corrections for mechanically held or nonspecifically bound haptens in the aggregates analyzed.

The decision to base the present research upon systems homologous to arsanilic acid and succinanilic acid was made after a careful evaluation of the factors discussed above. Their initial consideration was the result of a number of fortuitous circumstances: (1) Both systems had been extensively studied in these laboratories, and the background of knowledge and experience in their behavior would greatly lessen the

amount of preliminary investigation necessary before critical experiments could be set up. (2) There was no appreciable cross-reaction between the antigens of one system and the antibodies of the other. (3) A substantial supply of antisera of good titer was already prepared and available for use. The antisera had been prepared in quite comparable manners. Each had been prepared in rabbits against an antigen prepared by coupling bovine gamma globulin, of the same commercial lot, to the corresponding diazotized hapten. Lots of serum of each type were available that were of comparable age and initial titer.

The arsanilic acid system had the advantage that the arsenic in the antigen constituted a label that could be detected and measured with high specificity and precision. The succinanilic acid antigens and haptens were not susceptible to such ready analytical techniques, but the intensity of color of the haptenic dyes made direct colorimetric measurement of suitable sensitivity, although of somewhat less specificity than might be desired.

The analytical differentiation of the inhibiting from the precipitating haptenic groups required a second distinctive label. For such a label, iodine was chosen. Iodine can be measured with great sensitivity and specificity by chemical methods. The convenience of isotopic

iodine as a label is tempered by its short half-life, a disadvantage in part compensated for by its correspondingly high specific activity. Techniques for the synthesis of iodine-containing haptens analagous to the non-iodinated precipitating haptens were recorded in the literature.

The problem of the purification of the inhibiting haptens was approached with what turned out to be far too great optimism. Particularly in regard to the iodine-containing dyes, the attainment of a high degree of homogeneity proved to be a problem of great difficulty. For the arsanilic system at least, these problems were met, and for the succinanilic acid systems reasonable success was achieved for one of the haptens. The criteria finally chosen for the homogeneity of the haptens were based upon chromatographic methods, with paper chromatography proving the most convenient. A dye was considered pure if it (1) gave but a single color band on chromatographic analysis under two or more sets of development conditions, and if (2) the wave lengths of the absorption maxima and minima of the final preparation, and the ratios of the absorbancies at those points, accurately matched those of the dye eluted from the center of the band produced by that dye on a paper chromatogram.

Two general methods for the synthesis of iodine containing dyes were considered. Dyes containing suitable groupings, sufficiently removed from the haptenic portion of the molecule to minimize the influence of the halogen on the serological activity of the dye, could be directly iodinated. Alternatively, the dyes could be synthesized by coupling the diazotized haptenic group to a compound already containing suitably incorporated iodine. The latter technique proved to yield dyes most readily purified to the requisite standards, but intensive work was carried out on the former methods, in an effort to perfect a technique with the minimum total elapsed time and loss of iodine between the initial incorporation of inorganic iodine into an organic compound and the final purification of the hapten in a form ready to employ in the serological system.

This emphasis on speed was required because of the relatively short half-life of the readily available I^{131}. Delay between the receipt of the isotopic iodine and the final employment of the dye in the research results in a loss of sensitivity in the analytical phases accompanying the loss of specific activity of the iodine, and more seriously, the decay of significant quantities of the isotopic iodine in the haptens results in the accumulation in the preparation of altered molecules, no longer containing iodine, and of indeterminate

structure. Under such conditions, the hapten may no longer be considered to be homogeneous, and all the criticisms previously discussed become applicable to its use.

Studies were to be made with both specifically and nonspecifically purified antibody. It was beyond the scope of the present investigation to determine the degree to which haptens might be bound by serum albumin, and in general the studies were carried out in albumin-free systems. Most experiments with soluble antigens were performed with γ globulin fractions of the antisera prepared by precipitation with third-saturated ammonium sulfate. All specifically purified antibody employed in these studies was separated through the use of solid antigens, the "immune adsorbents" discussed in the following paragraphs.

Numerous studies have been carried out in the specific purification of antibodies against cellular antigens through the adsorption of the antibodies on the homologous cells, (55, 56, 57, 58 and 59). Campbell, Leuscher and Lerman (60) described an insoluble antigen, useful as an immune adsorbent, which they prepared by coupling the test protein to powdered cellulose* through

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* "Solka-floc", a product of the Brown Co. is short lengths of wood fiber obtained as a by-product in paper manufacture.

a diazotized p-aminobenzyl group that was bound to the cellulose through an ether linkage. Lerman (61) extended this technique to the preparation of a cellulose-based adsorbent containing azoarsanilic acid groupings. The properties of this adsorbent seemed to be such as to make the adsorbent of great utility to the present research, so a number of preparations, varying in their properties, were synthesized and characterized as detailed in the experimental section.

Aside from its usefulness in the preparation of specifically purified antibody by dissociation of that antibody from its union with the solid adsorbent, this antigen offered certain specific advantages. Because of its insolubility, aggregates of the adsorbent with antibody, in all antigen-antibody ratios up to the point of saturation of the adsorbent surface, are insoluble. Thus experiments may be carried out at any desired antigen-antibody ratio without the formation of soluble aggregates and the loss of antibody in the supernatant. The rigid shape and solid structure of the adsorbent should result in most of the adsorbed antibody lying on the exterior surface of the particles, with the likelihood of steric relationships being such that the outer ends of the adsorbed antibody molecules would not be in spatial contact with other haptenic groups of the adsorbent, and would be available for the binding of

inhibiting hapten. In other words, on the microscopic level, the situation is essentially that of antibody excess, in that haptenic groups are not available for the simultaneous binding of both ends of many of the antibody molecules, regardless of the average ratio of binding groups throughout the entire system. The advantage of working in regions of antibody excess in attempts to detect hapten binding has already been discussed, and the solid adsorbent is ideally adapted to this requirement.

Another advantage of the solid adsorbent is that the antibody-coated particles, washed free of serologically inactive (with respect to the haptenic group of the adsorbent) protein, constitutes a preparation of specifically purified antibody, and can be used to study hapten binding without any further processing. That is, by carrying out aggregate formation and hapten binding as successive rather than as simultaneous steps, the inhibiting hapten is never brought in contact with any protein other than that bound to the adsorbent, and interactions between the dye and other serum components cannot occur.

A third advantage of the cellulose-based adsorbent is its relatively great inert bulk, permitting it to be handled readily, and for the immune aggregate of a single experiment to be divided into precise aliquots by gravimetric measurement, so that analytical manipulations

can be performed on portions of a single preparation, rather than on "duplicate" lots that may vary somewhat in their properties due to experimental variations.

Specific purification of the antibody through such a solid adsorbent does not insure a homogeneous population of antibody molecules. There still remains variation in the strength of the bond which the individual antibody molecule is able to form with a given haptenic group. This factor has been difficult to evaluate by direct experimental measurement, but has long been recognized. The quality of "avidity" of antitoxic serum has been ascribed by Glenny and Barr (62) to such varying strength of antigen-antibody union. Boyd (63) has postulated that such variations are the determining factor in the structure of "low-grade", "incomplete", or "blocking" antibody. Lerman (64) using a columnof solid adsorbent of the kind described above, has claimed to fractionate the antibody of anti-azoarsanilic acid serum into fractions having differing characteristics that are consistent with a hypothesis of such varying bonding strength. Pauling, Pressman and Grossberg (46b) in their quantitative theory of hapten inhibition assumed such variation in antibody binding strength and introduced a parameter,

, representing an "error function" in the effective free energy of combination of antigen and antibody, into their equations. The value of for a given system

could be estimated from the nature of the plot relating precipitate inhibition to hapten concentration.

Another possible type of heterogeneity that may occur within a population of antibody molecules that have been purified by specific adsorption on an immune adsorbent is in regard to the number of combining sites. A single combining site, directed against the haptenic grouping with which the adsorbent is synthesized, should be adequate to permit that molecule to be bound to the adsorbent. If the antiserum contained antibody molecules of higher valency, they too will be adsorbed, so that the protein fraction prepared by immune adsorption should contain both univalent and multivalent antibody.

Among the univalent (with respect to the hapten of the adsorbent) antibody molecules, there exists the possibility of further heterogeneity with respect to the nature of the remainder of the antibody molecule's surface. Presumably, this may have the structure of "normal" globulin, or may contain combining sites directed against other antigenic areas of the immunizing antigen or against antigens of other origin.

With the solution of these problems in the preparation of materials, it was then proposed to investigate the composition of immune aggregates, particularly those formed in the region of effective antibody excess, between precipitating antigens and their homologous

antisera, in the presence of homologous or heterologous, iodine-labeled inhibiting haptens. Such haptens were to be chosen, as far as possible, with high inhibiting power (indicating a firm bond to the antibody molecule) and minimum nonspecific adsorption on protein or cellulose. Under such conditions, the ratio of bound to total hapten should be maximum, and the corrections for hapten present in physical solution in the water of the moist aggregate, or bound by non-immunological mechanisms to the protein (and in the case of solid adsorbents) cellulose, will be minimum. The sensitivity of the analytical techniques for measuring the iodine in the aggregate is adequate to determine total iodine in amounts of 0.1 μg. (10^{-9} moles) or greater with an average uncertainty of $\pm$ 5%, so that, by working with precipitates containing as little as 3 milligrams of antibody nitrogen (10^{-7} moles antibody), the hapten bound to as little as 1% of the total antibody present would constitute an adequate analytical sample. The physically held moisture in such a precipitate would be of the order of 0.1 ml., so that at hapten concentrations of 10^{-5} M or less, the correction for dissolved hapten would not be greater than the bound hapten at the 1:100 hapten:antibody ratio. Since earlier studies in this laboratory have indicated that in the arsanilic acid system, haptens of this concentration are capable of causing approximately fifty

percent inhibition to precipitation in antibody-synthetic antigen systems, the prospects of obtaining an unambiguous answer to the question of hapten binding in immune aggregates seemed to justify this approach.

Experimental

Materials

The antigens and haptens employed in this research have been described in earlier papers from these laboratories (34, 39, 44, 65, 66). For reasons of clarity and brevity, these are usually referred to by trivial or abbreviated designations, and this usage will be followed in the present discussion. The following list summarizes this nomenclature.

R = p-azophenylarsonic acid

R' = p-(p-azophenylazo)-phenylarsonic acid

S = p-azosuccinanilic acid

BGG = bovine gamma globulin, Cohn's fractions II and III

OA = crystalline (hen) ovalbumin

RBG = bovine gamma globulin coupled to diazotized p-arsanilic acid

ROA = ovalbumin coupled to diazotized p-arsanilic acid

SBG = bovine gamma globulin coupled to diazotized p-amino-succinanilic acid

R-adsorbent = the immune adsorbent prepared by coupling diazotized p-arsanilic acid to the resorcinol ether of cellulose.

S-adsorbent = the immune adsorbent prepared by

coupling diazotized p-aminosuccinanilic acid to the resorcinol ether of cellulose.

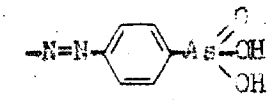
The structural formulae for the haptenic groups and their compounds that were used in these studies are shown in figures 2, 3 and 4.

The borate buffer solutions used in this work were 0.2 molar with respect to boric acid in 0.9% sodium chloride solution. Sufficient sodium hydroxide was then added to bring the pH to the desired value.

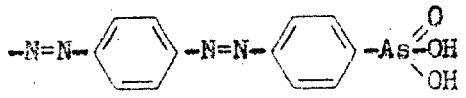
The antisera were all prepared in rabbits by prolonged immunization of the animals with the native or azoprotein antigen. The immunizations and bleeding were, except where otherwise noted, carried out by the departmental animal-room technicians. The sera from the individual bleedings were titrated against the appropriate test antigens and pooled according to the indicated antibody content. The pooled sera were stored without chemical preservatives at temperatures near -20°C. The frozen sera were thawed shortly before required for use, and stored at 4°C until used up. The antibody contents of the thawed sera were determined by the quantitative precipitin reaction.

The gamma globulin fractions of the antisera were separated by ammonium sulfate precipitation according to the following procedure:

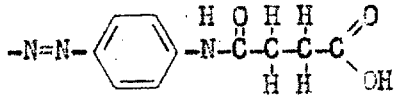
R =



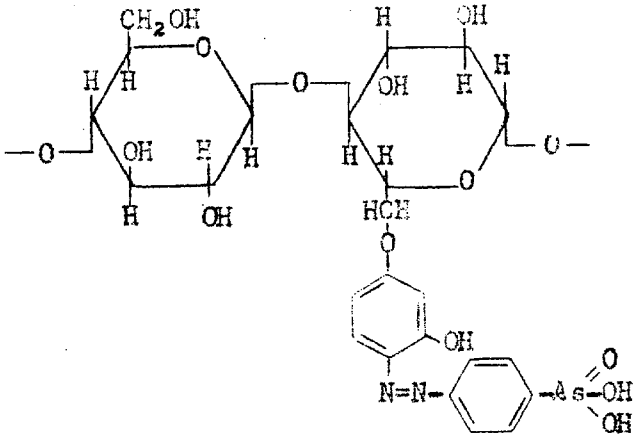
R' =



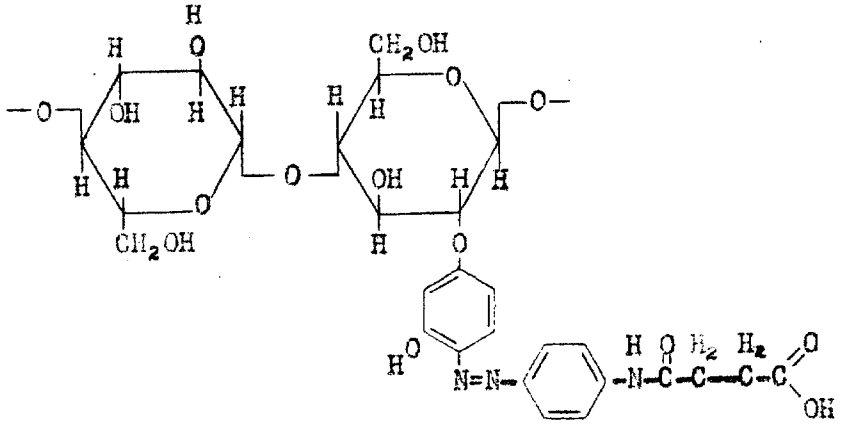
S =



R-adsorbant



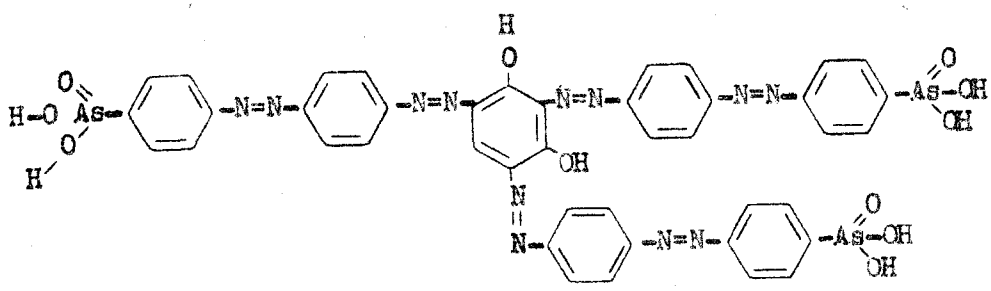
S-adsorbant



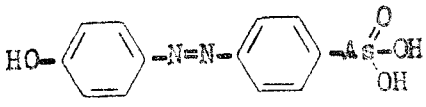
(The distribution of the ether linkages among the 2, 3 and 6 carbon atoms of the glucose rest is not known.)

Haptenic Groups and Immune Adsorbants

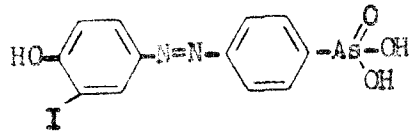
Figure 2



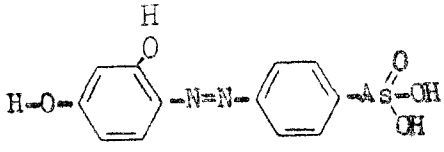
R'$_3$-resorcinol



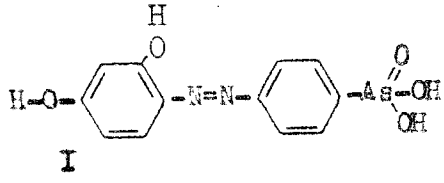
R-phenol



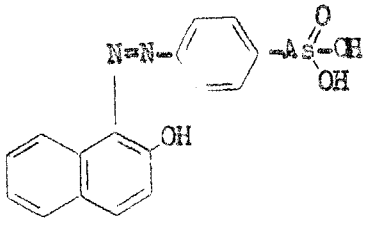
R-iodophenol



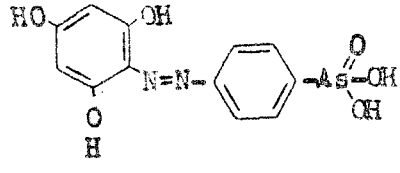
R-resorcinol



R-iodoresorcinol



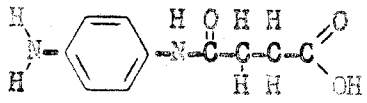
R-beta naphthol



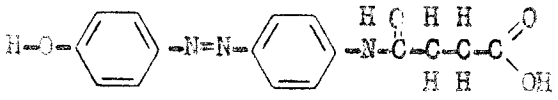
R-phloroglucinol

Arsanilic Acid Haptens

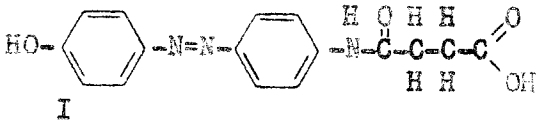
Figure 3



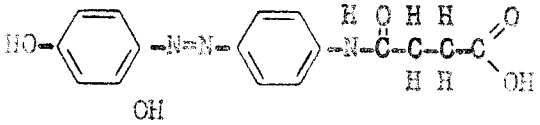
p-aminosuccinanilic acid



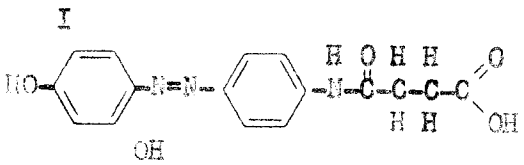
S-phenol



S-iodophenol



S-resorcinol



S-iodoresorcinol

Succinonilic Acid Haptens and Intermediates

Figure 4

One volume of antiserum, at room temperature, was precipitated by the gradual addition of one-half volume of saturated ammonium sulfate solution. The addition of ammonium sulfate solution was distributed over a period of five to fifteen minutes for the usual sized lots (fifty to three hundred milliliters of serum) and vigorous stirring without froth formation was achieved with a magnetically driven stirrer bar. The pH of the suspension was adjusted to 7.8 with sodium hydroxide. Stirring was continued for an additional hour, and then the precipitated globulins were spun down by centrifugation at approximately 2500 RPM for 30 minutes. The supernatant solution was decanted and removed as completely as possible by inverting the tube, draining, and rinsing down the walls of the tube (with the tube in an inverted position) taking care not to disturb the precipitate.

The precipitate was then dissolved in 0.9% saline, using sufficient saline to produce a final concentration of approximately 1% globulin. The precipitation and centrifugation were repeated twice more, and the final precipitate taken up in saline and dialyzed against buffered saline (one volume of borate buffer, pH 8.4 added to approximately ten volumes of 0.9% NaCl in the dialysis jar. The dialysis was carried out in the cold room at 4°C. The dialyzed solution was filtered through

Whatman No. 42 filter paper to remove traces of insoluble protein separating during the dialysis, and stored at 4°C until used. Each lot of γ globulin prepared was tested for antibody content by a constant antibody-variable antigen titration.

Antisera

Anti-RBG sera: The antigen against which these sera were prepared was made by coupling diazotized p-amino-phenylarsonic acid (arsanilic acid) to a commercial preparation of bovine gamma globulin*. The finished antigen contained about 30 haptenic groups per molecule of protein. The synthesis of the antigen and all phases of the immunizations and bleedings were done by departmental personnel. Antibody assays were carried out against the R'_3-resorcinol precipitating hapten.

There was a large volume of a pooled gamma globulin concentrate from low titer anti-RBG sera available in the department. This had been prepared from an assortment of various pools of low titer serum that had accumulated in the deep-freeze serum storage, and which were of too low potency to be attractive for most research programs. This preparation had a relatively low activity against the R'_3 resorcinol antigen, but a good titer against bovine globulin, particularly against the

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* Armour "Fractions II and III, lots C103 and C104"

immunizing antigen described below. The generous supply of a single pool of the material made it possible to carry out extensive tests on this preparation.

A typical precipitin curve of purified anti-RBG-antibody against R'_3-resorcinol is shown in Figure 5.

Anti-BGG sera: Two groups of rabbits were immunized against native bovine gamma globulin. The immunizing antigen was the soluble portion of the bovine globulin preparation* that had been used in the synthesis of the RBG antigen described above. Eighteen grams of the crude bovine globulin preparation was shaken repeatedly over a period of six hours with 600 ml. of 0.9% saline. The mixture was clarified by centrifugation and decantation through a rapid filter paper. This filtrate was assayed for nitrogen by Kjeldahl digestion and Nesslerization, and found to contain approximately 2.3% of protein. It was diluted to a concentration of 1%, and sterilized by passage through a Seitz filter. The final solution was assayed for protein by the biuret method of Kingsley (67) against a human globulin standard, and found to contain 1.04% globulin.

Five rabbits were immunized on the following schedule: One-half ml. antigen intraperitoneally semi-weekly for two injections, then one ml. intraperitoneally

*This material had partially denatured during years of storage, and only a portion of it could be dissolved in neutral saline.

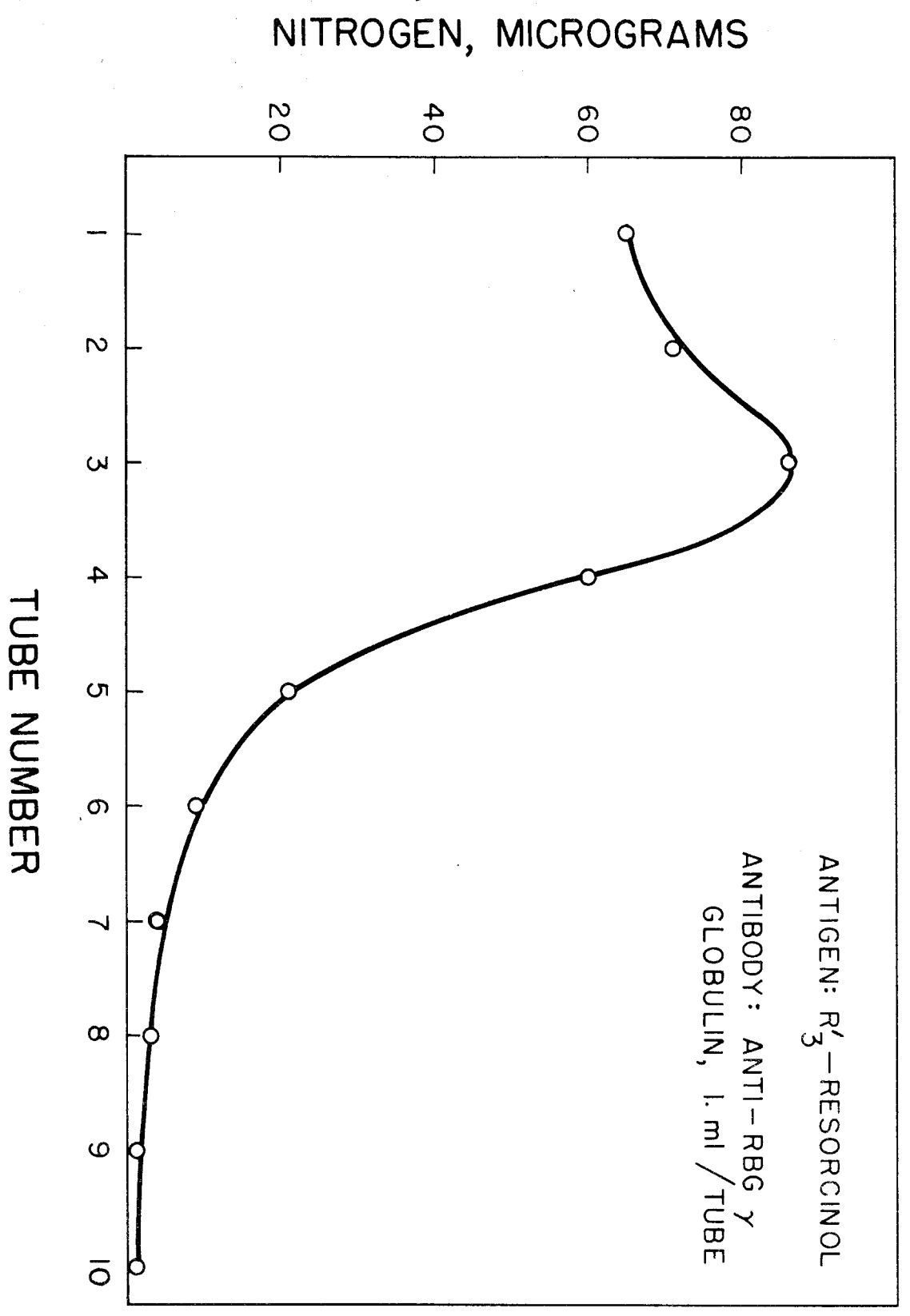
NITROGEN, MICROGRAMS
20
40
60
80
TUBE NUMBER
1
2
3
4
5
6
7
8
9
10
ANTIGEN: R'3—RESORCINOL
ANTIBODY: ANTI-RBG γ GLOBULIN, 1. ml / TUBE

FIGURE NO. 5

semiweekly for two injections, then one half ml. intravenous injection followed by 1 ml. intravenous injections on a three times a week schedule. After the second 1 ml. injection, one of the rabbits died in shock, and the injection schedule on the remaining rabbits was reduced to one-half ml. on the next injection, increasing the dosage by one-tenth of a ml. each succeeding injection until it was up to one ml. again. After six weeks injections and a one week rest period, test bleedings were made, following which the immunization program and bleedings were continued by the animal room technicians.

Later in the program an additional supply of anti-BGG serum was required. A new group of rabbits was immunized with a second lot of antigen prepared substantially as described above. All animal work was done by departmental personnel.

Figure 6 is the precipitin curve of a γ globulin fraction of an anti-BGG serum tested against lot 3 antigen.

Anti-OA serum: Preliminary test with an available γ globulin preparation from an anti-(egg white) serum being unsatisfactory, a single preparation of γ globulin from an anti-(crystallized ovalbumin) serum was worked up by the procedure described above. Approximately 90 ml. of a pool of high titer serum from the bleedings of 10-2-51 and 12-3-51 was obtained from the animal room,

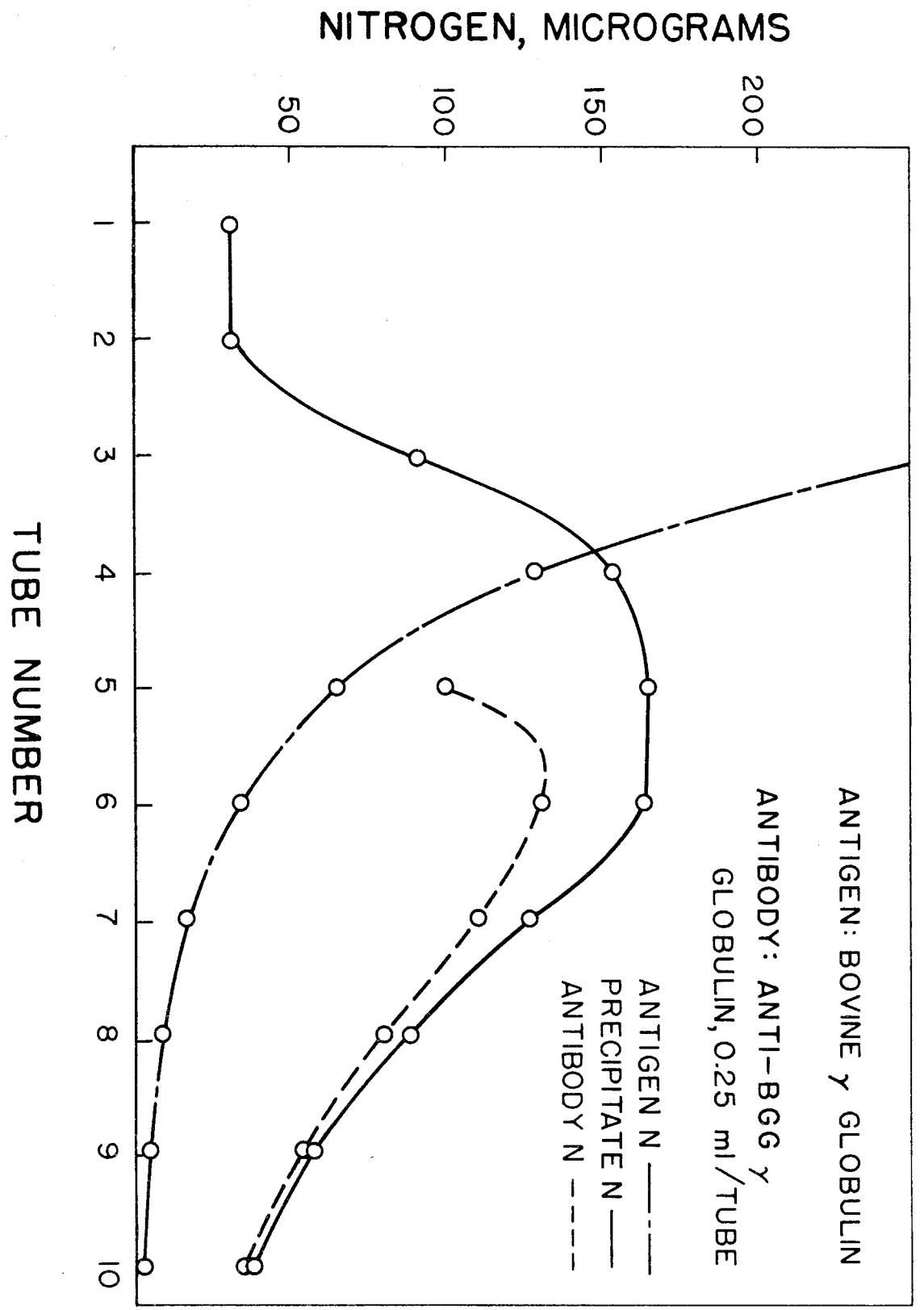
NITROGEN, MICROGRAMS
50
100
150
200
TUBE NUMBER
1
2
3
4
5
6
7
8
9
10
ANTIGEN: BOVINE γ GLOBULIN
ANTIBODY: ANTI-BGG γ
GLOBULIN, 0.25 ml/TUBE
ANTIGEN N
PRECIPITATE N
ANTIBODY N

FIGURE NO. 6

and the γ globulin made up to a final volume of 50 ml. The titration curve against 3 times recrystallized egg albumin is shown in Figure 7.

Anti-SBG serum: A lot of anti-succinanilic acid-azo-bovine globulin serum (Pool of 5-7-48, Seitzed 11-19-48) was obtained from the animal room for preliminary experiments with solid adsorbents. The serum did not yield significant amounts of precipitate with an old preparation of succinanilic acid-azo-ovalbumin as a test antigen but did give strong ring tests with a sample of the immunizing antigen. Laboratory records indicated that an adequate supply of the same pool was in storage, but when the sample lot first obtained was exhausted, search of the storage freezers failed to yield additional supplies of this antiserum. This shortage of serum, coupled with the extraordinary difficulties in purification of the succinanilic acid haptens and their chemical instability, led to the abandonment of the use of the succinanilic acid system.

Normal rabbit serum: Freshly prepared sera from normal rabbits was obtained from the animal room as required. Normal serum controls were in general employed only on whole-serum systems. No attempt was made to prepare normal γ globulin concentrates.

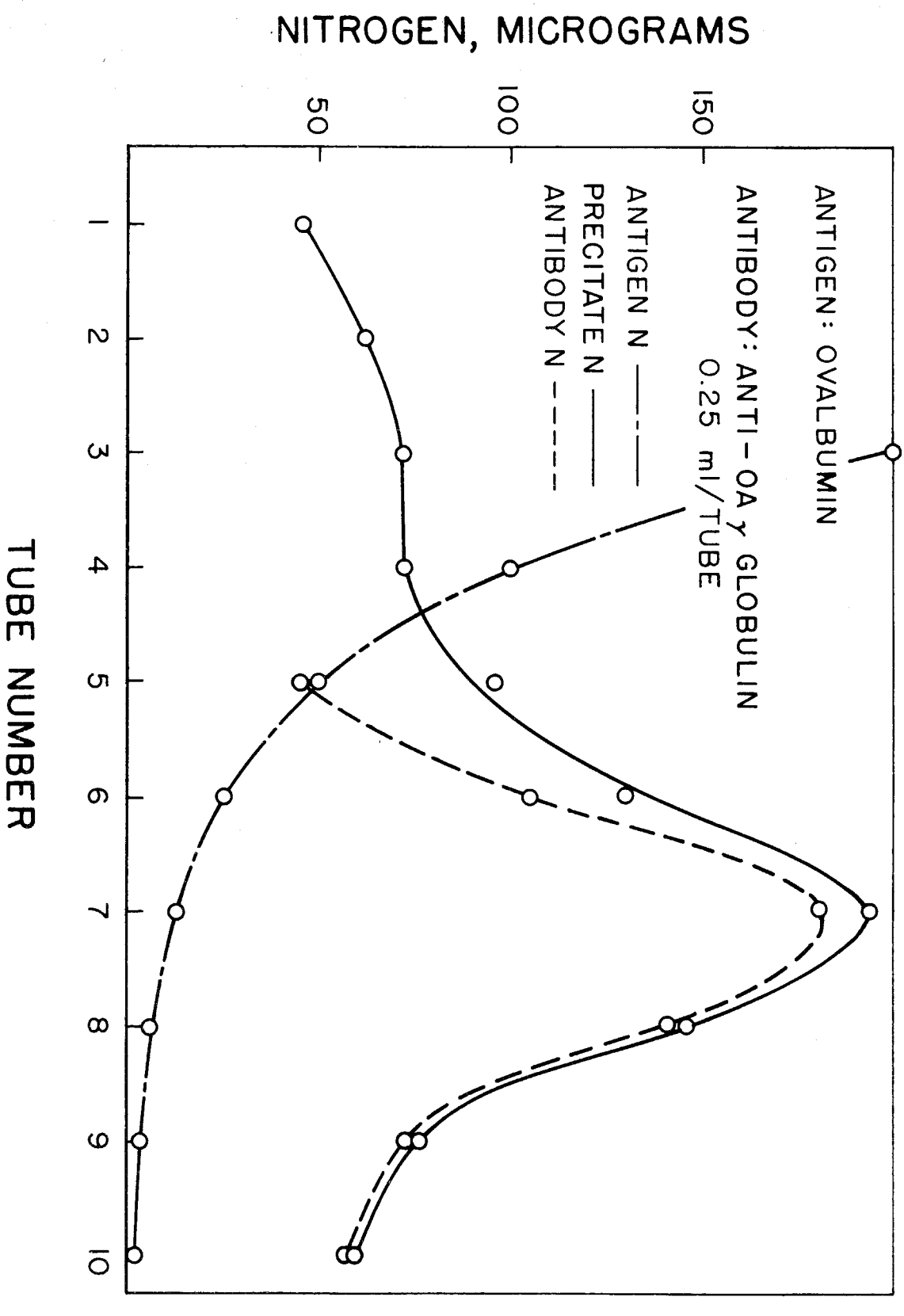
NITROGEN, MICROGRAMS
50
100
150
ANTIGEN: OVALBUMIN
ANTIBODY: ANTI-OA γ GLOBULIN
0.25 ml/TUBE
ANTIGEN N
PRECITATE N
ANTIBODY N
1
2
3
4
5
6
7
8
9
10
TUBE NUMBER

FIGURE NO. 7

Test Antigens

R'_3-resorcinol: The preparation and properties of 1,3, dihydroxy-2,4,6,tri-(p-(p-azophenylazo) phenylarsonic acid)-benzene have been described by Pardee and Pauling (66). The longer structure of the R' grouping is believed to be responsible for the superior precipitating properties of this material. The greater spatial separation resulting therefrom should permit all three of the haptenic groups to form bonds simultaneously with antibody molecules without undue steric hindrance. The material used in this research was obtained from laboratory stocks, and used without further purification.

R-ovalbumin: This antigen was prepared essentially as described by Boyd (29). One-half gram of p-arsanilic acid was dissolved in thirty-five ml. of approximately N/50 HCl, cooled in a salt ice-bath to 0°C, and diazotized by the gradual addition of a slight excess N $NaNO_2$ solution as determined with starch-iodide paper. The solution was allowed to stand for about one-half hour to insure complete diazotization, and then the excess nitrous acid was destroyed with ammonium sulfamate.

Three and a half grams of 3 times recrystallized ovalbumin was dissolved in 100 ml. of water. The protein solution was cooled to below 5°C, and, while stirring vigorously but without air entrainment by a magnetic stirrer, the diazotized arsanilic acid and N NaOH were

added concommitantly at such a rate as to keep the pH between 7.5 and 8.0, as measured with a Beckman model G pH meter. After all of the diazotized arsanilic acid was added, stirring was continued for an additional hour and a half, temperature control being maintained by surrounding the beaker with crushed ice. The solution was transferred to the cold room, and allowed to remain there over the weekend.

The azoprotein, precipitated at pH 3.5, was collected by centrifugation in a refrigerated centrifuge, washed three times with 0.01 N sodium chloride in 10% ethanol, and then dialyzed against distilled water until free of salts and traces of uncoupled dye. The resulting product was a reddish orange flocculant solid that dissolved very slowly in dilute sodium hydroxide. The volume was made up to 100 ml., the pH of the solution was adjusted to 9.0 and after filtration through Whatman No. 42 filter paper to remove traces of insoluble material, the solution was stored in the refrigerator.

Nitrogen (semimicro Kjeldahl) 0.37%.

Arsenic 0.10%.

Haptenic groups per molecule* of ovalbumin 26.

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* Based on a molecular weight of 44,000 (68)

Three different lots of bovine gamma globulin were tried as test antigen. The immunizing antigen will be referred to as Lot 1. Lot 2 was a commercial preparation of bovine gamma globulin**, Lot 3 was a fraction of Lot 1 that was purified by ammonium sulfate precipitation in a manner comparable to that used in preparing antibody concentrates.

Inhibiting Haptens

The dyes employed as inhibiting haptens were synthesized by techniques generally following those described by Fierz-David and Blangley (69). The directions given in this reference are well-written and have been found to be reliable. The suggestions made and background given enable the experimenter to apply the methods to related dyes with a minimum of difficulty and uncertainty.

The dyes were prepared in lots considerably larger than those synthesized by earlier workers in these laboratories. Purification techniques leading to rapid increases in purity generally give low yields of the purified product. Further, in studies on the iodination of the dyes, it was found that attempts to work up products of low initial purity were exhorbitantly time-consuming, and practically never led to final material

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** Armour "Fraction II, lot 1823B"

of satisfactory purity. Consequently, if an attempt at iodination led to an initial product of marked inhomogeneity or containing tarry materials, the entire lot was discarded. As a result, relatively large quantities of dyes were employed in this research, and the amounts of high-purity dyes prepared were sufficient for subsequent workers who may wish to repeat or extend the studies reported here.

In general, where a number of procedures have been tested in the preparation of any dye, only that found most satisfactory will be described in detail. Brief comments will be made on methods of iodination that were found to be unsatisfactory for the particular compounds tested.

