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CHARACTERIZATION OF ANTIBODIES TO PARASITES

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CHARACTERIZATION OF ANTIBODIES TO PARASITES*

In marked contrast to the voluminous literature describing immunochemical and physicochemical characterization of antibodies formed in response to infection with a variety of bacteria and viruses, as well as to numerous pure antigens, relatively little is known about the characteristics of antibodies formed in response to infection with protozoa and helminths. Multiple serologic tests are available for diagnosis of parasitic diseases, but we lack knowledge of the nature of the antibodies which participate in these reactions. Such characterization of antibodies, formed in response to parasitic infestation, would not necessarily be of academic interest only but might shed additional light on the mechanisms of parasitism and host response.

When I saw the proposed title of my talk, I realized that by necessity the information discussed would be largely prospective in nature rather than both prospective and retrospective as can be formulated from the subjects covered by the other discussants. In many ways this is an advantage--in an area of immunology of parasites as embryonic as this it is often useful to direct one's attention to what might be accomplished in the future.

Characterization of antibodies is a vast area of research and includes definition of primary, secondary, tertiary and quaternary structure, molecular and genetic variations, determination of protein-bound carbohydrates, synthesis, distribution and turnover, serologic reactivity and specificity, etc. Here we shall concern ourselves solely with methods for immunochemical and/or physicochemical characterization and thus with

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recognition of the three major immunoglobulin classes of antibody, IgG, IgA and IgM. Suitable references are given for each method.

First I would like to mention data available in regard to characterization of antibodies in three parasitic infections. In 1965 Abele, Tobie, Hill, Contacos and Evans published their findings on alterations in serum proteins and 19S antibody production during the course of induced malarial infections in man.(1) These authors noted a consistent increase in IgM in immunoelectrophoretic patterns of serum obtained from volunteers infected with Plasmodium vivax and Plasmodium cynomolgi. Sera were separated by gel filtration on columns of Sephadex G-200, and the fractions tested for antibody activity by means of the fluorescent antibody test using a fluorescein-labeled rabbit anti-human immunoglobulin antiserum. In addition, reductive cleavage with mercaptoethanol was used to help differentiate IgG and IgM antibodies. Antibody activity was confined to the IgM fractions of serum during the first few days of antibody production in several of the volunteers. Thereafter antibody activity was found in both IgM and IgG. Approximately 40 days after infection antibody activity was present only in IgG. Of significance for our discussion here is the use by the authors of both blood-induced and sporozoite-induced infections and the variability in response in the different volunteers. For example, in one case of sporozoite-induced infection antibody activity was demonstrable only in IgG and never in IgM fractions. Characterization of the antibodies in these cases has revealed previously unrecognized parameters requiring further exploration; e.g., the relative efficacy of these different classes of antibodies in protection

against infections,[2] their reactivity and specificity in different serologic tests, the antigenic structures stimulating their production, relation of inoculum size to class of immunoglobulin response, etc.

Recently we have demonstrated the presence of IgM toxoplasma antibodies in humans with acute acquired and congenital toxoplasmosis.[3] A complete review of our data has recently been submitted for publication and will not be described here.[4] Characterization of toxoplasma antibodies was performed to determine if demonstration of IgM toxoplasma antibodies would prove of diagnostic significance. Since IgG toxoplasma antibodies freely traverse the placenta, their presence in the newborn may only reflect maternal infection.[5] IgM antibodies do not normally traverse the placenta but may be formed <u>in utero</u> in response to infection. Their demonstration in a newborn infant, in the absence of a placental leak, has proven to be diagnostic of congenital toxoplasmosis. Initially, demonstration of IgM toxoplasma antibodies was accomplished by use of sucrose density gradient ultracentrifugation and reductive cleavage. Recently we developed a modification of the indirect fluorescent antibody technique, using a fluorescein-tagged antiserum specific for IgM. This technique can be employed in any laboratory which has a fluorescent microscope and can be completed in a period of approximately two hours. Hopefully it will prove useful in studies of characterization of antibodies to other parasites and in diagnosis of other infections--especially in the newborn. We are now employing the technique in a prospective study of 5000 newborns to determine the incidence of congenital toxoplasmosis in the San Francisco Bay Area. Because of the wide clinical spectrum of congenital toxoplasmosis in the newborn[7] and because of the presence of maternally transmitted antibodies in the newborn, such a study could not be performed previously.

