injection. This status before the administration of the glucose load could explain the evident maintenance of the plasma level in these rats. Consideration, also, should be given to the possibility that the epinephrine-pretreatment had resulted in metabolic adjustments related to increased liver glycogenolysis and peripheral utilization of glucose in these animals so that the sudden imposition of a glucose load *per os* could be taken into the metabolic pool without seriously impairing the functions of the animal.

Summary. 1. The survival rate, and the results of tissue composition are presented for rats showing signs of failure and those surviving the administration of a glucose load *per os* (2 ml– 50% solution) 60 minutes after epinephrine-pretreatment (0.04 mg/100 g wt) and those which were not pretreated. 2. Signs of mortal failure in a large percentage of the non-pretreated series of rats shortly after glucose *per os* was associated with a significant increase in the plasma potassium concentration and a significant decrease in the liver potassium composition only. 3. In the epinephrine-pretreated series a large percentage of the rats survived the glucose load *per os*. It was shown that the level of plasma potassium and the liver potassium concentration were unchanged from the control values. The muscle potassium concentration was found significantly increased after glucose in this series. 4. Representative EKG records of non-protected and epinephrine-protected rats are shown. The EKG changes in the former group are consonant with the analytical results indicating that hyperkalemia was responsible for failure of these animals. 5. The results were discussed as suggestive of: a) the sequence of changes in the non-pretreated series which led to mortal failure and low survival rate shortly after the glucose *per os*; and b) the possible manner in which epinephrine-pretreatment had altered the metabolic status of these rats before the glucose *per os* was given and thereby "protected" them from the changes in tissue composition found in the former group.

1. D'Silva, J. L., *J. Physiol.*, 1937, v90, 303.
2. Brewer, G., Larson, P. S., and Schroeder, A. R., *Am. J. Physiol.*, 1939, v126, 708.
3. Keys, A., *Am. J. Physiol.*, 1938, v121, 325.
4. Dury, A., Holler, J. W., and Smith, C., (MMs submitted, *J. Clin. Invest.*).
5. Tum Suden, C., *Am. J. Physiol.*, 1947, v149, 589.
6. Dury, A., and Johnston, T. N., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 425.
7. Dury, A., PROC. SOC. EXP. BIOL. AND MED., 1951, v77, 199.
8. Van Slyke, D. D., *J. Biol. Chem.*, 1923, v58, 523.
9. Schales, O., and Schales, S. S., *J. Biol. Chem.*, 1941, v140, 879.
10. Kingsley, G. R., and Reinhold, J. G., *J. Lab. and Clin. Med.*, 1949, v34, 713.

Received January 14, 1952. P.S.E.B.M., 1952, v79.

Specific Inhibition of Precipitation as an Aid in Antigen Analysis with Gel Diffusion Method.* (19364)

BERTIL BJÖRKLUND. (Introduced by J. L. Melnick.)

From the State Bacteriological Laboratory and the Department of Virus Research, Karolinska Institutet, Stockholm, Sweden.

Antigenic material derived from bacteria, animal tissues, and similar sources is usually immunologically extremely heterogenous. Even chemically pure synthetic antigens almost always exhibit a complex immunological pattern giving rise to a number of distinct antibodies. The heterogeneity of antigens is not always demonstrable in the ordinary flocculation, agglutination or complement fixation procedures. More information can be gained by the use of the quantitative precipitation reactions performed according to the princi-

* This study was aided by a grant from Karolinska Institutet, Stockholm.