Preparation of p-(p-azophenylarsonic acid)-phenol (R-phenol): Forty-three and four-tenths grams (0.2 moles) of p-arsanilic acid (Eastman White Label) is dissolved in 300 ml. of water containing 24 g. (0.23 moles) of Na_2CO_3 and the solution is filtered to remove traces of insoluble impurities. Sufficient concentrated HCl is added to the filtrate to just dissolve the precipitated arsanilic acid (Approximately 100 ml.). The solution is cooled to 2 -4°C and 200 ml. of 1.00 N $NaNO_2$ solution, previously cooled to below 5°C, is added dropwise over a period of one-half hour to the well-stirred solution. The temperature is not

permitted to rise above 8° C at any time during the diazotization. The reaction mixture is tested with starch-iodide paper to insure the presence of a slight excess of nitrous acid, and after an additional half-hour to insure completion of the diazotization, solid ammonium sulfamate is added to destroy remaining traces of nitrous acid.

Eighteen and eight-tenths grams (0.2 moles) of reagent grade phenol is dissolved in 200 ml. of ice water containing 53 g. (0.5 moles) of Na_2CO_3 and 20 g. of NaOH*. The solution of diazotized arsanilic acid is added from a separatory funnel to the alkaline phenol solution. The temperature is kept below 4° C and local excesses of the uncoupled diazo compound are avoided by vigorous stirring during the slow addition. One hour is allowed for the completion of coupling after all of the reagent has been added.

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* The amounts of Na_2CO_3 and NaOH are adjusted to the excess acid in the diazotization solution, using such amounts that after the two solutions have been mixed, there will be no evolution of CO_2 (with its attendant frothing) but that the composition of the final solution will be essentially NaCl and $NaHCO_3$. In the initial stages of the addition of the diazo solution to the phenol, the coupling solution will be strongly alkaline, but with the large excess of phenol present at that time, the concentrations of free diazonium compound will be kept so low that there is little opportunity

The solution of the dye is allowed to rise to room temperature spontaneously, then poured into a large porcelain evaporating dish, placed on a tripod, and heated with a large, soft flame until the solution is brought to a slow boil. Three hundred grams of fine crystalline NaCl* is slowly added to the boiling solution with stirring and the mixture is held at the boiling point for fifteen minutes. Remove the flame, and allow the preparation to cool gradually. As the temperature drops, crystals of the disodium salt of the dye begin to separate. When the temperature reaches 40-45° C;** the suspension is poured onto a large Büchner funnel fitted with a fast filter paper (Whatman No. 52) and the slurry of dye and sodium chloride crystals is pressed as dry as possible. The filter cake is washed twice with saturated sodium chloride solution and once with ice-cold distilled water.

The filtrate, although darkly colored, contains little recoverable dye, and that is of low purity. It is discarded.

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for self-coupling. At the end of the reaction, the pH will be close enough to neutral that self-coupling will be effectively inhibited.

* A slight excess over that required to saturate the solution.

** It is important, particularly in the first crystallization, to filter the material at the temperature given. Filtration at higher temperatures leads to low recover-

The crude preparation contains significant amounts of sodium chloride as an impurity, but relatively small amounts of colored impurities. It can be further purified by a repetition of the process described above; dissolving it in a minimum quantity of boiling water, saturating with sodium chloride, cooling to about 40° C and filtering and washing. A final recrystallization can be made from boiling water without salting out.

The yield of crude dye is about 70 g. (95% of theory). The succeeding purification steps will reduce the over-all yield to approximately 75% of the theoretical.

Analysis: Arsenic: found* 20.4% theory 20.46%
Chloride: 3.85% (as NaCl)
Assay: 95.8%

The dye was tested for homogeneity by column chromatography on neutrol filtrol-celite (7:3) as described by Pauling (70) and by ascending paper chromatography.

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ies and clogging of the filter by material coming out of solution during the filtration process. If the slurry is allowed to cool to lower temperatures, amorphous materials begin to separate out of solution, and the mixture becomes slimy, very difficult to filter and wash, and the purity of the product is greatly diminished. Attempts to purify such an unsatisfactory initial product have been disappointing.

* Corrected for NaCl content.

In each instance the dye migrated in a simple well-defined band, without evidence of the presence of colored impurities. The description of the technique used in the paper chromatography follows.

A few milligrams of the sodium salt of the dye is converted into the free acid by solution in water and precipitation with dilute hydrochloric acid. The dye is spun down in the centrifuge, washed with distilled water, and taken up in a minimum quantity of hot methyl cellosolve. The resulting solution is applied to the base line of a strip of Schleicher and Schull filter paper no. 598 and the chromatogram developed in the organic phase of a mixture of n-butanol-pyridine-water (3:1:1.5) at room temperature.

The center third of the colored band of such a chromatogram was cut out, eluted in M/15 disodium phosphate, and the absorption ratios determined at the maxima and minima shown. The corresponding values for the bulk preparation indicate excellent agreement and the substantial homogeneity of the dye.

The ionization constants are given for arsenic acid (71) as 5×10^{-3} for the first hydrogen and 4×10^{-5} for the second hydrogen. That for phenol is given as 1.3×10^{-10}. Assuming these values to be reasonable approximations of those for the equivalent groupings in this dye, it may be seen that the two hydrogens of the

arsonic acid are of too nearly the same strength to permit the monosodium salt to exist in solution without there being an appreciable concentration of either the free acid or the disodium salt in equilibrium with it in any buffer system that might be chosen. At the pH of the serological systems being studied, the dye is substantially all in the form of the disodium salt. At pH values above twelve, the phenolic hydrogen has been substantially all replaced with sodium. Spectrophotometric curves run at pH 8 and pH 13 may therefore be anticipated to be characteristic of the secondary salt and phenolate forms respectively, with minimum hybridization between the desired forms and lower or high levels of ionization.

Spectrophotometric curves for this dye, determined with a Beckman model DU spectrophotometer, at the indicated pH values, are shown for the spectral region of 220 to 620 mμ, in Figure No. 8. Additional spectrophotometric data are recorded in Table No. 1.

Preparation of 2-hydroxy-1-(p-azophenylarsonic acid)-naphthalene (R-β naphthol): Forty-three and four-tenths grams (0.2 moles) of p-arsanilic acid is diazotized as described for the phenol dye. The coupling is carried out in a similar manner, substituting 29 g. of β naphthol for the phenol. The initial product is filtered from the hot slurry at 50° C, and is purified by one salting

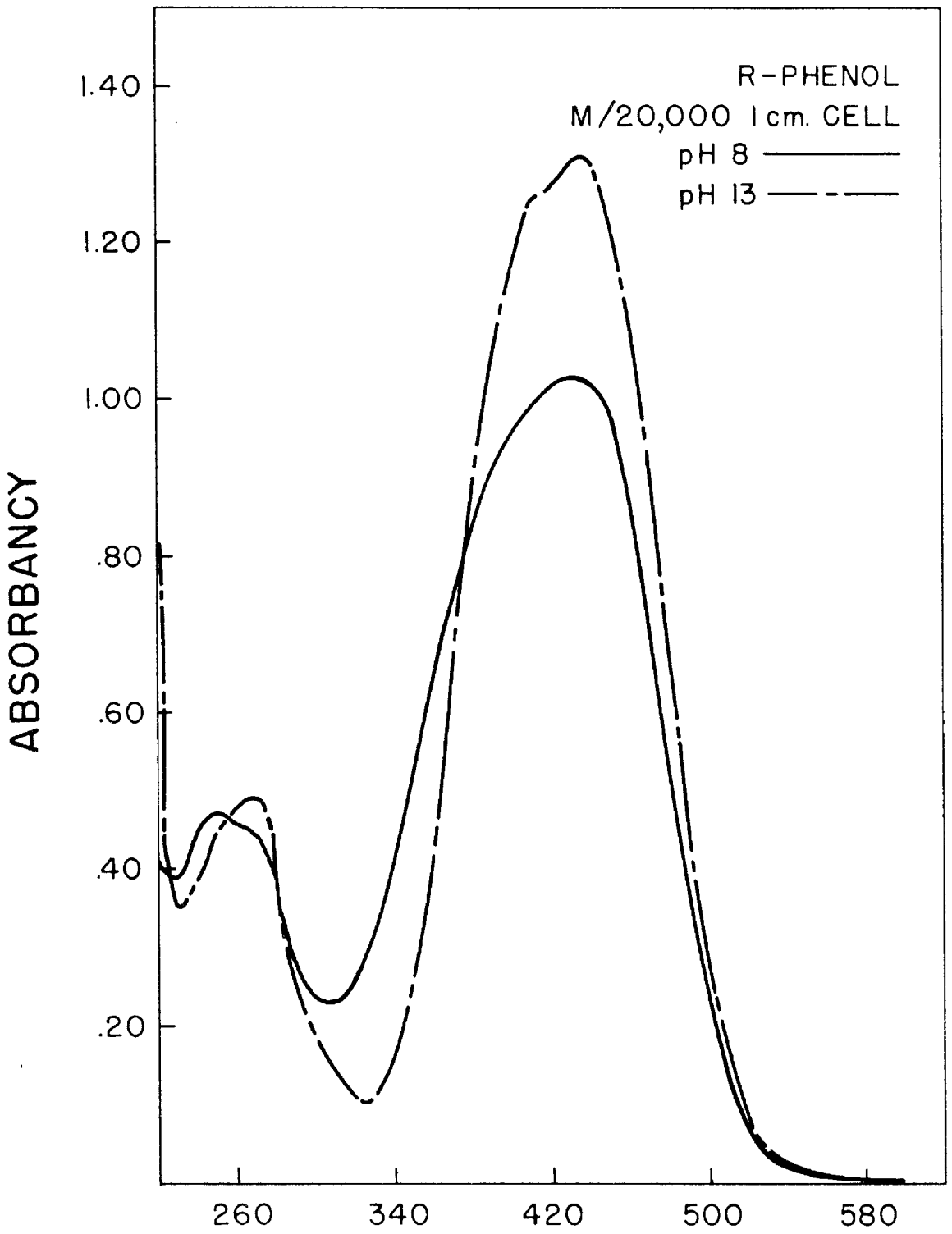
R-PHENOL
M/20,000 1 cm. CELL
pH 8
pH 13
1.40
1.20
1.00
.80
.60
.40
.20
ABSORBANCY
260
340
420
500
580
WAVELENGTH mμ

FIGURE NO. 8

out step and two recrystallizations from boiling water. The dye has an intense red color, and recrystallizes cleanly. The purified dye shows no evidence of inhomogeneity on either column or paper chromatography.

The ease of synthesis and purification of this dye is undoubtedly related to the extreme specificity of the coupling in the α position of the naphthol. Thus there are no isomeric dyes formed as side reactions. A corrolary of this property of the naphthol is its resistance to iodination. All attempts to prepare an iodinated dye by direct iodination resulted either in failure of addition of iodine, or if conditions were more rigorous, to the formation of badly degraded products that could not be resolved into pure, homogeneous iodinated compounds. The desirable attributes of the 1-azo β naphthol dyes are sufficient to justify the synthesis of β naphthol iodinated at some position other than the α. Such an iodonaphthol should yield haptens of great purity and homogeneity.

Analysis:

Arsenic: found 17.8% theory 18.00%

Chloride: 1.20% (as NaCl)

Assay: 97.7%

The spectrophotometric curves for the dye at pH 8.0 and 13.0 are shown in Figure 9, and additional spectrophotometric data in Table No. 1.

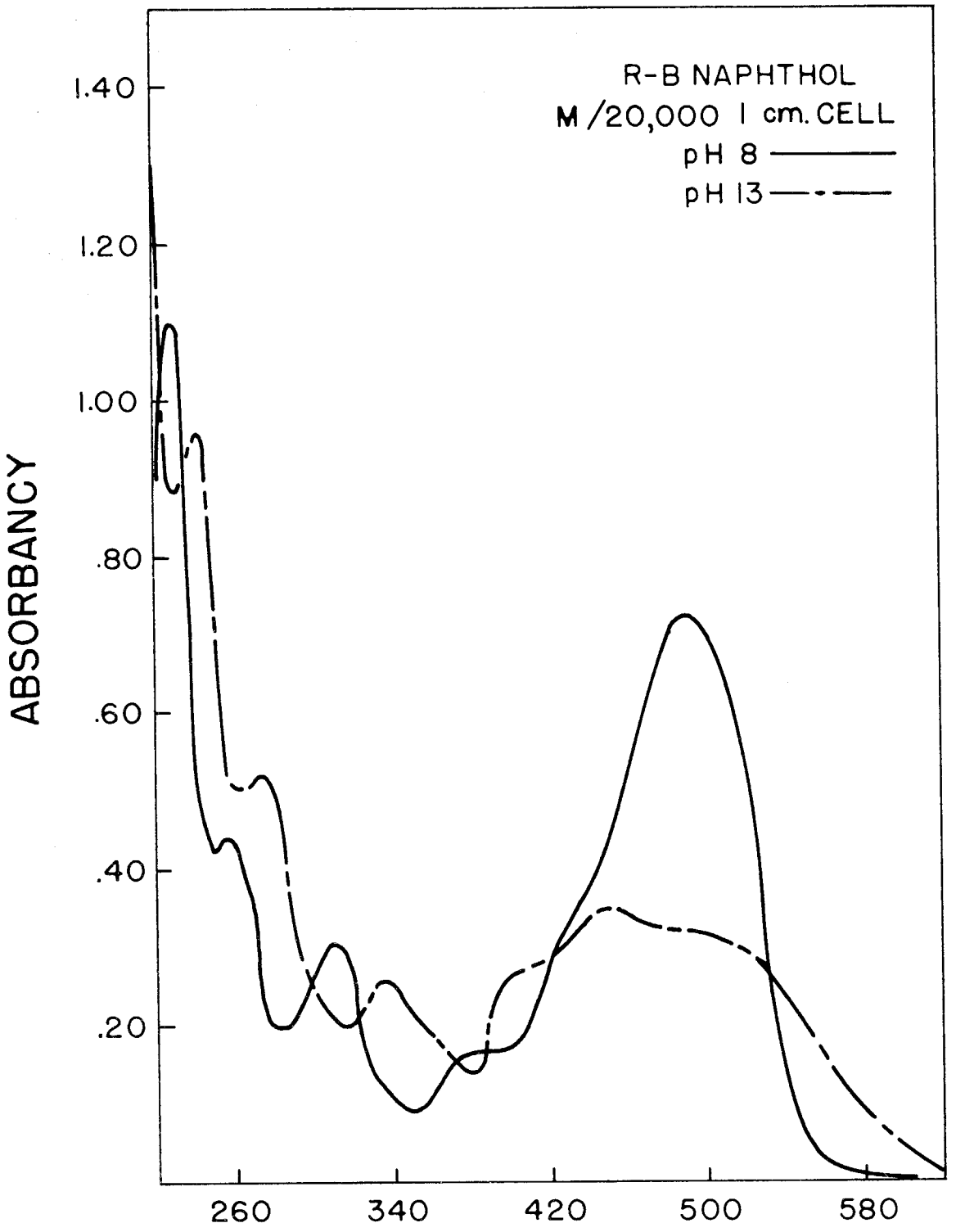
R-B NAPHTHOL
M/20,000 1 cm. CELL
pH 8
pH 13
1.40
1.20
1.00
.80
.60
.40
.20
ABSORBANCY
260
340
420
500
580
WAVELENGTH, mμ

FIGURE NO. 9

Preparation of 1,3-dihydroxy-4-(p-azophenylarsonic acid)-benzene (R-resorcinol): Resorcinol couples to diazotized arsanilic acid very smoothly in acid solution. Since the resorcinol is water soluble, the reaction can be carried out in solution. An excess of resorcinol is employed to minimize the formation of polyazo dyes.

Forty-three and four-tenths grams of arsanilic acid (0.20 moles) is dissolved in 60 ml. of concentrated HCl and 200 ml. of N $NaNO_2$ added as previously described. The solution of the diazonium compound is added dropwise with stirring to an ice-cold solution of 30 g. (0.27 moles) of reagent grade resorcinol in 200 ml. of water. The dye precipitates out in a granular form that filters and washes very readily. The clear yellow dye yields sharp, homogeneous bands on both column and paper chromatography. The dye is sufficiently pure to use as a starting point for synthesis of the iodinated dye without further purification. For the determination of its analytical constants, a small aliquot was purified by recrystallization from alcohol.

Analysis:

Arsenic: found 22.0% theory 22.15%.

Spectrophotometric curves are shown in Figure No. 10, and additional spectrophotometric data in Table No. 1.

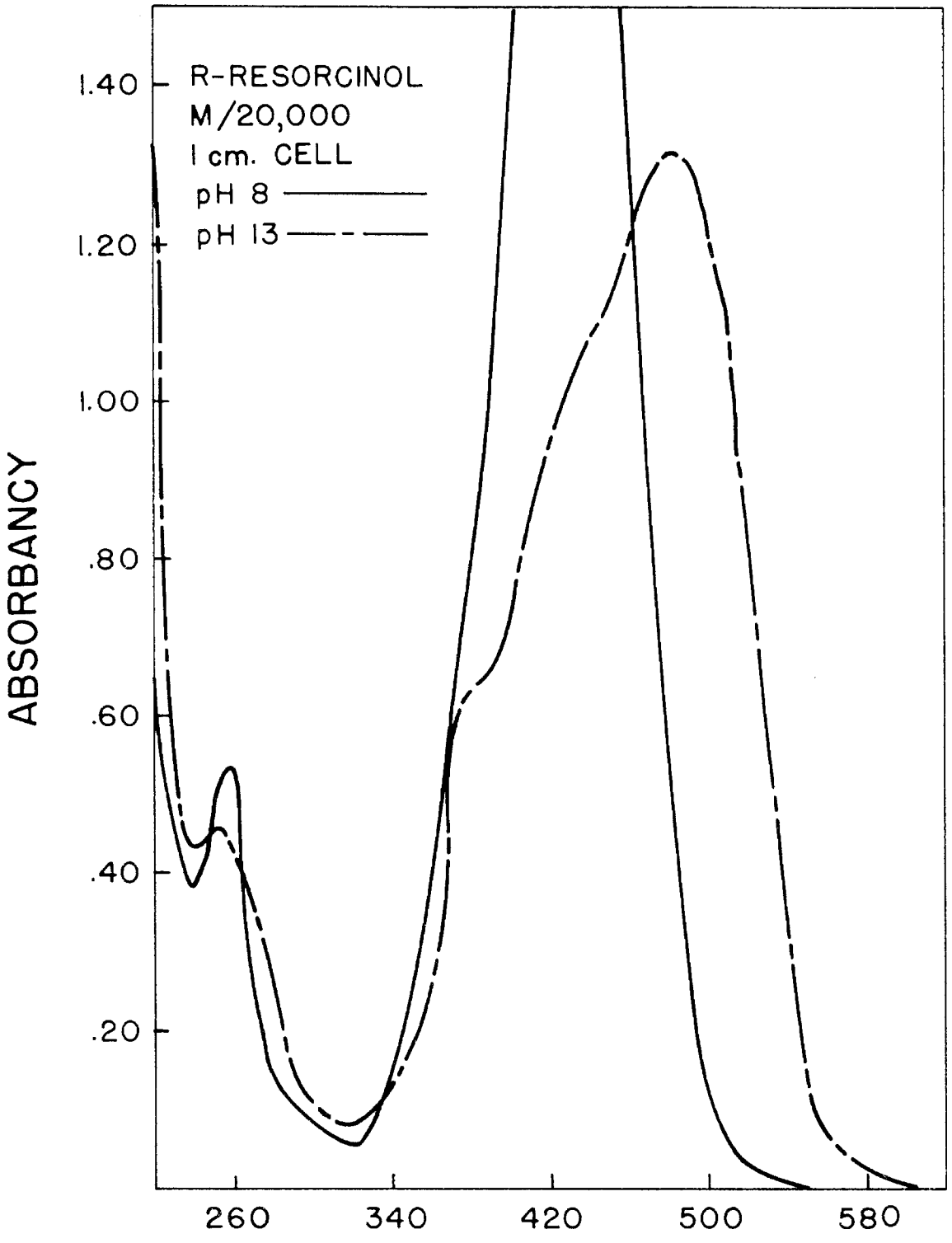
R-RESORCINOL
M/20,000
1 cm. CELL
pH 8
pH 13
1.40
1.20
1.00
.80
.60
.40
.20
ABSORBANCY
260
340
420
500
580
WAVELENGTH, mμ

FIGURE NO. 10

Preparation of 1,3,5-trihydroxy-2-(p-azophenylarsonic acid)-benzene (R-phloroglucinol): Seventeen and four-tenths grams (0.08 moles) of arsanilic acid was diazotized as described for the resorcinol dye, and added with stirring to a cold solution of 12.6 g. (0.10 moles) of reagent grade phloroglucinol. A finely divided, dark red precipitate separated from a very dark supernatant. Chromatographic examination showed the supernatant to contain a hopelessly complex mixture of colored materials, and no attempt was made to work it up. The precipitated dye was washed with boiling water, hot alcohol, hot methyl cellosolve, and finally with cold acetone. None of the solvents used extracted significant quantities of colored material, and after each extraction the insoluble residue became more homogeneous on chromatography.

The final product showed only one band on chromatography, but the trailing edge was quite diffuse. It was not determined whether this was due to impurities or whether it was a characteristic known as "tailing" that some compounds exhibit. Preliminary attempts at iodination of the phloroglucinol dye indicated that this technique was not satisfactory, so no further efforts were expended on the intermediate.

Analysis:

Arsenic: found 21.4% theory 21.15%

spectrophotometric curves are shown in Figure No. 11 and additional spectrophotometric data in Table No. 1.

Synthesis of p-amino-succinanilic acid: p-Nitro-succinanilic acid was prepared by the method described by Pauling et al (65). Sixty-nine grams (0.5 moles) of p-nitro-aniline (Eastman White Label) and 50 g. (0.5 moles) of succinic anhydride were each dissolved in 125 ml. portions of hot dioxane, mixed, heated in a boiling waterbath for five minutes, the heat removed from the bath and the entire system allowed to cool slowly to 40° C. A light cloudy precipitate of impurities that had developed to that point was removed by quickly filtering through a coarse sintered glass filter. The filtrate rapidly crystallized from seeding initiated in the stem of the funnel, and after storage over night in the refrigerator, the crop of crystals was filtered off, washed with cold dioxane and dried.

Yield 72 g. (60% of theory) m.p. 192-193° C.

A second crop of somewhat less pure material was recovered by partial evaporation of the mother liquor and cooling overnight. Yield, 17 g., m.p. 190-193° C.

The combined first and second crops were recrystallized from 800 ml. of an equal volume mixture of dioxane and methyl-ethyl-ketone. Yield, first crop, 45 g.,m.p. 198-199° C.

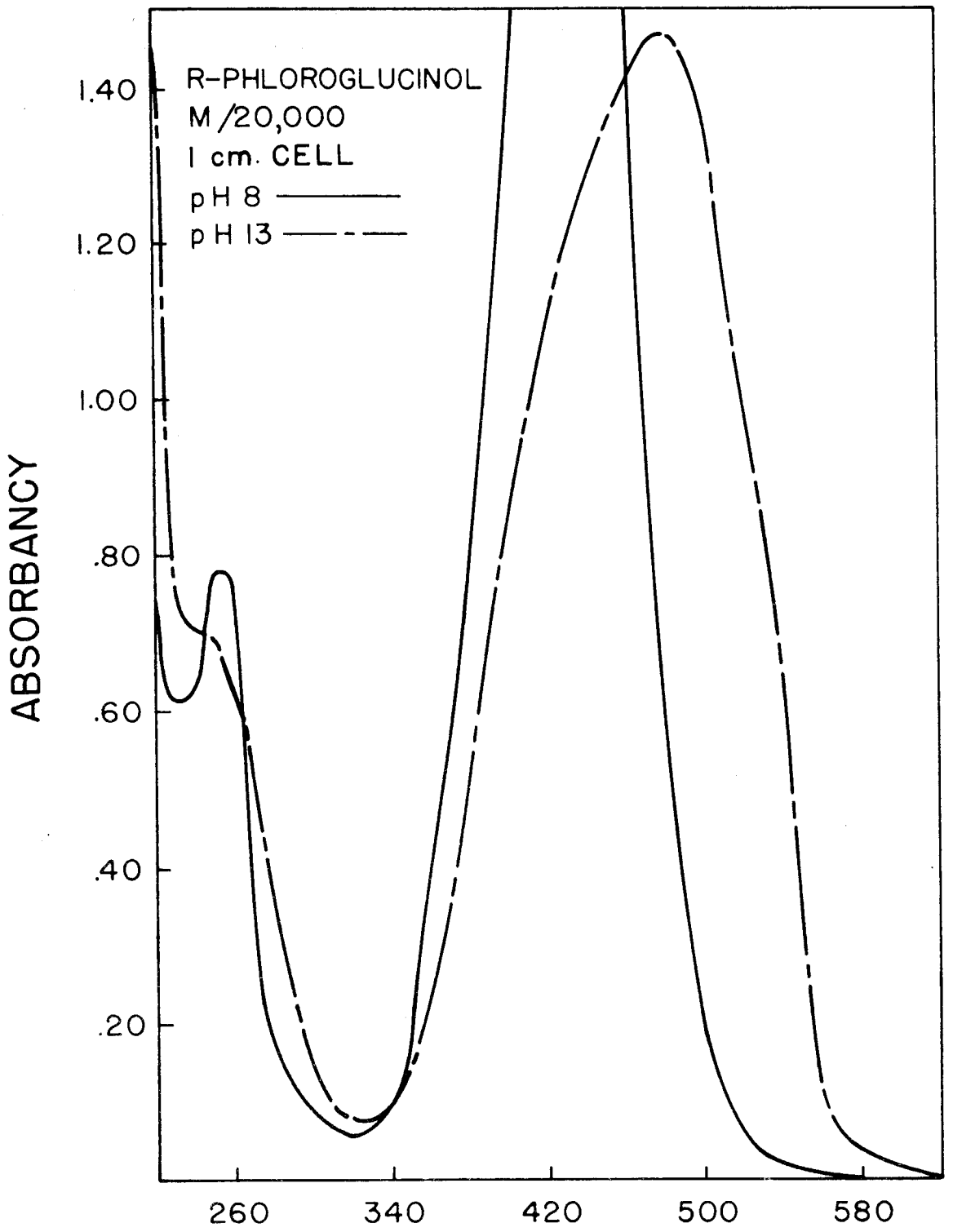
R-PHLOROGLUCINOL
M/20,000
1 cm. CELL
pH 8
pH 13
1.40
1.20
1.00
.80
.60
.40
.20
ABSORBANCY
260
340
420
500
580
WAVELENGTH, mμ

FIGURE NO. 11

The mother liquor was treated with 5 g. of vegetable charcoal (Norite A), evaporated to a third of its original volume, and a second crop of adequate purity obtained.

Yield 33 g., m.p. 196-198° C.

Reduction of p-nitrosuccinanilic acid: The nitro compound was reduced to the corresponding amine by hydrogenation over Adam's platinum-black catalyst (72) at room temperature and moderate pressure. The reaction was carried out in a Burgess-Parr low pressure hydrogenation apparatus. The hydrogen tank was stated by the manufacturers to have a volume of 4000 ml.

Twenty-three and eight-tenths grams (0.1 mole) of p-nitro-succinanilic acid was partially dissolved in 150 ml. of absolute ethanol (solution not quite complete at room temperature) and 0.2 g. of platinum-black catalyst added. The ambient temperature was 25° C. The reduction was started with a tank pressure of 48 pounds per square inch gauge, and when the pressure fell to 35 pounds, the reservoir was refilled to 59.5 psig. Absorption of hydrogen was complete in one hour and twenty-five minutes. The initial temperature rise was moderate, but was sufficient to bring all of the substrate into solution. Towards the end of the reduction large amounts of the p-amino-succinanilic acid crystallized out in the pressure flask, making the agitation rather ineffective

in keeping the system well-mixed. Subsequent batches were reduced in 0.05 mole lots.

The course of the hydrogenation is shown in Figure No. 12.

The entire contents of the reduction flask was poured into hot 90% methanol, and the spent catalyst filtered off. On cooling, the filtrate deposited a crop of very lightly colored crystals of p-aminosuccinanilic acid.

Yield, first crop 6 g., m.p. 184° C with decomposition.

The mother liquor was combined with that from other lots, decolorized with Norite A, concentrated, and cooled. The total recovery of the p-aminosuccinanilic acid was approximately 75%, based on the nitro compound used.

Preparation of 1,3-dihydroxy-4-(p-azosuccinanilic acid) benzene (S-resorcinol): At the stage of this research program at which the succinanilic dyes were being prepared, previous experience had indicated the resorcinol dye was the most likely starting material for direct iodination. Ortho-iodophenol was also on hand for the direct synthesis of its labeled hapten. Consequently the resorcinol dye was the only dye prepared from the succinanilic acid hapten in amounts adequate for iodination experiments.

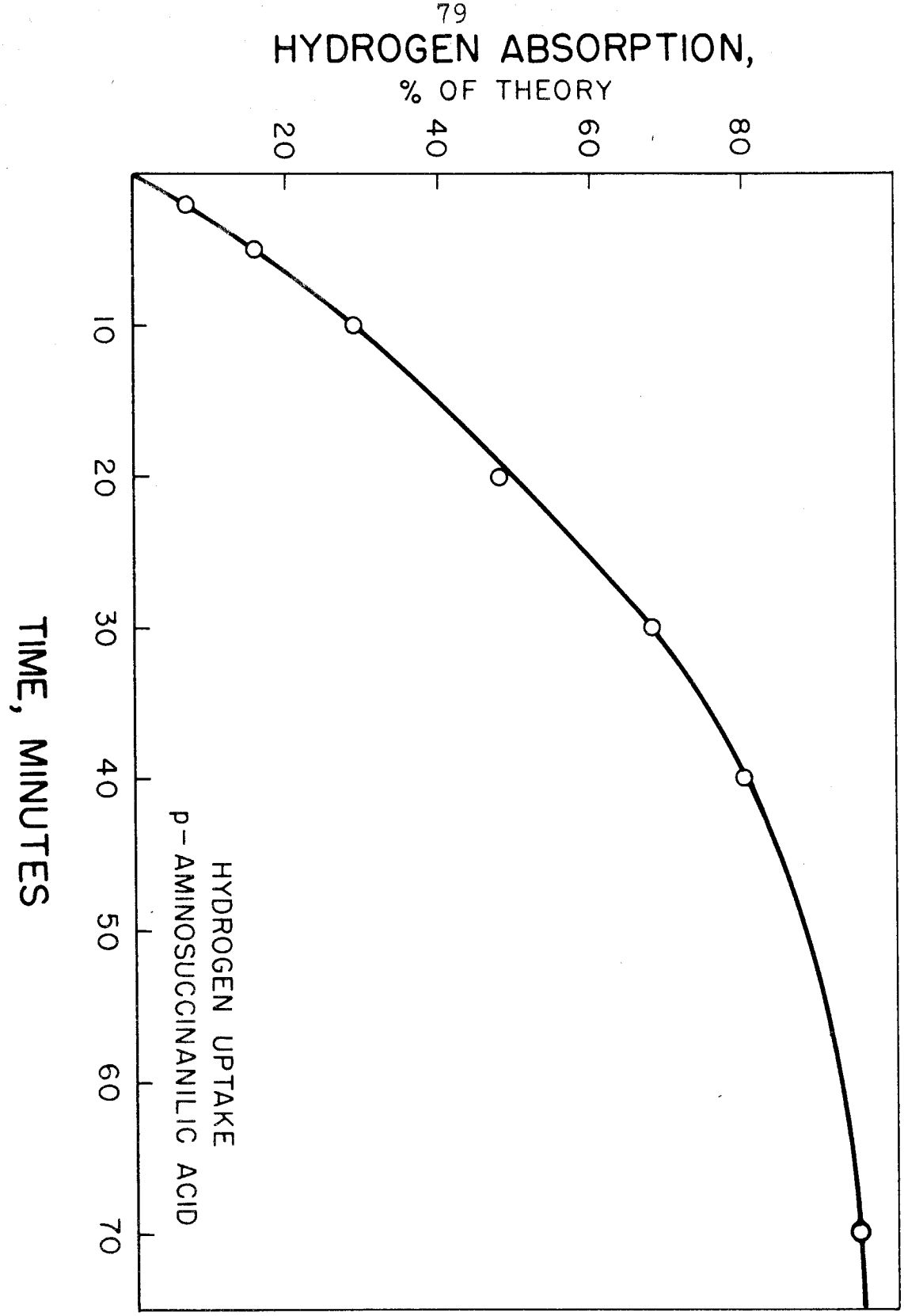
HYDROGEN ABSORPTION,
% OF THEORY
20
40
60
80
10
20
30
40
50
60
70
TIME, MINUTES
HYDROGEN UPTAKE
p-AMINOSUCCINANILIC ACID

FIGURE NO. 12

Four and eight-tenths grams (0.02 moles) of p-aminosuccinanilic acid was suspended in 50 ml. of water plus 4 ml. of concentrated HCl. To the ice-cold solution N $NaNO_2$ was added dropwise with stirring. After each 5 ml. addition of the nitrite, the addition was interrupted until the starch-iodide test for free HNO_2 became negative. A permanent blue endpoint was obtained after the addition of 20.5 ml. of the nitrite solution. The p-aminosuccinanilic acid gradually goes into solution during the diazotization process.

The solution of diazotized p-aminosuccinanilic acid was added dropwise with constant stirring to a cold solution of 3.3 g. (0.3 moles) of resorcinol dissolved in 100 ml. of 5% Na_2CO_3 solution. Near the end of the reaction the entire mass set to a stiff, deep red gel. Acidification of the gel led to its separation into a colored supernatant and a finely divided precipitate that filtered and washed with only moderate difficulty.

The crude dye was dissolved in dilute NaOH and then precipitated out by the gradual neutralization of the alkali with a stream of CO_2 passed into the flask. The CO_2 was generated by the volatilization of dry ice. The flask of dye was kept immersed in an ice bath and stirred with a magnetic stirrer during this process. As the alkali was neutralized, the sodium salt of the

dye*, separated out in a finely granular precipitate. Attempts to filter this mixture at room temperature led to rapid gelatization of the precipitate, with complete clogging of the filter. It is possible that this filtration could be carried out in the cold room, but the success of the salting out procedure led to the abandonment of further efforts with the carbon dioxide precipitation.

The gel described above was dissolved in a final volume of approximately 200 ml. of water at a temperature of 70° C. Fifty grams of NaCl was added, with the formation of a finely crystalline precipitate of the dye. The slurry was filtered hot, and washed with a small amount of 1% NaCl. This dye could not be recrystallized from nor washed with distilled water, because of the invariable formation of slimy gel-like masses. It was recrystallized once from 1% sodium chloride solution. The free acid form of the dye is readily soluble in n-butanol, in which form it may be applied to paper strips for chromatographic analysis. The chromatogram, when developed in n-butanol-pyridine-water (3:1:1.5) showed a single, well-defined color band. The spectrophotometric curves are shown in Figure No. 13 and absorption ratios are given in Table No. 1.

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* It is the phenolate form that is extremely soluble.

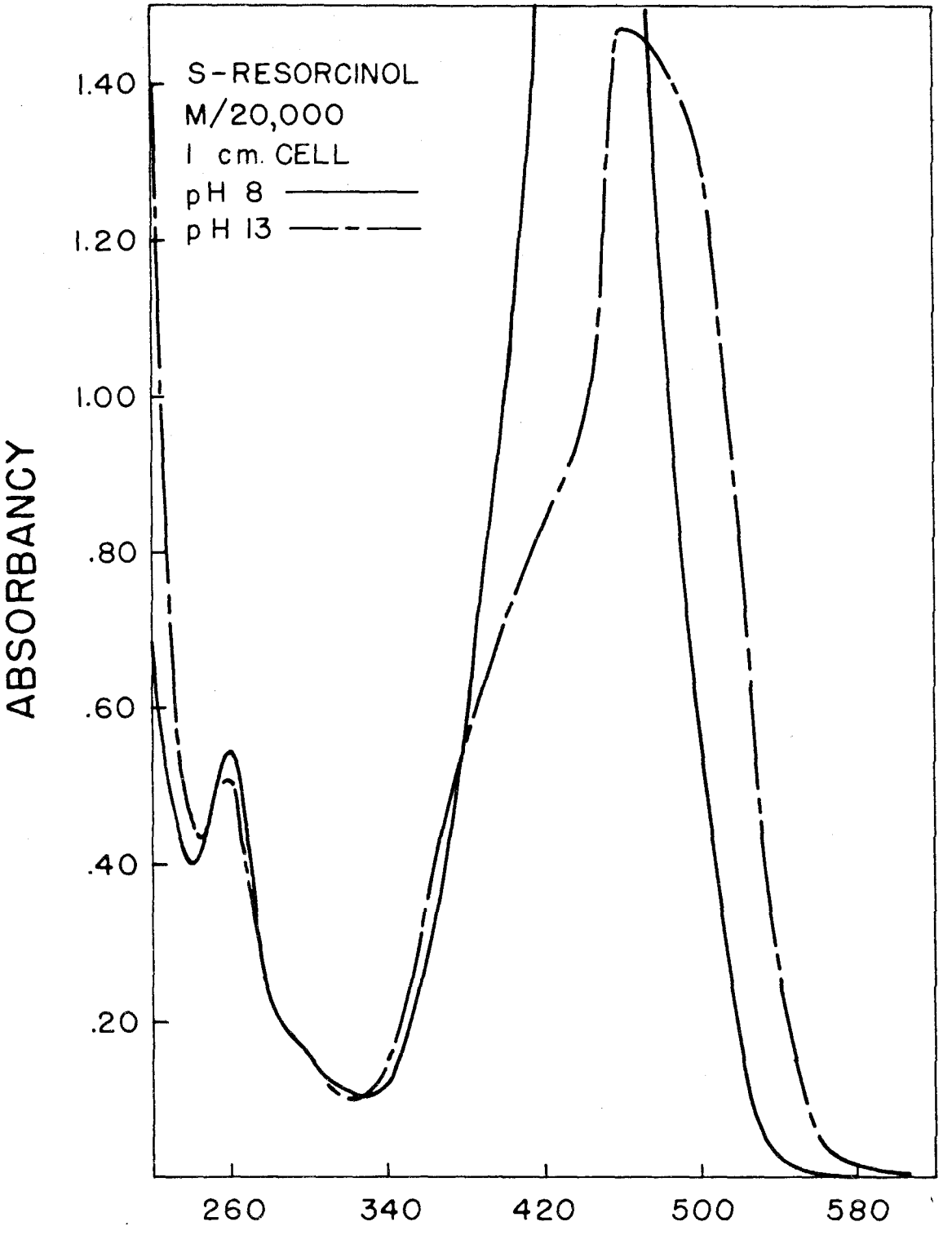
S-RESORCINOL
M/20,000
1 cm. CELL
pH 8
pH 13
1.40
1.20
1.00
.80
.60
.40
.20
ABSORBANCY
260
340
420
500
580
WAVELENGTH, mμ

FIGURE NO. 13

Preparation of p-hydroxy-(p-azosuccinanilic acid) -benzene (S-phenol): A small lot of this dye was prepared for comparison with the corresponding dye from o-iodophenol. Four and eight-tenths grams (0.02 moles) of p-amino-succinanilic acid was diazotized and coupled to phenol in a manner entirely equivalent to that described for the resorcinol dye. Attempts to crystallize either the free acid or the sodium salt from the coupling solution were unsuccessful. The method finally found to be most satisfactory follows:

The reaction mixture, after allowing an hour for completion of coupling, is acidified with hydrochloric acid and extracted with normal butanol*.