Crandall, Cebra and Crandall have recently described the relative proportion of IgG-, IgA- and IgM-containing cells in rabbit tissues during experimental trichinosis.[8] Direct counting of cells containing two different immunoglobulin classes in a single tissue section was accomplished by staining with pairs of immunofluorescent reagents, specifically reactive with γ-, μ- or α-immunoglobulin heavy chains and labeled with contrasting fluorochromes.

By employing two different pairs of reagents on adjacent sections the relative numbers of cells containing IgG, IgM and IgA were calculated. The most striking change in relative proportions of immunoglobulin-containing cells in the intestinal mucosa of infected animals was an increase in cells containing IgM in early infections. Later in infection, cells containing IgG made up a relatively larger proportion of the total IgM plus IgG population. This change in relative proportions of IgM and IgG cells with time after infection correlates with the sequence of appearance of the immunoglobulin classes of humoral antibody after antigenic stimulation. The number of immunoglobulin-containing cells in the diaphragm suggested that skeletal muscle is an important site for immunoglobulin production in Trichinella infections. The authors detected humoral IgG and IgM antibodies to Trichinella but not IgA. Although this study was oriented mainly toward study of cellular production of the various immunoglobulin classes of antibody, it can readily be seen that such an experimental approach to characterize antibodies and antibody formation might be used in the study of host response to a variety of parasites.

There are large numbers of techniques now available for characterization of antibodies. No attempt will be made here to review or even mention all of them. Information about many of the techniques may be found in references (5), (9), and (10).

The characterization of antibodies in the three parasitic infections mentioned above illustrate what can be done in this area in the field of parasitology. Each of the techniques used in those studies had been previously employed to characterize antibodies formed against many unrelated organisms and antigens. One of them, density gradient ultracentrifugation, is shown diagramatically in Figs. 1a and 1b. For separation of the various molecular species of immunoglobulins, gradients of sucrose are usually employed. In Fig. 2 are shown the results obtained by this method in a serum of a patient with acute acquired toxoplasmosis. Only IgM or 19S antibodies were demonstrable by this method at five weeks of illness, and only IgG or 7S antibodies were demonstrable at 9 and 53 weeks. This technique requires the use of a preparative ultracentrifuge which is expensive and not readily available in most laboratories now involved in research in parasitology. Another method for separation of the immunoglobulins is gel filtration on columns of Sephadex.(11,12) An example of results obtained using this method is shown in Fig. 3. Using this technique we were able to show not only the absence of cross reactivity between heterophile and dye test antibodies but also learned something of the molecular size of these two antibodies in the serum samples employed.(13) Although IgA antibodies were not looked for in this experiment, they usually reside in eluates collected between the first and second peaks.

To actually define an antibody as IgG, IgA or IgM one must usually resort to studies employing antisera specific for the heavy chains of these three immunoglobulins. (There is a fourth class, IgD, but antibody activity has not yet been reported in this immunoglobulin.) Very adequate reviews of this area of immunology have been covered previously and will not be discussed here.[5,9,10,14] The techniques are simple and usually immunodiffusion in agar is employed. The antisera are available from a number of supply houses in the United States and Europe. Great care must be exercised to verify the stated specificity of any purchased antiserum. For this purpose controls (specific immunoglobulins) may be purchased or obtained by chromatographic procedures. A number of investigators in the field are often willing to supply small amounts of these immunoglobulins and/or specific antisera.

It is a relatively simple matter to obtain serum IgG and IgM by chromatography on columns of diethylaminoethyl cellulose (DEAE). We first separate serum by starch block electrophoresis, an easy and inexpensive technique.[10,15] The γ-portion is then applied to a column of DEAE previously equilibrated with 0.0175 M phosphate buffer pH 6.3. The fraction eluted with that buffer contains only IgG. Then elution with 0.1 M phosphate buffer pH 5.8 results in a fraction containing IgA contaminated with IgG and IgM. Finally, elution with 0.4 M phosphate buffer in 2 M NaCl pH 5.2 will elute a very purified IgM. Recycling and use of DEAE Sephadex followed by Sephadex G-200 will produce a purified IgA preparation.[16] These immunoglobulins may then be used to immunize animals for obtaining specific

antisera for controls in immunodiffusion studies, for adsorption of non-specific antisera and for inhibition and fluorescent antibody studies.