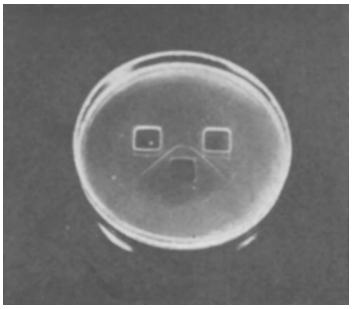

FIG. 1. Antigen analysis according to Ouchterlony. In the agar, poured in a Petri dish, three basins are spared. On the picture one sharp line of precipitate can be seen giving reaction of identity.

ples laid down by Heidelberger, but this method is complicated, requires comparatively large amounts of antigen and immune serum, and is not always sufficiently sensitive. The gel precipitation method as developed by Ouchterlony presents some advantages: very high resolving power, as practically any number of components can be individually demonstrated, and sensitivity of the same order as any of the other serological methods. This method is based upon the observation that antigens and their corresponding antibodies, diffusing towards each other in a gel, react by forming sharply defined lines of precipitation, the sites of which are dependent upon the diffusion rates and the concentrations of the reactants. By the introduction of 3 diffusion centers, 2 different antigen or antibody systems can be directly compared by means of phenomena of interaction indicating immunologic identity, partial identity or non-identity of individual components (Fig. 1-4). When working with preparations containing numerous antigenic components, it may be difficult to analyze and identify the individual components. Fusion of the lines and the very multiplicity of precipitates may give rise to analytical difficulties which can be hard to overcome. For certain analytical purposes, there is thus a need for a modification, which makes it possible to study in detail one or some of the components. The present paper reports some experiments concerning the principle and possibilities of such a modification.

Materials. Antigens. Diphtheria toxin produced on the medium of Philippe and Loiseau, dialyzed and concentrated by ultrafiltration. Tetanus toxin produced on the broth of Martin, passed through a Seitz filter and concentrated by ultrafiltration. Normal rabbit

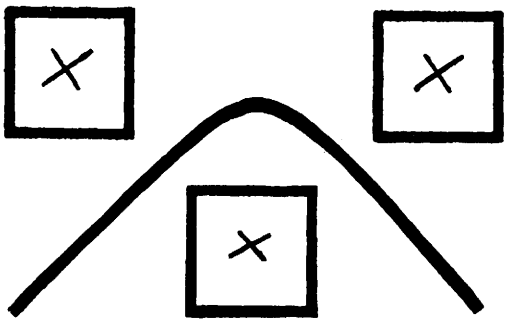

FIG. 2. Reaction of identity: If 2 antigenic preparations, both containing the antigen X, diffuse from the top basins, they will form a line of precipitate with the anti-X-serum diffusing from the bottom basin.

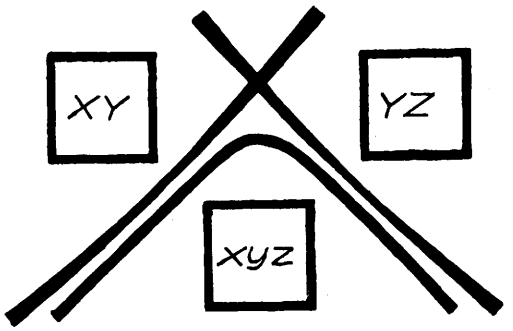

FIG. 3. Reaction of non-identity: If 2 antigenic preparations, both containing an antigenic component not in common with the other, diffuse from the top basins each one will form a line of precipitate with the corresponding antibody from the bottom basin. The lines do not interfere with each other. Further, common components give rise to a reaction of identity.

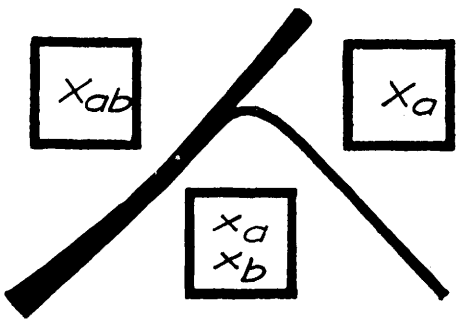

FIG. 4. Reaction of partial identity: If one of the antigens has 2 antigenic properties, one of which is in common with the other antigen, a partial fusion of the precipitates will occur.

plasma from healthy animals. Bone-marrow extract: bone-marrow from rabbits was extracted with ether, lyophilized, ground in a ball mill, and again extracted with ether. The dry powder, obtained after removal of the ether, was extracted with saline, and centrifuged. The clear supernatant was used as antigen(1).