The butanol extracts are combined and extracted with sodium bicarbonate (5%) twice**. The sodium bicarbonate extracts are combined and acidified, and the S-phenol dye precipitates out. It is collected by centrifugation, washed with distilled water, taken up in a small volume of NaOH and precipitated as the normal salt with CO_2, as described for the resorcinol dye. The precipitated

---

* The colored material in the reaction mixture falls into two broad classes, one of which is readily butanol soluble and the other of which is but slightly butanol soluble. Two extractions with equal volumes of n-butanol remove most of the former with minimum carry over of the latter.

** This extracts the carboxylic acid dye from the butanol but leaves free phenols and amino compounds behind.

dye is washed with a small volume of ice-cold distilled water, and recrystallized from hot 1% NaCl solution.

Paper chromatography indicates that the dye is substantially free from colored impurities. The spectrophotometric absorption ratios show that the bulk preparation is slightly less pure than the center band of the chromatogram. R_f in n-butanol:pyridine:water = 0.67.

Spectrophotometric curves are shown in Figure No. 14 and additional data in Table No. 1.

The Direct Iodination of Phenolic Dyes: The iodination of dyes containing a phenolic nucleus has been reported by a number of workers. The studies recorded here are largely based on three general techniques: (1) Iodination with ICl in glacial acetic acid (73, 74, 75). (2) Iodination with nitrogen triiodide in ammonia (76) and (3) Iodination with potassium triiodide in weakly alkaline solution, such as $NaHCO_3$, Na_2CO_3 or $Na_2B_4O_7$ (77, 78).

Difficulty was experienced in getting theoretical amounts of iodine to add to the dyes with the iodine monochloride technique. The halogenated dyes formed were found to be heterogeneous in composition, and difficult to purify. While this work was in progress a communication from Roe et al (79) confirmed these experiences with this method. A brief summary of the

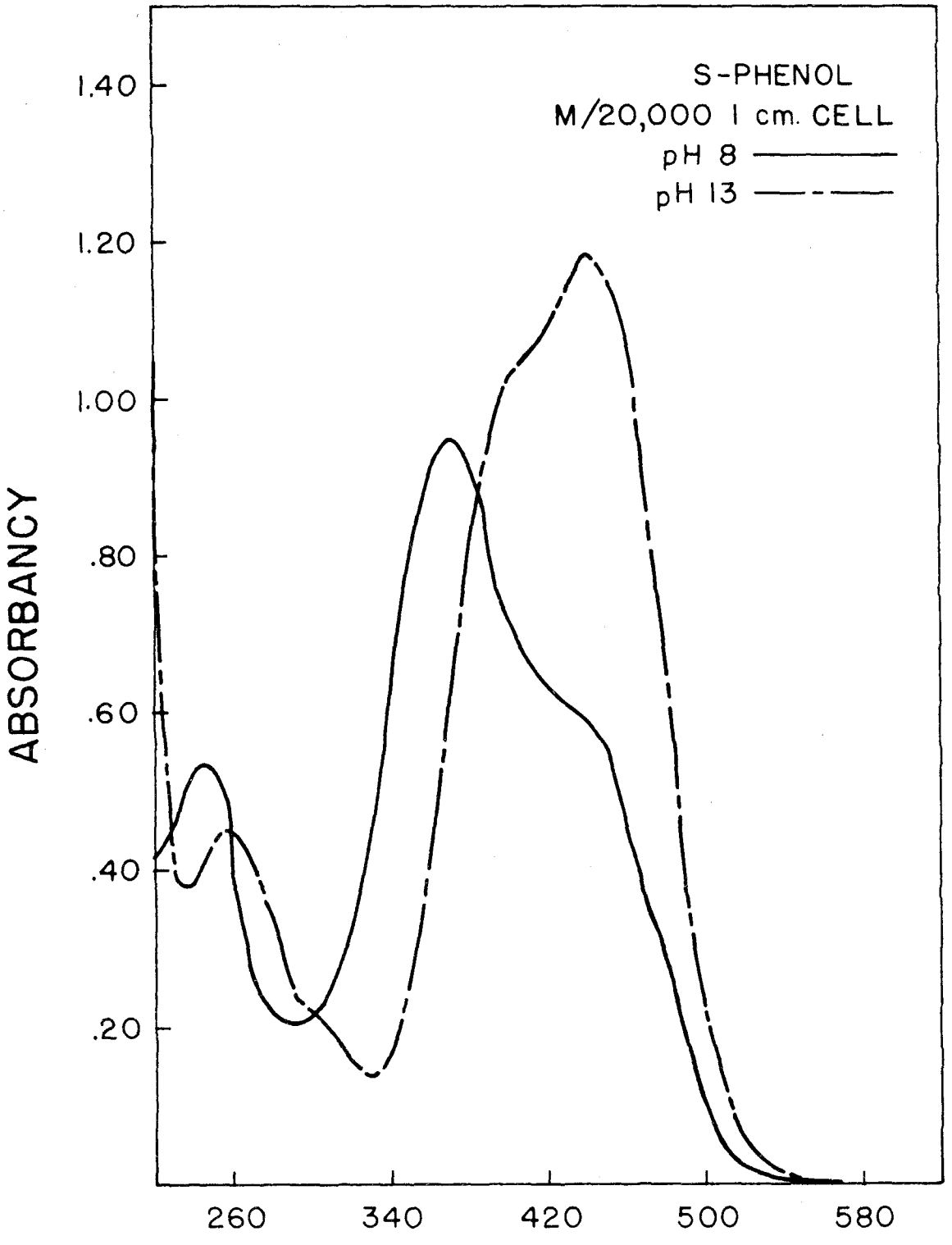
S-PHENOL
M/20,000 1 cm. CELL
pH 8
pH 13
1.40
1.20
1.00
.80
.60
.40
.20
ABSORBANCY
260
340
420
500
580
WAVELENGTH, mμ

FIGURE NO. 14

more typical experiments with this technique will be given to illustrate the results observed. In no case was any dye prepared by this method employed in serological systems.

A single attempt was made with the iodine-ammonia method. The consumption of iodine was far below the theory and the crude product had only a small amount of iodine. The method was not pursued further.

The potassium triiodide-weak alkali was found to be the most useful method. Compounds resisting the addition of iodine in a $NaHCO_3$ medium could often be iodinated at higher pH values, but in such cases the iodination was invariably accompanied by undesirable side reactions leading to products of great complexity, often containing tarry materials, which were extremely difficult to resolve into homogeneous fractions. The further difficulty in determining which of the many fractions contained the desired product led to the abandonment of iodination in solutions more alkaline than sodium bicarbonate.

Iodination with iodine monochloride: Four hundredths moles of the R-phenol dye was placed in a 500 ml. round-bottomed flask with a standard taper glass joint on the neck, 150 ml. of glacial acetic acid was added to the flask and a straight reflux condenser fitted to the neck, and 4 ml. (0.04 moles) of iodine monochloride was

added through the condenser over a period of approximately 10 minutes. The condenser was then rinsed down with glacial acetic acid and the flask slowly heated with a microburner. The boiling point was attained in fifteen minutes, and gentle refluxing continued for two hours. Then 125 ml. of water was slowly added through the condenser, and refluxing continued for an additional four hours. Another 125 ml. of water was then added, and the contents of the flask were allowed to cool.

The solution was freed from traces of insoluble material by filtration. Dilution of the filtrate with a liter of ice water precipitated most of the colored substances. The precipitate was filtered out, washed, and dried. A rough check on the iodine content indicated that somewhat over one-half of the theoretical amount of iodine was in this fraction of the dye. Chromatography revealed marked heterogeneity, with at least three reasonably distinct bands plus considerable "smear" over the remainder of the strip.

Attempts to purify this material by a number of methods were all of limited success. Repeated solution in alkali and reprecipitation with acid resulted in a loss of iodine with no significant improvement in the chromatogram. Attempts at recrystallization from alcohol, methyl cellosolve, acetic acid, o-dichlorobenzene and n-butanol all gave similar results, characterized

by difficulty in getting the material into solution and virtual impossibility in getting it back out. The most nearly satisfactory method involved solution in strong alcohol, followed by gradual evaporation of the alcohol over a warm water bath. The loss of alcohol, plus the slow absorption of water from the bath vapors, resulted in a reprecipitation of the dye in a process gradual enough that it could be stopped, and precipitate and solution separated, under some semblance of control. Before any satisfactorily pure fractions were worked up, the problem was eliminated by the direct synthesis of the dye from o-iodophenol.

No significant uptake of iodine would be obtained with the R-β naphthol dye in the iodine monochloride-acetic acid system. Addition to the resorcinol and phloroglucinol dyes went smoothly but the product in each case showed marked heterogeneity on the paper chromatogram. It is quite possible that these dyes can be iodinated satisfactorily with iodine monochloride, but with the early success of the KI_3-bicarbonate technique with the former, and the tendency of the phloroglucinol dyes to adsorb on cellulose, neither synthesis was further investigated.

Iodination with the KI_3-bicarbonate procedure worked well with the resorcinol dyes. The phenol dyes did not take up theoretical amounts of iodine (for the

mono-iodo derivitive) at room temperature, and if they were heated to 50 -60° C, the dye was badly degraded. β-Naphthol dye slowly took up iodine at 50° C, but when a lot iodinated at this temperature for a twenty-four hour period was worked up, the only pure fraction that could be obtained was some of the starting material. The phloroglucinol dye suffered extensive degradation under mild conditions. The crude product showed almost continuous muddy-colored diffuse bands and general background on the paper chromatogram. The resorcinol dye could be iodinated and purified with little difficulty, once the details of the procedure were worked out. Because of the potential usefulness of this technique in preparing I^{131} labeled hapten for immunochemical research in the arsanilic acid system, it is given below in some detail.

Iodination of 1,3-dihydroxy-2(p-azophenylarsonic acid)-benzene: Nine and five-tenths grams (0.1 moles) of the disodium salt of the R-resorcinol dye is suspended in 250 ml. of water containing 20 g. (0.2 moles) of $KHCO_3$. Molar KI_3 in M KI is added dropwise with good stirring. The iodine is taken up rather rapidly at first, but as the theoretical value of one mole of KI_3 per mole of dye is approached, the rate of addition must be reduced*. Continue the gradual addition of the KI_3 until the starch test remains positive for a half-

*At no time should there be enough free iodine in the reaction mixture to give it more than a faint iodine color.

hour without the addition of more iodine. At this stage all the dye will have gone into solution, but there may be some traces of a light yellow-colored insoluble impurity. Filter. Precipitate the dye by the gradual addition of 15 ml. of concentrated hydrochloric acid with vigorous stirring. Filter off the precipitated dye, wash well with distilled water, dissolve in sodium carbonate, and repeat the acid precipitation and washing.

The crude dye contains approximately the theoretical amount of iodine (one atom per mole of dye) and the paper chromatogram shows most of the color concentrated in a single band, with two or three weak, but discrete, side bands. There is a gratifying absence of a "smeared" background.

This product may be purified satisfactorily by two different techniques. The free acid may be recrystallized from fifty times its weight of 50 volumes percent ethanol in water. Two or three recrystallizations will yield a product of high purity with a final recovery of approximately one-third of the starting material. This method is most convenient for small lots of the iodinated dye, such as might be handled in working with isotopic iodine.

Larger lots of the dye may be purified somewhat more readily by recrystallization as the sodium salt. The crude dye is dissolved in ten times its weight of water containing a slight excess of Na_2CO_3 and brought to the boiling point. Sufficient solid NaCl is added to saturate the solution. Boiling is continued for several minutes and the slurry is allowed to cool slowly. When the temperature has reached 40° C, the mixture is filtered, the filter cake pressed as dry as possible, washed with saturated NaCl solution, and finally with a little ice-cold distilled water. This dye will usually contain some sodium chloride as an impurity; this will not ordinarily interfere with its use in serological systems. If its homogeneity is still not adequate, the salt precipitation can be repeated and a final recrystallization from distilled water will yield a satisfactory product. There is some tendency for the dye to separate out in a colloidal form in this last recrystallization if enough NaCl has not been introduced into the solution as an impurity in the salted-out dye. The addition of a few tenths of a percent salt to the water will control this, without leading to significant amounts of NaCl in the final product.

Characterization of the R-iodoresorcinol dye:

Analysis:

Arsenic: found* 14.3, theory 14.74

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* Corrected for NaCl content.

Iodine: 24.0 24.97

Chloride: 2.55% (as NaCl)

Assay: 94%

Chromatography:

R_f (n-butanol:pyridine:water:, 3:1:1.5) 0.28

Spectrophotometric Data:

Absorption curves, Figure No. 15

Absorbance ratios, Table No. 1.

Iodination of 1,3-dihydroxy-4 (p-azosuccinanilic acid): Although no highly satisfactory preparation was completed, it is believed that direct iodination of this dye can yield pure material in reasonable yield. The failure of the supply of anti-SBG serum during the experiments with this dye led to their abandonment. The dye is not stable on storage, but the stability is at least of the same order as the useful life of isotopic iodine, so that it is of potential utility in immunochemical research.

Six and eight-tenths grams (0.02 moles) of the sodium salt of the S-resorcinol dye is suspended in 50 ml. of water containing 3.5 g. (0.04 moles) of sodium bicarbonate. Twenty milliliters of KI_3 (1 M in M KI) is added dropwise, never letting the addition get more than a little ahead of the iodine uptake. As the iodine is added, the dye goes into solution and the color deepens rapidly. After iodination is complete,

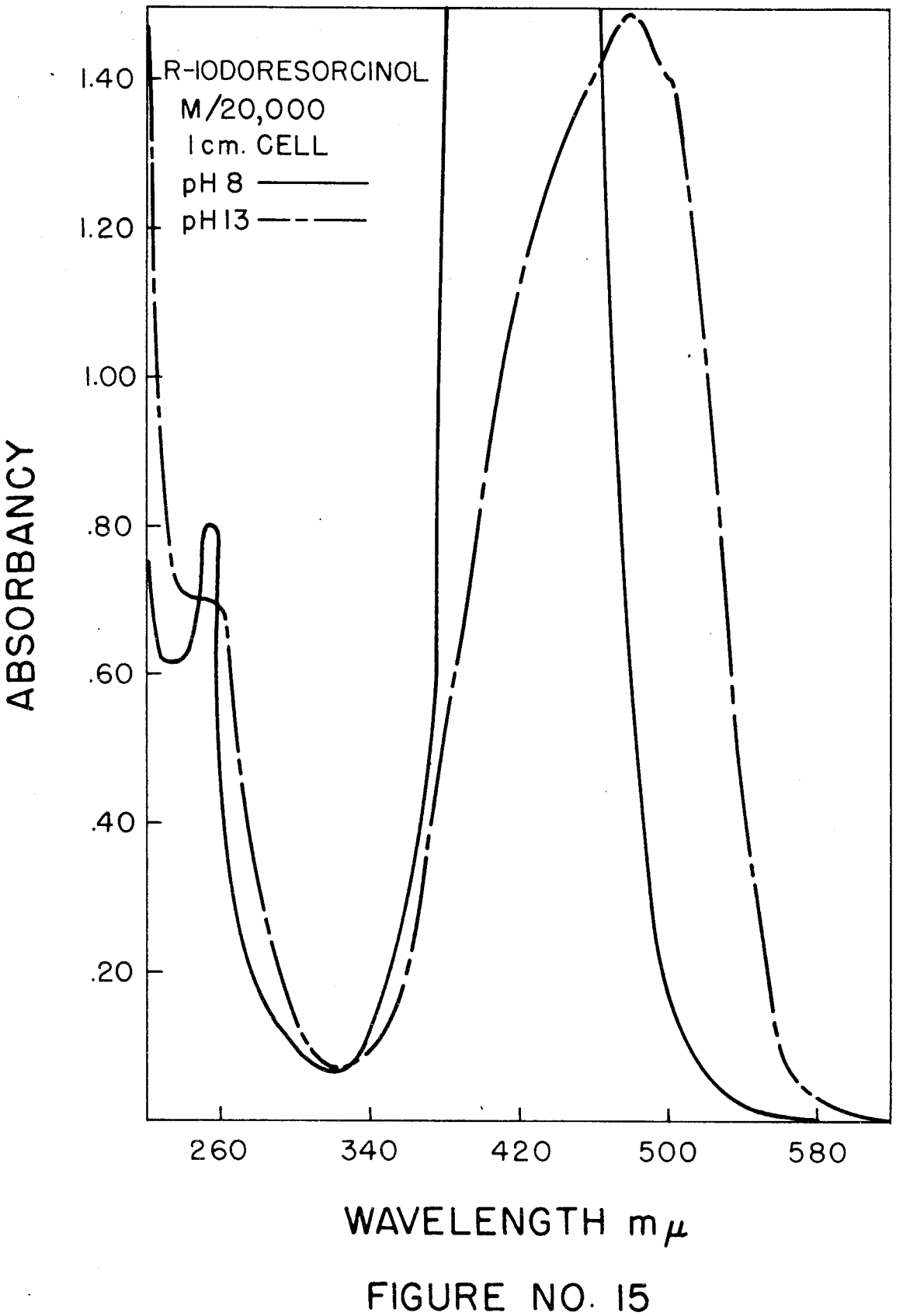
R-IODORESORCINOL
M/20,000
1 cm. CELL
pH 8
pH 13
1.40
1.20
1.00
.80
.60
.40
.20
ABSORBANCY
260
340
420
500
580
WAVELENGTH mμ

FIGURE NO. 15

as shown by a starch endpoint permanent for 15 minutes, the neutral solution is extracted twice with equal volumes of n-butanol, then acidified with hydrochloric acid, and the acid form of the dye is extracted into n-butanol by two more such extractions. The dye is extracted out of the butanol into sodium bicarbonate solution, precipitated with dilute hydrochloric acid, and collected on a filter. The dye is air-dried*, taken up in dry n-butanol, and passed through a column of neutrol filtrol and celite. The adsorbent removes a substantial amount of a muddy-colored impurity (oxidation products?) that adhere to the upper portion of the column. The eluate, on chromatographic analysis, is found to contain one strong band of dark red dye, and a lighter-colored band of a minor constituent that seems to be unchanged starting material.

The butanol solution of the dye is extracted with sodium bicarbonate solution (5%), and the dye is precipitated out with dilute hydrochloric acid. The precipitate is taken up in a minimum volume of hot 5% sodium bicarbonate solution. The hot solution is

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* The best lot prepared in this study contained traces of the brown impurities that should have been removed by the neutrol filtrol column. The difficulty seemed to be the result of too much moisture in the n-butanol solution that was passed through the column. This solution was obtained by extraction into butanol from the

saturated with sodium chloride, digested at 90 - 100° C for several minutes, and then cooled to about 45° C. The warm slurry is filtered, the filter cake is pressed out firmly, washed with saturated NaCl solution and finally with a small volume of ice-cold 1% saline. If this product is not sufficiently pure, the salting out may be repeated, and a final recrystallization from 1% NaCl may be employed to reduce the salt contamination in the final product.

Characterization of the S-iodoresorcinol dye:

Analysis:

Iodine: found* 27.1%, theory 26.59%

Chloride: 6.25% (as NaCl)

Assay: 92%

Chromatography:

R_f (n-butanol:pyridine:water; 3:1:1.5) 0.47.

Spectrophotometric Data:

Absorption curves, Figure No. 16.

Absorbancy ratios, Table No. 1.

Preparation of 1-hydroxy-2-iodo-4-(p-azophenyl-arsonic acid)-benzene (R-iodophenol): Ortho-iodophenol

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acidified sodium bicarbonate solution. The directions given above have been modified to insure elimination of this difficulty. The impurity referred to contains a relatively high concentration of iodine, and its propensity for nonspecific adsorption on cellulose or proteins makes its presence intolerable in dyes to be used in serological systems.

* Corrected for NaCl content.

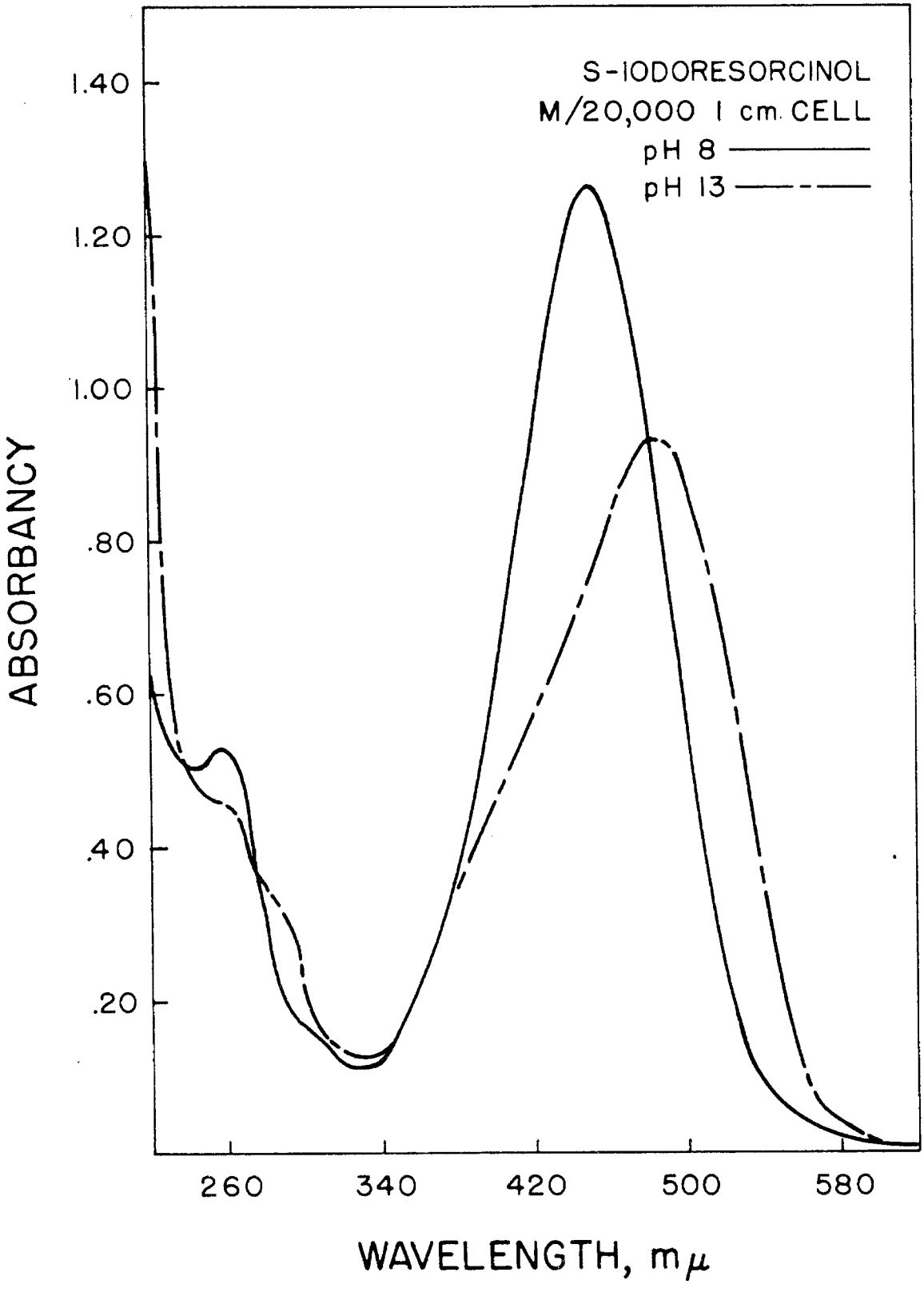
S-IODORESORCINOL
M/20,000 1 cm. CELL
pH 8
pH 13
1.40
1.20
1.00
.80
.60
.40
.20
ABSORBANCY
260
340
420
500
580
WAVELENGTH, mμ

FIGURE NO. 16

(Eastman White Label) was purified by vacuum sublimation.

Two and sixteen-hundredths grams (0.01 moles) of p-arsanilic acid is diazotized as previously described in a solution containing 5 ml. of concentrated HCl. Two and two-tenths grams (0.01 moles) of purified o-iodophenol is dissolved in 60 ml. of water containing 0.4 g. (0.01 moles) of NaOH and 9 g. (0.085 moles) of Na_2CO_3. The solution is cooled to 3-5° C, and the diazotized arsanilic acid added with stirring over a period of half an hour. Allow the reaction mixture to stand for an additional half hour, then add HCl just to the point of CO_2 evolution. Heat to boiling*, saturate with solid NaCl, cool gradually to 40 - 45° C, and filter. Press the mother liquor from the filter cake as completely as possible, wash with saturated saline and then with a little ice-cold distilled water. If the crude dye has any odor of iodophenol, allow to dry and then wash with benzene. The dye may be purified by an additional salting out followed by one or two recrystallizations from hot distilled water.

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* Carry out this step in a good hood: any free iodophenol in the preparation will be volatilized.

Characterization of the R-iodophenol dye:

Analysis:

Arsenic:	found* 15.0,	theory 15.22%
Iodine:	25.5	25.78
Chloride:	0.69%	(as NaCl)
Assay:	.98%	

Chromatography:

R_f (n-butanol:pyridine:water, 3:1:1.5) 0.67.

Spectrophotometric Data:

Absorption curves, Figure No. 17.

Absorbancy ratios, Table No. 1.

Preparation of 1-hydroxyl-2-iodo-4-(p-azosuccinanilic acid)-benzene (S-iodophenol): Four and eight-tenths grams (0.02 moles) of p-aminosuccinanilic acid is dissolved in 50 ml. of water plus 4 ml. concentrated HCl. One normal $NaNO_2$ solution is added dropwise to the ice-cold solution, interrupting the addition after each 5 ml. portion has been added until the starch-iodide test is negative. When a slight excess (approximately 20.5 ml.) of nitrite has been added, allow half an hour to ensure complete diazotization and then destroy the excess nitrous acid with solid ammonium sulfamate.

Four and four-tenths grams (0.02 moles) of vacuum-sublimed o-iodophenol is dissolved in 75 ml. of water containing 2 g. of NaOH and 2 g. of Na_2CO_3. The phenol solution is cooled to below 5° C, and the diazotized

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* Corrected for NaCl content.

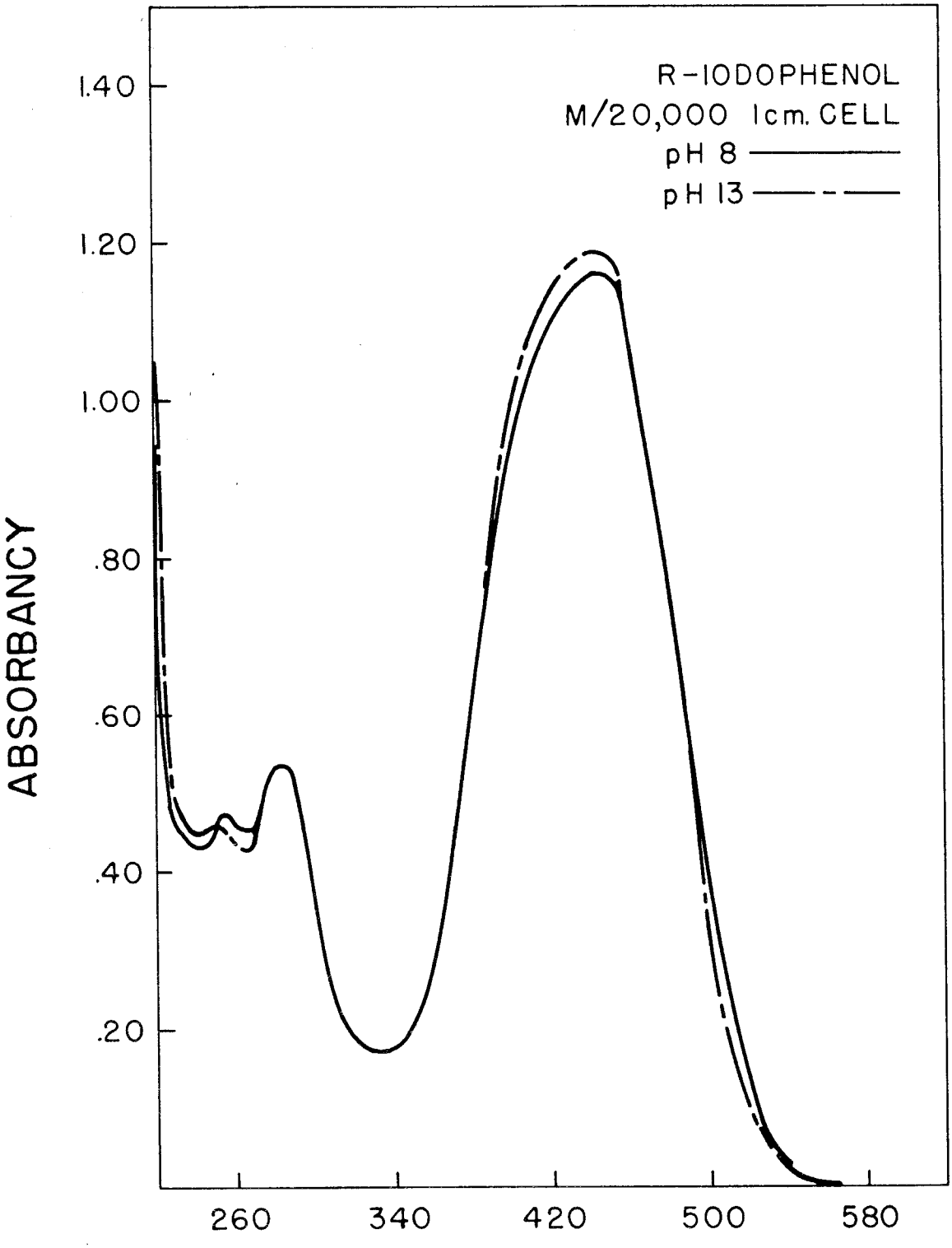
R-IODOPHENOL
M/20,000 1cm. CELL
pH 8
pH 13
1.40
1.20
1.00
.80
.60
.40
.20
ABSORBANCY
260
340
420
500
580
WAVELENGTH mμ

FIGURE NO. 17

p-amino-succinanilic acid is added slowly with stirring over a period of half an hour. An additional hour is allowed for coupling, at which time little odor of o-iodophenol remains. Hydrochloric acid is added just to the point of CO_2 evolution, the mixture is brought to the boiling point*, and the dye is salted out with solid NaCl.

The crude dye, contaminated with considerable NaCl, is collected on a filter and washed with saturated saline and ice-cold distilled water. The dye may be recrystallized by salting out and finally from 1% NaCl solution.

One lot of this dye, after the first salting out, was washed with acetone on the filter to dry the filter cake. The moist acetone dissolved the dye and eluted it from the sodium chloride in the filter. The acetone solution was evaporated to small volume with a stream of air at room temperature, and fine yellow crystals in the form of long needles began to separate out. After cooling over night in the refrigerator, the crystals were filtered off, pulled free from acetone, washed with a few drops of ice-cold distilled water, and dried. This fraction when applied to the paper chromatogram in the form of an n-butanol solution of the free acid, and developed in the n-butanol-pyridine-water solvent, showed no inhomogeneity.

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* Use the hood.

Characterization of S-iodophenol dye:

Analysis:

Iodine: found 27.7, Theory 27.52

Chloride: none

Assay: 100%

Chromatography:

R_f (n-butanol:pyridine:water; 3:1:1.5) 0.33

Spectrophotometric Data:

Absorption curves, Figure No. 18

Absorbancy ratios, Table No. 1

Preparation of Immune Adsorbents: The immune adsorbents employed in this study were first described, for the arsanilic acid system, by Lerman (80). Experimental details were not given, but the synthesis was described as involving the following steps (1) purification of Solka-floc by alkaline and acid washes, (2) bromination of the cellulose with phosphorus tribromide, (3) etherification with mono-sodium resorcinolate, and (4) coupling with diazotized arsanilic acid. A number of modifications of experimental conditions were investigated, and the adsorbents synthesized correspondingly vary somewhat in their chemical composition and serological properties. The procedure described in detail represents that which, in the opinion of the writer, produces the most generally useful adsorbent. The characterization of the different lots

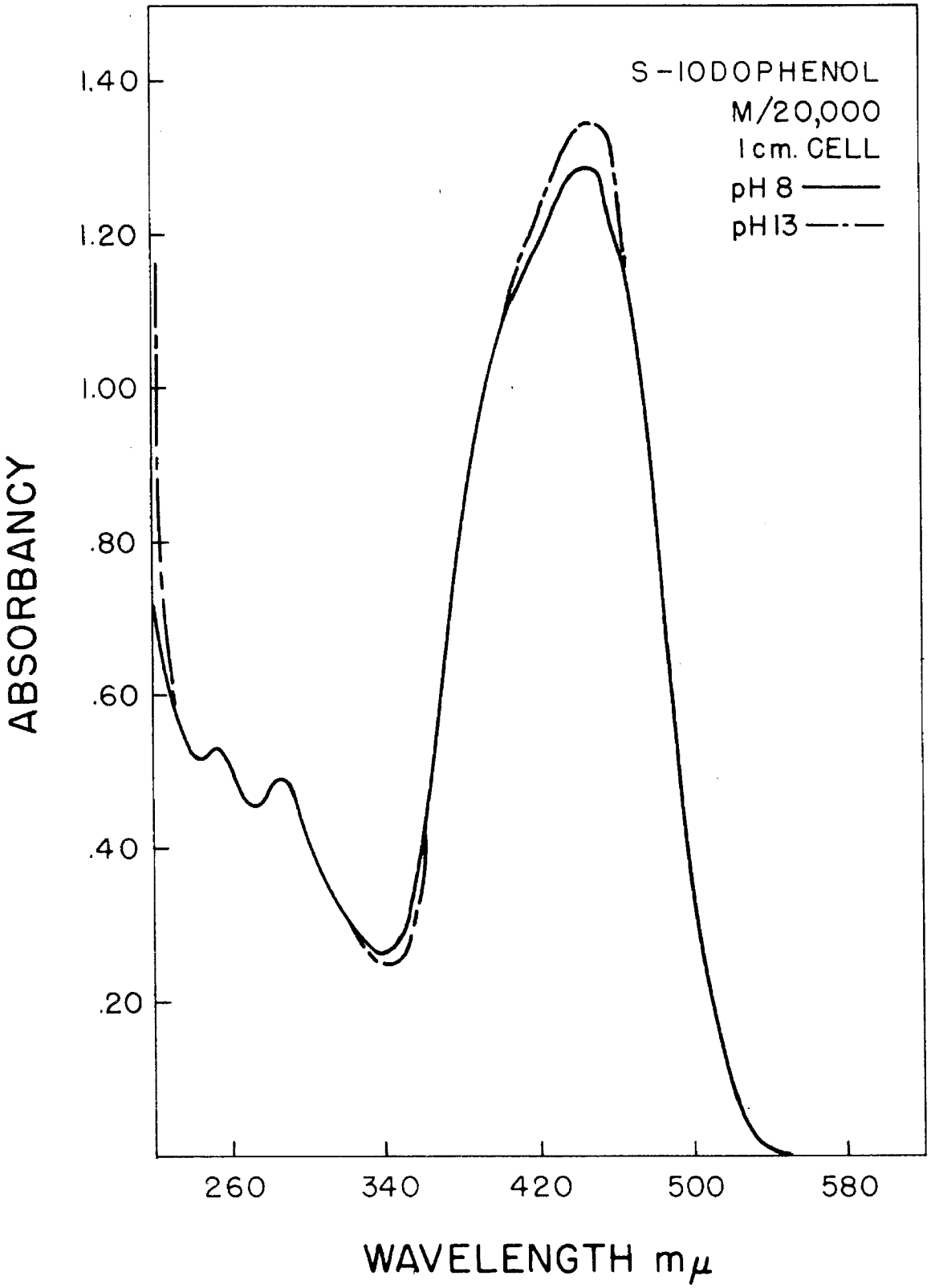
S-IODOPHENOL
M/20,000
1 cm. CELL
pH 8
pH 13
1.40
1.20
1.00
.80
.60
.40
.20
ABSORBANCY
260
340
420
500
580
WAVELENGTH mμ

FIGURE NO. 18

Table No. 1

Hapten	pH	Max-I λ mμ.	Max-I a_M* x10^{-4}	Min-I λ mμ.	Min-I a_M* x10^{-4}	Max-II λ mμ.	Max-II a_M* x10^{-4}	Min-II λ mμ.	Min-II a_M* x10^{-4}	Absorbancy Ratios Max-I:Min-I a**	b***
R-phenol	8	431	2.06	300	.470	251	.946	228	.780	4.38	
"	13	433	2.61	324	.204	269	.982	230	.696	12.8	13.0
R- naphthol	8	490	1.44	347	.180	311	.616	280	.394	8.02	
"	13	452	.700	380	.350	335	.610	318	.400	2.00	2.02
R-resorcinol	8	427	4.17	319	.104	258	1.74	238	.762	40.1	
"	13	481	2.64	310	.170	255	.908	240	.896	15.5	15.7
R-phloroglucinol	8	435	4.67	320	.120	256	1.56	232	1.22	38.9	
"	13	482	2.88	327	.150					19.2	21.1
S-resorcinol	8	445	3.88	325	.200	262	1.09	240	.796	19.4	
"	13	487	2.64	315	.220	261	1.01	245	.894	12.0	12.6
S-phenol	8	370	1.90	290	.408	246	1.07			4.66	
"	13	441	2.37	328	.272	258	.900			8.72	8.90
R-iodoresorcinol	8	433	4.82	324	.122	255	1.60	232	1.23	39.5	
"	13	481	2.99	323	.140					21.3	21.9
S-iodoresorcinol	8	449	2.54	326	.222	258	1.06			11.4	
"	13	484	1.97	328	.248					7.52	11.3
R-iodophenol	8	446	2.32	333	.342	275	1.08	266	.916	6.79	
"	13	442	2.38	333	.342	275	1.08	265	.856	6.96	7.07
S-iodophenol	8	445	2.59	338	.522					4.96	
"	13	447	2.61	341	.578					5.67	5.82

* a_M = molar absorbancy index

** a = absorbancy ratio in bulk preparation.

*** b = absorbancy ratio in hapten eluted from center of band on paper chromatogram.

of adsorbents is given in Table No. 2*

The cellulose fiber employed by Campbell et al (60) and by Lerman is a proprietary product of the Brown Co. It is a by-product of paper manufacture and consists of the "fines" from that process which are too short to incorporate into the paper structure. It is extremely heterogeneous in size, and the individual particles are of irregular shape and contain numerous folded surfaces and internal voids. The unclassified Solka-floc packs very tightly in absorption columns, and if the adsorbent is designed for such use, the Solka-floc should be cleared of fines by repeated suspension and fractional decantation.

The size classification can be combined conveniently with chemical purification. The procedure described in that employed for the material used in the preparation of Lot III of the R-adsorbent and Lot I of the S-adsorbent.

Five hundred grams of commercial Solka-floc is placed in a 9 l. tall-form Pyrex bottle, which is then almost completely filled with water. The bottle is stoppered and thoroughly shaken to disperse aggregates of the fibers. The suspension is allowed to settle for approximately one hour, at the close of which the supernatant is decanted as completely as possible from the sediment. This process is repeated until a reason-

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* Page 111, this thesis.

ably sharp demarcation is obtained between the sediment and the supernatant. At this stage the suspension medium is changed to N/2 NaOH. It is convenient to prepare this in the bottle by adding a small amount of strong alkali to the water and Solka-floc in the bottle*. The alkali wash is continued until the supernatant no longer shows any colored impurities extracting into the alkali.