A widely used and simple method for characterization of IgM antibodies is reductive cleavage of disulfide bonds with mercaptoethanol, which destroys IgM antibody activity.[17,18] In most instances such treatment of IgG antibodies results in no loss of antibody activity. We have used this method to differentiate between IgG and IgM dye and hemagglutination test antibodies for toxoplasma. The sera were incubated at 37°C for one hour in 0.02 M 2-mercaptoethanol. Following incubation the samples and controls were immediately diluted and run in the serologic tests. Alkylation with iodoacetamide is not necessary if the tests are run soon after treatment with 2-mercaptoethanol. Fig. 4 shows the results of such treatment in the hemagglutination test in a serum from a case of acute acquired toxoplasmosis.[4]

In laboratories without a preparative or analytical ultracentrifuge an alternative method which may be used for determining molecular weights of antibodies or their fragments obtained by enzymatic degradation is gel filtration (Sephadex).[19] There is an excellent linear correlation between the logarithim of the molecular weight of a protein and the ratio of its elution volume to the void volume of the columns. Using various forms of Sephadex, this relationship holds true for molecular weights from 13,000 to 225,000, and probably considerable extension beyond this is possible.

The availability of antisera for the different classes of human immunoglobulins allows for the use of the indirect fluorescent antibody technique

for demonstration of IgG, IgA or IgM antibodies as mentioned above for toxoplasma. Such a technique might be applied to trypanosomiasis, malaria and shistosomiasis as well as to many other parasitic diseases of man. In animal studies the problem is more involved since specific antisera for the various classes of animal immunoglobulins are not readily available. Here one would have to resort to preparing the purified immunoglobulins and their respective antisera.

The above mentioned techniques lend themselves not only to a study of serum antibody and cellular production of immunoglobulins but also to study of antibodies of body secretions and excretions.[15,20,21,22] Relatively little is known of the characteristics of coproantibody response to intestinal parasites. Approximately 90% of immunoglobulin producing cells of the lamina propria of the duodenum and jejunum produce IgA.[23] What is the role of this immunoglobulin in parasitic infections of the intestine? What is the sequence of antibody formation in the intestine to various parasites? Since little is known of the importance and function of IgA as exocrine antibody, studies employing amoeba and certain helminths may help clarify this subject.

Although it was not within the scope of this manuscript to cover extensively those techniques which might be applied to such studies, sufficient references are given to allow one to become acquainted with them. It is unfortunate that these newer techniques, many of them relatively simple to perform, have not been utilized by more laboratories in the study of parasites and of parasitism. For years the immunology of parasitic diseases

has been associated with histopathology and study of antigens for diagnosis and immunization. In recent years tremendous advances have been made in the technology of immunochemistry--and thereby in our knowledge of immunity. In this country laboratories such as those of Drs. Elvio Sadun and Irving Kagan have served as an impetus to other workers in parasitology not previously oriented to immunology. With laboratory methods now at hand for characterization of antibodies to parasites, it is hoped that such studies will be performed at least in part by, or in laboratories of, those with the greatest knowledge of the field of parasitology and appreciation of its problems--parasitologists.

CAPTIONS FOR FIGURES

FIGURE

1a Diagrammatic presentation of the sedimentation of particles in a swinging bucket centrifuge tube. The distribution of particles initially is shown in tube A. Tubes B to E show the sedimentation of particles during centrifugation and illustrate the sources of cross contamination. The sedimentation rates of particle boundaries are indicated by the angled lines. The bars at the right indicate the distribution of solvent and particles in the last tube.

1b Rate-zonal centrifugation in a conventional swinging bucket centrifuge tube. Initially a thin sample zone is layered over a liquid density gradient (A) with the tube at rest. After centrifugation (B) particles having different sedimentation rates are separated into zones at different density levels in the gradient.