Hyperimmune sera. Diphtheria antitoxin from a horse immunized with crude toxoid. Tetanus antitoxin from a horse immunized with toxoid. Anti-bone-marrow serum from a horse immunized with a total amount of 32.75 g dry powder of rabbit bone-marrow during a period of 4 months. (For further information see the same reference as given above).

Diffusion medium. Good quality agar was dissolved in 30 parts of distilled water, precipitated when hot with 0.5% calcium chloride, filtered, and left to congeal. It was then cut into small pieces, and rinsed in running tap water for 72 hours. After melting, an equal amount of 1.6% saline was added, and further merthiolate and methyl orange to final concentrations of 1:10,000 and 3:100,000, respectively. This agar solution was poured in Petri dishes to form a thin layer. After congelation a second layer was established, in which 3 basins were spared with the aid of a metal matrix (Fig. 1).

Methods. All diffusion plates were handled at 20°C. In order to limit evaporation of water from the gel, the plates were kept under glass cover. Sterile conditions were maintained. All plates were made in duplicate. If the precipitation patterns were not identical, both plates were discarded. For immunoprecipitations, concentration gradients were established in the diffusion medium by filling antigen preparations in the two lateral basins and immune serum in the bottom one. The basins were filled once daily for 3 consecutive days. Specific inhibition of precipitation was achieved by introduction of antigen or antibody into the gel before the precipitation experiment was started. For this purpose all 3 basins were filled for 3 consecutive days with the preparation, which was then allowed to diffuse completely into the gel. Precipitation was recorded photographically after 10 days. The plates were illuminated obliquely by reflected short wave light from a mercury lamp. Through the Tyndall effect the precipitates stood out distinctly against the orange background and pictures of high contrast were obtained with blue-sensitive film.

Experimental. In varying concentrations, the following antigens were tested for their suitability for the present experiments: diphtheria toxin, tetanus toxin, and staphylotoxin. These preparations gave distinct lines of precipitation with their corresponding antisera. As the precipitation patterns of the diphtheria and tetanus systems were the most typical and distinct ones, easy to reproduce, and could be produced simultaneously on the same side in the same plate, they were considered the most suitable ones for this study. In diffusion plates the bottom basins were filled with a mixture of diphtheria and tetanus antitoxins, the top left basins with diphtheria toxin, and the top right ones with tetanus toxin. Two distinct groups of precipitates appeared, showing the pattern of a "non-identity" reaction: on the left side that of the diphtheria system, and on the right side that of the tetanus system (Fig. 5). Although the precipitates of both preparations were complex, each pattern presented such a typical configuration that it was readily recognizable. Next, the diffusion plates were prepared beforehand with diphtheria antitoxin of a certain concentration, as described under methods, following which precipitation was attempted with the same reactants as in the previous experiment. As demonstrated in Fig. 6, the diphtheria precipitates in this case appeared closer to the top

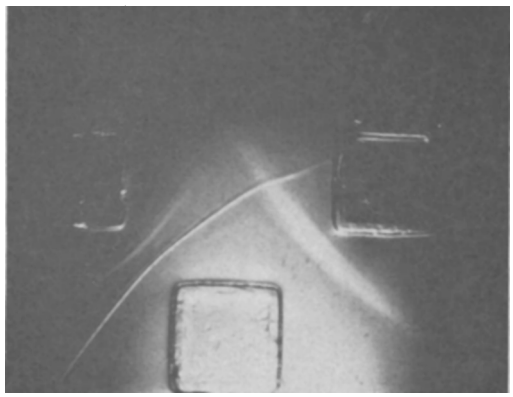

FIG. 5. Precipitation patterns of the diphtheria (left) and the tetanus (right) systems.