The classification is then continued with approximately N/2 HCl through six to ten washings, and then with distilled water until the washings are neutral. At the end of this time, the surface of the layer of sedimenting fibers should form a sharp boundary between the sediment and the supernatant. The minimum-sized particle to be retained in the sediment may be controlled by variation in the settling time. The lot of Solka-floc used in this work exhibited a fairly well-marked division into "fines" and coarser fibrous material, and the classification described here is designed to make the separation at this division.

The sediment from the last washing is collected on a large Büchner funnel, sucked as dry as possible and then washed with acetone to dry the fibers and to

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* Care should be taken that the alkali is well-diluted before it comes into contact with the cellulose, to prevent swelling of the cellulose fibers and resulting poor sedimentation.

remove colored impurities. Suction is then discontinued and the filter cake leached with acetone under gravity head. When the acetone comes through colorless, the mat is pulled dry under vacuum. The dried Solka-floc is then ready for use in preparation of the adsorbent.

The physical structure of the Solka-floc, although giving a favorable ratio of surface to volume, make the material difficult to handle in analytical columns. Density of the column is hard to control, and the voids and re-entrant surfaces favor trapping of the test fluids so that prolonged washing is necessary to remove all substances not specifically bound to the column. The low porosity of the column makes this washing extremely tedious and there is no certain method of telling when the washing is complete.

Rayon flock has certain advantages as a form of cellulose suitable for the preparation of these adsorbents. It requires no preliminary purification. It is uniform in particle size and shape and packs into absorption columns of readily controlled density. It is free of voids or surface irregularities that might entrap liquids, and its columns may be easily washed free of non-adsorbed material. Its greatest disadvantage, particularly from the standpoint of preparative columns, is its relatively low ratio of surface to volume. The longer cuts of the flock have a tendency to "felt",

especially when the diameter of the column is not large in comparison with the length of the flock. The lowest denier* flock currently available is still somewhat coarser than would be optimum for an immune adsorbent, but the outstanding advantages of the rayon flock from the standpoint of the "clean" performance of its columns more than compensate for its reduced capacity when the adsorbent is used as an analytical tool.

Preparation of cellulose bromide: One hundred grams of dry cellulose fiber (purified Solka-floc or rayon flock) is placed in a three-necked two-liter flask. The center neck is equipped with a motor-driven stirrer passing through a close-fitting stuffing box. One side neck is fitted with a dropping funnel and the other with a Dean and Stark moisture trap and reflux condenser. The upper end of the condenser is protected with a calcium chloride tube. One liter of dry trichloroethylene** is added, the stirrer started, and the flask heated through an oil bath by a closed-element hotplate. The mixture is refluxed until all the moisture in the system has been distilled out by azeotropic distillation and removed through the moisture trap.

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*The diameter of rayon fiber is expressed in deniers. The denier of a filament is equal to the weight of 9000 meters of the filament, expressed in grams.

** The high density of the trichloroethylene assists in the suspension of the cellulose and minimizes bumping,

The suspension of cellulose in trichloroethylene is allowed to cool to approximately 40° C* and 20 ml. of phosphorus tribromide (0.1 mole per glucose rest) added slowly with stirring. The reaction is permitted to continue without additional heating for two hours. The mixture is then filtered onto a dry Büchner funnel and washed with dry benzene to remove unreacted phosphorus tribromide. The fibers are washed with absolute methanol until the wash is acid-free. The methanol is displaced with absolute ethanol, and the alcohol-wet cellulose is returned to the reaction flask for the next step in the synthesis.

Preparation of cellulose-resorcinol ether: Twelve hundred milliliters of absolute ethanol and 500 ml. of dry benzene are added to the cellulose in the flask, the stirrer started, and the benzene-alcohol-water triple azeotrope distilled off. The efficiency of the still is low, but by the time 500 ml. of distillate have been taken off through the moisture trap, the distillate does not cloud on dilution with kerosene. Twenty-two grams (0.2 moles) of resorcinol is added to the flask and then 3.5 g. (0.15 moles) of sodium in absolute

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and its relatively high boiling point(87° C) and low water solubility make it an efficient drying solvent.

* If the temperature is too high, charring of the cellulose results.

ethanol is added through the dropping funnel. Stirring and gentle reflux are continued over night (approximately 16 hours) and then the mixture is allowed to cool and the cellulose-resorcinol ether collected on a large Büchner funnel.

The fibers are washed with ethanol (1 liter), ethanolic sodium hydroxide (2 l. N/10) and distilled water. The uncoupled resorcinol* can be removed by prolonged alternate soaking in 5% Na_2CO_3 solution followed by distilled water washing until the filtrate is neutral**.

Coupling with diazotized hapten: The purified cellulose-resorcinol ether is suspended in 1.5 l. of water containing 21 g. (0.25 moles) of $NaHCO_3$ and cooled

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* At this stage it is imperative that all uncoupled resorcinol be washed out of the fibers. If any is left, it will couple with the diazonium compound in the next step of the synthesis, and the larger molecule will diffuse out of the fiber with extreme slowness. This residual dye, which is almost impossible to remove completely, will act as an inhibiting hapten when the adsorbent is used in a serological system.

** The washing is not adequate until the Na_2CO_3 solution, after standing in contact with the fibers for 48 hours, shows no trace of resorcinol when tested with diazotized sulfanilic acid. The length of washing required to achieve this degree of purity will depend upon the starting material. Solka-floc will require not less than a week, with nightly Na_2CO_3 leaching and exhaustive washing each day. Rayon-based adsorbents can be washed in two or

to 10° C. A solution of 0.05 moles of diazotized hapten, prepared as described in the synthesis of the inhibiting haptenic dyes, is gradually added to the vigorously stirred suspension and coupling is allowed to proceed for an additional 45 minutes. The mixture is filtered with suction on a Büchner funnel and washed with a series of Na_2CO_3 and water cycles as described for the previous step until the Na_2CO_3 washings, after forty-eight hour leaching of the adsorbent, show no more than a faint trace of color.* The adsorbent is then washed with dilute hydrochloric acid to convert it into the free acid form, distilled water until the washings are neutral, and dried with acetone.

The lots of adsorbents prepared, and their analytical properties, are recorded in Table No. 2.

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three days, using shorter leaching and washing cycles.

* The amount of washing required to achieve this will be critically dependent upon the thoroughness with which the resorcinol was washed out of the fibers in the preceding step. If this washing was inadequate, a high degree of freedom of the adsorbent from free dye cannot be secured.

Adsorbents that seem to be reasonably free from uncoupled dye when synthesized, may, after storage for some months, "bleed out" considerable quantities of dye when wet with buffer preparatory to being used in serological experiments. This is presumed to be the result of diffusion of the dye to the surface of the fiber during the storage period. A relatively brief washing with buffer will remove all but traces of this dye, and may conveniently be done directly in the adsorption column.

Table No. 2

Adsorbent	Cellulose Base	Hapten Nitrogen mg./g.	Arsenic mg./g	Glucose Rests per Hapten Group
R-I	Purified Solka-floc	0.79	4.9	94*
R-II	Purified Solka-floc	0.30	2.1	220
R-III	Purified and Size-classified Solka-floc	0.46	2.8	165
R-IV	Rayon flock 15 denier 3 mm. cut	0.05	0.24	1925
R-V	Rayon flock 1.5 denier 0.25 mm. cut	0.26	1.7	272
S-I	Purified and Size-classified Solka-floc	0.48		325**

* Calculated from the arsenic content.

** Calculated from the nitrogen content, assuming 40% recovery of azo nitrogen (the average obtained in the analysis of the arsanilic acid adsorbents) and complete recovery of the amide nitrogen.

Methods

Analytical

Determination of Arsenic: Arsenic was determined in haptenic dyes, immune adsorbents and immunological aggregates by the method of Chaney and Magnuson (81) or by preliminary wet digestion followed by arsine evolution and color development as described by Kingsley and Schaffert (82), according to the nature of the sample.

In the Chaney-Magnuson procedure, organic matter is destroyed by perchloric-nitric-sulfuric digestion, and the arsenic in the digest is separated from interfering materials by distillation as arsenic trichloride from the acid digest in a constant volume still developed by Chaney (83). The arsenic trichloride is trapped in a dilute solution of KIO_3, which hydrolyzes the arsenic trichloride and oxidizes the arsenic to the pentavalent state. The arsenic-containing distillate is treated with ammonium molybdate and hydrazine sulfate. The heteropoly blue developed upon heating is measured in a Beckman Model DU spectrophotometer at 840 mμ. The procedure gives satisfactory recoveries and precision in the range of 5 to 100 μg. of arsenic per sample.

In the analysis of highly purified haptenic dyes, in which interfering materials, such as phosphorus, are known to be absent, the acid digest, after strong

fuming to insure complete removal of perchloric and nitric acids, may be diluted to a known volume, and a suitable aliquot taken for color development without distillation. This modification eliminates the somewhat time-consuming distillation procedure with the additional sources of mechanical losses associated with it.

For amounts of arsenic below ten micrograms, the Kingsley and Schaffert procedure offers greater sensitivity and lower blanks. The HCl digestion described by these authors does not give good recoveries on many pentavalent arsenic compounds, and for the purposes of this investigation, all samples were digested according to the Chaney and Magnuson technique. The digest is diluted, treated with stannous chloride and potassium iodide to reduce the arsenic to the trivalent state, and metallic zinc and HCl added. The hydrogen and arsine evolved are bubbled through a dilute solution of iodine in KI. The arsine is oxidized by the iodine to arsenate and final color development resembles that in the Chaney and Magnuson procedure. Since the final volume can be half to a fifth of that obtained in the distillation procedure, and as the blanks are lower, the method has definite advantages for small samples that are low in arsenic.

Calibration graphs for the two methods are shown in Figure No. 19.

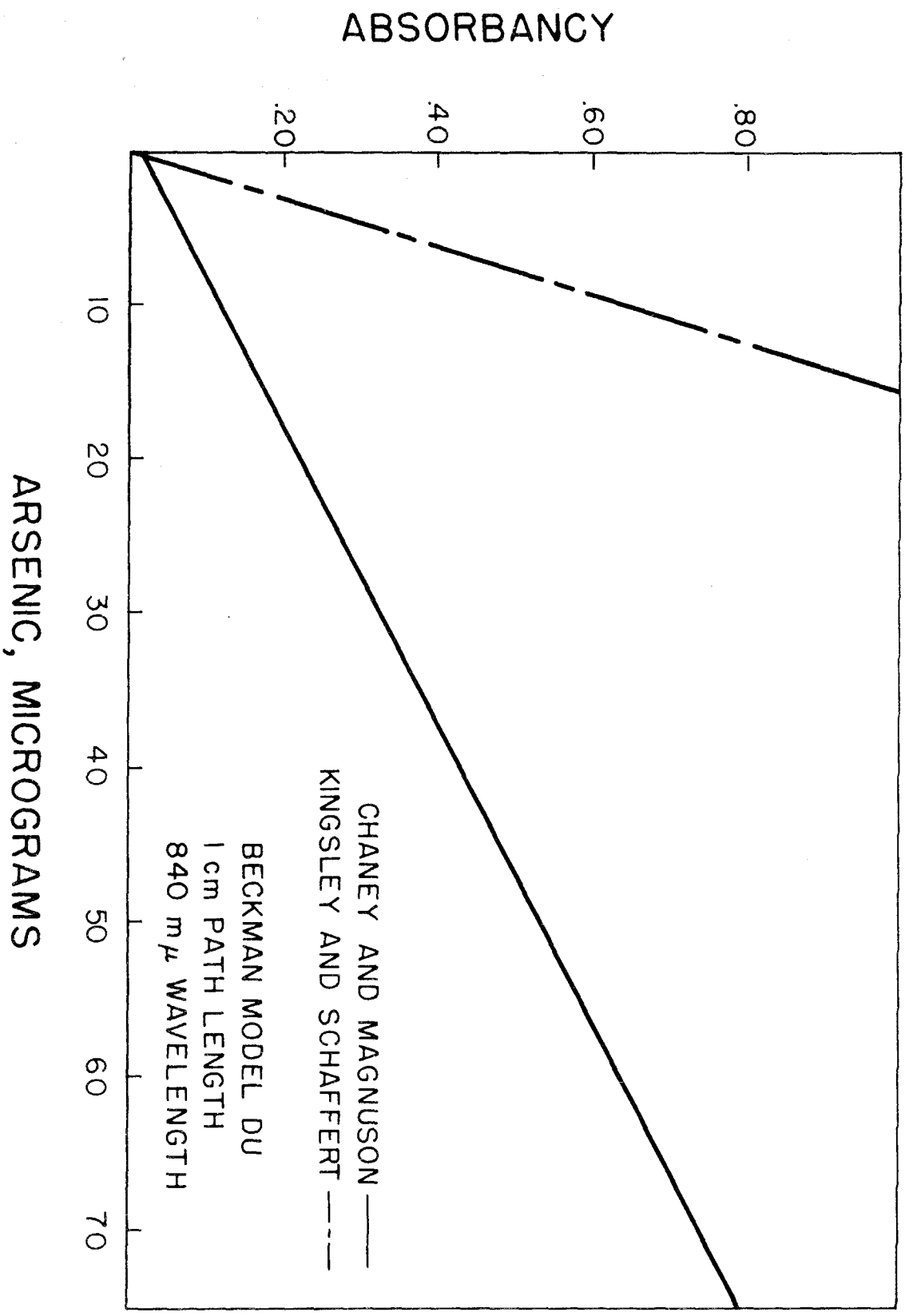
ABSORBANCY
.20
.40
.60
.80
10
20
30
40
50
60
70
ARSENIC, MICROGRAMS
CHANEY AND MAGNUSON ——
KINGSLEY AND SCHAFFERT — - —
BECKMAN MODEL DU
1 cm PATH LENGTH
840 mμ WAVELENGTH

FIGURE NO. 19

Determination of Chloride: The principal impurity in many of the haptenic dyes is sodium chloride from the saline in which they were recrystallized. This was determined by the method of Sendroy (84) as modified by Van Slyke and Hiller (85). The acid chloride-containing solution is shaken with solid $AgIO_3$. The free HIO_3 liberated by metathesis with the HCl is determined in the filtrate from the reaction mixture by titration with $Na_2S_2O_3$ after addition of KI.

Determination of Iodine: The iodine label of the haptenic dyes was measured in the immune aggregates by the method of Chaney (83, 86) with unpublished improvements of the author. The method involves the wet oxidation of the iodine-containing material with chromic-sulfuric acid. The digest is reduced and the iodine distilled in the special still developed for this method. The iodine in the vapors passing through the trap of the still is absorbed in dilute sodium hydroxide, and the iodine concentration in the distillate is determined by its catalytic activity upon the rate of reduction of the cerate ion by arsenite.

The sample is digested in a 300 ml. round-bottomed, two-necked flask, with 35 ml. of 70% H_2SO_4 and an excess of CrO_3. Three hundred milligram samples of cellulosic adsorbents required 3 g. of CrO_3. Digestion

is carried to the stage of incipient fuming, and the digest allowed to cool. Then 15 ml. of distilled water is added and again boiled off to insure complete removal of volatile products of the digestion.

The digest, containing the iodine in the form of non-volatile iodate, is diluted with 15 ml. of water and the flask is connected to the still, which is designed to scrub the vapors with dilute NaOH solution and then return the condensed steam to the flask via a capillary reflux-return. In rapid succession 1 ml. of 45% (W/V) phosphorus acid, 1 ml. of 0.45 N arsenious acid, and 1 ml. 1.5% H_2O_2 (prepared by dilution of Becco brand 90% H_2O_2) are added to the boiling contents of the flask through a funnel in the side-neck. The function of the phosphorous acid is to reduce the small amount of chromic acid undecomposed during the digestion, and the arsenious acid and hydrogen peroxide reduce the iodate to hydriodic acid and/or free iodine. The iodine is absorbed by the dilute sodium hydroxide in the trap of the still. In six minutes distillation is complete and the trap contents and rinsings are made up to a volume of 12 ml.

A 4 ml. aliquot of the distillate is added to 0.5 ml. of 0.15 N arsenious acid in 1.5 N. H_2SO_4 in a 13 × 100 mm. Pyrex test tube selected for optical and

dimensional characteristics suitable for use as a cuvette. The tube is placed in a thermostated aluminum block at approximately 30° C until temperature equilibrium is attained, and 0.5 ml. of 0.1 N ceric sulfate in 3.5 N H_2SO_4 is added and well mixed. The tube is then placed in the thermostated cuvette holder of a recording colorimeter.

The colorimeter operates from an electronically controlled power supply and the vacuum type phototube produces a signal proportional to the light passing through the cuvette. The output of the phototube passes through a vacuum tube impedance changer and power amplifier, which furnishes the signal that actuates a modified Leeds and Northrup "Micromax" recording potentiometer. The displacement of the potentiometer pen is linear with respect to the percent transmission of the cuvette.

The kinetics of the catalytic reaction are such that the percentage transmission increases linearly with time and at such a rate that the cotangent of the angle between the pen trace and the horizontal is proportional, within the range of iodine and reagent concentrations employed, to the iodine concentration in the cuvette. The relationship between the cotangent and the iodine concentration may be varied over a reasonable range by the choice of (1) wave length of the

light used, (2) cuvette diameter, (3) chart width between zero and 100% transmission, (4) chart speed, (5) temperature of the reacting system, and (6) acid concentration. The conditions chosen, 9 inch effective chart width, Corning No. 554 blue filter, and the reagent concentrations and temperatures given, lead to a conversion factor of 0.01. That is, the cotangent of the angle made by the trace with the horizontal, when multiplied by 0.01, yields a product that is equal to the concentration of iodine, in µg. per ml., in the solution in the cuvette. A typical calibration chart, photographically reproduced from an actual recorder tracing, is shown in Figure No. 20.

The extreme sensitivity of this procedure to interference by mercury is a definite handicap in a laboratory in which Nessler's solution is used extensively. In spite of care to use glassware that had been cleaned with chromic-sulfuric cleaning solution for all experiments in which iodine analyses were required, the analytical results for iodine were not in all cases satisfactory. Under normal conditions over a period of many years during which the author has been concerned with the determination of iodines by this method in a commercial laboratory, recoveries of iodine from known solutions have averaged from 85 to 95%, and the probable error of the average of duplicate determinations

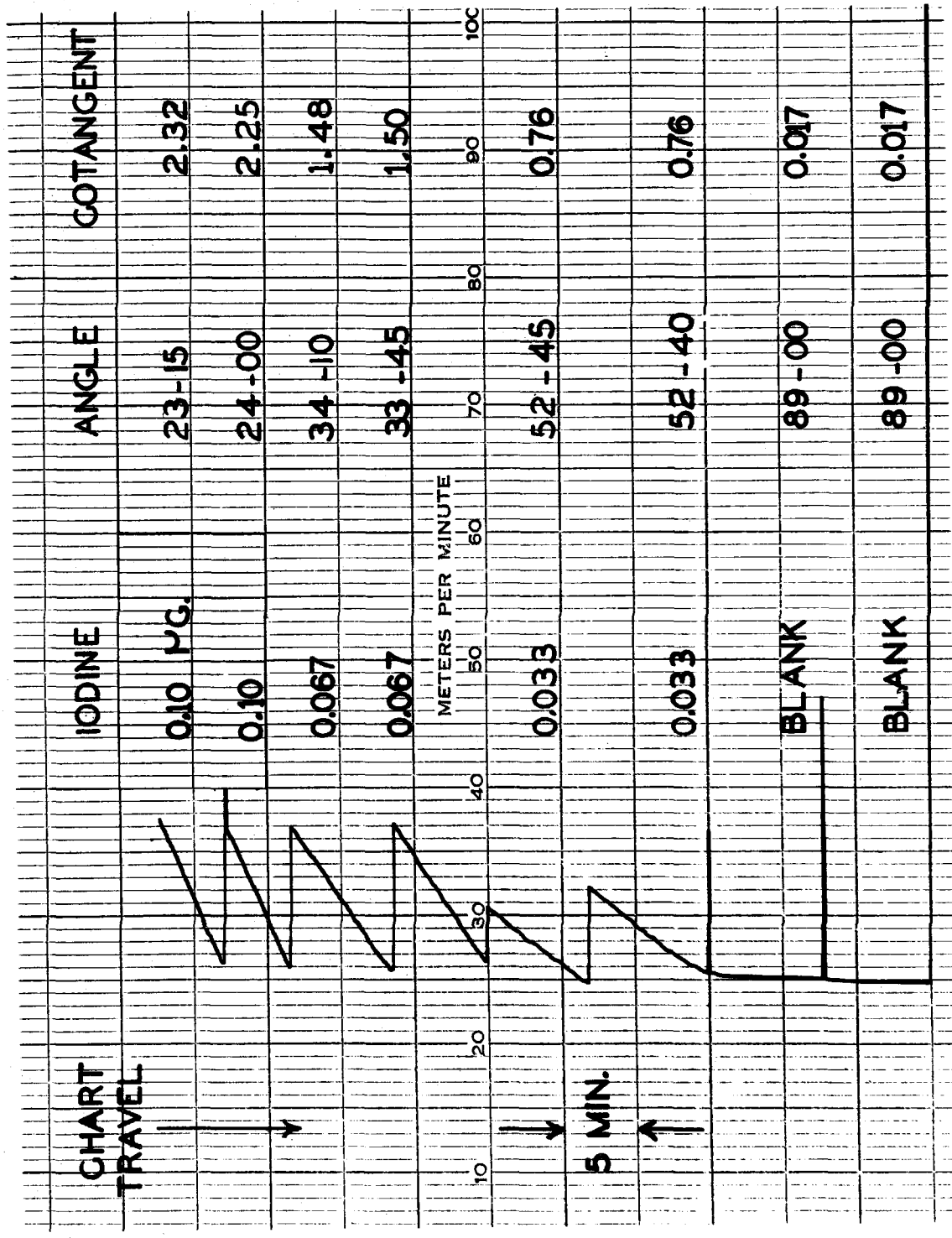
CHART TRAVEL
IODINE
ANGLE
COTANGENT
0.10 MG.
0.10
0.067
0.067
0.033
0.033
BLANK
BLANK
23-15
24-00
34-10
33-45
52-45
52-40
89-00
89-00
2.32
2.25
1.48
1.50
0.76
0.76
0.017
0.017
METERS PER MINUTE
10
20
30
40
50
60
70
80
90
100
5 MIN.

Figure No. 20

has been less than 5%. In some of the experiments reported here, the precision is definitely less than was expected, and it is felt that mercury contamination was largely responsible.

Determination of Nitrogen: Nitrogen in the immune aggregates and adsorbents was determined by Kjeldahl digestion and Nesslerization with or without preliminary distillation. Total nitrogen in the precipitates obtained in the precipitin tests was determined by the method of Lanni and Campbell (42). The entire washed precipitate is digested with 0.5 ml. of concentrated H_2SO_4. Completion of the digestion is hastened by the addition of one or two drops of 30% H_2O_2 (reagent grade "Superoxol" Merck) to the charred material in the hot sulfuric acid. Digestion is continued to ensure complete destruction or volatilization of the H_2O_2. The digest is cooled, diluted to about 33 ml., and 1 ml. of 5 N NaOH is added, followed by 15 ml. of Nessler's solution. The final volume is adjusted to 50 ml. and the yellow to amber color measured with a suitable photoelectric colorimeter.

The rather large volumes of sulfuric acid required to digest the cellulose-based immune adsorbents made direct Nesslerization of the digests impractical and of too low a sensitivity for the nitrogen content associated with them. They were analyzed by a semi-

micro Kjeldahl procedure based on that described by Redemann (87). The sample was digested in a 100 ml. Kjeldahl flask with 3 to 5 ml. of concentrated H_2SO_4 and 2.5 g. of Pregl $CuSO_4$-K_2SO_4 catalyst. The digest was diluted with 15 ml. of water and the flask was connected to the distilling apparatus described in this paper. It was alkalinized with 30% NaOH and the ammonia liberated was steam distilled and collected in 5 ml. of 0.01 N H_2SO_4. The distillate was made up to 100 ml. in a volumetric flask, and then Nesslerized with 10 ml. of Nessler's solution.

The intensities of the colors resulting from the Nesslerization of the ammonia-containing solutions were determined by one of three different techniques. Initially, in accordance with the custom in these laboratories, readings were made on a Klett-Summerson photoelectric colorimeter equipped with a No. 42 filter (420 mμ. effective peak transmission). The disadvantages of this method were the large and somewhat variable blank readings, and the poor sensitivity of the colorimeter to the low intensity of light from the tungsten source that could pass through the filter, making the precise determination of the null point difficult.

The substitution of a No. 47 filter (470 mμ. effective peak transmission) greatly improved the performance of the colorimeter in both of these respects.

This improvement was accompanied by a decrease in scale spread for a given range of nitrogen concentrations that partially neutralized the advantages, but the over-all improvement in precision and accuracy, for the usual range of nitrogen concentrations, was gratifying.

Late in the research program, a prototype of Beckman Instrument's Model C colorimeter became available. This instrument has a photoemissive sensing element and a precise optical system designed for cylindrical cuvettes. It was used with the No. 47 filter and values obtained upon it have considerably less instrumental error than those read on the Klett-Summerson colorimeter.

Calibration charts for the two colorimeters are given in Figures Nos. 21 and 22.

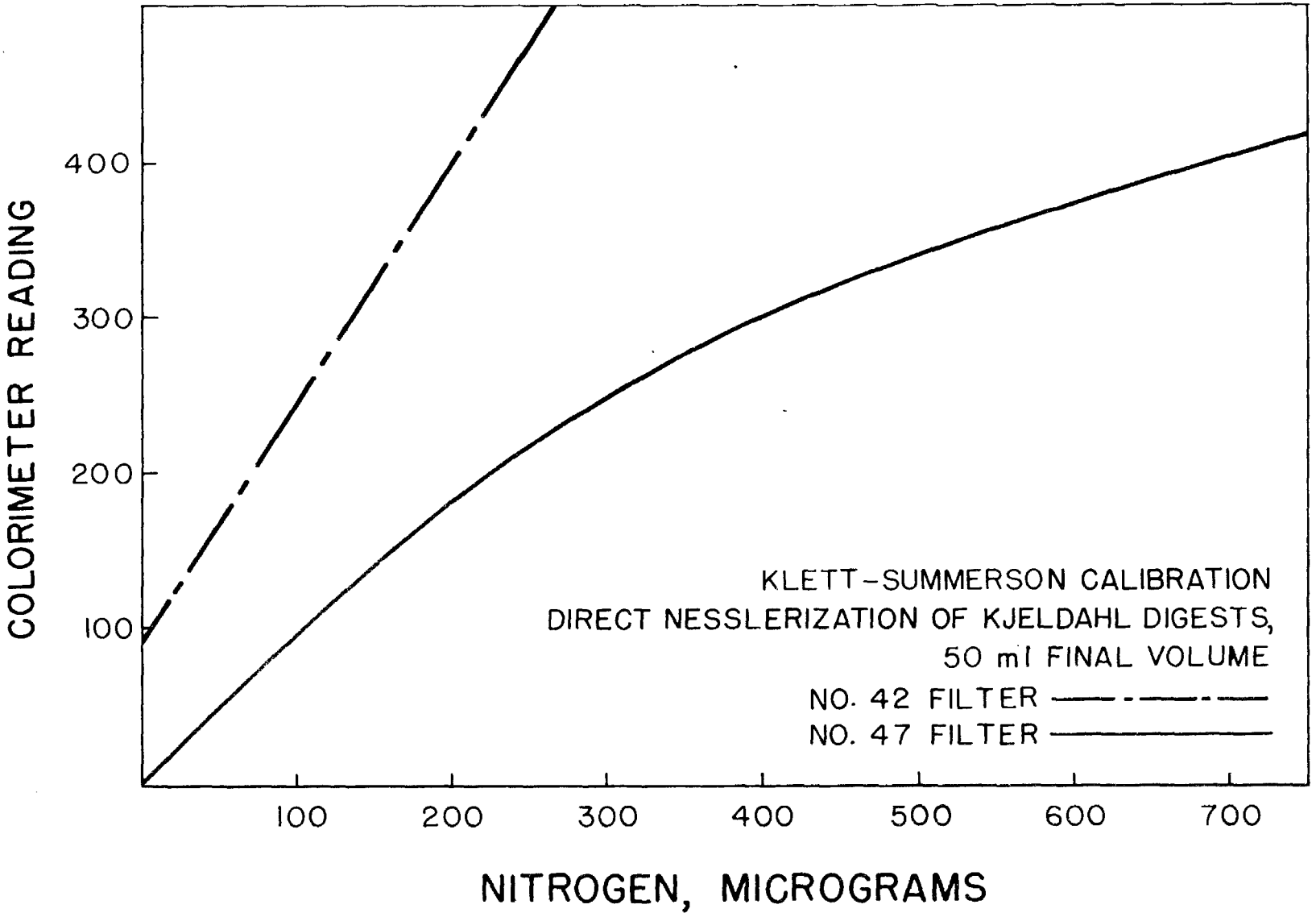
COLORIMETER READING
400
300
200
100
100
200
300
400
500
600
700
NITROGEN, MICROGRAMS
KLETT-SUMMERSON CALIBRATION
DIRECT NESSLERIZATION OF KJELDAHL DIGESTS,
50 ml FINAL VOLUME
NO. 42 FILTER
NO. 47 FILTER

FIGURE NO. 21

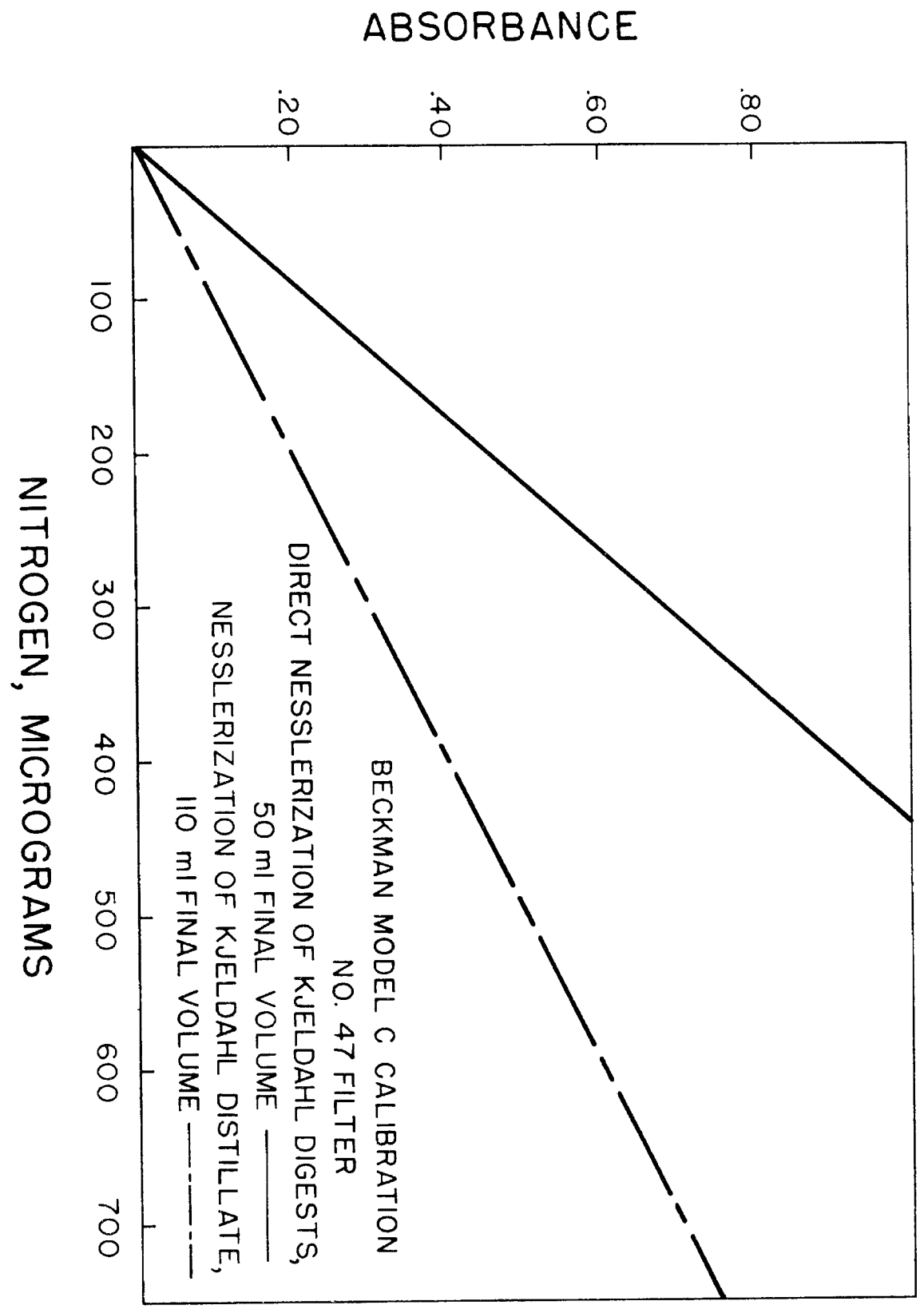
ABSORBANCE
.20
.40
.60
.80
100
200
300
400
500
600
700
NITROGEN, MICROGRAMS
BECKMAN MODEL C CALIBRATION
NO. 47 FILTER
DIRECT NESSLERIZATION OF KJELDAHL DIGESTS,
50 ml FINAL VOLUME
NESSLERIZATION OF KJELDAHL DISTILLATE,
110 ml FINAL VOLUME

FIGURE NO. 22

Immunochemical

Determination of the antibody content of a serum: The antibody contents of all antisera employed in this study were carefully determined by the "constant antibody-variable antigen" titration of Heidelberger and Kendall (88). The total nitrogen was determined in the precipitate of each tube and from this the nitrogen of the antigen was subtracted for those tubes in the zones of antibody excess and equivalence, assuming that all of the antigen added to the system was precipitated. The remaining nitrogen, multiplied by 6.25 was taken as the measure of the antibody protein in the precipitates. The precipitated antibody is graphed as ordinates against the antigen added to the system as abscissae. The peak of this graph is taken as a measure of the antibody in the system of the kind precipitable by the test antigen employed.

The protocol for a typical antiserum titration is given in some detail below:

Antiserum: gamma globulin fraction from pooled low-titer anti-RBG serum.

Antigen: bovine gamma globulin, ammonium sulfate purified fraction of Armour's fractions II and III, lots C103 and C104.

A set of ten 13 x 100 mm. Pyrex test tubes is arranged in a rack. Two milliliters of 1.2% antigen in buffered saline is placed in the first tube and 1.00 ml. of buffered saline, pH 8.4 is placed in each of the remaining tubes. One milliliter of antigen solution is transferred from the first tube to the second, the contents are thoroughly mixed by drawing the solution into the pipette and forcibly expelling several times, and then 1.00 ml. of this dilution are transferred to the third tube, and the process repeated until the last tube is reached. One milliliter of the final dilution is withdrawn from this tube and discarded. One-half milliliter of antibody solution is added to each tube and the tubes are well-shaken to mix the contents. The initial stage of the antigen-antibody reaction is allowed to take place at room temperature. Within a few minutes cloudiness develops in tubes three to five and this turbidity slowly develops into a flocculent precipitate. The development of precipitation in the tubes on either side of this optimum proportions zone is slower, and the final amount is less, with the retardation related to the extent to which the tube contents differ from those in the region of maximum precipitation. The tubes are placed in a refrigerator at 4° C after three hours at room temperature, and aggregation of the primary particles is allowed to proceed for 72 hours.

The precipitin tubes are then filled with cold 0.9% NaCl solution and placed in precooled carriers in a refrigerated centrifuge. They are centrifuged at 2500 RPM for 40 minutes, the supernatant carefully decanted from the precipitate, and the tubes inverted on a pad of filter paper and allowed to drain for fifteen minutes. The mouths of the tubes are wiped dry with clean tissue, and the precipitate washed with a gentle stream of cold saline delivered from a polyethylene wash bottle. The centrifugation and decantation are repeated and the precipitate washed through a third cycle.

To each of the well-drained precipitates, 0.5 ml. of concentrated H_2SO_4 is added and the tubes are placed in a brass digestion rack (89). This rack has a heavy gauge bottom member, into which a series of depressions have been machined with a spherically contoured milling tool. The bottoms of the precipitin tubes fit rather closely in these depressions, and the closeness of fit assures good heat transfer between the plate and the tubes. The shallowness of the depressions restricts strong heating to that portion of the tube below the meniscus of the sulfuric acid.

The digestion rack and precipitin tubes are placed on a tripod over a Meker burner and heated with a rather soft flame that distributes the heat uniformly over the bottom surface of the rack. The protein in the tubes

chars and slowly oxidizes. After about 30 minutes digestion, the rack is removed from the flame and allowed to cool for several minutes. One drop of 30% H_2O_2 is added to each tube so that it falls directly into the sulfuric acid*. The rack is then returned to the flame and digestion continued for another 30 minutes.

The tubes are permitted to cool to below 100° C and the clear contents are transferred quantitatively by multiple rinsings to 25 x 200 mm. test tubes calibrated at 35 and 50 ml. The total volume of washings is kept to less than 33 ml. One milliliter of 5 N NaOH is added to each tube, the contents are mixed, and then 15 ml. of Nessler's solution is added from a volumetric pipette** with an enlarged tip for rapid delivery. The volume in each tube is made up to exactly 50 ml. and the contents thoroughly mixed. Blanks and standards (250 and 500 µg. N) are carried through in parallel with the protein determinations.

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* If this addition is made at the correct stage of cooling, the reaction between the hot digest and the hydrogen peroxide will be vigorous, with almost instantaneous clearing of the suspended carbon particles, but there will be no strong spattering likely to result in loss of material from the tube.

** If the color is to be read with a No. 42 filter, the addition of Nessler's solution must be made with precision, since small variations in the amount of Nessler's

The yellow to amber solutions resulting from the Nesslerization of the diluted digests must be free from turbidity*. The colors are read on a Beckman Model C clinical colorimeter, equipped with a Klett-Summerson No. 47 (effective peak transmission 470 mμ) filter. The colorimeter readings and calculations are shown in Table No. 3 and the results are graphed in Figure No. 23.

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solution will result in significant changes in the blank due to the color of the Nessler's solution itself.

* The colors should be read promptly for turbidity tends to develop on standing, particularly in the samples containing larger amounts of nitrogen. Nessler's solution is very sensitive to carbonyl compounds and traces of acetone or aldehyde vapors produce intense turbidity, so that care should be taken to avoid contamination of the laboratory atmosphere with such vapors.

Table No. 3

Tube No.	Precipitating System: Ml. Antibody	Mg. Antigen	% Trans.	d	$d - d_B$	Nitrogen in Precipitate µg.: Total	Antigen (total in system)	Antibody*
Blank			96.8	.014				
Std. No. 1			50.5	.297	.283	250		
Std. No. 2			26.3	.580	.566	500		
1	0.5	12.00	91.2	.040	.026	23	1,920	
2	0.5	6.00	77.4	.111	.097	86	960	
3	0.5	3.00	27.6	.559	.545	481	480	
4	0.5	1.50	19.1	.719	.705	622	240	382**
5	0.5	.75	26.2	.582	.568	502	120	382**
6	0.5	.375	38.8	.411	.397	351	60	291
7	0.5	.188	46.8	.330	.316	279	30	249
8	0.5	.094	57.8	.238	.224	198	15	183
9	0.5	.047	68.3	.166	.152	134	8	126
10	0.5	.023	81.0	.091	.077	68	4	64

* Assuming that all of the antigen nitrogen in the system is included in the precipitate.

** .382 mg. N per 0.5 ml. of antibody solution: $.382 \times \frac{1}{0.5} \times 6.25 = 4.78$ mg. antibody protein per ml. of globulin solution.