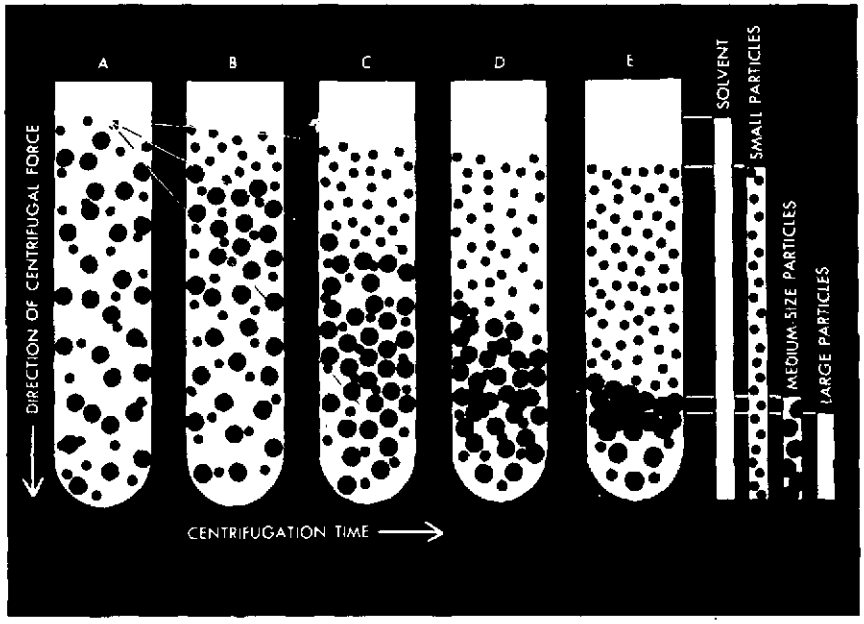
(Figures 1a and 1b reproduced by permission from Fractions, No. 1, 1965, published by Spinco Division of Beckman Instruments, Inc. Article title: "Zonal Ultracentrifugation" by Norman G. Anderson.)

2 Separation of 19S and 7S dye test antibodies by sucrose density gradient ultracentrifugation in a case of acute toxoplasmosis. Ordinate = sucrose gradient fractions. 1 through 6 = IgM. 7 through 19 = IgG.

3. Elution pattern from Sephadex G-200 of a pool of sera containing heterophile and dye test antibodies. Eluting solvent: 0.15 M NaCl.

FIGURE

4. Hemagglutination pattern in serum of case of acute acquired toxoplasmosis. Top--no treatment of serum, titer = 1:400. Bottom--serum treated with 2-mercaptoethanol, titer = <1:50.


A
B
C
D
E
SOLVENT
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MEDIUM-SIZE PARTICLES
LARGE PARTICLES
DIRECTION OF CENTRIFUGAL FORCE
CENTRIFUGATION TIME