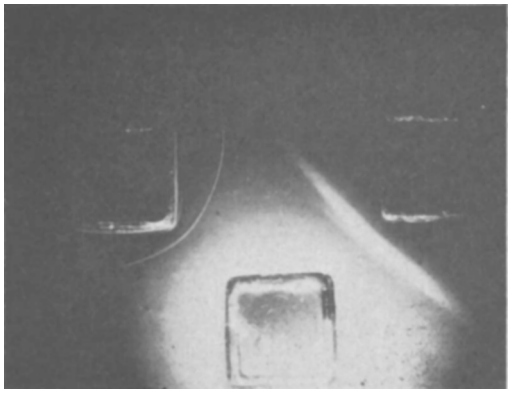

FIG. 6. Incomplete inhibition of the diphtheria lines (left).

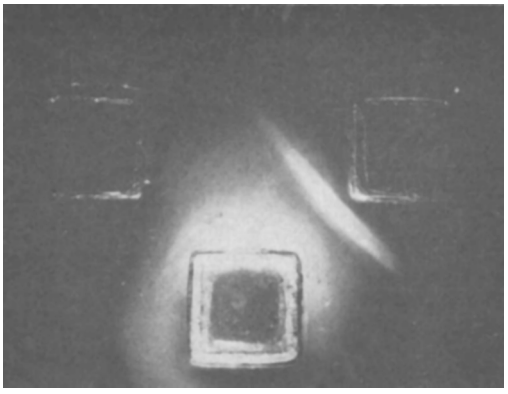

FIG. 7. Complete inhibition of the diphtheria lines.

left basin. The tetanus lines were not influenced. If in preparation of the plates a higher concentration of diphtheria antitoxin was employed the diphtheria lines could be completely inhibited (Fig. 7).

In principally the same way a systematic study of the inhibiting effect of each one of the 4 components of the present antigen-antibody system was carried out. The results are summarized in Table I. In each case the preparation of the medium with either one of a pair of reactants, antigen or antibody, in a sufficient concentration resulted in a complete inhibition of all precipitates belonging to that particular reaction, whereas the site and appearance of the precipitates of the non-inhibited reaction were not influenced. The results so far obtained indicate that the method might be useful in the antigen analysis of highly complex biological materials. This possibility was tested on the immunoprecipitation of rabbit bone-marrow and anti-bone-marrow hyper-immune horse serum.

Preliminary experiments had shown that this system presented a very complex precipitation "spectrum" with Ouchterlony's method. Furthermore, with rabbit plasma as antigen the same hyper-immune serum produced a number of precipitate lines (Fig. 8). Although some "reactions of identity" between the two antigens were obvious, a thorough analysis of the complicated pattern was not feasible.

During the preparation of the bone-marrow antigen, small amounts of blood are unavoidably retained in the material. The appearance in the hyper-immune serum of antibodies to plasma constituents is thus readily explained. It is reasonable to presume, however, that the bone-marrow contains additional cellular antigens that are not demonstrable in normal plasma. By addition of plasma to the diffusion medium in concentrations sufficient to suppress the appearance of lines belonging to the plasma-antiserum system it should be possible to bring out and analyze the reactions of the non-humoral, precipitate-forming components of the bone-marrow. Accordingly, diffusion plates were pretreated with rabbit plasma. After completed diffusion, precipitation tests were set up with anti-bone-marrow serum in the bottom basins, a preparation of rabbit bone-marrow in the top left basins, and rab-

TABLE I. Inhibition of Appearance of Precipitation. Arrangements and results.

Inhibitors	Diphtheria serum	Tetanus serum	Mixed sera	Antigens, toxins
	D		D	Diphtheria
	D	T	D+T	Mixed
		T	T	Tetanus
Diphtheria toxin				Diphtheria
		T	T	Mixed
		T	T	Tetanus
Tetanus toxin	D		D	Diphtheria
	D		D	Mixed
				Tetanus
Diphtheria serum				Diphtheria
		T	T	Mixed
		T	T	Tetanus
Tetanus serum	D		D	Diphtheria
	D		D	Mixed
				Tetanus

D = Appearance of precipitation pattern of the diphtheria system.
T = Appearance of precipitation pattern of the tetanus system.