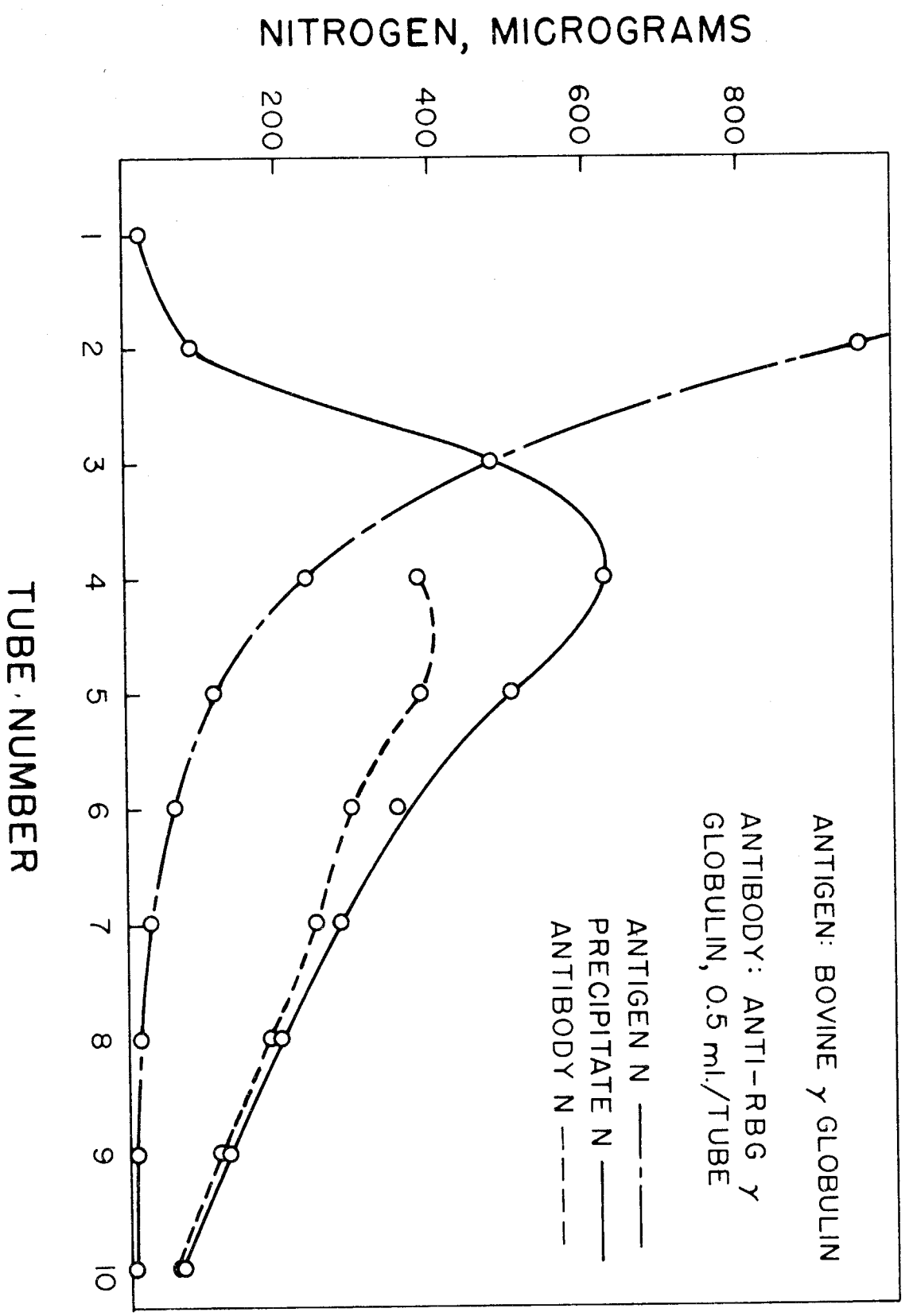

NITROGEN, MICROGRAMS
200
400
600
800
1
2
3
4
5
6
7
8
9
10
TUBE NUMBER
ANTIGEN: BOVINE γ GLOBULIN
ANTIBODY: ANTI-RBG γ GLOBULIN, 0.5 ml./TUBE
ANTIGEN N
PRECIPITATE N
ANTIBODY N

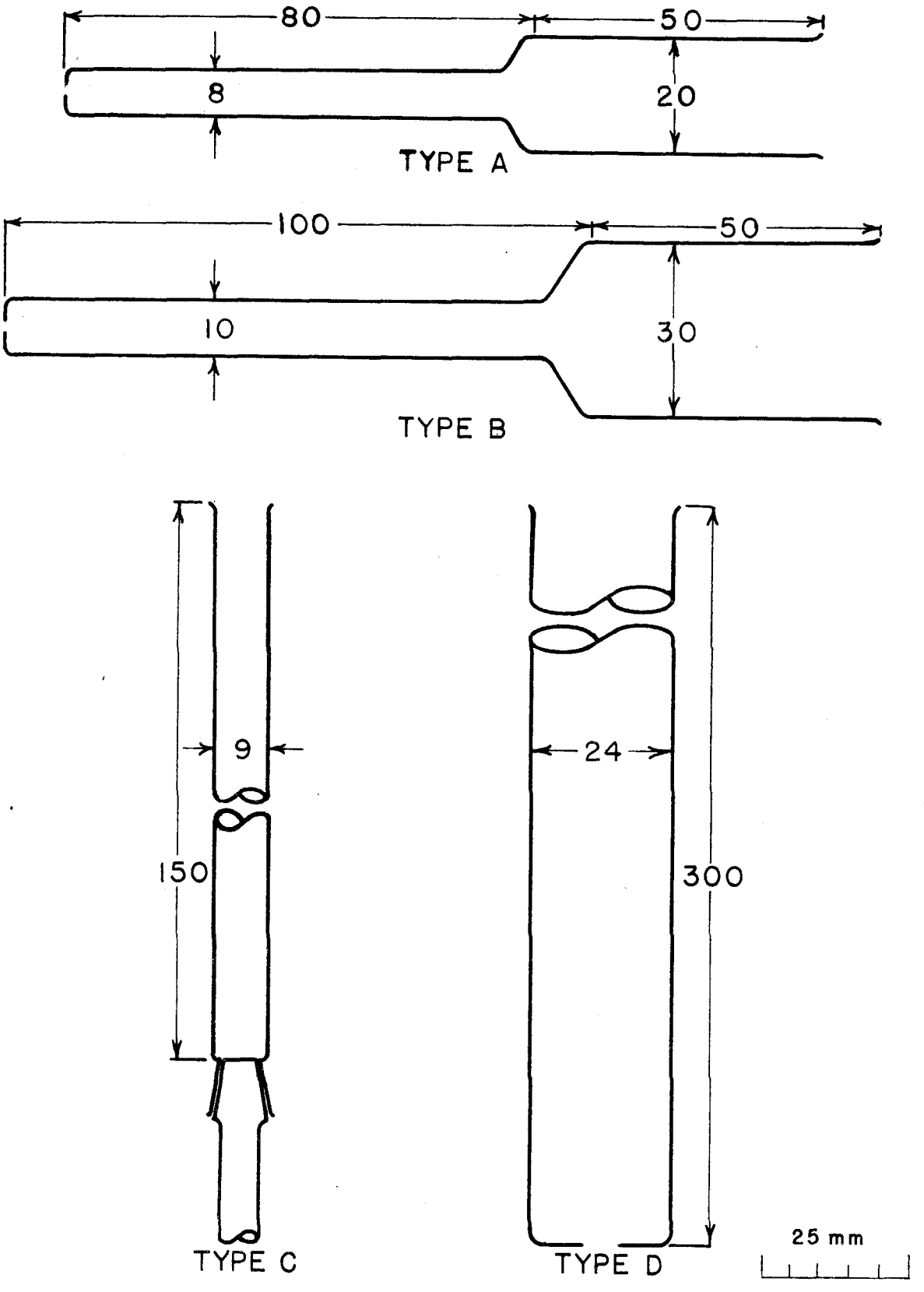
FIGURE NO. 23

Properties of Immune Adsorbents

The ability of the immune adsorbents to adsorb homologous antibody out of solutions with which it was treated was investigated under a number of conditions, as were the conditions under which the antibody could be eluted by solutions of inhibiting haptens. These studies were designed primarily to establish the conditions optimum for hapten binding experiments, and no attempts were made to investigate systematically the properties of these adsorbents. In particular, no effort was made at a direct comparison of the different lots of adsorbents under conditions that would permit the establishment of quantitative relationships among them.

The column experiments were performed with a number of different sizes and styles of chromatographic columns. Figure No. 24 shows these various styles of columns, and reference to these columns will be made, in the experiments to be described, in terms of the designations given in this figure.

Adsorption of antibody from serum by R-adsorbent: R-adsorbent, Lot I, and purified Solka-floc were set up in parallel columns in 20 mm. packed sections in type "A" chromatographic columns. The columns were washed with borate buffer, pH 8.0, and 10 ml. of a 1:1 dilution of an anti-RBG serum in buffer was percolated



ADSORPTION COLUMNS

FIGURE NO. 24

through each column. The effluents were tested for antibody activity by a qualitative titration against R'_3-resorcinol. The degree of precipitation was read after 3 hours at room temperature and 2 days in the refrigerator.

Table No. 4

Tube No.	Antigen Concentration	Control Serum	Precipitation Solka-floc Effluent	R-Adsorbent Effluent
1	1:10,000	+++	++	-
2	1:20,000	+++	++	-
3	1:40,000	++++	+++	-
4	1:80,000	++++	+++	-
5	1:160,000	+++	+++	-
6	1:320,000	+++	++	-
7	1:640,000	++	+	-
8	1:1,280,000	++	+	-

The moderate decrease in activity of the effluent from the Solka-floc column as compared to the untreated control serum is ascribed to the dilution of the serum with buffer solution held in the interstices of the column.

Saturation of adsorbent with antibody: Three hundred milligram aliquots of R-adsorbent (Lot II) or purified Solka-floc were packed in columns (type "A") by suspending in water and collecting on a glass wool plug at the bottom of the tube by filtration under vacuum. The columns were flushed with borate buffer, pH 8.0, and then serum diluted with an equal volume of buffer

was flowed through the column under gravity. The columns were washed with 10 ml. portions of buffer and then the adsorbents were forced out into 100 ml. Kjeldahl flasks, digested, and distilled. The ammonia was collected in dilute sulfuric and determined by Nesslerization. The results are summarized in Table No. 5.

These results show that the R-adsorbent was beginning to show saturation and incomplete adsorption of antibody at somewhere between 4 and 8 ml. of antiserum per 300 mg. of adsorbent. Extrapolation of the adsorbed nitrogen curve suggests that at complete saturation, the adsorbent would hold about 2 mg. of antibody nitrogen per gram of adsorbent. The undesirably high retention of nonspecifically bound protein is attributed to (1) filtration of suspended lipo-proteins that made a haze in the immune sera* and (2) incomplete washing of the column with serum proteins remaining in the interstices of the column and in the voids within the cellulose fibers.

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* The immune sera had been stored in the frozen state and had considerable turbidity that could not be removed by filtration through retentive filter paper (Whatman No. 42). The normal serum was fresh and quite clear.

Table No. 5

Column Packing	Serum Volume	Serum Kind	Nitrogen, µg. Total	Nitrogen, µg. Adsorbent	Nitrogen, µg. Protein	Protein N/ µg. per ml. serum
R-adsorbent	2 ml.	anti-RBG	327	130	197	98
"	4 ml.	"	588	130	428	107
"	4 ml.	"	541	130	411	103
"	8 ml.	"	649	130	519	65
"	4 ml.	anti-SBG	289	130	159	40
"	4 ml.	"	206	130	76	19
"	8 ml.	normal	177	130	47	6
Solka-floc	1 ml.	anti-RBG	76	35	41	41
"	2 ml.	"	91	35	56	28
"	2.5 ml.	"	108	35	73	30
"	3 ml.	anti-SBG	216	35	181	60
"	4 ml.	"	291	35	256	64
"	4 ml.	normal	113	35	78	19
"	4 ml.	"	157	35	122	30

As a check on the filtration factor, future column experiments were set up with a layer of Solka-floc above the immune adsorbent and separated from it by a disc of filter paper the size of the internal diameter of the column. The filter plug could then be separated from the adsorbent for independent analysis or discard.

Parallel tubes (Type "A") were set up with 300 mg. aliquots of R-adsorbent (Lot II) or Solka-floc, each covered with a 150 mg. layer of Solka-floc as a filter pad. An additional filter paper disc was placed over the filter pad to protect it from resuspension by the turbulence of the fluid being added to the column. The columns were rinsed with buffer and then three ml. of anti-RBG serum, diluted with an equal volume of buffer, was passed through each. The columns were washed with 10 ml. portions of buffer and the layers analyzed separately.

Table No. 6

	Nitrogen, µg.			µg. Protein N
	Total	Adsorbent	Protein	per ml. serum
Filter layer, R column	56	18	38	13
Adsorbent layer R column	291	130	161	54
Filter layer control column	57	18	39	13
Solka-floc layer control column	61	35	26	9

This experiment demonstrates that both factors are involved in the nonspecific protein retention of the columns and suggests that of the 54 µg. of protein N retained in the R-adsorbent per ml. of serum passed through the column, 9 were held as a physical contaminant and 45 were bound by antibody-hapten interactions.

Distribution of antibody in an adsorbent column: A column (type "C") was prepared containing 10 layers of R-adsorbent (Lot III) covered by a filter pad of purified Solka-floc. Each layer contained 100 mg. of fiber and the layers were separated from each other by filter paper discs. The column was washed with borate buffer, pH 8.0, and 12.5 ml. of anti-RBG serum (66 micrograms of precipitable N per ml. of serum) diluted with an equal volume of buffer was drawn through the column with gentle suction. Approximately 2 hours were required for passing through column. The column was washed with twenty-five ml. of buffer and the adsorbent was extruded from the column and the layers separated for nitrogen analysis by Kjeldahl digestion and distillation followed by Nesslerization.

Table No. 7

Nitrogen, μg.

Layer		Total	Adsorbent	Protein
Solka-floc		56	5	51
R-adsorbent No.	1	173	51	122
	2	152	51	101
	3	137	51	86
	4	135	51	84
	5	117	51	66
	6	105	51	54
	7	107	51	56
	8	93	51	42
	9	99	51	48
	10	89	51	38
Total protein N retained				698
Antibody N put on (12.5 × 66)				825
Recovery of antibody				84%

The absence of a sharp break or concentration front on the column is probably the result of too rapid flow of the antiserum through the column. The calculated antibody recovery is not corrected for non-antibody nitrogen mechanically bound to the column. The correction for this error is of undetermined magnitude, but, judging from the results with the experiments described just previously, may amount to from 10 to 15%. The distribution of serum nitrogen on the column is graphed in Figure No. 25.

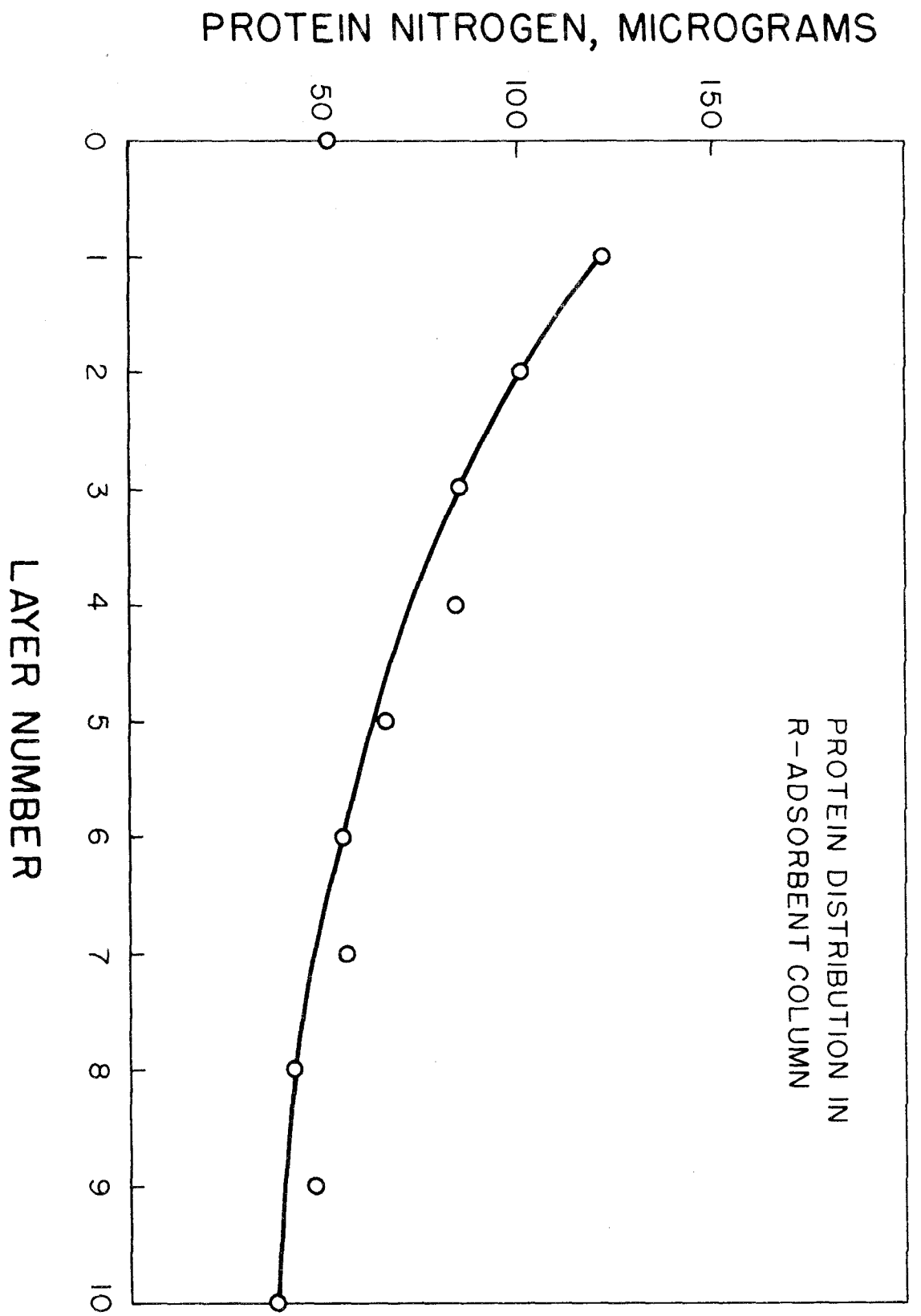
PROTEIN NITROGEN, MICROGRAMS
50
100
150
0
1
2
3
4
5
6
7
8
9
10
LAYER NUMBER
PROTEIN DISTRIBUTION IN
R-ADSORBENT COLUMN

FIGURE NO. 25

Preparation of purified antibody by elution from R-adsorbent, Experiment I: One gram of R-adsorbent (Lot I) in a (type "B") column was prewashed with borate buffer, pH 8.0, and 12.5 ml. of anti-RBG serum (66 micrograms of precipitable N per ml.) diluted with an equal volume of buffer was allowed to flow through it. The column was washed with 10 ml. of buffer and then eluted with a saturated solution of R-β naphthol in borate buffer pH 8.6. The first and second 10 ml. portions of eluate were dialyzed against buffer until only traces of hapten remained, and were then analyzed for total nitrogen and for antibody nitrogen precipitable by R'_3-resorcinol.

Table No. 8

Antibody Nitrogen put on column	(12.5 × 66)	= 825 μg.
Total protein nitrogen in first eluate	(10 × 19.5)	= 195
Total protein nitrogen in second eluate	(10 × 1.3)	= 13
Total eluted nitrogen		= 208
Precipitable N in first eluate* (not less than)		87
Precipitable N in second eluate		trace
Recovery of antibody nitrogen		= 10.5%
Purity of eluted protein		= 45%

* The optimum proportions zone was missed in the precipitation curve so that the amount of precipitable N shown is a lower limit.

Preparation of purified antibody by elution from R-adsorbent, Experiment II: Two grams of R-adsorbent, Lot V, was packed in a type "D" column, covered with a 300 mg. layer of purified Solka-floc, washed with buffer over a period of three days until no more hapten-bleeding was detectable, and 10 ml. of fresh high titer anti-RBG serum (300 µg. N per ml.) diluted with an equal volume of borate buffer, pH 8.4, was allowed to percolate through the column at room temperature under gravity head. A period of four hours was required for the serum to pass through the column. The column was then washed with ice-cold saline under strong suction. A total of 100 ml. of saline was used and a wash time of about one-half an hour.

M/1000 R-resorcinol in borate buffer was then percolated through the column at room temperature under gravity head. A total of 50 ml. of hapten passed through the column in about 18 hours. The eluate was placed in a dialysis casing and simultaneously dialyzed and pervaporated until the volume had decreased to less than 10 ml. The dialysis was continued in the cold-room for forty-eight hours. At the end of this time the solution was almost colorless. The dialysate was made up to a volume of 10 ml. and analyzed for total and specifically precipitable protein.

Table No. 9

Total protein N (10 ml. × 320 micrograms/ml.)	= 3200
Antibody N (10 ml. × 290 micrograms/ml.)	= 2900
Purity of eluted antibody	= 90%
Recovery of total antibody	= 72%

Preparation of purified antibody by elution from R-adsorbent, Experiment III: Two grams of R-adsorbent (Lot III), was washed with buffer until free from hapten bleed, and then equilibrated in a 250 ml. centrifuge bottle with 25 ml. of anti-RBG serum (66 micrograms precipitable N per ml.) by rolling on miniature ball-mill rollers for two hours at room temperature. The adsorbent was centrifuged down and the supernatant poured off and tested for antibody content. The adsorbent was washed twice with 50 ml. portions of buffer and eluted with three successive portions of M/1000 R-resorcinol hapten. The eluates were dialyzed until substantially hapten-free* and were analyzed for total nitrogen by Kjeldahl digestion and Nesslerization. Precipitin tests were set up against R'_3-resorcinol to determine the amount of antibody nitrogen in each eluate. The analytical results are summarized in Table No. 10.

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* The last traces of hapten were very difficult to remove and denaturation of the antibody was observed on prolonged dialysis of dilute antibody solutions.

Table No. 10

Sample	Volume ml.	Total N µg./ml.	Total N µg.	Antibody N µg./ml.	Total Antibody N, µg.	Antibody Purity %
Original serum	25			66	1650	
First eluate	24	28	670	23.5	564	84
Second eluate	38	11.5	435	5.2	188	43
Third eluate	22	4.3	95	1	22	23
Total eluates			1200		774	65
Recovery of antibody (based onoriginal antibody content of serum) = 47%						
Supernatant from adsorption	50			4	200	
Recovery of antibody (based on antibody taken up by the adsorbent)= 53%						

Preparation of purified antibody by elution from R-adsorbent, Experiment IV: Three grams of R-adsorbent (Lot III) was washed with buffer until free of hapten bleed and then equilibrated with 20 ml. of medium titer anti-RBG serum diluted with an equal volume of buffer. Equilibration was accomplished by tumbling in a 250 ml. centrifuge bottle on the rollers of a miniature ball-mill for five days in the cold-room. The bottle was then filled with cold saline and the adsorbent centrifuged down. The supernatant was decanted and the adsorbent washed three times with 200 ml. portions of cold saline.

The antibody was eluted with two portions of M/1000 R-iodophenol dye. Elution was performed by tumbling on the rollers of the ball-mill for periods of three to five days each. The eluates were combined, dialyzed against buffer, pH 8.0, and pervaporated to a final volume of 25 ml. During the dialysis considerable protein precipitated out of solution. The precipitate was separated from the supernatant and each was analyzed for protein and hapten. The hapten was measured by analysis for iodine. Antibody was determined in the supernatant by the precipitin test against R'_3-resorcinol.

Table No. 11

Supernatant	
Total protein nitrogen	2010 µg.
Antibody nitrogen	750 µg.
Purity of antibody protein	37%
Hapten concentration	.48 µg./ml.
Precipitate	
Total protein nitrogen	870 µg.
Total hapten	1.70 µg.

Hapten Binding on Aggregates of Antibody and Immune Adsorbent

Hapten binding in columns of immune adsorbents: Five hundred milligram aliquots of immune adsorbent were packed in type "A" columns prewashed with 10 ml. of borate buffer pH 8.0, and treated with 5 ml. of test serum. The non-bound proteins were washed through the column with 20 ml. of borate buffer, and 10 ml. of M/1000 haptenic dye percolated through the column. Unbound dye was then flushed through with an additional 20 ml. of buffer. The adsorbents used were R-adsorbent Lot II, and S-adsorbent Lot I. The sera employed were anti-RBG, anti-SBG, normal and buffer control. The haptens used were R-iodophenol, R-iodoresorcinol, S-iodophenol and S-iodoresorcinol. Each adsorbent was tested with the four serum systems and each adsorbent-serum combination was tested with each hapten. Systems in which the anti-serum was homologous to the adsorbent were run in duplicate. The column contents, after the final buffer wash, were analyzed for iodine. Table No. 12 tabulates the combinations and gives the tube number and iodine content of each. The analytical data are summarized in Table No. 13.

Table No. 12

Serum	R-adsorbent								S-adsorbent							
	R-hapten				S-hapten				R-hapten				S-hapten			
	Iodo-phenol		Iodore-sorcinol		Iodo-phenol		Iodore-sorcinol		Iodo-phenol		Iodore-sorcinol		Iodo-phenol		Iodore-sorcinol	
	No.	Iodine	No.	Iodine	No.	Iodine	No.	Iodine	No.	Iodine	No.	Iodine	No.	Iodine	No.	Iodine
Anti-RBG	2	0.40	5	0.55	4	11.0	8	19.7	34	0.55	35	0.65	36	0.71	37	48.3
	3	0.25	6	0.57	7	4.49	9	20.3								
Anti-SBG	14	0.28	15	0.60	16	0.56	17	22.1	22	0.55	24	0.77	26	0.74	28	28.3
									23	0.49	25	0.64	27	0.72	29	27.7
Normal	10	0.61	11	0.80	12	0.56	13	18.5	30	0.60	31	0.71	32	0.84	33	25.8
Buffer Control	18	0.20	19	0.61	20	0.80	21	14.9	38	0.51	39	0.43	40	1.34	41	32.8

All iodine values are given in micrograms of iodine in entire tube contents.

Table No. 13

Specimen	Iodine Trace Angle	Cot. of Angle	Cot. minus Blank	Iodine in Aliquot µg.	Aliquot	Iodine in Sample µg.
Tube No.						
2	20.6	2.660	2.569	.134	4/12	0.40
3	31.3	1.645	1.554	.081	4/12.5	0.25
4	9.5	5.976	5.885	.306	1/48	11.0
5	15.5	3.606	3.515	.183	4/12	0.55
6	15.3	3.655	3.564	.189	4/12	0.57
7	55.3	.692	.601	.031	1/144	4.49
8	20.2	2.718	2.627	.137	1/144	19.7
9	19.6	2.808	2.717	.141	1/144	20.3
10	14.0	4.011	3.920	.204	4/12	0.61
11	10.9	5.193	5.102	.265	4/12	0.80
12	15.3	3.655	3.564	.185	4/12	0.56
13	21.3	2.565	2.474	.129	1/144	18.5
14	27.8	1.897	1.806	.094	4/12	0.28
15	14.4	3.895	3.804	.198	4/12	0.60
16	15.3	3.655	3.564	.185	4/12	0.56
17	18.2	3.042	2.951	.153	1/144	22.1
18	35.8	1.387	1.296	.068	4/12	0.20
19	14.0	4.011	3.920	.204	4/12	0.61
20	11.2	5.050	4.914	.256	4/12	0.77
21	25.7	2.078	1.987	.103	1/144	14.9
22	15.6	3.582	3.491	.183	4/12	0.55
23	17.2	3.230	3.139	.163	4/12	0.49
24	11.3	5.005	4.914	.256	4/12	0.77
25	13.4	4.918	4.107	.213	4/12	0.64
26	11.7	4.829	4.738	.246	4/12	0.74
27	12.0	4.705	4.614	.240	4/12	0.27
28	14.5	3.867	3.776	.196	1/144	28.3
29	14.8	3.785	3.694	.192	1/144	27.7
30	14.3	3.923	3.832	.199	4/12	0.60

Table No. 13
(cont.)

Specimen	Iodine Trace Angle	Cot. of Angle	Cot. minus Blank	Iodine in Aliquot µg.	Aliquot	Iodine in Sample µg.
Tube No.						
31	12.2	4.625	4.534	.236	4/12	0.71
32	10.4	5.449	5.358	.279	4/12	0.84
33	15.8	3.534	3.443	.179	1/144	25.8
34	15.4	3.630	3.539	.184	4/12	0.65
35	13.1	4.297	4.206	.219	4/12	0.65
36	12.2	4.625	4.534	.236	4/12	0.71
37	8.7	6.535	6.444	.335	1/144	48.3
38	16.6	3.354	3.263	.170	4/12	0.51
39	19.4	2.840	2.749	.143	4/12	0.43
40	51.2	.840	.713	.037	1/36	1.34
41	12.6	4.474	4.383	.228	1/144	32.8
R-iodo-phenol						
0.002 ml.	34.8	1.439	1.348	.0702	4/12	0.21
"	33.3	1.522	1.431	.0745	4/12	0.22
R-iodo-resorcinol						
0.002 ml.	46.1	.962	.871	.0453	4/12	0.14
"	48.6	.882	.791	.0411	4/12	0.12
S-iodo-phenol						
0.002 ml.	24.3	2.215	2.124	.1105	4/12	0.33
"	24.7	2.174	2.083	.1084	4/12	0.33
S-iodo-resorcinol						
0.002 ml.	34.9	1.434	1.343	.0699	4/12	0.21
"	33.1	1.534	1.443	.0751	4/12	0.23

Table No. 13
(cont.)

Specimen	Iodine Trace Angle	Cot. of Angle	Cot. minus Blank	Iodine in Aliquot µg.	Aliquot	Iodine in Sample µg.
Iodine Standard 0.15 µg.	44.0	1.036	.945	.050	4/12	0.15
Iodine Standard 0.45 µg.	18.3	3.024	2.933	.150	4/12	0.45
Reagent Blank	84.8	.091				

Calculation of hapten concentrations:

R-iodophenol $\frac{.215}{1.27 \times 10^8} \times \frac{1000}{.002} = .00085$ M

R-iodoresorcinol $\frac{.130}{1.27 \times 10^8} \times \frac{1000}{.002} = .00051$ M

S-iodophenol $\frac{.330}{1.27 \times 10^8} \times \frac{1000}{.002} = .00130$ M

S-iodoresorcinol $\frac{.220}{1.27 \times 10^8} \times \frac{1000}{.002} = .00087$ M

Pre-incubation of hapten with serum: As a check upon the possibility that the failure of the hapten to adsorb on the anti-RBG R-adsorbent aggregate was the result of inadequate exposure time of the hapten to the aggregate, the hapten and the antiserum were incubated for three days in the refrigerator before the mixture was passed through the column. Fifteen milliliters of serum was mixed with 1 ml. of M/1000 R-iodophenol and 14 ml. of buffer, pH 8.0, allowed to stand in the cold for three days and then filtered through Whatman No. 42 filter paper. Anti-RBG, anti-SBG and normal sera were run in parallel. Ten milliliter aliquots of each serum mixture were run through 300 mg. R-adsorbent and S-adsorbent columns (type "A") which had been pre-washed with 5 ml. of buffer. After the serum had run through under moderate suction (approximately half an hour for the serum to pass through the tube), the column was washed with 20 ml. of buffer and the contents analyzed for iodine.

The third 10 ml. aliquot of the anti-RBG serum mixture was run through an R-adsorbent column and washed as above and the column analyzed for nitrogen. Total nitrogen found, 279 micrograms; adsorbent nitrogen, 97 micrograms; protein nitrogen (by difference) 182 micrograms. Antibody = 7.1×10^{-9} moles.

The analytical data for the iodine determinations are detailed in Table No. 14.

Table No. 14

Specimen	Iodine Trace Angle	Cotangent of Angle	Cotangent minus Blank	Iodine Aliquot µg.	Aliquot	Iodine in Specimen µg.
Tube No. 1 anti-RBG serum R-adsorbent	71.6	.333	.242	.0128	4/12	.038
Tube No. 2 anti-SBG serum R-adsorbent	65.2	.462	.371	.0196	4/12	.059
Tube No. 3 normal serum R-adsorbent	64.6	.475	.384	.0203	4/12	.061
Tube No. 4 anti-RBG serum S-adsorbent	57.3	.642	.551	.0292	4/12	.088
Tube No. 5 anti-SBG serum S-adsorbent	61.3	.547	.456	.0241	4/12	.072
Tube No. 6 normal serum S-adsorbent	65.2	.462	.371	.0196	4/12	.059
Reagent Blank	84.8	.091				
Iodine Standard 0.15 µg.	44.0	1.036	.945	.050	4/12	.150

Elution of antibody protein from adsorbent by iodinated haptens: To check on the possibility of the failure of the experiments pages 147 to 151 inclusive being the result of the elution of the antibody from the columns by the hapten solution employed, the following experiment was set up: Three hundred milligram portions of R-adsorbent, Lot II, were packed in type "A" columns and charged with 10 ml. of a 1:1 dilution of anti-RBG serum with borate buffer, pH 8.6, and washed with 10 ml. buffer. The column was then eluted with 20 ml. of hapten solutions varying from M/4000 to M/1000 in strength, rinsed with a final 5 ml. of buffer, and analyzed for nitrogen.

Table No. 15

Hapten Concentration	Protein Nitrogen Found, μg.
0 (buffer control)	316
M/4000	137
M/2000	130
M/1333	123
M/1000	151

The protein nitrogen figure for the M/1000 hapten is presumably in analytical error, but it is apparent that under the conditions of this experiment, the major portion of the antibody nitrogen is removed at very low hapten concentrations, but that somewhat less than half of the antibody is relatively resistant to hapten elution under these conditions.

Distribution of antibody and hapten on cellulose adsorbent columns; Experiment I: A type "C" column was charged with twenty layers of R-adsorbent (Lot III) each containing 100 mg. dry weight of the adsorbent. The column was topped with a 100 mg. layer of purified Solka-floc. Discs of filter paper were used to separate the successive layers.

The column was washed with 20 ml. borate buffer, pH 8.0, and then 18 ml. of antibody (68 μg. antibody N/ml.) diluted with an equal volume of buffer, was run through the column under 1 atmosphere at room temperature. Initial flow rate was approximately 0.2 ml./minute. The column "ran dry" during the night and became partially plugged. Washing with buffer was very slow and difficult. With full vacuum the maximum flow rate that could be attained was 1 ml./hour. A total buffer wash of 35 ml. was secured over the course of three days.

The total antibody placed on the column was estimated to be approximately 5×10^{-8} moles. A one hundred percent excess (10^{-7} moles) of R-iodophenol hapten in 10 ml. of buffer was run through the column and the non-adsorbed hapten was washed through with an additional 20 ml. of buffer.

Alternate layers were analyzed for nitrogen and iodine. The analytical data are shown in Table No. 16.

Table No. 16

Analytical Data, Column Experiment I

Column Layer	Total N µg.	Adsorbent N µg.	Protein N µg.	Moles Protein × 10⁹	Iodine Trace Angle	Cotangent of Angle	Cotangent - Blank	Hapten Iodine µg.	Moles Hapten × 10⁹	Protein:Hapten Molar Ratio
Solka-floc	96	5	91	3.5						
1				3.5*	71.8	.326	.148	.025	.20	17.5
2	137	51	86	3.4						
3				3.4*	75.5	.259	.081	.041	.11	31
4	115	51	64	2.5						
5				3.2*	76.0	.249	.071	.012	.10	32
6	130	51	79	3.1						
7				2.9*	74.4	.279	.101	.017	.14	20
8	120	51	69	2.7						
9				2.4*	75.6	.257	.079	.013	.11	22
10	103	51	52	2.0						
11				1.8*	23.3	2.32	2.14	.33	2.3	**
12	90	51	39	1.5						
13				1.4*	75.7	.255	.077	.013	.10	14
14	82	51	31	1.2						
15				1.2*	76.3	.244	.066	.011	.09	13
16	82	51	31	1.2						
17				1.2*	81.7	.146	-.032	none	none	--
18	82	51	31	1.2						
19				1.1*	77.8	.214	.036	.006	.05	22
20	77	51	26	1.0						

*Value interpolated from smoothed curve of nitrogen data for even-numbered layers.

** Specimen contaminated, discard this value.

Protein nitrogen, iodine, and antibody:hapten ratios are plotted in Figure No. 26.

Distribution of antibody and hapten on cellulose adsorbent columns, Experiment II: A second column of the same structure was set up, but care was taken to avoid too firm compacting of the adsorbent. A buffer flow rate of 15 ml./hour could be obtained under full vacuum on this column. The buffer-washed column was treated with 15 ml. of anti-RBG serum diluted with an equal volume of buffer at a rate of flow such that approximately four days in cold-room was required for the antiserum to pass through the column, (gravity head). The column was washed with 50 ml. buffer with suction, and 0.1 ml. of M/1000 R-iodophenol diluted in 10 ml. of buffer was passed through the column in two days in the cold-room, under gravity head. The column was then washed with 60 ml. of buffer at room temperature, and pressed as dry as possible. The adsorbent was extruded from the column and alternate sections were analyzed for nitrogen and iodine.

The analytical data are tabulated in Table No. 17 and the protein, hapten, and protein:hapten ratios are plotted against column position in Figure No. 27.