Fig. 1 a

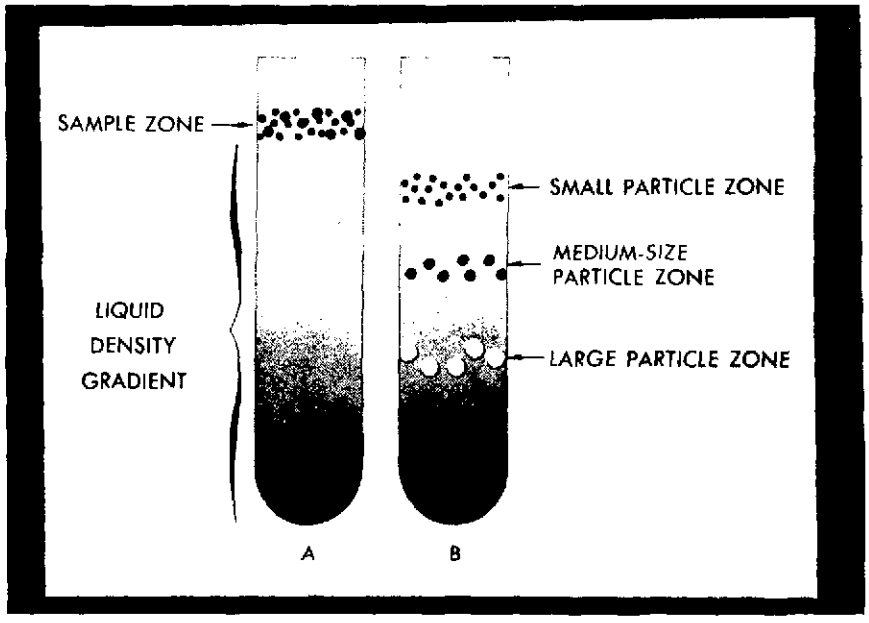
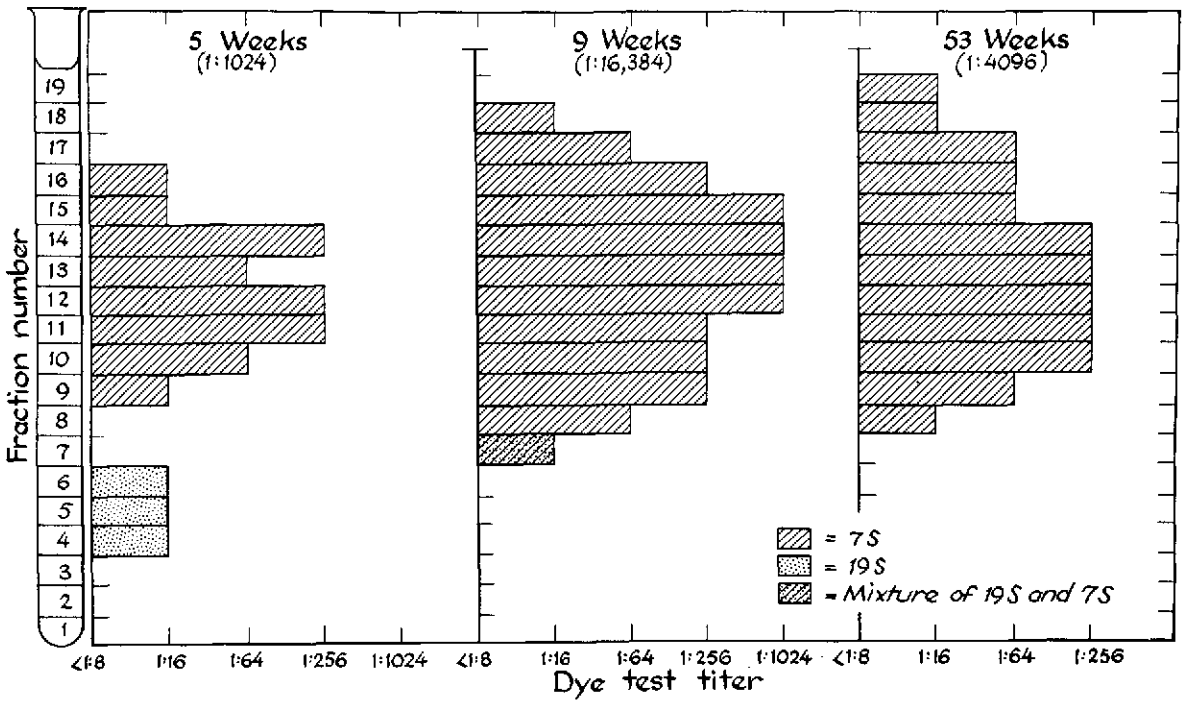

SAMPLE ZONE
SMALL PARTICLE ZONE
MEDIUM-SIZE PARTICLE ZONE
LIQUID DENSITY GRADIENT
LARGE PARTICLE ZONE
A
B

Fig. 1 b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9 Weeks
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53 Weeks
(1:4096)
Fraction number
19
18
17
16
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14
13
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11
10
9
8
7
6
5
4
3
2
1
= 7S
= 19S
= Mixture of 19S and 7S
<1:8
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1:64
1:256
1:1024
Dye test titer