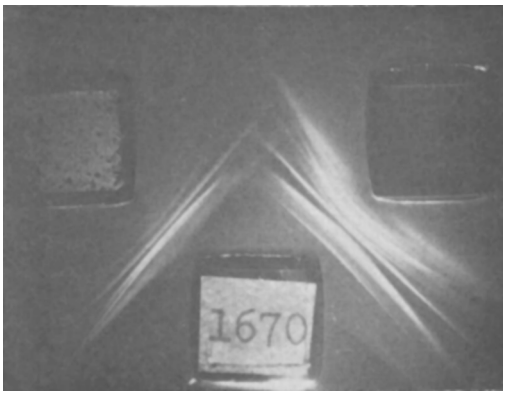

FIG. 8. The complete precipitation patterns of bone-marrow (left) and plasma (right).

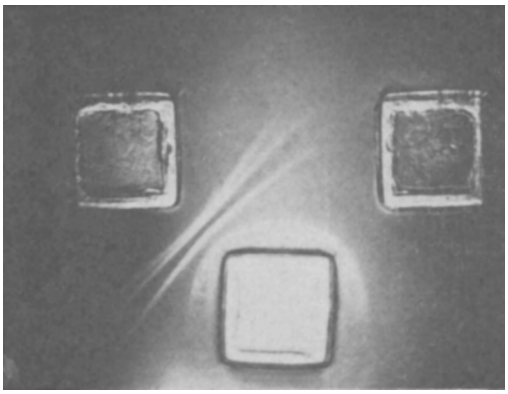

FIG. 9. Four lines of the bone-marrow (left) and incomplete inhibition of the plasma lines.

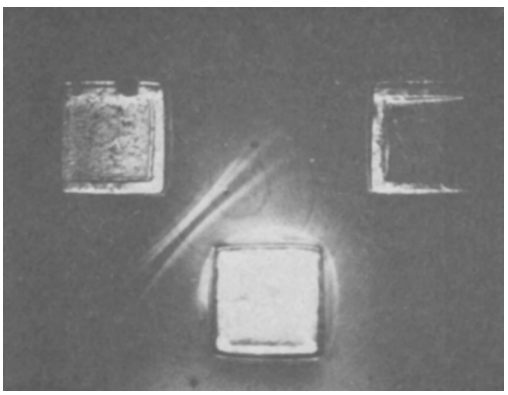

FIG. 10. Four lines of the bone-marrow and almost complete inhibition of the plasma lines.

bit plasma in the top right basins. As controls untreated plates were also used.

The appearance of lines corresponding to plasma antigens was not completely inhibited as shown by concentric precipitates around the antibody basins. However, the development of 4 distinct precipitates was evident on the left side, whereas no corresponding lines were seen on the other side (Fig. 9), a fact indicating that the bone-marrow preparation contained antigens not present in normal plasma.

It was possible by increasing the concentration of rabbit plasma in the medium to suppress almost completely the formation of plasma lines without any visible effect upon the 4 precipitates characteristic of the bone-marrow preparation (Fig. 10).

Discussion. Ouchterlony (2-8) showed that the site of an immunoprecipitate in the diffusion medium was determined i.a. by the ratio of concentrations of antigen and antibody in the diffusion centers. By variation of the concentration ratio the precipitate was displaced towards the basin with the lower relative concentration. Theoretically it should be possible to establish such conditions that the precipitate will appear not in the gel, but virtually within the basin with the lower concentration.

Introduction of one reactant in the medium prior to addition of the other is no doubt the best method to secure the high concentrations necessary to retain the precipitate within the boundaries of the basin. In this way a barrier is formed in the very wall of the basin preventing diffusion of the reactant into the medium. In principle this is equivalent to specific absorption of a serum, provided that the diffusion of other reactive constituents is not influenced by the formation of specific precipitates in the wall of the basin. If the latter condition is fulfilled the procedure would provide a simple means of eliminating selectively certain lines from a complicated precipitation spectrum, thus facilitating an antigen analysis.