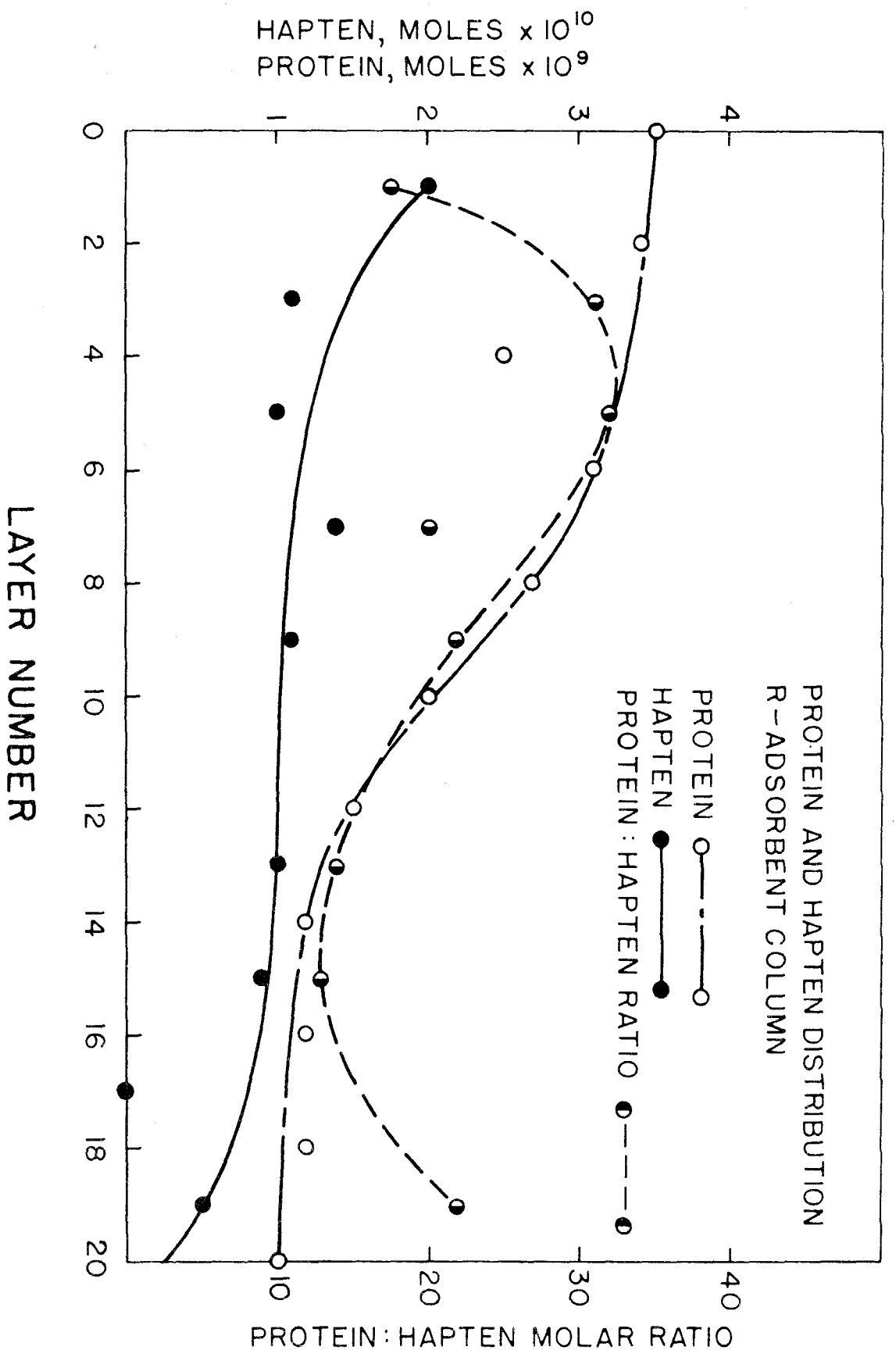
HAPTEN, MOLES x 10^10
PROTEIN, MOLES x 10^9
1
2
3
4
0
2
4
6
8
10
12
14
16
18
20
LAYER NUMBER
PROTEIN AND HAPTEN DISTRIBUTION
R-ADSORBENT COLUMN
PROTEIN
HAPTEN
PROTEIN : HAPTEN RATIO
10
20
30
40
PROTEIN : HAPTEN MOLAR RATIO

FIGURE NO. 26

Table No. 17

Analytical Data, Column Experiment II

Column Layer	Total N µg.	Adsorbent N µg.	Protein N µg.	Moles Protein × 10^9	Iodine Trace Angle	Cotangent of Angle	Cotangent - Blank	Hapten Iodine µg.	Moles Hapten × 10^9	Protein:Hapten Molar Ratio
Solka-floc	65	5	60	2.3						
1				3.8*	76.4	.242	.064	.011	.09	42
2	146	51	95	3.7						
3				3.6*	77.8	.214	.036	.006	.05	72
4	137	51	86	3.4						
5				3.1*	75.8	.251	.073	.013	.10	31
6	123	51	72	2.8						
7				2.5*	77.2	.227	.049	.008	.06	40
8	99	51	48	1.9						
9				1.9*	77.4	.224	.046	.008	.06	32
10	96	51	45	1.8						
11				1.8*	78.3	.207	.029	.005	.04	45
12	97	51	46	1.8						
13				1.7*	78.6	.202	.024	.004	.03	57
14	90	51	39	1.5						
15				1.5*	77.4	.224	.046	.008	.06	25
16	88	51	37	1.4						
17				1.3*	76.7	.236	.058	.010	.08	16
18	78	51	27	1.1						
Reagent Blank					79.8	.178				
Iodine Standard					43.5	1.050	.872			

* Value interpolated from smoothed curve of nitrogen data for even-numbered layers.

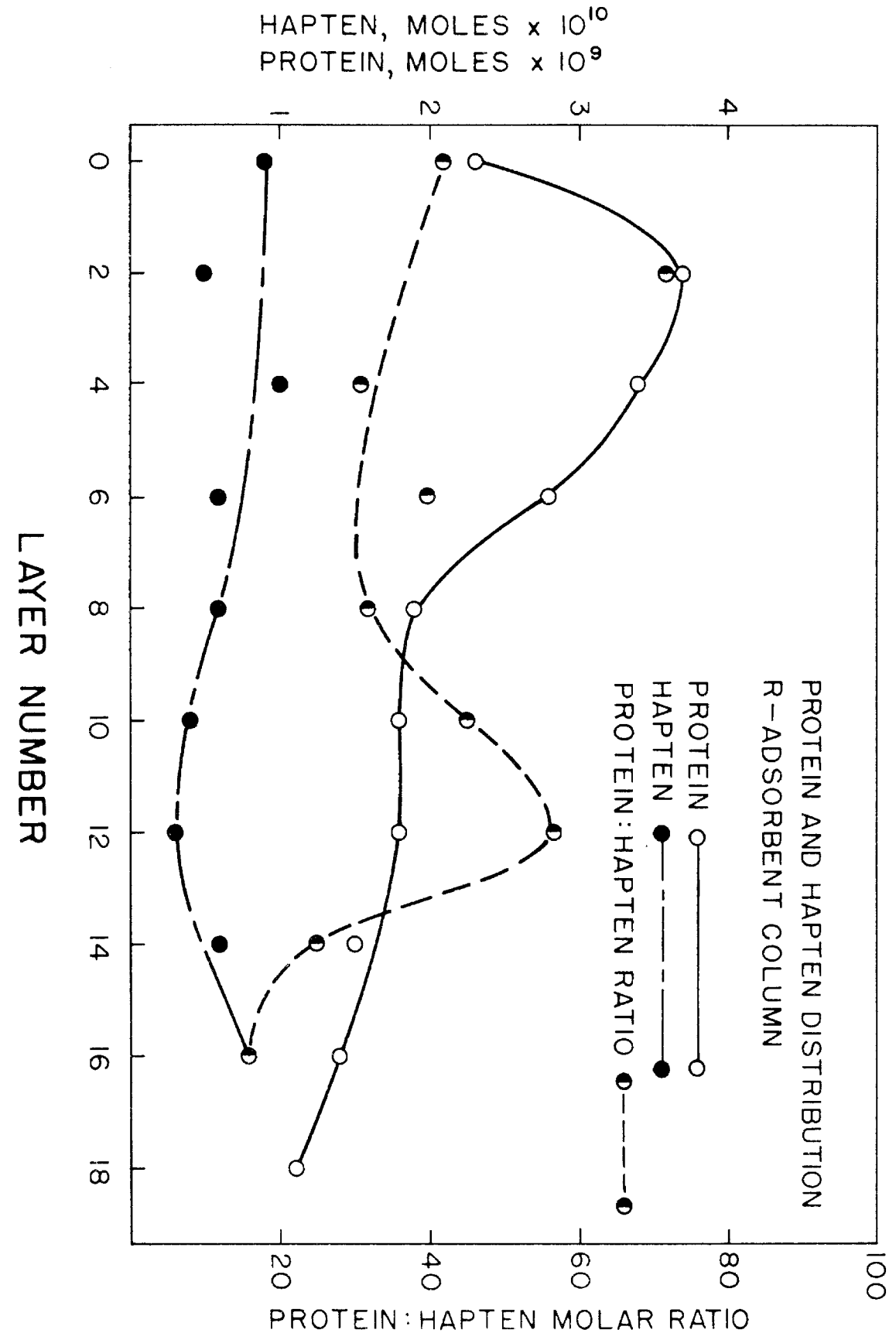
HAPTEN, MOLES x 10^10
PROTEIN, MOLES x 10^9
1
2
3
4
LAYER NUMBER
0
2
4
6
8
10
12
14
16
18
PROTEIN AND HAPTEN DISTRIBUTION
R-ADSORBENT COLUMN
PROTEIN
HAPTEN
PROTEIN:HAPTEN RATIO
20
40
60
80
100
PROTEIN:HAPTEN MOLAR RATIO

FIGURE NO. 27

Distribution of antibody and hapten on cellulose adsorbent columns, Experiment III: A third column was prepared in the same manner as the second, but the γ globulin fraction from low-titer anti-RBG serum (15 ml. globulin solution, 88 μg. antibody N/ml.) diluted with an equal volume of buffer was used instead of whole serum. A higher concentration of hapten was tried in this experiment (30 ml. M/2000 R-iodophenol) and a final wash of 50 ml. buffer.

The analytical data are tabulated in Table No. 18 and protein N, hapten, and protein:hapten ratios are plotted against column position in Figure No. 28.

Batch equilibration of adsorbent with serum and with hapten, Experiment I: One gram of R-adsorbent (Lot II) was washed twice in a 50 ml. serum-bottle type centrifuge tube with 40 ml. portions of buffer. Forty milliliters of a 1:1 dilution of anti-RBG serum in buffer (serum titer 0.24 mg. antibody N/ml.) was added to the moist adsorbent and the tube, which was filled to within a ml. or two of the top, was stoppered and placed in a tumbling machine* and very gently tumbled for two hours at room temperature and 90 hours in the cold-room at 4° C. The adsorbent was centrifuged down in a refrigerated centrifuge and washed twice with 40 ml. portions of ice-cold saline.

* The tumbling machine consists of a turntable, rotating on an axis inclined at about 45° and driven at a speed of about 25 RPM by an electric motor. The specimen

Table No. 18

Analytical Data, Column Experiment III

Column Layer	Total N µg.	Adsorbent N µg.	Protein N µg.	Moles Protein × 10^9	Iodine Trace Angle	Cotangent of Angle	Cotangent − Blank	Hapten Iodine µg.	Moles Hapten × 10^9	Protein:Hapten Molar Ratio
Solka-floc	50	5	45	1.8						
1	91	51	40	1.6						
2			40*	1.6	70.5	.354	.192	.033	.26	6.1
3	82	51	31	1.2						
4			39*	1.5	70.9	.346	.184	.032	.25	6.0
5	91	51	40	1.6						
6			37*	1.4	71.7	.331	.169	.029	.23	6.1
7	96	51	45	1.8						
8			35*	1.4	73.1	.304	.142	.025	.20	7.0
9	88	51	37	1.4						
10			32*	1.2	72.2	.321	.159	.028	.22	5.5
11	76	51	25	1.0						
12			29*	1.1	73.0	.306	.144	.026	.20	5.5
13	74	51	23	0.9						
14			24*	0.9	74.8	.272	.110	.019	.15	6.0
15	78	51	27	1.1						
16			17*	0.7	74.3	.281	.119	.021	.17	4.2
17	68	51	17	0.7						
18			9*	0.4	75.6	.257	.095	.017	.13	3.0
19	55	51	4	0.2						
20	53	51	2	0.1						
Iodine blank					80.6	.162				
0.15 µg. Iodine standard					44.2	1.028	.866			

* Value interpolated from smoothed curve of nitrogen data for odd-numbered layers.

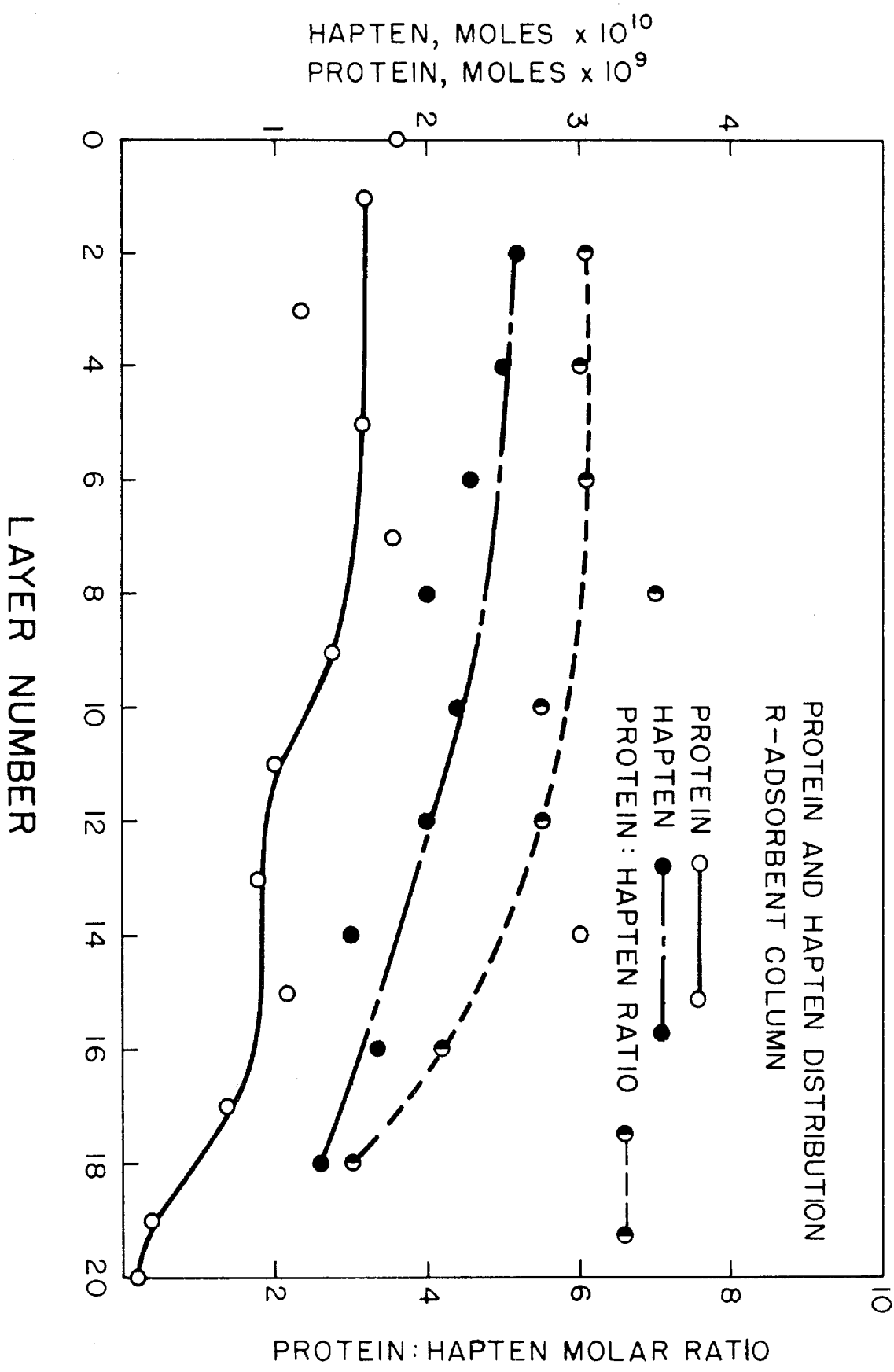
HAPTEN, MOLES x 10^10
PROTEIN, MOLES x 10^9
1
2
3
4
LAYER NUMBER
0
2
4
6
8
10
12
14
16
18
20
PROTEIN AND HAPTEN DISTRIBUTION
R-ADSORBENT COLUMN
PROTEIN
HAPTEN
PROTEIN: HAPTEN RATIO
2
4
6
8
10
PROTEIN: HAPTEN MOLAR RATIO

FIGURE NO. 28

The washed aggregate was suspended in 35 ml. of saline and divided into aliquots as follows: (1) Ten milliliters was collected in a type "A" column, washed with 10 ml. of borate buffer, pH 8.0, and analyzed for nitrogen by Kjeldahl digestion and distillation. (2) Ten milliliters was collected as above, but treated successively with 5 ml. of M/20,000 R-iodophenol dye and 5 ml. of buffer, and run for nitrogen. (3) Ten milliliters was run as a duplicate of aliquot No. 2, but was analyzed for iodine. (4) Five milliliters was diluted to 10 ml. with buffer, treated with 0.25 ml. of M/1000 R-iodophenol hapten, mixed gently for a period of an hour, centrifuged, washed with saline and analyzed for iodine. The supernatant and washings (4a) from aliquot No. 4 were combined and analyzed for eluted protein.

The analytical data are tabulated in Table No. 19.

Batch equilibration of adsorbent with serum and with hapten, Experiment II: Two grams of R-adsorbent (Lot IV) was washed with alkali and buffer until free of hapten bleed. The adsorbent was placed in a 250 ml. centrifuge bottle with 25 ml. of γ globulin from low titer anti-RBG serum diluted with 25 ml. of borate buffer, pH 8.0, and equilibrated by agitation on rollers for 24 hours in the cold-room.

---

tubes are mounted on this turntable by clipping to the walls of a 6"×6"×6" basket that is fastened to the turntable concentrically with its axis. By varying the angle at which the tube is mounted, i. e., the skew angle between the tube and the axis of rotation of the turntable, any degree of agitation may be obtained between simple rotation of the tube and end-over-end tumbling.

Table No. 19

	Aliquot No.1	Aliquot No. 2	Aliquot No.3	Aliquot No. 4	Aliquot No. 4a
Total N, μg.	469	444	444*	128**	107
Adsorbent N, μg.	100	100	100	50	
Protein N, μg.	369	344	344*	78	107
Antibody, moles x 10^9	14.4	13.4	13.4*	3.0	4.2
Iodine Analysis***					
Iodine, μg.			.237	.273	
Hapten, moles x 10^9			1.86	2.15	
Antibody:hapten ratio			7.1	1.3	

* From analysis of duplicate Aliquot No. 2.

** By subtracting supernatant nitrogen (Aliquot No. 4a) from one-half of total nitrogen of Aliquot No. 1. This assumption is open to considerable uncertainty, since settling out of adsorbent from the suspension during division into aliquots may well have left the last 5 ml. of suspension more dense than the calculated value, and the indicated nitrogen content for this aliquot may be too low.

***The trace angles for these analyses were 29.0 and 25.4° respectively. The blank trace angle was 81.4° and the .15 μg. standard, 40.2°.

The bottle was filled with cold saline, the adsorbent centrifuged down and washed twice with 200 ml. portions of cold saline. One milliliter of M/1000 R-iodophenol and 50 ml. of buffer were added to the washed adsorbent and equilibrated on the rollers for four days. The adsorbent was then centrifuged down and washed three times with 250 ml. portions of cold saline and transferred to a Büchner funnel with an additional 100 ml. of saline. The adsorbent was dried and divided into aliquots for nitrogen and iodine analysis.

Table No. 20

	Results per gram of adsorbent
Total nitrogen	234 µg.
	220 µg.
Average	227 µg.
Adsorbent nitrogen	50 µg.
Protein nitrogen	177 µg.
Moles antibody x 10^9	7.0
Iodine	.443 µg.
	.309 µg.
Average	.376 µg.
Moles hapten x 10^9	3.0
Antibody:hapten ratio	2.3

Hapten binding on antibody-adsorbent aggregates under equilibrium conditions, Experiment I: Five grams of R-adsorbent, Lot IV, was placed in a 250 ml. centrifuge bottle and washed with buffer, centrifuged down, and resuspended in 50 ml. of borate buffer, pH 8.0, and 25 ml. of γ globulin from low-titer anti-RBG serum. The bottle was placed on rollers in the cold-room for three days, centrifuged, and the supernatant decanted. The antibody added to the system was 2200 μg. (25 ml. × 88 μg. N/ml.) of antibody nitrogen. The supernatant was tested against R'_3-resorcinol and found to contain 800 μg. precipitable N (160 ml. × 5 μg./ml.). The antibody nitrogen bound to the adsorbent is thus estimated to be not more than 1400 μg.

The aggregate was washed 3 times with 200 ml. portions of cold saline and then distributed into eight 15 ml. centrifuge tubes in approximately equal aliquots. These were treated as tabulated below:

1 and 2: Wash four times with 12 ml. portions of cold saline.

3 and 4: Agitate with 5 ml. M/1000 R-iodoresorcinol plus 7 ml. borate buffer for 15 minutes, centrifuge, decant, and wash 5 times with cold saline.

5 and 6: Agitate with 1 ml. M/1000 R-iodoresorcinol plus 11 ml. borate buffer and wash four times with cold saline.

7 and 8: Agitate with 0.1 ml. M/1000 R-iodoresorcinol plus 12 ml. borate buffer, centrifuge and wash four times with saline.

The aggregates were transferred to sintered glass filter crucibles with distilled water washing, pulled dry as possible, and then dried in a vacuum oven at 60° C. Each lot was divided by weight into aliquots for nitrogen and iodine analysis. The analytical data are recorded in Table No. 21.

Hapten binding on antibody-adsorbent aggregates under equilibrium conditions, Experiment II: Five grams R-adsorbent (Lot V) was treated with 50 ml. buffer-antibody mixture for four days in the cold-room. The adsorbent was then washed on a Büchner funnel with approximately 2 l. cold saline and distributed into sintered-glass filter crucibles. The aliquots were treated with the indicated volumes and concentrations of hapten for 20-25 minutes, filtered and pulled dry as possible but avoiding prolonged suction with drying of the aggregate.

Lots A, B, and C: 15 ml. 10^{-5} molar R-iodoresorcinol.

Lots D and E: 20 ml. 10^{-6} molar R-iodoresorcinol.

Lots F and G: 20 ml. 10^{-7} molar R-iodoresorcinol.

Lot H (control adsorbent): 20 ml. 10^{-5} molar iodoresorcinol.

Lot J (control adsorbent): 20 ml. 10^{-7} molar iodoresorcinol.

The moist aggregates were transferred to tared aluminum-foil weighing cups, dried in a vacuum oven at 60° C., and divided into suitable aliquots for nitrogen and iodine analysis.

Aliquot weights and moisture contents are given in Table No. 22.

The nitrogen data are given in Table No. 23.

The iodine data are given in Table No. 24.

Table No. 22

Aliquot Weights* and Moisture Contents.

Lot No.	Tare Weight	Gross Wet Weight	Gross Dry Weight	Net Dry Weight	Water Loss
A	1.208	3.832	2.127	.919	1.705
B	1.218	4.036	2.203	.985	1.833
C	1.219	3.437	1.999	.780	1.438
D	1.217	2.274	1.646	.429	.628
E	1.224	2.369	1.643	.419	.726
F	1.219	2.065	1.539	.320	.526
G	1.210	2.865	1.780	.570	1.085
H	1.227	2.585	1.820	.593	.765
J	1.212	2.557	1.768	.556	.789

* All weights are given in grams.

Table No. 23

Nitrogen Analyses

Sample No.	Dry Weight Grams	Free Liquid Grams	Hapten Concentration Moles/Liter	Moles Hapten $\times 10^9$	Total Nitrogen µg.	Adsorbent Nitrogen µg.	Hapten Nitrogen µg.	protein Nitrogen µg.	Protein Nitrogen per g. Adsorbent µg.	Protein moles per g. Adsorbent $\times 10^9$
A-1	.254	.47	10^{-5}	4.7	310	78	1.3	231	.91	35.5
A-2	.305	.57	10^{-5}	5.7	368	94	1.6	272	.89	34.7
B-1	.260	.38	10^{-5}	3.8	313	80	1.1	232	.89	34.7
B-2	.253	.37	10^{-5}	3.7	303	77	1.0	225	.89	34.7
C-1	.372	.69	10^{-5}	6.9	430	114	1.9	314	.84	32.8
D-1	.126	.18	10^{-6}	.18	148	39		109	.87	34.0
E-1	.150	.26	10^{-6}	.26	178	55		123	.82	32.1
G	.570	1.09	10^{-7}	.11	605	175		430	.76	29.7
H-1	.233	30	10^{-5}	3.0	91	72	0.8	18		
Average										34

Table No. 24

Sample Number	Iodine Trace Angle	Cotangent of Angle	Cotangent minus Blank	Total Iodine in Sample, µg.	Moisture of Sample, g.	Iodine in Moisture µg./g.	Iodine in Moisture of Sample, µg.	Bound Iodine in Sample, µg.	Dry Weight of Sample, g.
A-1	17.6	3.312	3.163	.474	.234	.540	.126	.348	.126
A-2	20.5	2.675	2.526	.387	.226	.540	.122	.216	.122
A-3	19.9	2.763	2.614	.399	.282	.540	.152	.247	.152
B-1	14.7	3.812	3.663	.549	.341	.540	.184	.365	.183
B-2	16.6	3.354	3.205	.480	.349	.540	.188	.292	.187
B-3	25.3	2.116	1.967	.312	.231	.540	.125	.187	.124
C-1	18.3	3.024	2.875	.441	.206	.540	.111	.330	.112
C-2	19.0	2.904	2.753	.423	.297	.540	.160	.263	.161
C-3	18.9	2.921	2.772	.423	.273	.540	.147	.276	.148
H-1	26.7	1.988	1.839	.294	.159	.540	.085	.209	.123
H-2	27.4	1.929	1.780	.282	.129	.540	.070	.153	.100
H-3	20.7	2.646	2.497	.381	.199	.540	.108	.273	.154
D-1	27.0	1.963	1.814	.288	.450	.062	.028	.260	.307
E-1	31.0	1.664	1.515	.240	.468	.062	.029	.211	.270
F	36.2	1.366	1.217	.198	.526	.004	.002	.196	.320
J	28.9	1.812	1.663	.264	.789	.004	.003	.261	.556
Hapten 10^{-5} M .25 ml.	45.8	.973	.824	.135					
Hapten 10^{-6} M 3 ml.	35.7	1.392	1.243	.201					
"	39.3	1.222	1.073	.174					
Hapten 10^{-7} M 10 ml.	51.5	.795	.646	.105					

Table No. 24

(cont.)

Sample	Iodine Trace Angle	Cot. of Angle	Cot. minus Blank	Total Iodine in Sample, µg.
Iodine Standard 0.15 µg.	45.4	.986	.837	.150
Iodine Standard 0.45 µg.	16.8	3.133	2.984	.450
Reagent Blank	81.5	.149		

	Iodine per gram Adsorbent, µg.					
Sample Number	Total Bound Iodine	Cellulose-Bound Iodine	Protein-Bound Iodine	Hapten* Moles $\times 10^9$	Protein** per g. Adsorbent M. $\times 10^9$	Protein: Hapten Molar Ratio
A-1	2.76	1.67	1.09			
A-2	2.14	1.67	0.47			
A-3	1.63	1.67	-0.04			
B-1	1.99	1.67	0.22			
B-2	1.56	1.67	-0.11	2.66	34	13
B-3	1.51	1.67	-0.16			
C-1	2.94	1.67	1.27			
C-2	1.64	1.67	-0.03			
C-3	1.87	1.67	0.20			
H-1	1.70					
H-2	1.53	1.67				
H-3	1.77					
D-1	0.847	0.59***	0.25	1.73	34	20
E-1	0.782	0.59***	0.19			
F	0.613	0.47	0.143	1.13	34	30
J	0.470	0.47				

* Average value for replicate specimens.

** From data in Table No. 16.

*** Interpolated value.

Hapten Binding in Immune Precipitates

Hapten binding in immune precipitates formed in the presence of inhibiting hapten, Experiment I: Gamma globulin prepared from anti-RBG serum was used as antibody solution in three sets of precipitin tubes, with RBG, the immunizing antigen, used as the test antigen. The composition of precipitates formed between anti-RBG γ globulin and the immunizing antigen, in the presence of, and in the absence of, inhibiting hapten was investigated, with particular reference to hapten binding.

Series A: One-half ml. of antibody solution was set up against 1:2 serial dilutions of RBG antigen, with 1 ml. of 0.5% antigen in the first tube of the series. No inhibiting hapten was used.

Series B: Precipitin tests were set up in duplicate using the same concentrations of antibody and antigen as in series A, but the serial dilutions of the antigen were made in M/1000 R-iodoresorcinol hapten in borate buffer.

The precipitin tubes were allowed to stand three hours at room temperature and four days in the cold-room. They were then filled with cold saline, centrifuged at 2200 RPM for 30 minutes, decanted, drained, and washed twice with cold saline. The set of series B tubes for iodine analysis was triple washed. Nitrogens were determined on the A series and one set of the B

series in the usual manner, reading the final color on the Klett-Summerson colorimeter with a No. 42 filter. Iodine analyses were carried out on the duplicate set of series B.

Analytical data are summarized in Table No. 25 and presented in graphical form in Figures Nos. 29 and 30.

Hapten binding in immune precipitates formed in the presence of inhibiting hapten, Experiment II: Gamma globulin fractions of anti-RBG and anti-OA sera were mixed with solutions of their homologous antigens in the presence of R-iodophenol. The amounts employed were chosen to obtain quantities of precipitate adequate for nitrogen and iodine analysis. The anti-RBG preparation contained 400 μg. of precipitable nitrogen per ml. and the antigen concentration at equivalence was 1:16,000. The anti-OA preparation contained 725 μg. antibody nitrogen, and at the equivalence point combined with 310 μg. of crystalline ovalbumin per ml. of solution.

The samples designed for iodine analysis are designated by number and those for nitrogen analysis by letter. The protocol for the experiment is given in Table No. 26.

The precipitin tubes were allowed to stand at room temperature for 3 hours and then transferred to the cold-room for 10 days. The tubes were filled with cold 10^{-6} molar R-iodophenol in borate buffer, centrifuged one-

Table No. 25

Series A

Tube No.	Nitrogen, µg. Total	Antigen	Antibody
1	371	800	
2	860	400	460
3	772	200	572
4	680	100	580
5	520	50	470
6	366	25	341
7	207	13	194
8	122	6	116
9	74	3	71
10	39	1	38

Series B

Tube No.	Nitrogen, µg. Total	Antigen	Antibody	Antibody Moles $\times 10^9$
1	162	800		
2	594	400	194	7.6
3	554	200	354	13.8
4	446	100	346	13.5
5	317	50	267	10.4
6	227	25	202	7.9
7	153	13	140	5.5
8	96	6	90	3.5
9	50	3	47	1.8
10	22	1	21	0.8

Table No. 25
(cont.)

Iodine Analyses Series B

Tube No.	Trace Angle	Cot.	Cot.-Bl.	µg. I	Hapten Moles $\times 10^9$	Protein:Hapten Molar Ratio
1	43.3	1.061	.984	.138	1.09	
2	58.3	.618	.541	.076	.60	12.7
3	53.6	.737	.660	.093	.73	18.9
4	62.1	.529	.452	.064	.50	27
5	59.5	.589	.512	.072	.57	18.3
6	62.6	.518	.441	.062	.49	16.1
7	64.7	.473	.396	.056	.44	12.5
8	68.4	.396	.319	.045	.35	10.0
9	72.1	.323	.246	.035	.28	6.4
10	71.1	.342	.265	.037	.29	3.8
Blank	85.6	.077				
.15 µg. Std.	41.2	1.142	1.065			
.45 µg. Std.	17.8	3.115	3.038			

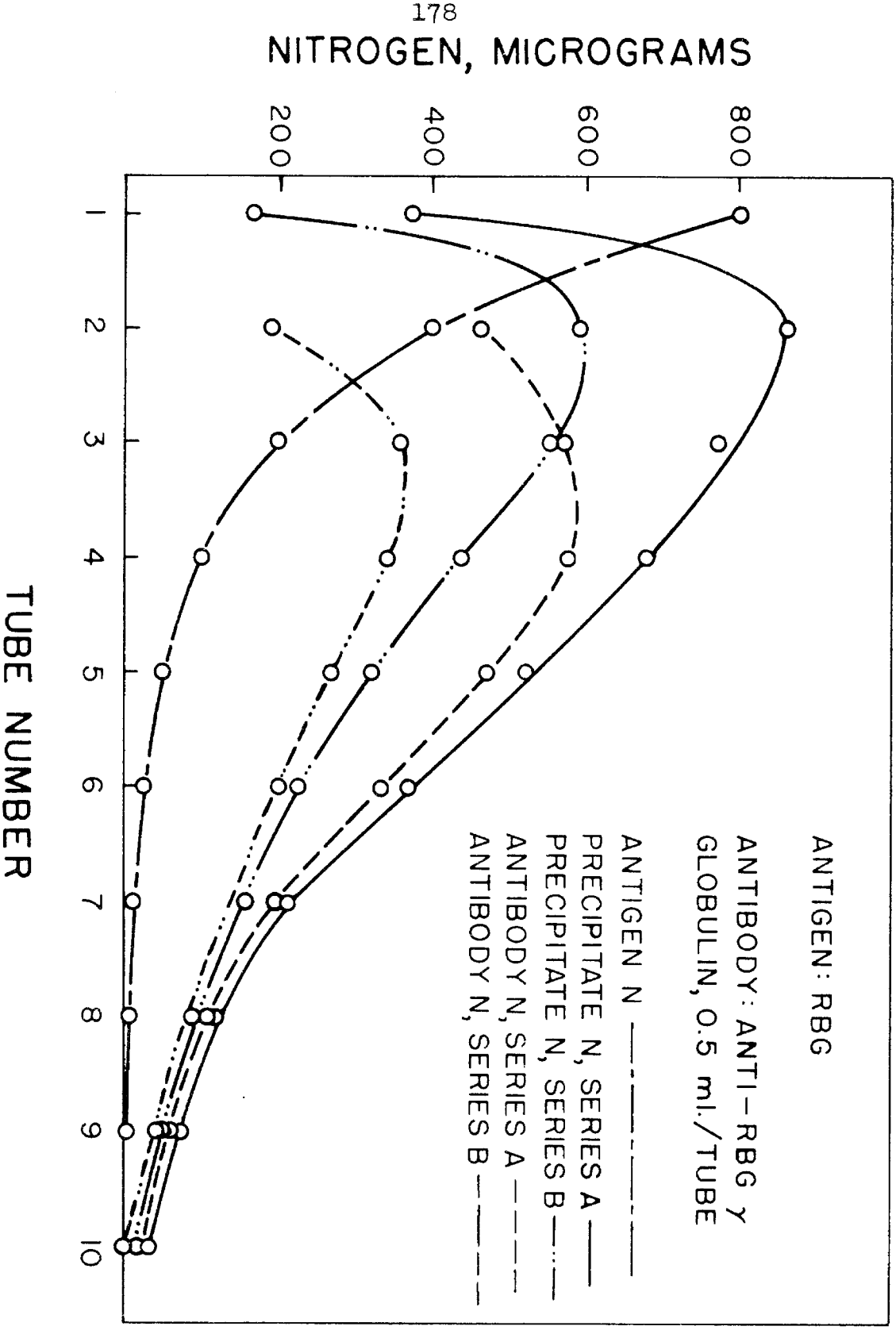
NITROGEN, MICROGRAMS
200
400
600
800
1
2
3
4
5
6
7
8
9
10
TUBE NUMBER
ANTIGEN: RBG
ANTIBODY: ANTI-RBG γ GLOBULIN, 0.5 ml./TUBE
ANTIGEN N
PRECIPITATE N, SERIES A
PRECIPITATE N, SERIES B
ANTIBODY N, SERIES A
ANTIBODY N, SERIES B

FIGURE NO. 29

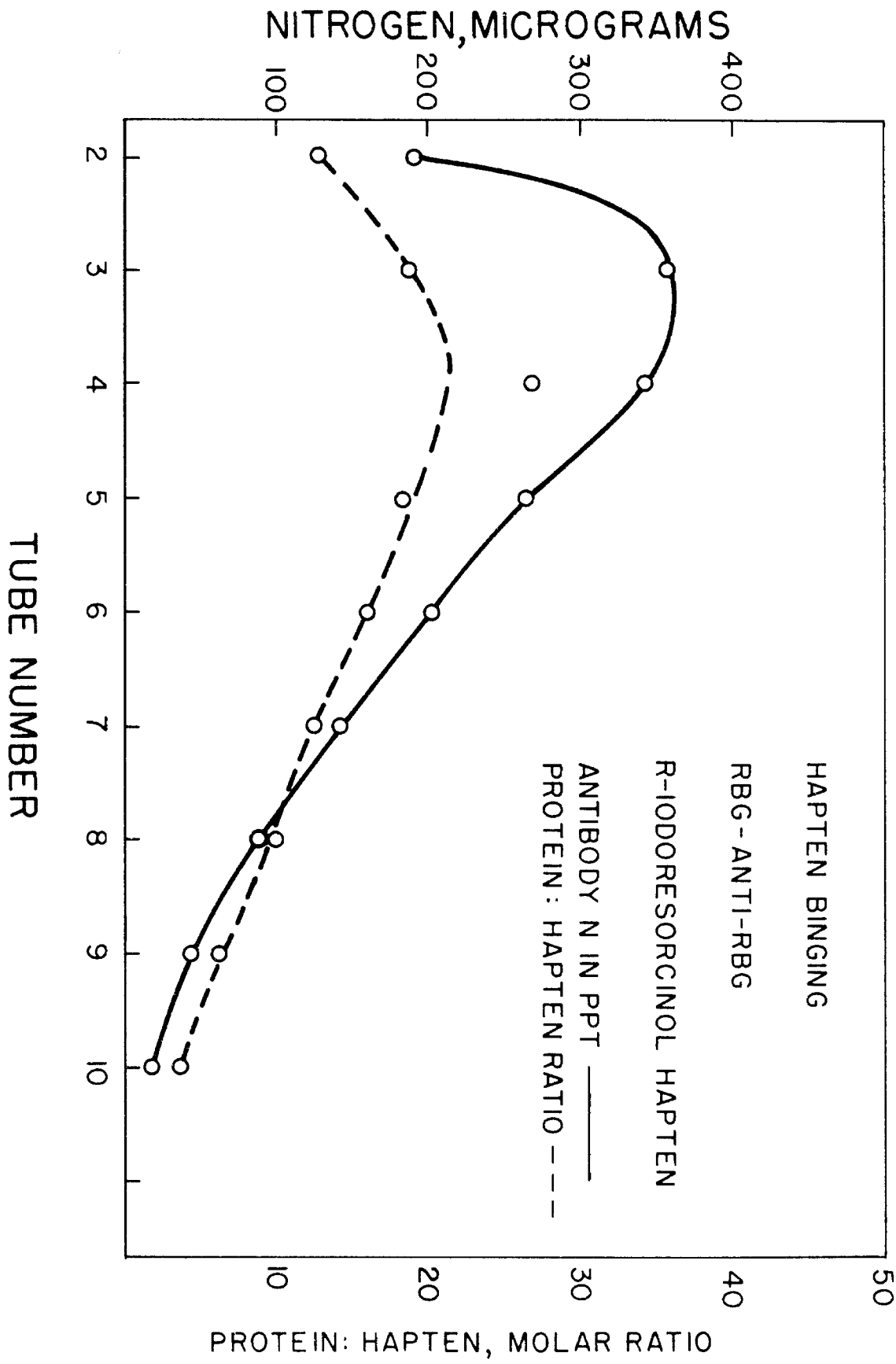
NITROGEN, MICROGRAMS
100
200
300
400
TUBE NUMBER
2
3
4
5
6
7
8
9
10
HAPTEN BINGING
RBG-ANTI-RBG
R-IODORESORCINOL HAPTEN
ANTIBODY N IN PPT ——
PROTEIN: HAPTEN RATIO - - -
10
20
30
40
50
PROTEIN: HAPTEN, MOLAR RATIO

FIGURE NO. 30

half hour at 2500 RPM in a refrigerated centrifuge, drained, washed twice with 10^{-6} molar hapten, drained, the outside of the tubes carefully wiped, and the tubes and contents weighed. After the contents had been removed for iodine analysis, the tubes were weighed back to determine the total contents, so that the free water present in the precipitates could be calculated.

The lettered samples were similarly washed and drained and then analyzed for nitrogen.

The analytical data for nitrogen are given in Table No. 27 and that for iodine in Table No. 28.

Table No. 26

Tube No.	Antibody	Antibody Volume	Antigen	Antigen Volume	Hapten* Volume	Buffer Volume
1 2 3	Anti-OA	3.0 ml.	1:1000 OA	1.0 ml.	1.0 ml.	5.0 ml.
4 5 6	Anti-RBG	3.0 ml.	1:10,000 R'$_3$-resorcinol	1.0 ml.	1.0 ml.	5.0 ml.
A B	Anti-OA	0.3 ml.	1:1000 OA	0.1 ml	0.1 ml.	0.5 ml.
C D	Anti-RBG	0.3 ml.	1:10,000 R'$_3$-resorcinol	0.1 ml.	0.1 ml.	0.5 ml.