Fig. 2

Fig. 3

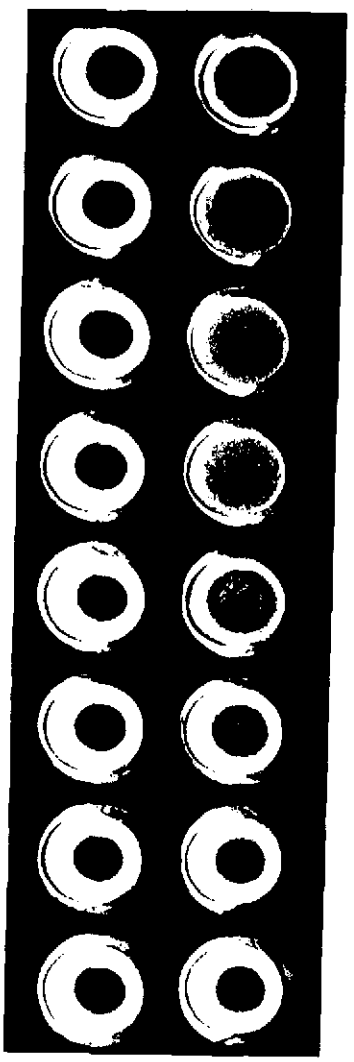
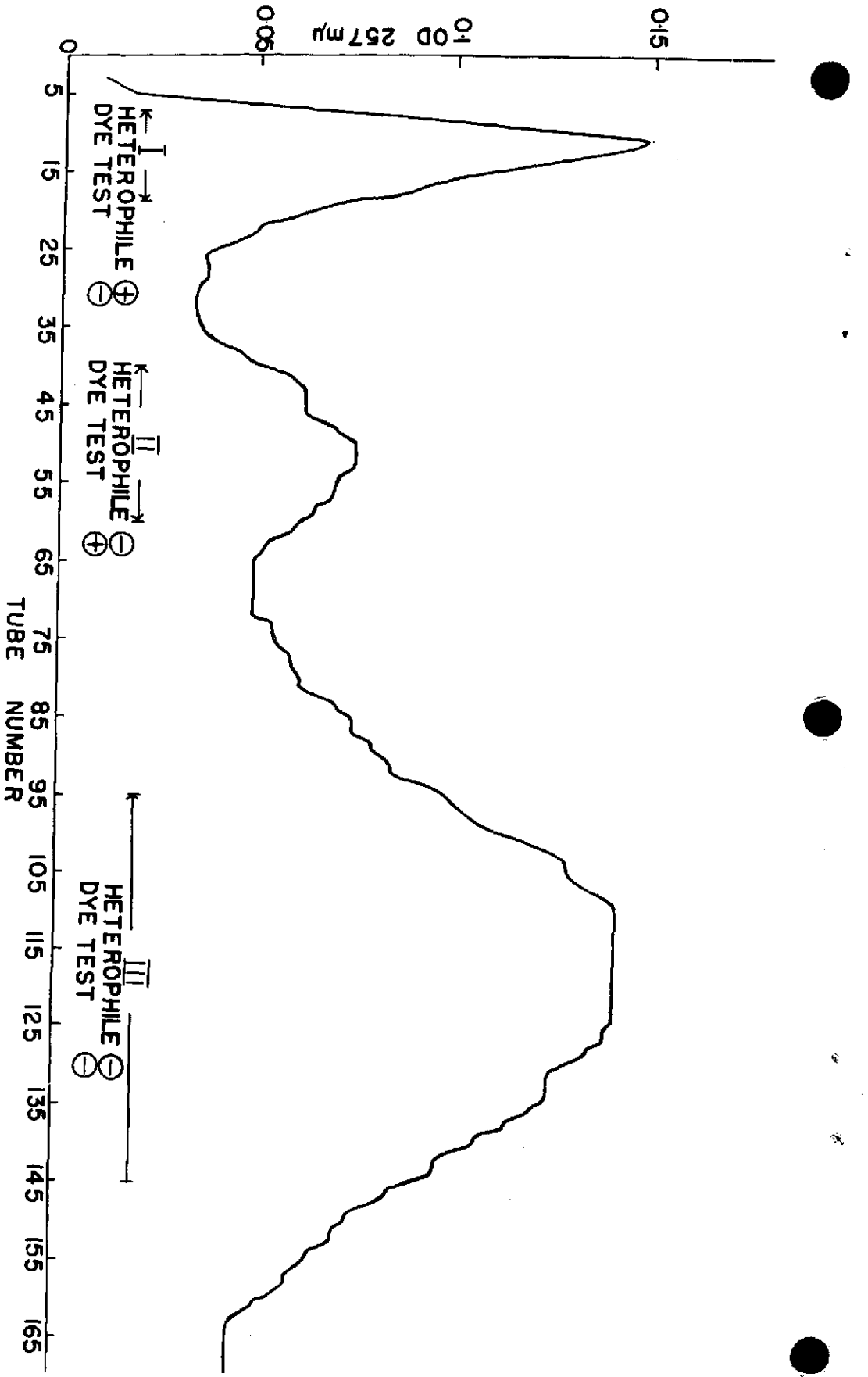


Fig. 4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105
115
125
135
145
155
165
TUBE NUMBER
HETEROPHILE DYE TEST I
HETEROPHILE DYE TEST II
HETEROPHILE DYE TEST III

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