* Hapten = 10^{-5} M R-iodophenol

Table No. 27

Sample No.	Klett Rdg.*	Total Nitrogen, µg.	Precipitate Nitrogen, µg.	Protein mg.
Blank	7	3		
Blank	9	5		
A	208	238	234	1.49
B	215	248	244	
C	119	128	124	0.81
D	129	138	134	

* The nitrogens were read on a Klett-Summerson colorimeter with a No. 47 filter, at a final volume of 50 ml.

Table No. 28

Sample No.	Trace Angle	Cotangent	Cotangent - Blank	Iodine μg.	Free Water mg.	Iodine in Water, μg.	Protein-bound Iodine, μg.	Protein-bound Hapten Moles x 10^9	Protein Moles x 10^9	Protein: Hapten Ratio
Blank	77.9	.214								
Iodine Standard	39.5	1.213	.999	.150						
Iodine Standard	15.8	3.534	3.320	.450						
3 ml. Hapten	32.8	1.552	1.338	.201	0.065 g. iodine per ml. hapten solution					
3 ml. Hapten	34.5	1.455	1.241	.186						
1	66.9	.427	.213	.032	.260	.018	.014			
2	67.4	.416	.202	.030	.262	.018	.012	.126	93.2	740
3	64.2	.483	.269	.040	.258	.018	.022			
4	54.7	.708	.494	.074	.083	.005	.069			
5	53.7	.735	.521	.078	.082	.005	.073	.560	49.3	88.2
6	53.9	.729	.515	.077	.080	.005	.072			

Discussion

The haptenic dyes employed in this research proved to be adequate for the problem at hand. In particular, the arsanilic acid dyes were obtained in yields and purity that left little to be desired. There was some tendency of the dyes, when in solution, to deteriorate with the formation of products that had a greater tendency to adsorb onto protein and cellulose bodies in a nonspecific manner. This was particularly evident in the increase in nonspecific cellulose adsorption of the dye in some of the later experiments with the immune adsorbents as compared with the low blanks obtained in the first series of tests, at which time the hapten solutions were all freshly prepared.

The chemical analyses of the dyes for arsenic and iodine were not of adequate precision to constitute a true measure of the purity of the haptens. The percent of arsenic or iodine in related dyes or isomers that might have been present as an impurity would be too near that of the desired molecular species to make such analysis a sensitive method of detection of impurities of this type. This is particularly true of the colorimetric methods employed, which cannot be considered as adequate assay methods. The partition methods were of much greater utility, as well as being much less time consuming. One disadvantage of the developing

solvent used in the paper chromatography is that the pyridine in the solvent is difficult to remove completely from the dye, and spectrophotometric curves of the dye eluted from the chromatogram show the presence of the pyridine bands in the ultraviolet.

The measurement of the absorbancy ratios at the maxima and minima of the absorption spectrum furnished a sensitive technique for the detection of dye impurities, particularly for those dyes having high values of such ratios and sharply peaked maxima and minima. The separation of monohaptenic dyes from the di and tri azo analogues is very sharp on the paper chromatogram or in column chromatography, so that the use of a chromatographically purified reference sample assures a basis of comparison that is free from these materials. It is these polyazo compounds that have the greatest tendency to associate and to adsorb nonspecifically upon proteins and surfaces. Their presence in the dye is readily detected by chromatographic methods, and samples showing traces of such material in the initial preparation invariably showed marked decreases in the absorbancy ratios. Preparations that were satisfactory from the standpoint of both the chromatographic tests and the spectrophotometric measurements are believed to be adequately free from polyhaptenic impurities.

After experiments described in this report had been completed, a synthetic magnesium silicate preparation, "Florisil"* came to the attention of the author. This is available in a fine granular form that handles well in columns. A few preliminary tests indicated that this adsorbent is at least as satisfactory, from the standpoint of adsorption characteristics, as the neutrol filtrol previously used, and much superior from the standpoint of physical structure. Its columns are of open structure and uniform porosity. Its capacity is adequate, and purification of small lots of dyes by column fractionation on this material may well be superior to recrystallization techniques.

The nature of the impurities developing in the dyes during storage has not been investigated, but these impurities are resolvable on chromatographic columns. The passage of the hapten solutions through "Florisil" columns immediately before their employment in immune systems may well be a protection against interference by such impurities.

The succinanilic acid haptens proved to be of especially poor chemical stability. Even in the dry form, the development of inhomogeneities proceeded rather rapidly. The failure of the supply of antisera

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* Manufactured by the Floridin Co., Warren, Pa.

for this hapten led to abandonment of the work on these dyes before the problems in the purification and use of these dyes had been solved, but chromatographic purification of the solutions immediately before use would seem to be imperative.

The analytical methods were generally satisfactory. The analysis of the cellulose-based adsorbents for adsorbed proteins proved to be somewhat troublesome. In those cases in which the amount of protein nitrogen was of the same order of magnitude as the hapten nitrogen in the adsorbent, variation between duplicates was undesirably large. This is in large part due to the inconsistency with which the azo nitrogen in the adsorbent was recovered. Azo nitrogen is not ordinarily measured by the usual Kjeldahl technique (90), but the large quantities of carbohydrate in these adsorbents apparently act as a reasonably efficient reducing agent for the azo nitrogen. The analysis of the pure adsorbents for nitrogen and arsenic indicate that about 40% of the azo nitrogen is recoverable as ammonia. The percentage of recovery is quite variable, depending upon such factors as the rate of digestion, and the length of the digestion process before charring commenced. Since the azo nitrogen constituted a blank that had to be sub-

tracted from the total nitrogen found, this uncertainty in the blank correction resulted in a corresponding unreliability in the protein values.

The immune adsorbents are of great potential usefulness in immunochemical investigations. Those used in this research do not represent the ultimate in desirable characteristics. Each lot prepared had some disadvantage not inherent in the adsorbent itself. Lot V, prepared with 1.5 denier, one-quarter millimeter flock, was the most generally useful, but contained somewhat more uncoupled hapten than was anticipated. Lot IV, prepared with the coarse rayon flock, had far too small a ratio of surface to mass. The adsorbents prepared with Solka-floc were too difficult to handle in columns and too hard to wash free from excess reagents.

The powdered cellulose prepared for column chromatography* may be much superior to either of these forms of cellulose as a starting point for the synthesis of immune adsorbents. It would require no preliminary purification, its physical structure is favorable for column work, and its specific surface is greater than that of currently available rayon flock.

The preparation of specifically purified antibody through the use of solid immune adsorbents of the type

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* Made by the makers of Whatman filter papers.

described here was not a direct object of the present research. None of the adsorbents prepared were ideal for such use. They were either too difficult to handle in columns and to wash free from mechanically held protein, or they had too low a surface to mass ratio with resulting loss in capacity. The powdered cellulose adsorbents ought to be more suitable for such purposes.

The characterization of the proteins adsorbed to the immune adsorbents was sufficient to establish that they were a highly purified fraction of antibody directed against the haptenic group coupled to the adsorbent. The percentage of precipitable protein in the eluates from the columns varied considerably from experiment to experiment, to a large degree with the opportunity for denaturation of the protein between elution and the performance of the precipitin test. A suggested modification of the procedure of concentrating the eluate and freeing it from hapten would be ultrafiltration through a semipermeable membrane, which could rather quickly concentrate the antibody to a point at which the solution would have increased resistance to denaturation and much less hapten to be removed by dialysis.

The loss of the anti-succinanilic acid system has deprived many of the experiments reported here of the most valid type of controls. The preliminary experiments run wth the only lot of the antiserum available did not include adequate antibody assay. No precipitate was

obtained with the test antigens available, but the serum did react with the immunizing antigen. Examination of the data summarized in Table No. 7 for the first experiment on hapten binding with adsorbent-antibody aggregates suggests that the serum did not react with the succinanilic acid adsorbent. The S-iodophenol hapten was not bound in excessive quantities to the adsorbents in the absence of a surface layer of antibody, as shown by the values of iodine found in the columns treated with buffer and hapten solutions only. It was, however, bound to protein, as indicated by the high iodine values found in those tubes that contained R-adsorbent coated with anti-RBG antibody (tubes 4 and 7). The failure of the S-iodophenol to be adsorbed on the S-adsorbent that had been treated with anti-SBG serum strongly suggests that the adsorbent had not adsorbed any antibody from the serum. The lack of activity of the serum against S-ovalbumin, S_3-resorcinol, and the S-adsorbent is evidence that the precipitate formed between the serum and the immunizing antigen is the result of antibodies against the native groups of the bovine globulin. If the "lost" antisera were of the same type as the lot used in these tests, it would have been of little utility in these experiments.

Hapten Binding on Aggregates of Antibody and Immune Adsorbent

The early experiments attempting to measure hapten binding on aggregates of adsorbent and antibody were disappointing. The amount of nonspecific retention of the haptens on the adsorbent was large, and attempts to reduce this by exhaustive washing dissociated any hapten from the aggregates. These tests were made with Solka-floc based adsorbents, and it is felt that much of the hapten retention was the result of mechanical trapping of reagent in the voids of the adsorbent fibers. The first experiment (Tables Nos. 7 and 8) demonstrated that the succinanilic acid dyes were unsatisfactory and that the anti-succinanilic serum was of doubtful activity. The amounts of hapten used were undoubtedly far too great. Of the 10^{-5} moles of hapten used in each tube, not more than 2×10^{-8} moles could have been specifically bound to the approximately 4 mg. of antibody that would be required to saturate the 0.5 g. of adsorbent used in each tube. The amounts of iodine found, averaging about 4×10^{-9} moles for all the tubes in which arsanilic acid haptens were used, represent only 0.04% of that put through each column. This illustrates the dangers of trace impurities in the dyes. In the absence of adequate controls, this amount of dye uptake might well have been considered significant.

That this dye retention was not entirely due to mechanical entrapment is indicated by the significantly higher retention of iodine from the R-iodoresorcinol tubes (average iodine found per tube = 0.63 μg.) than from the tubes treated with R-iodophenol (average 0.44 μg. iodine per tube) in spite of the lower assay of the R-iodoresorcinol solution, which had been prepared from a lot of the dye that contained considerable sodium chloride. The cause for this difference is not known. It is believed to be due at least in part to the more polar nature of the resorcinol, but the possibility cannot be ruled out that traces of polyazo dyes were present in the reagent.

The next experiment, in which the dye was preincubated with the serum, also gave negative results. Here the ratio of hapten to antibody was much lower, with 10^{-6} moles of hapten being used with about 5×10^{-8} moles of antibody in the case of the anti-RBG serum. This was sufficient to inhibit partially the adsorption of the antibody on the adsorbent, but nitrogen analysis on a duplicate column showed that adequate amounts of protein had been retained to have bound measurable quantities of iodine.

The unexpected observation that the iodine retention on R-adsorbent that had been treated with anti-RBG serum was less than that by the control adsorbent that had been

buffer washed only, raises the question as to what kind of a mechanism could be responsible. A possible explanation is the coating of the adsorbent fiber with a film of adsorbed antibody protein, lessening the diffusion of the haptenic dye into the voids of the fiber.

The ready elution of the antibody from a column of the immune adsorbent by relatively dilute solutions of the hapten, as shown in the data of Table No. 15, emphasizes the need to avoid large excesses of hapten in these experiments. The reason for the resistance of a portion of the protein to elution has not been determined, but is presumably related to the heterogeneity of the population of antibody molecules.

The experiments on the distribution of antibody and hapten in the layered columns must be regarded as inconclusive. The first of this series was unsatisfactory because of the difficulties experienced in the column manipulations. The nitrogen and iodine values for the successive layers show wide random variations, and the ratios of protein to hapten are still more disordered. In experiment I the iodine bound to the upper layers is significantly greater than that bound to the lower layers to an extent that rules out the possibility that all of the retention of the hapten on the column is due to binding to cellulose, but the data are not suitable for quantitative treatment.

In experiment II the protein values are much better ordered, but the iodine values show very poor correlation with those for the protein. The average bound hapten does not exceed one molecule for every forty molecules of antibody in these two experiments.

Experiment III of this series was carried out with a γ globulin fraction of anti-RBG serum. The higher hapten concentration used undoubtedly eluted some of the antibody from the column and resulted in an increase in mechanically held hapten, but the analytical data for nitrogen and iodine correlate well, and clearly show the amount of hapten found in a layer is related to the protein content of that layer. The increase in the ratio of bound hapten to protein in the lower layers of the column is at least in part due to the greater influence of the cellulose-bound dye upon the ratio in these layers that were low in protein. The assumption of a value of approximately 0.01 μg. of iodine per layer for this correction would lead to a value of about 10 throughout the column for the ratio of protein to bound hapten. That is, approximately 10% of the antibody molecules on the immune adsorbent are bound to their homologous hapten. This value represents a lower limit, for not all of the protein on the column is antibody. The experiments on the properties of the Solka-floc based adsorbents indicated that the physically held proteins retained

by the adsorbents is significant. However, in this experiment, the use of a γ globulin antibody concentrate minimized the amount of non-antibody protein in the column. The very low values for nitrogen in the bottom layers of this column indicate that this correction would not be over 10% of the measured amounts.

The first experiment with batch equilibrium of an antibody-adsorbent aggregate with hapten gave indications of hapten binding of a similar order of magnitude. Particularly for Aliquot No. 3, the amounts of both nitrogen and iodine were adequate for reliable analytical determination. The concentrations of hapten that contacted the adsorbent (M/30,000 or less) were low enough to minimize both antibody elution and nonspecific dye adsorption. The nitrogen data for Aliquot No. 4, being obtained as a difference between numbers of questionable precision, cannot be considered as precise, but the total iodine found for this fraction represents an acceptable demonstration of hapten binding for a much larger quantity of antibody.

Experiment II of this series was carried out with a rayon-based adsorbent (Lot IV) with a low concentration of hapten. The total amount of hapten iodine found was thought to be significant, and the more elaborate experiment, maintaining equilibrium conditions throughout,

was designed to verify these conclusions. This experiment demonstrated that, either as a result of deterioration of the hapten, or because of the greater affinity of the rayon for the dye as compared to the Solka-floc, the possibility of nonspecific binding of the observed amounts of hapten to the adsorbent could not be excluded, and the results of this experiment must be considered as inconclusive.

The experiment on hapten binding under equilibrium conditions, although suffering from the large amount of hapten adsorption on the untreated adsorbent and a somewhat larger than usual scatter to the iodine analyses on replicate specimens, is nevertheless, statistically significant. The nine replicates for iodine analysis in the series using 10^{-5} molar hapten, when taken as a group and compared with the three replicates of the control group, using the Student "t" test of the significance of the difference between means, yields a value of $t = 1.74$, which with the 10 degrees of freedom, gives a probability value of 0.11.

The experiment at the 10^{-6} molar hapten concentration, with $t = 4.26$ and one degree of freedom, gives a probability of 0.17. The experiment with 10^{-7} molar hapten, having no replicates does not lend itself as well to statistical analysis, but making reasonable estimates of the uncertainty of the analytical procedures,

based upon previous experiences, the significance of the results is of the same order of magnitude.

The probability of the positive findings at all three levels being the result of chance is equal to the product of the three individual probabilities, which is less than 0.01, a value which is considered "highly significant". The manner in which the calculated antibody:hapten ratios fall on a smooth curve lends further support to their validity.

Hapten Binding in Immune Precipitates

The precipitin curve determined in the presence of iodinated hapten presents a concordant picture of the hapten-antigen-antibody relationship. The degree of inhibition of precipitation is consistent with that reported by the earlier workers in these laboratories for non-iodinated haptens of similar structure. The amounts of iodine found were less than optimum for analytical purposes, but the ratios of protein to hapten found in the precipitates fall on a smooth curve that is in qualitative agreement with the results that would be predicted by the alternation theory of precipitate formation.

These tests were run at a stage of the research before the appreciation of the ease with which hapten elution occurred had been obtained. The failure of this washing to remove the hapten is attributed to the type of

washing employed, with a minimum of disturbance of the precipitate. This washing should presumably lead to maximum nonspecific retention of the hapten in the bulkiest precipitate, from which the dye would diffuse most slowly. The fact that the lowest hapten to protein ratio was found at the region of equivalence, as would be expected on theoretical grounds, indicates that inadequate washing of the precipitates with mechanical retention of the hapten, cannot be the explanation for the hapten found.

The increased hapten binding in regions of antigen excess is a little more difficult to explain on immunochemical grounds. A possible explanation involves the heterogeneity of the antigen and the antibody, and the concept of heteroligating antibody, which will be discussed in part II of this dissertation.

The measurement of hapten binding under equilibrium conditions, further confirms the ability of immune aggregates to bind hapten. The corrections for physically retained hapten solutions were made with a care and precision that made the uncertainty due to this factor negligibly small. The analytical findings were in satisfactory agreement, and the difference in the bound hapten between the homologous system and the ovalbumin control is sufficient that there can be no question as to its mathematical significance.* The ratio of hapten

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* For this experiment $t = 30$, $f = 4$ and $a < .001$.

to antibody is low, 1:88, but this may be due in part to the failure to secure an optimum antigen-antibody ratio at which to demonstrate hapten binding. The high ratio of antigen to antibody (in terms of haptenic groups of the R'_3-resorcinol) at equivalence in systems of purified antibody means that even in the region of moderate antibody excess where this experiment was performed, the haptenic groups in the precipitating antibody exceeded by a factor of more than ten the inhibiting hapten added to the systems.*

* The 1: 10,000 R'_3-resorcinol is approximately 1.1×10^{-4} molar.

II

HETEROLIGATING ANTIBODY IN ANTI-ARSANILIC ACID SERA

INTRODUCTION

The theory of antibody formation propounded by Pauling (27) predicts a degree of heterogeneity among the globulin molecules modified by synthesis in spatial contact with the antigen. The predicted heterogeneity with respect to specificity (91), strength of antigen-antibody bonding (44, 64) and antibody valence (30) have been experimentally verified. An additional type of heterogeneity, that with respect to the nature of the antigenic groupings against which the two ends of the globulin molecule are directed, has heretofore eluded demonstration.

Considering the importance of the question to the mechanism of antibody synthesis and the structure of immune aggregates, little effort has been directed to the solution of the problem. Many of the data bearing on it have been obtained in the course of researches on other problems. Most of the quantitative data have been lacking adequate sensitivity and precision to detect heteroligating antibody in the systems under study unless such material were present in relatively large amounts.

The previous studies in this field may be divided into two groups, according to whether the dissimilar antigenic groupings being investigated were in separate molecules of antigen or were portions of a single molecular species. Most of them have belonged in the former class, and have resulted in a failure to detect antibody molecules capable of reacting with two or more antigenically distinct substances. In some cases no evidence for their existence was found, and in others the results were inconclusive, but in no case were the data adequate to support an affirmative conclusion. The theory of Pauling would predict much greater likelihood of the formation of heteroligating molecules of the second kind, but the study of Haurowitz and Schwerin (92) is the only significant one on this type of system.

Hektoen and Boor (93) reported a study on the capacity of the rabbit to produce antibodies against a large number of antigens simultaneously administered. From one animal they obtained a serum capable of reacting with 34 of the 35 antigens administered. In general, they found that absorption of the serum with one antigen failed to remove any antibody to unrelated antigens that did not cross react with the absorbing antigen. They estimated antibody concentrations by determining the highest dilution of antigen capable of precipitating with the serum, a technique without theoretical justification and of

questionable experimental validity (16). They concluded from their results that the polyvalent serum could be described as a mixture of separate antibody entities. If it be assumed that the distribution of antibody binding sites against the various antigens be distributed among the antibody molecules on a statistical basis, a serum as complex as this one could hardly be anticipated to have enough heteroligating antibody of any single kind to be detectable by much more refined techniques than used by these authors.

Heidelberger and Kendall (94) and Heidelberger and Kabat (95) examined polyvalent antipneumococcus sera for the presence of heteroligating antibody in the course of studies on the specific purification of antibody. Although they found that anti-SI antibody was carried down in a precipitate with SII polysaccharide, and that anti-SII antibody was carried down with the precipitate formed by the SI polysaccharide, they attributed this to a weak cross reaction. They believed that the experiment in which the antibody eluted from such a precipitate was found to be precipitable by the homologous polysaccharide without cross reaction, as shown by the independence of the results upon the order in which the reprecipitations were made, ruled out heteroligating antibody as an explanation for the observed phenomenon. Heidelberger and Kabat found slight differences in the amounts of precipitates obtained with antibody purified from a polyvalent

bovine serum active against types I, II and III pneumococci, depending upon the order in which the antigens were taken. These differences were small and of questionable significance, but the authors did not exclude the possibility of small amounts of heteroligating antibody being present, and concluded that "in a polyvalent serum most, if not all, of the antibodies to each pneumococcus type occur as molecules distinct from those of other types".

Goodner and Horsfall (96) found a similar degree of nonspecific inclusion of heterologous antibody in precipitates formed with mixtures of monovalent sera, in which the possibility of heteroligating antibody did not exist.

Dean, Taylor and Adair (97) made an investigation designed to determine whether the globulin in an immune precipitate was in fact the antibody, or merely a nonspecific globulin indicator. The serum from rabbits immunized simultaneously with crystalline ovalbumin and crystalline horse serum albumin was subjected to a number of quantitative adsorption experiments planned to detect the removal of any protein by one antigen that could be precipitated by the second. They too failed to demonstrate the presence of any such cross reacting protein in their system. Their data with one serum would admit the possibility of a small amount of such material, but were inadequate to justify a positive conclusion in favor of such an existence, and the authors themselves attached no significance to them.

Lanni and Campbell (42) have reported the most extensive studies in this field, and have presented the most lucid discussion of the practical and theoretical implications that the demonstration of such heteroligating antibody would carry. The term itself was introduced by these authors. Their experiments were designed along two different lines. One series was what they described as "a quantitative mixing experiment (which) involves

> the determination of the amount of precipitate which forms when identical aliquots of polyvalent serum are allowed to react in constant volume with (a) one antigen, (b) a second, serologically dissimilar antigen, and (c) a mixture containing each antigen in the quantities present in (a) and (b). If a comparison of the amount of the mixed antigen precipitate and the sum of the amounts of the two single-antigen precipitates reveals an analytically significant deviation from identity, one may tentatively suppose that heteroligating antibody is present. If, on the other hand, one finds no significant deviation from identity under a variety of experimental conditions, one is justified in describing the polyvalent serum as a mixture of two unlinked groups of homoligating antibodies, each group having a capacity to react with only one of the two antigens."

Their second series of experiments involved absorption of the polyvalent serum with one of the antigens and a comparison of the absorbed and unabsorbed sera with respect to their activities against the second antigen.

Their results agree closely with those of Heidelberger et al. They observed the same type of cross-reaction between the SI and SII antibodies. They ascribed this phenomenon to a nonspecific physical adsorption which could be treated with the Langmuir adsorption equation, rather than as a true case of serological related-antigen type of cross reaction. They concluded from their data that the antibody molecules in the polyvalent serum were synthesized by a single-antigen mechanism. Their conclusions were restricted to this extent, and were not extrapolated to systems in which a serological heterogeneity might exist on a single antigen molecule.

Haurowitz and Schwerin examined the serum prepared in rabbits against an antigen of arsanil azo sheep serum globulin for the presence of antibody capable of reacting with either the arsanil grouping or the native globulin. Their method was successively to "exhaust" the serum with the three test antigens, arsanil azo ovalbumin, sheep serum globulin, and the immunizing antigen, and to compare the amounts of precipitates (determined gravimetrically) obtained with each antigen as a function of the previous treatment of the serum. The method of "exhausting" the serum of antibody against a given antigen lacked somewhat of theoretical justification. It consisted in the treatment of a given volume of the serum with a portion of antigen in a small volume of saline,

freezing the mixture for 24 hours, thawing, and centrifuging out the precipitate. The supernatant was again treated with the same amount of antigen, and the process repeated until only a small but quite variable amount of precipitate was obtained. The process was then continued with the second antigen.

They found that about a third of the total antibody in the serum was precipitable with neither the arsanil azo ovalbumin nor the native globulin, but was precipitable with the immunizing antigen. They ascribe this fraction to antibody molecules directed against a portion of the antigenic surface containing both the arsonic acid and native globulin groupings.

As none of their experiments were performed in replicate, it is difficult to know how much significance can be ascribed to the relatively small differences shown in their tables, and as there is no assurance that antigen-antibody equivalence was ever attained at any stage of the fractional precipitations, it is questionable whether the sums of the weights of the precipitates obtained are a valid measure of the amount of antibody against that antigen in the system. A further criticism is that the contribution of the antigen protein to the total weight of the precipitate is not allowed for, and with the widely variable antigen-antibody ratios in the systems at the time of precipitation, this correction may be quite

variable from one experiment to another.

These authors conclude that:

> "These experiments indicate that bivalent as-antibodies* containing two different bonding groups are absent from immune sera to arsanil-sheep serum globulin."

They further interpret their results as support of the theory of the univalence of precipitating antibody, a position which they argue is the only one that adequately explains the absence of precipitation inhibition by excess antibody.

The researches reported in Part I of this dissertation were primarily designed to provide tools for the investigation of the problem of heteroligating antibody in anti-arsanilic acid sera. If it could be shown that hapten was bound to homologous antibody binding sites in immune aggregates, then the presence of heteroligating antibody in a precipitate formed in an anti-RBG serum by the native bovine globulin antigen should be detectable by the ability of that antibody to carry with it, into the precipitate, arsanilic acid haptens. If these haptens were labeled with an atom that could be detected with great specificity and sensitivity, then the presence of very small proportions of heteroligating antibody could be demonstrated, a demonstration not

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* In this paper "a" is used to designate the haptenic grouping, and "s" the native protein.

dependent upon the significance of small differences in the total amounts of precipitable antibody in absorbed and unabsorbed sera. A further advantage of the hapten-binding technique in this case, as compared with the demonstration of hapten-binding in homologous systems, is that the concentration of hapten in the system can be determined on the basis of analytical convenience only, without having to be concerned with hapten inhibition of the precipitate.

The development of the use of solid immune adsorbents presented an additional technique suitable for the investigation of this problem. The absorption of an anti-RBG serum with the adsorbent should remove from the serum all molecules with at least one active site directed against the arsanilic acid group. This would involve no experimental uncertainties regarding antigen-antibody ratios or the formation of soluble complexes not precipitated from a fluid system, and need not alter the volume of the serum. Furthermore, the antibody eluted from such a column by a solution of arsanilic acid hapten should contain only antibody molecules of the type described above. If such an antibody solution, when added to a preparation of anti-bovine globulin antibody, results in a specific increase in the amount of antibody protein precipitable by the native antigen, then it must be concluded that the column eluate con-

tained antibody molecules active against both the native protein and the coupled hapten, i.e., heteroligating antibody. The demonstration of the presence of such an antibody would be consistent with the "single antigen molecule" synthesis of antibody protein, and would furnish additional evidence in support of the Pauling theory of antibody formation and the bivalence of antibodies.

The experiments reported here employ each of these techniques. It has been possible to show a loss in anti-globulin activity in sera absorbed with R-adsorbents, and to show that the antibody eluted from such adsorbents contained molecules precipitable by bovine globulin antigen. The binding of hapten by immune precipitates formed between anti-RBG γ-globulin and the bovine globulin native antigen has been demonstrated. The plan of additional experiments to test these results is also given.

Experimental

Loss of titer of anti-RBG γ globulin against BGG on absorbing with R-adsorbent: Two grams of R-adsorbent (Lot V) was packed in a chromatographic column (type D) and washed with borate buffer, pH 8.6, until free of hapten bleed. The column, so prepared, contained 3.8 g. of free water, as determined by weight gain. A solution of γ globulin from rabbit anti-RBG serum was then passed through the column under gravity head. The effluent of the column was tested with trichloracetic acid, and that portion coming through before protein appeared in the effluent (approximately 2-3 ml.) was discarded. The effluent was then collected in 10 ml. portions and titrated against BGG antigen. The serial dilutions of antigen (1:2) were prepared in large batches, and then distributed to the corresponding tubes in the precipitin tests. The precipitable antibody nitrogen was determined for each aliquot of the effluent in the usual manner. The antibody nitrogen found for each aliquot is tabulated below:

Table No. 29

First aliquot	210 µg. N per ml.
Second aliquot	Analysis lost*
Third aliquot	322 µg. N per ml.
Fourth aliquot	323 µg. N per ml.
Fifth aliquot	339 µg. N per ml.
Control**	339 µg. N per ml.

Recovery of anti-BGG antibody in eluted antibody from R-adsorbent column, Experiment I: The R-adsorbent column of the preceding experiment was washed with borate buffer, pH 8.6 until the wash tested negative to protein by ring test with trichloracetic acid. Total buffer wash was 70 ml. The antibody was then eluted from the column with 10 ml. of 1:1000 solution of R-resorcinol in borate buffer. The eluate tested negative for anti-BGG precipitating antibody by the ring test. The eluate was tested for the presence of "univalent" or "incomplete" antibody activity against bovine γ globulin by adding it to a portion of γ globulin from high-titer anti-BGG rabbit serum, and determining the increase in precipitable antibody nitrogen.

Three series of precipitin tubes were set up. In the first, anti-BGG γ globulin diluted with two volumes of buffer was employed as antibody solution. In the second, the γ globulin was diluted with an equivalent

---

* Turbidity in Nesslerized solutions.

** Original antibody solution.

volume of the R-resorcinol dye used in eluting the R-adsorbent column, and in the third, with the hapten-eluted material from the test column itself.

The antigen dilutions were prepared by serial dilutions in bulk lots, with a dilution ratio of $1:\sqrt{2}$. Each precipitin tube contained 1.000 ml. of antibody solution. The aliquots were distributed with volumetric precision by the use of the Chaney (98) modification of the Krogh syringe-pipette. The protein content of the antigen dilutions was checked by direct nitrogen determination.

The precipitin tests were allowed to stand three hours at room temperature, and then aged one week in the cold-room at 4° C. Washings and nitrogen determinations were carried out in the usual manner. Final nitrogen determination was made by reading the colors resulting from Nesslerization with a Klett-Summerson colorimeter with a No. 47 filter. The analytical data are tabulated in Table No. 30 and presented in graphical form in Figure No. 31.

Test for heteroligating antibody in eluate from R-adsorbent column, Experiment II: Experiment I was repeated, saturating the 2 g. R-adsorbent column with the antibody contained in 20 ml. of γ globulin from low-titer anti-RBG serum. The column was then washed with 100 ml. of saline by gravity flow in the cold-room.

Table No. 30

BUFFER CONTROL	Klett Rdg.	µg. Total N	µg. Antigen N	µg. Antibody N
1	32	32	636	
2	26	25	450	
3	30	30	318	
4	47	49	225	
5	73	78	159	
6	132	142	113	
7	155	169	80	89
8	147	160	67	93
9	141	153	40	113
10	132	142	33	109
HAPTEN CONTROL				
1	27	26	636	
2	27	26	450	
3	32	32	318	
4	53	56	225	
5	73	78	159	
6	129	139	113	
7	146	159	80	79
8	143	155	67	88
9	133	144	40	104
10	126	135	33	102
COLUMN ELUATE				
1	33	33	636	
2	33	33	450	
3	40	41	318	
4	54	56	225	
5	92	98	159	
6	140	152	113	
7	154	168	80	88
8	147	160	67	93
9	138	149	40	109
10	129	139	33	106

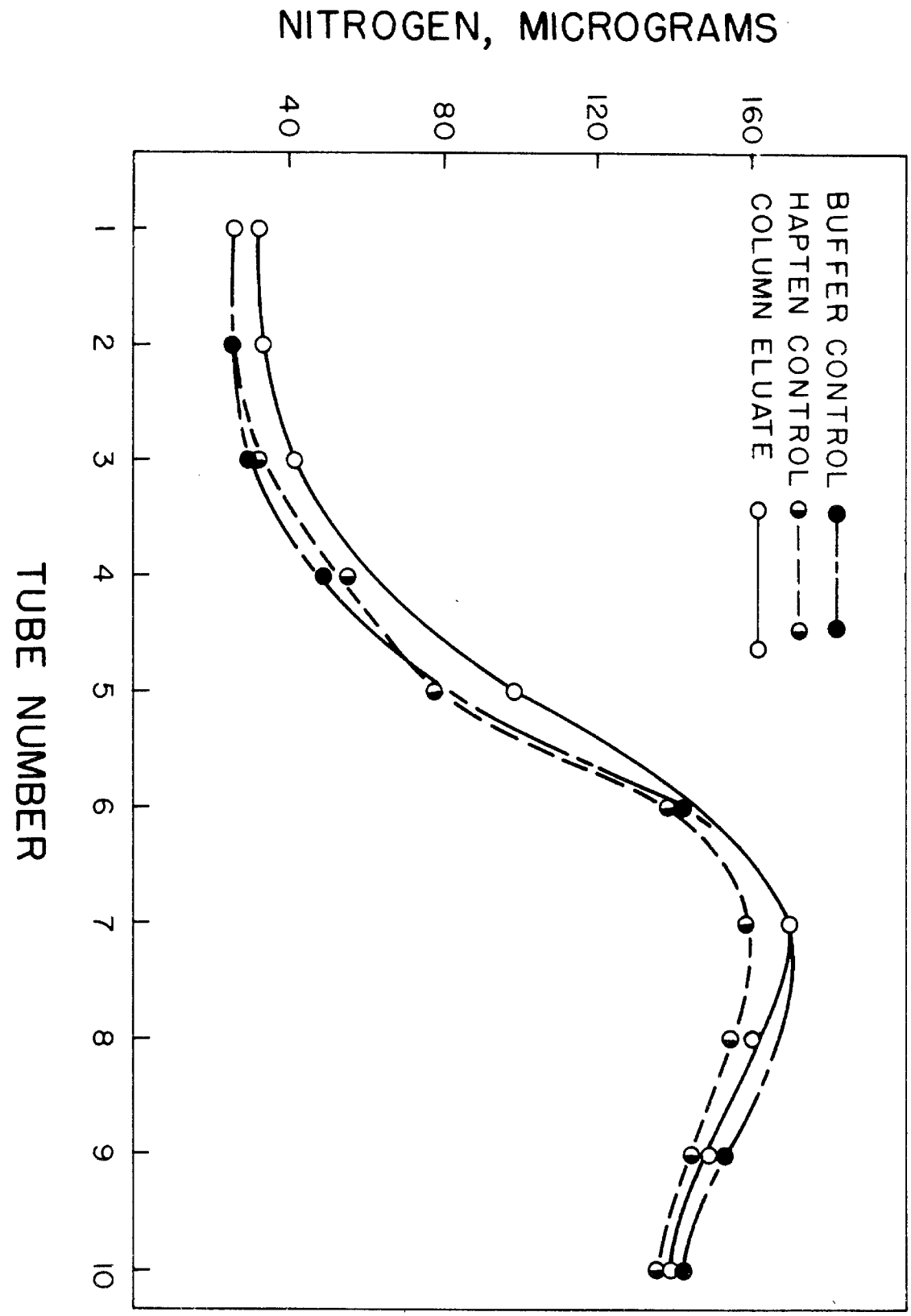
NITROGEN, MICROGRAMS
40
80
120
160
BUFFER CONTROL
HAPTEN CONTROL
COLUMN ELUATE
TUBE NUMBER
1
2
3
4
5
6
7
8
9
10

FIGURE NO. 31

Two days were required to complete the washings until protein tests were negative. The antibody was eluted from the column with 12 ml. of 1:1000 R-resorcinol dye, and the dye washed through with 9 ml. of borate buffer. The combined dye and buffer eluates, totalling 21 ml., were added to 10 ml. of γ globulin from high-titer anti-BGG rabbit serum. Mixtures of 12 ml. plus 9 ml. buffer, and of 21 ml. buffer, each added to 10 ml. of the antibody solution, were set up as controls.

Precipitin tests were set up in triplicate, with serial dilutions of bovine γ globulin (Armour's fraction II, lot 1823B) as antigen. The concentration of protein in the antigen solution in the first tube was 0.04% and serial dilutions were in the ratio of 1: $\sqrt[3]{2}$. Each tube contained 1.000 ml. of antibody solution and 1.000 ml. of antigen solution. The precipitin tests were completed and analyzed in the usual manner. The nitrogen data are summarized in Table No. 31 and presented in graphical form in Figure No. 32.

During the time that the nitrogens of first group of precipitin tubes of the column eluate series was being read, a drop in the line voltage in the laboratory resulted in a shift in the zero point of the colorimeter that was not immediately detected. The high nitrogen values found in the first few tubes of this series are at least in part the result of this instrumental error.

Table No. 31

Series	Tube No.	Nitrogen in ppt. µg.			Antigen Nitrogen µg.	Antibody N µg.
Buffer Control	1	16	16	17	64	
	2	20	17	19	51	
	3	22	22	22	40	
	4	27	29	27	32	
	5	34	35	35	26	
	6	38	39	39	20	19
	7	40	42	41	16	25
	8	38	37	—	13	24
	9	33	34	35	10	24
	10	29	30	—	8	21
Hapten Control	1	17	16	16	64	
	2	18	19	19	51	
	3	22	21	22	40	
	4	25	27	28	32	
	5	31	33	33	26	
	6	37	39	39	20	19
	7	40	41	41	16	25
	8	36	—	40	13	25
	9	31	34	35	10	23
	10	29	29	31	8	22
Column Eluate	1	25	19	19	64	
	2	31	24	24	51	
	3	32	30	28	40	
	4	36	32	32	32	
	5	41	37	39	26	
	6	45	40	45	20	23
	7	47	46	47	16	31
	8	45	42	44	13	31
	9	39	38	39	10	29
	10	36	35	36	8	28

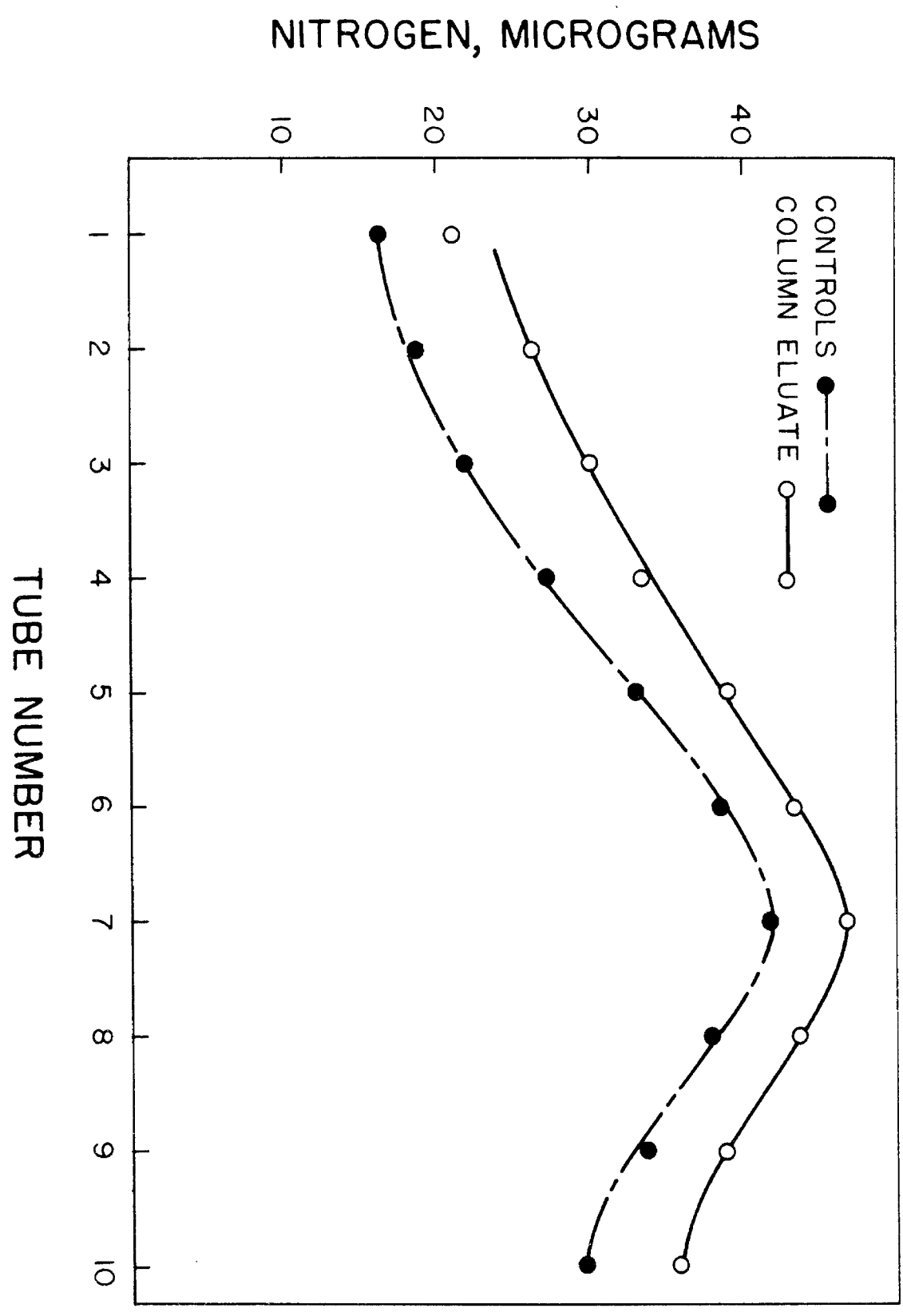
NITROGEN, MICROGRAMS
10
20
30
40
CONTROLS
COLUMN ELUATE
TUBE NUMBER
1
2
3
4
5
6
7
8
9
10

FIGURE NO. 32

Test for heteroligating antibody in eluate from R-adsorbent column, Experiment III: To determine whether the added nitrogen found in the precipitates formed in BGG anti-BGG systems to which eluates from R-adsorbent column have been added are the result of (1) heteroligating antibody, or (2) anti-BGG antibody mechanically retained in the column, or (3) complement or other protein specifically bound in the adsorbent-antibody aggregate, the following experiment was performed.

Three grams portions of R-adsorbent were prepared in type "D" columns, washed with water and buffer, and treated with (a) γ globulin from anti-RBG serum, or (b) γ globulin from anti-BGG serum, and the eluates tested for precipitable nitrogen by addition to BGG-anti-BGG and OA-anti-OA systems. The detailed protocol follows:

The prepared columns were treated with 30 ml. of antibody solution diluted with 30 ml. buffer, allowing the solutions to flow through the columns under gravity head in the cold-room. Two days were required for this step. The columns were then washed with three 100 ml. portions of cold saline under vacuum, and the adsorbent was then strongly compacted and pulled as dry as possible. Each column was then eluted with 20 ml. R-resorcinol dye 1:1000 in pH 8.0 borate buffer followed by 10 ml. of additional buffer. The combined eluates from

each column were tested in the precipitin systems.

Series A: Twelve milliliter eluate from the column treated with the anti-RBG antibody was added to 6 ml. of anti-BGG γ globulin. One milliliter portions of this antibody solution were set up in triplicate with 1:2 serial dilutions of an ammonium sulfate fractionated preparation of the immunizing antigen. Four dilutions were employed. Four milliliters of the remaining solution was set up in a single tube with an appropriate amount of antigen (chosen to represent slight antigen excess).

Series B: Twelve milliliters of diluted hapten (8 ml. 1:1000 dye plus 4 ml. buffer) plus 5 ml. of anti-BGG γ globulin was set up in parallel with the A series, using the identical antigen dilutions.

Series C: Twelve milliliters buffer plus 5 ml. anti-BGG γ globulin was set up to parallel the A series, but the "large scale" tube was omitted.

Series D: Twelve milliliters of the eluate from the R-adsorbent column treated with anti-BGG antibody was added to 6 ml. of anti-BGG globulin and set up in parallel to the A series.

Series E: Twelve milliliters of the eluate used in the A series was added to 6 ml. of γ globulin from anti-OA serum, and the resulting antibody solution was set up in triplicate against a series of four 1:2 serial dilu-

tions of the immunizing antigen. Four milliliters were set up in a single "large scale" tube.

Series F: Twelve milliliters of the hapten solution described for the "B" series was added to 6 ml. of anti-OA γ globulin and set up in parallel with series E.

Series G: Twelve milliliters buffer was added to 6 ml. of anti-OA γ globulin, and set up in parallel with series E, but the "large scale" tube was omitted.

Series H: Twelve milliliters of the eluate used in the D series was set up in parallel with series E.

The use of 5 ml. portions of the anti-BGG γ globulin in series B and C, was necessitated by the exhaustion of the supply of this lot of antibody.

Nitrogen determinations of the precipitates treated and analyzed in the usual manner are given in Table No. 32.

Table No. 32

Series		Total N, µg.			Avg.	Antigen N µg.	Antibody N µg.
A	1	44	44	42	43.3	260	
	2	54	62	60	61.9	130	
	3	85	84	87	85.1	65	
	4	86	85	85	85.1	32.5	52.6
B	1	39	36	35	36.5	260	
	2	49	48	47	47.4	130	
	3	78	76	76	76.6	65	
	4	81	79	78	79.4	32.5	46.9
C	1	33	34	35	34.2	260	
	2	43	51	48	47.4	130	
	3	72	75	75	73.8	65	
	4	75	79	--	77.0	32.5	44.5
D	1	40	43	41	41.4	260	
	2	57	57	59	57.7	130	
	3	82	85	86	84.4	65	
	4	84	86	87	85.7	32.5	53.2
"Large Scale" tubes							
	A				375	260	115
	B				320	260	60
	D				384	260	124

Table No. 32 (cont.)

Series		Total Nitrogen µg.			Avg.	Antigen N, µg.	Antibody N, µg.
E	1	90	93	95	92.7	32	
	2	280	291	284	285	16	269
	3	224	230	219	224.3	8	216
	4	143	146	145	144.7	4	141
F	1	---	87	77	82	32	
	2	---	289	284	286.5	16	270
	3	213	223	225	217	8	209
	4	136	142	142	140	4	136
G	1	91	70	70	77	32	
	2	284	293	290	289	16	273
	3	219	220	225	221	8	213
	4	141	142	143	143	4	138
H	1	76	99	91	89	32	
	2	293	291	287	290	16	274
	3	227	234	230	230	8	222
	4	144	146	147	146	4	142
"Large Scale" tubes*							
E					760	96	664
F					760	96	664
H					760	96	664

* The nitrogen content of these tubes was over the optimum amount for determination by the technique used, and the calibration curve for nitrogen is considerably flattened in this region, so that deviations of $\pm$ 1% in the nitrogen content could not have been detected with certainty.

Test for heteroligating antibody in eluate for R-adsorbent column, Experiment IV: The columns of R-adsorbent used in Experiment III were stripped of all protein and dye with dilute sodium hydroxide wash, then equilibrated with buffer at pH 8.4.

Series A: A quantity of γ globulin from anti-RBG serum containing 5 mg. of antibody nitrogen precipitable by R'_3-resorcinol (31 ml. of a preparation containing 161 µg. precipitable N per ml.) in a final volume of 50 ml., buffered with borate to pH 8.4, was allowed to flow under gravity head through one column in the cold-room. The column was washed with buffer for three days under gravity flow and then eluted with 30 ml. 0.1% R-resorcinol hapten in borate buffer. Eluate contained 25 µg. protein N per ml.

The column eluate (25 ml.) was added to 8.5 ml. of a γ globulin preparation from anti-BGG serum, and precipitin tests were set up in triplicate against bovine globulin as the precipitating antigen. The first tube contained 250 µg. antigen N, and successive tubes contained serial dilutions at a $1 : \sqrt{2}$ ratio. Antigen and antibody solutions were distributed with a precision syringe-pipette, and antigen dilutions for all series of this experiment were made up in a single series of bulk dilutions.

Series B: The second adsorbent column was treated with anti-BGG γ globulin containing 5 mg. of precipitable nitrogen (9.6 ml. of a preparation containing 520 µg. pre-

cipitable N per ml.). The column was washed and eluted as in (A), and the eluate containing 4 μg. protein N per ml. was added to anti-BGG γ globulin and precipitin tubes set up as in (A).

Series C: Twenty-five ml. of R-resorcinol hapten in borate buffer, of a strength equal to that in the column eluates, was set up as a control.

The precipitin tubes were allowed to stand at room temperature for three hours and then in the cold-room for three days. The tubes were then filled with cold saline, spun down for 30 minutes at 2400 RPM, decanted, and twice washed with cold saline. The digestions and Nesslerizations were carried out in the usual manner, and the final colors read on a Beckman Model C colorimeter with a No. 47 Klett-Summerson filter.

The analytical data are summarized in Table No. 33.

Test for heteroligating antibody in eluate for R-adsorbent column, Experiment V: Ten grams of R-adsorbent (Lot V) in a "D" type column was exhaustively washed with borate buffer (pH 8.4), until free of hapten bleed, and then 75 ml. of γ globulin from low-titer anti-RBG serum (66 μg. precipitable N per ml., vs. R'$_3$-resorcinol) diluted with 25 ml. of borate buffer was allowed to flow through the column under gravity head at room temperature. Approximately eight hours were required for the passage

of the antibody solution. The column was then washed with buffer at room temperature under vacuum until 500 ml. of buffer had passed through the column. No protein could be detected in the effluent during the last half of this washing period. The column was strongly compressed to squeeze out as much mechanically held fluid as possible, and then 50 ml. of 0.1% R-resorcinol was allowed to flow through the column under gravity head. The next morning a volume of 47 ml. of eluate, containing 42 μg. of protein N per ml., had passed through the column.

Series A: Seven volumes of the column eluate was added to one volume of anti-BGG globulin, and precipitin tubes were set up in duplicate, each tube containing 2.5 ml. of the antibody solution and one ml. of bovine γ globulin antigen. Serial antigen dilutions were in the ratio of $1:\sqrt{2}$.

Series B: Control series, using hapten solution in place of the column eluate used in series A.

The precipitin tubes were set up, processed and analyzed as in experiment No. 4. The analytical data are summarized in Table No. 34 and Figure No. 33.

Test for heteroligating antibody in eluate for R-adsorbent column, Experiment VI: Five grams of R-adsorbent (Lot V) was thoroughly washed with buffer in a type "D" column, and 30 ml. of γ globulin from anti-RBG

Table No. 33

Series	Tube No.	Set. 1	Set. 2	Set. 3	Ave.	Antigen N µg.	Antibody N µg.
A	1	19.8	25.7	25.5	23.7	250	
	2	49.7	57.3	60.4	55.8	176.7	
	3	132.7	136.2	140.9	136.6	125	
	4	156.0	161.5	167.3	161.6	88.4	73.2
	5	174.3	173.3	175.8	174.5	62.5	112.0
	6	176.5	-----	181.3	178.9	44.2	134.7
	7	176.2	181.2	181.8	179.7	31.3	148.4
	8	173.0	179.0	177.6	176.5	22.1	154.4
	9	158.2	159.8	162.0	160.0	15.6	144.4
	10	125.0	129.9	130.2	128.4	11.0	117.4
B	1	23.3	15.6	22.1	20.3	250	
	2	47.4	47.1	47.8	47.4	176.7	
	3	135.6	135.6	135.6	135.6	125	10.6
	4	164.2	157.6	164.2	162.0	88.4	73.6
	5	172.7	175.0	175.4	174.4	62.5	111.9
	6	179.5	179.8	177.9	179.1	44.2	134.9
	7	178.4	178.1	177.9	178.1	31.3	146.8
	8	177.5	177.0	177.3	177.3	22.1	155.2
	9	154.9	159.8	158.5	157.7	15.6	142.1
	10	127.2	125.0	125.4	125.9	11.0	114.9
C	1	19.6	28.3	28.7	25.5	250	
	2	51.7	57.2	60.2	56.4	176.7	
	3	131.6	138.2	131.9	133.9	125	8.9
	4	163.4	166.5	158.5	162.8	88.4	74.4
	5	169.2	176.0	168.7	171.3	62.5	108.8
	6	181.7	167.8	169.9	173.0	44.2	128.8
	7	181.4	163.0	173.2	172.5	31.3	141.2
	8	180.8	170.8	169.9	173.8	22.1	152.7
	9	163.0	150.3	150.0	154.4	15.6	138.8
	10	126.0	122.3	120.9	123.1	11.0	112.1

NOTE

Pages 227 and 231 are absent in this thesis because of an error on the part of the typist in allowing for figures. There is no material missing.

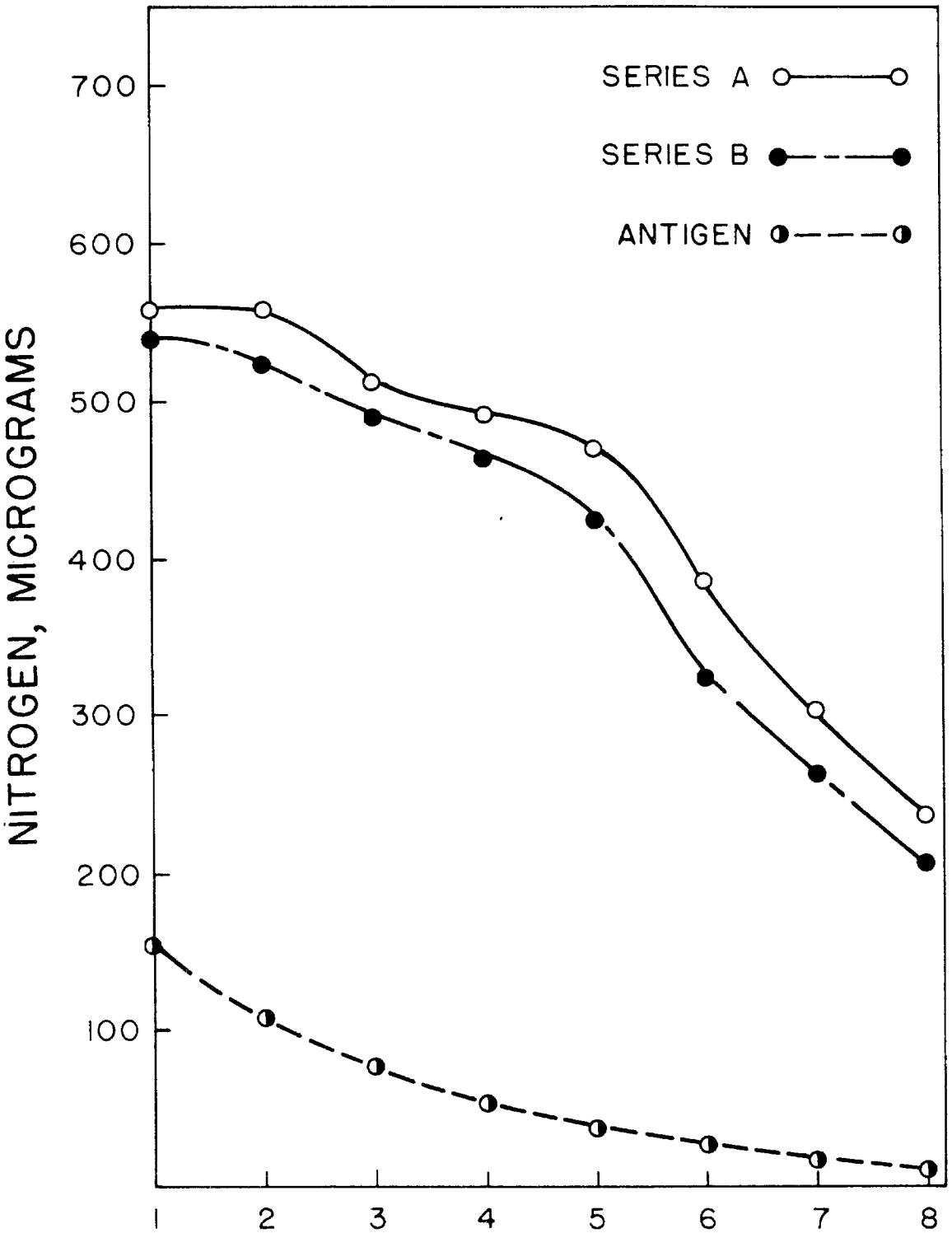
SERIES A
SERIES B
ANTIGEN
700
600
500
400
300
200
100
NITROGEN, MICROGRAMS
1
2
3
4
5
6
7
8
TUBE NUMBER

FIGURE NO. 33

serum (79 µg. precipitable N per ml. vs. R'_3-resorcinol, unlabeled lot obtained from laboratory stocks) allowed to flow through column under gravity head at room temperature. Three days were required for the passage of the antibody solution. The column was thoroughly washed with buffer under vacuum until the eluate tested negative for protein by trichloracetic acid, and then for an additional 100 ml. Then the column was eluted with 30 ml. 0.1% R-resorcinol hapten in borate buffer, pH 8.4. The eluate obtained contained approximately 50 micrograms of protein nitrogen per ml.

Series A: One volume of globulin from anti-BGG serum plus seven volumes of eluate from the R-adsorbent column described above was used as antibody solution. Two ml. of antibody solution plus 1 ml. of bovine γ globulin antigen (the same dilutions as used in experiment No. V) were added to each precipitin tube. Tube No. 4 was set up in triplicate; the remaining tubes were not duplicated because of the limited amount of eluate available.

Series G: Control series were set up in duplicate using hapten dye solution in place of the column eluate.

The precipitin tests were processed and analyzed as previously described. The analytical data are summarized in Table No. 35 and Figure 34.

Table No. 34

Series	Tube No.	Total Nitrogen, µg. Set 1	Set 2	Avg.	Antigen N µg.	Antibody N, µg.
A	1	545	568	557	154	403
	2	568	544	556	109	447
	3	502	525	514	77	437
	4*493	484	500	492	54	438
	5	461	479	470	38	422
	6	374	396	385	27	358
	7	298	307	303	19	284
	8	230	245	238	14	224
B	1	540			154	386
	2	524			109	415
	3	490			77	413
	4*462	469	476	469	54	415
	5	425			38	387
	6	348			27	319
	7	261			19	242
	8	207			14	207

Increments in antibody nitrogen per tube:

1	17 µg.
2	32
3	24
4	25
5	35
6	39
7	42
8	17
Avg.	29 µg.

Increment in equivalence zone, tubes 2 - 4 incl. 27 µg.

Protein added to each tube in eluate: $2.5 \times 7/8 \times 42 = 92$ µg.

Added protein recovered in precipitate, (27 µg.) = 29%

* Tube No. 4 was set up in triplicate for each series.

Table No. 35

Series	Tube No.	Total Nitrogen in Ppt, µg. Set 1	Set 2	Set 3	Avg.	Antigen N, µg.	Antibody N, µg.
A	1	462				154	308
	2	441				109	332
	3	427				77	350
	4	404	416	406	409	54	355
	5	388				38	350
	6	349				27	322
	7	260				17	243
	8	210				14	196
B	1	450	448		449	154	295
	2	408	415		412	109	303
	3	380	400		390	77	313
	4	377	371		374	54	320
	5	356	348		352	38	314
	6	323	311		317	27	290
	7	259	249		254	17	237
	8	188	187		188	14	174

Increments in antibody nitrogen per tube:

1	13
2	29
3	37
4	35
5	36
6	32
7	6
8	22
Avg.	26 µg.

Increments in equivalence zone (tubes 2 - 6): 34 µg. N./ tube.

Protein added to each tube in eluate: $2 \times 7/8 \times 50 = 88$ µg.

Added protein recovered in precipitate (34 µg.) = 39%

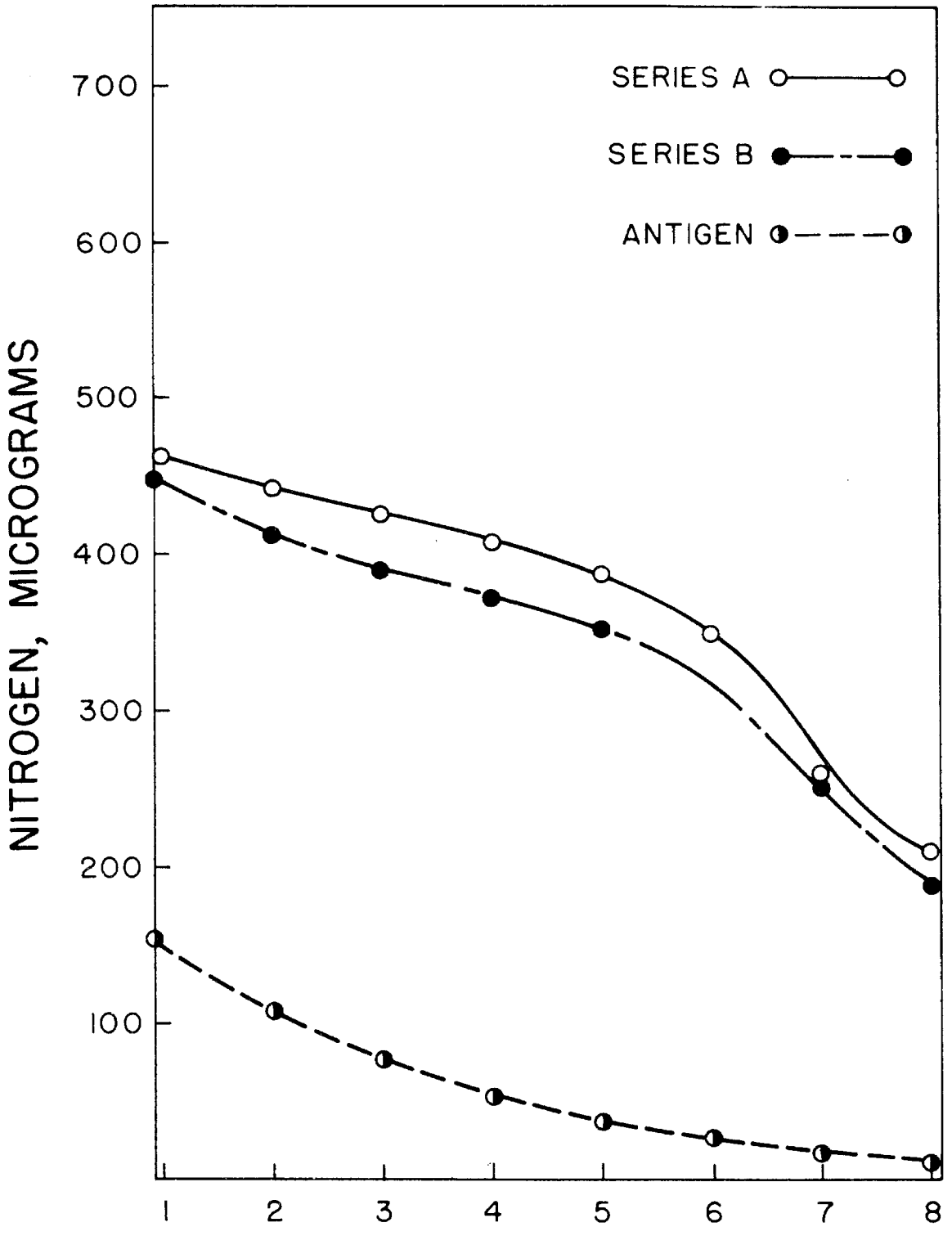
SERIES A
SERIES B
ANTIGEN
700
600
500
400
300
200
100
NITROGEN, MICROGRAMS
1
2
3
4
5
6
7
8
TUBE NUMBER

FIGURE NO. 34

Hapten binding by immune precipitates between bovine globulin antigen and anti-RBG γ globulin: In conjunction with the second experiment on hapten binding in fluid systems, described in Part I of this dissertation, an examination was made of the hapten binding in the system described above, using identical techniques to those there described. The system of anti-BGG γ globulin versus the immunizing antigen and the ovalbumin system were both treated as controls on nonspecific hapten binding. The protocol of the experiment is given in Table No. 36, and the nitrogen and iodine analytical data are summarized in Tables Nos. 37 and 38.

Table No. 36

Tube No.	Antibody	Antibody Volume	Antigen	Antigen Volume	Hapten* Volume	Buffer Volume
1						
2	Anti-OA**	3 ml.	1:10,000 OA	1 ml.	1 ml.	5 ml.
3						
7	Anti-BGG***	3 ml.	1:1,000 BGG	1 ml.	1 ml.	5 ml.
8						
9						
10						
11	Anti-RBG****	5 ml.	1:1,000 BGG	2 ml.	1 ml.	2 ml.
12						

One-tenth these quantities were set up in duplicate for nitrogen analyses.

---

* The hapten was 10^{-5} M R-iodophenol.
** The anti-OA γ globulin contained 725 μg. precipitable N per ml.
*** The anti-BGG γ globulin contained 520 μg. precipitable N per ml.
**** The anti-RBG γ globulin contained 254 μg. precipitable N per ml. active against the bovine globulin test antigen.

Table No. 37

Sample	Klett Rdg.	Precipitate Nitrogen, µg.	Protein in Precipitate	Protein in Large Sample
A	208	234	1.49 mg.	14.9 mg.
B	215	244		
E	126	132	.83	8.3
F	127	133		
G	149	159	1.03	10.3
H	156	166		

Table No. 38

Sample	Total I, µg.	Free water mg.	I in water µg.	Protein-bound I, µg.	Protein-bound I µg./mg.	Protein: Hapten Molar Ratio
1	.032	260	.018	.014	.00094	
2	.030	262	.018	.012	.00081	740
3	.040	258	.018	.022	.00147	
7	.018	139	.009	.009	.00108	
8	.012	132	.009	.003	.00036	487
9	.037	143	.009	.028	.00337	
10	.041	137	.009	.032	.00311	
11	.043	140	.009	.034	.00330	280
12	.029	132	.009	.020	.00194	

The blanks, standards, and hapten analyses are the same as those recorded in Table No. 28, in Part I.

Discussion

The loss in titer of the anti-RBG globulin against the bovine globulin test antigen on absorption with the R-adsorbent is of a magnitude outside of the experimental errors associated with this experiment. The total buffer in the column at the start of the experiment was 3.8 g. Not less than 2 ml. of protein-free buffer was discarded before the effluent of the column was collected for antibody titration. Of the first 10 ml. of effluent collected, not less than 8.2 ml. represents absorbed antibody solution. Since the initial material contained 339 μg. of precipitable N per ml., this effluent, had there been no adsorption of the anti-BGG antibody by the column, would have contained 278 μg. of antibody nitrogen. The value found, 210 μg. indicates a loss of about 25% of the total antibody activity against bovine globulin.

This decrease is far greater than the uncertainty of the analyses, which in this series of precipitin tests showed unusually small variations. The variation in nitrogens found in the corresponding tubes of the precipitin tests for the fifth aliquot of the effluent and the control antibody solution was less than 2% of the total nitrogen found for all tubes except the last two in which the amounts of precipitate were only a few micrograms.

The loss of the nitrogen analyses for the second aliquot as the result of contamination of the Nesslerized solution with acetone vapors deprives the experiment of a most valuable item of data, but the asymptotic approach of the effluent titer to that of the original globulin solution is in complete accord with what would be expected for this system if the presumption of the existence of heteroligating antibody in the antibody solution be made. The total amount of such heteroligating antibody adsorbed by the column was not less than 4 mg. This represents a significant fraction of the total capacity of the column for anti-arsanilic acid antibody, and suggests that the pool of antibody used in this experiment contained amounts of heteroligating antibody approaching that which would be predicted by a statistical distribution of binding sites of the antibody molecules.

The antibody solution used in this experiment contained 66 μg. of precipitable N against R'_3-resorcinol and 339 μg. active against bovine globulin. If the assumption is made that all of the heteroligating antibody is precipitable by the protein antigen, but that only a portion of it is precipitable by the polyhaptenic dye, both because of the poorer precipitating power of that antigen and because there is not enough of the bivalent anti-R antibody present to facilitate the pre-

cipitation of all of the antibody monovalent against this antigen, a prediction can be made on the basis of such a statistical distribution of antibody binding sites, of the nature of the population of antibody molecules in the solution. Such a calculation leads to an estimate of only about 8 μg. of homoligating anti-R per ml. of solution, a value which is obviously far less than the true value. The assumption of a statistical distribution is in itself unlikely, and would in most instances lead to amounts of heteroligating antibody that could hardly have been missed by earlier workers.

Experiments on the recovery of anti-BGG activity in the hapten eluates from the R-adsorbent columns gave somewhat discordant results. In all cases where the eluate contained substantial amounts of total protein, significant increases in the protein precipitable by bovine globulin were observed when the eluate was added to the precipitating system. In no case could precipitating antibody against bovine globulin be detected in the eluates by ring or tube tests.

In experiment I, the increase in precipitate was significant in tubes 5 and 6 only. These tubes were in the region of moderate antigen excess, in which region the antibody, univalent with respect to the antigen, might be thought most likely to be included in the precipitate. The increases in the other tubes, particularly

when compared with the hapten control, are suggestive but hardly large enough to be significant in an experiment performed without replicates.

Experiment II, carried out with each series in triplicate, yielded amounts of precipitate that were considerably less than optimum for the nitrogen analyses. The agreement between replicates was in general very satisfactory, and the percentage increase in precipitable nitrogen impressive. The increase in nitrogen throughout the precipitin curve is more uniform in this case, a uniformity that is in part due to the smaller range of antigen concentrations encompassed by the serial dilutions in the ratio of $1:\sqrt[3]{2}$.

In experiment III, the unfortunate shortage of the anti-BGG led to an unwise decision to decrease the amount of antibody in series B and C. This made it impossible to draw precise comparisons between these series and the A and D series, for which they would otherwise have served as additional controls. The increase in nitrogen found in the A series, for tubes 1 and 2, is certainly suggestive, and for tube 2, is statistically significant. In this experiment, as in the first, this increase in precipitable nitrogen was observed in the region of moderate antigen excess. The large scale tubes unfortunately fell at the equivalence point, where no increment in precipitated nitrogen was observed.

The absence of precipitating antibody in the effluent from the R-adsorbent column that had been treated with anti-BGG γ globulin (as established by negative ring tests for such antibody) is evidence that the anti-BGG antibody obtained in the eluate from the test column is not homoligating antibody that has resisted flushing out during the preparation of the column.

The control tests set up with the ovalbumin system to detect the possible presence of some nonspecific indicator protein, such as a complement fraction, that might be the cause of the nitrogen increase in the BGG system, failed to show any such nonspecific nitrogen increase. Unfortunately, the amounts of the increases observed in the BGG system were not large enough to have been detected with certainty in the large precipitates obtained in the control experiments.

Experiment IV must be considered as negative. The small amount of total protein present in the eluate from the R-adsorbent column of series A (25 μg. of protein N per ml.), when diluted with antibody solution, contributed less than 19 μg. of nitrogen per precipitin tube. The analytical uncertainty in this experiment was of the order of several micrograms for many of the tube sets. The small amount of nitrogen in the eluate from the R-adsorbent column that had been treated with anti-BGG antibody (4 μg. of protein N per ml.) was all recoverable

in the precipitate, and this indicates that the method of column washing used in this test is only moderately effective in flushing the non-adsorbed protein from the interstices of the column.

Experiments V and VI constitute the most convincing evidence for the existence of heteroligating antibody in the column eluates. The washing of the columns was most thorough, and the freedom of the wash buffer from traces of protein was rigorously established. The protein in the eluate represented only a small portion of the total antibody placed on the column. In experiment V approximately 5 mg. of antibody nitrogen was put on the column, and less than 2 mg. of protein nitrogen was present in the eluate. The capacity of the column was adequate to adsorb all the anti-R antibody from the material run through it. The relatively mild conditions of elution presumably selectively removed those antibody molecules least firmly bound to the column. From a statistical standpoint, the antibody molecules univalent to R should be more readily eluted than those with two sites capable of binding to the column, for the former molecules not only have no opportunity to be bound simultaneously at both ends, but their chances of readsorbing on the column at a lower level after an initial elution are less than for the bivalent molecules. The percentage of heteroligating antibody in the eluates, 29% in experiment V and 39% in experiment VI, is certainly

greater than that existing in the original serum. The quantities of eluate were not adequate to permit all the precipitin tests to be set up in triplicate, but the duplicate values are in good agreement, and the values for tube 4, which in each case was set up in triplicate, lie on the curves established by the other tubes of their respective sets.

The attempt to demonstrate hapten binding by heteroligating antibody in the precipitate formed between bovine globulin and the anti-RBG γ globulin met with only qualified success. The amount of hapten bound to the precipitate was considerably less than optimum for the iodine analysis, and the resulting analytical uncertainty was a large percentage of the total iodine found. Consequently the differences between the iodine bound per milligram of precipitate for the control systems and for the heteroligating antibody experiment are of questionable significance.

Taking the three ovalbumin precipitates and the three bovine globulin precipitates together as a set of six control measurements, and comparing them with the three precipitates obtained with the BGG-anti-RBG system, the differences in hapten binding, when analyzed by the Student "t" test, yield a value of $t = 2.09$. With 7 degrees of freedom, as in this experiment, this value of "t" corresponds to a probability of 0.08. That is,

there is about one chance in twelve that these results could have been the consequence of random variations in the measurements. A probability of this magnitude is certainly suggestive, and cannot be dismissed completely, but it lacks something of being "significant" in the usually accepted sense of the term, which is ordinarily restricted to a degree of probability in which the chances of the results being due to random variation in the sample are less than one in twenty. This test ought to be repeated, using larger aliquots of serum and more replicates, since the amount of hapten binding indicated, if real, could be established with considerable certainty under such conditions.

Since the amount of hapten binding observed in the homologous system under the conditions used in this experiment corresponded to only one hapten molecule to 88 antibody molecules, the amount of hapten binding found in the heteroligating antibody experiment, which, when corrected for the amounts of nonspecific hapten adsorption shown in the control systems, corresponds to about one hapten molecule for every 500 antibody molecules, may represent an appreciable amount of heteroligating antibody in the precipitate. The formation of the precipitate in the presence of somewhat higher concentrations of hapten should result in a higher level of hapten binding by the heteroligating antibody. If it did not also result in too great an increase in nonspecific

binding on the control precipitates, the analytical data should show improvement, since the uncertainties of the corrections for the iodine in the free water of the precipitate are, at the hapten level used in this experiment, still but a very small part of the total probable error of the analysis. The total water content of the precipitate can be determined with considerable precision, and the iodine content of the water can be known with relative precision either by careful preparation of the solution from haptenic dye of known purity, or by replicate analyses of the actual solution used, and ideally by both.

A few words are in order regarding the first experiment on hapten binding in fluid systems of Part I of this thesis. The hapten contents of the precipitates in the antigen-excess portion of the curve were larger, per milligram of precipitate, than were those of precipitates formed at the equivalence point. The precipitating antigen in this experiment was the immunizing antigen against which the serum had been prepared, i.e., RBG. As such it was a highly complex antigen, containing antigenic groupings of at least three different kinds, the R groups, native bovine globulin groups, and compound groups containing elements of each.* The equivalence

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* This antibody preparation contained 66 μg. per ml. of antibody nitrogen precipitable by R'_3-resorcinol, 254 μg.

point for each type of antibody in the antiserum γ globulin preparation would not be expected to fall at the same ratio of total antigen to total antibody. Therefore the point at which the maximum total amount of antibody is precipitated does not necessarily correspond to any true equivalence point. Furthermore, in the aggregate the framework of antigen and antibody molecules would tend to make a relatively rigid three-dimensional network, a lattice of sufficiently close spacing that any heteroligating molecule, attached within the lattice at one end by a native protein antigen but so situated that no protein-coupled R group in the network was available for binding the other end, would be protected from other protein antigens by the steric hindrance afforded by the lattice, and could bind hapten without reference to competition of the hapten with protein antigens.

The reasoning above must be regarded as completely speculative, and the phenomenon discussed may be nothing but an experimental artifact, but if the phenomenon is real, the elucidation of its mechanism should shed considerable light on the structure of immune aggregates.

A technique for the demonstration of heteroligating

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per ml. of antibody nitrogen precipitable by bovine globulin, and 1180 μg. per ml. of antibody nitrogen precipitable by the immunizing antigen.

antibody that occurred to the author after the conclusion of the experimental portion of this research is described below: If an anti-RBG serum containing heteroligating antibody molecules directed against both the R and the native bovine globulin groupings be adsorbed on a column of R-adsorbent, all molecules containing at least one binding site directed against the R group will be bound to the column. All unbound protein may then be washed from the column. If then a solution of bovine globulin, labeled with either stable or isotopic iodine to a degree that does not greatly alter its native antigenicity (17), be passed through the column, this iodinated globulin should be bound to the distal end of all such heteroligating antibody on the column. The globulin solution should not tend to elute the antibody from the column, and the union between the antibody and the globulin antigen should be strong enough to resist dissociation when the unreacted globulin was washed from the column. The analysis of the column for iodine, either by chemical or counting techniques, would give a measure of the heteroligating antibody on the column.

Such an experiment should have two advantages from the standpoint of the iodine analysis alone. The blanks for the nonspecifically held iodine should be low, both because of the decreased tendency of the protein to nonspecific adsorption on the column as compared to the

haptenic dyes, and because the columns could be more exhaustively washed without loss of large amounts of antigen. The total amounts of iodine found would be greater, for each antigen molecule would contribute more iodine to the system and the greater strength of the antibody-antigen bond would lead to a higher level of saturation of the heteroligating sites with antigen.

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PROPOSITIONS

1. An azoprotein having a single haptenic group coupled to each molecule of the protein would be a valuable tool in investigating both antibody formation and the structure of immune aggregates. The electrophoresis-convection technique of Kirkwood et al. should make it possible to separate the mono azo proteins from the more or less highly coupled molecules in the reaction mixture if the coupled hapten has a high electronic charge.

2. Potent precipitating antisera for chorionic and pituitary gonadotropins would permit the differentiation and assay of these hormones by immunochemical means. Search should be made for animals capable of producing such a serum.

3. Substantially pure heteroligating antibody could be separated from a suitable serum if immune adsorbents capable of of reacting with both kinds of binding sites were available, by the choice of suitable azoproteins as immunizing antigens, using haptenic groups that do not cross-react and with which immune adsorbents can be synthesized.

4. A quantitative comparison of the amounts of heteroligating antibody produced against a mixed antigen under the conditions (1) that both haptens are present as a part of the same antigen molecule, and (2) the two haptens occur on separate molecular antigens, should throw light on the mechanism of antibody synthesis.

5. The "incomplete" or "blocking" antibody often observed early in the course of an immunization is variously interpreted as "univalent" or "weakly binding". The fractionation

of the antibody on columns of immune adsorbents should permit precise determination of the binding strength of the antibodies, and the hapten binding power of the aggregate would be a measure of their valence.

6. Additional work needs to be done on the influence of selective mechanisms on the composition of immune aggregates formed in heterogeneous systems.

7. The protection of the professional position of the chemist in the fields relating to public health demands that the chemical profession take the initiative in securing licensure under the control of the profession.

8. The use of microspheres of glass or ceramics containing silver or some other non-toxic element that is well adapted to detection and measurement by neutron activation analysis would constitute a technique for the study of the distribution and dissemination of particulate air pollutants. This technique would approach in sensitivity and specificity the use of radioisotopes without the disadvantages associated with the latter.

9. It is known that the partially burned fuels in automobile exhausts contain molecular species that enter into the chain reactions involved in the production of smog. Research is needed to determine what fuel molecules contribute to these products, and to determine the economic feasibility of producing motor fuels low in these components.

10. Currently available techniques for the measurement of velocities of gases flowing in ducts or flues are poorly

adapted to the determination of low flow rates. Sonar techniques should be adaptable to this problem.