From Ouchterlony's studies it would appear that the formation of an immunoprecipitate does not prevent the diffusion of constituents not involved in the specific reaction. However, before application of the procedure outlined in this paper to any actual problem it was deemed necessary to study the effect on artificial mixtures of antigens and antibodies, respectively. The system chosen for this study contained diphtheria and tetanus toxins and their antitoxins. The individual precipitation patterns of each toxin was found to

be so characteristic that identification was possible beyond doubt. The results showed clearly that elimination of the lines belonging to one component by pretreatment of the medium with either antigen or antibody had no influence whatever upon the formation of the precipitate characteristic of the other. It would seem, therefore, that the procedure can be safely applied in the analysis of complex antigenic systems.

Preliminary experiments along these lines with antigens prepared from rabbit bone-marrow gave promising results. In a subsequent paper the application of the procedure in the analysis of the antigenic structure of different organs will be described(9).

Summary. A modification of Ouchterlony's gel diffusion method for antigen analysis is described. It is based on the following observations: 1. By pretreatment of the diffusion medium with sufficient amounts of one component of a complex immunologic system the subsequent appearance in the medium of precipitates corresponding to this particular component could be completely inhibited. 2. The diffusion of the other components of the same system and the formation of other immunoprecipitates was not influenced. 3. By incorporation in the diffusion medium of individual components of a complex immunologic system it is thus possible, by elimination of some of the lines, to simplify the precipitation spectrum and facilitate the antigen analysis.

1. Björklund, B. and Hellström, L., *Acta Med. Scand.,* 1951, v139, 122.

2. Ouchterlony, Ö., *Ark. för Kemi, Mineralogi och Geologi,* 1948, v26B, 1.

3. ———, *Ark. för Kemi,* 1949, v1, 43.

4. ———, *Ark. för Kemi,* 1949, v1, 55.

5. ———, *Lancet,* 1949, v256, 346.

6. ———, *Dissertation, Karol. Inst.,* Stockholm, 1949.

7. Ouchterlony, Ö., Ericsson, H., Neumüller, C., *Acta Med. Scand.,* 1950, v138, 76.

8. Ouchterlony, Ö., *Bull. Soc. Chim. biol.,* 1951, v33, 758.

9. Björklund, B., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 324.

Received January 3, 1952. P.S.E.B.M., 1952, v79.

Serological Analysis of Components in Hemopoietic Tissue.* (19365)

BERTIL BJÖRKLUND. (Introduced by J. L. Melnick.)

From the State Bacteriological Laboratory and the Department of Virus Research, Karolinska Institutet, Stockholm, Sweden.

In a previous paper(1) by Björklund and Hellström the biological effects of a horse hyperimmune serum against rabbit bone-marrow were described. Rabbits, after receiving intravenous injections of this serum, displayed symptoms referable on one hand to a histopathologically demonstrable aplasia of the hemopoietic system and degeneration of the circulating blood cells, on the other hand to a general toxic effect. Administration of large serum doses invariably caused the death of the animals. It seemed probable that antibodies were primarily responsible for the effects observed.

It might be assumed that the preparations of bone-marrow used for immunization of the horses contained a number of substances with distinct immunological properties. Among these, some are presumably specific for different elements of the hemopoietic system, while others originate in capillary endothelium, connective tissue, serum and similar sources not intrinsically connected with the hemopoietic apparatus proper. By means of serological *in vitro* studies it should be possible to differentiate between the antigenic components and examine their distribution in different organs and tissues. The same methods might also be applied as a guide in attempts at isolation and purification of the biologically interesting antigens. The present paper reports

* This study was aided by a grant from Karolinska Institutet, Stockholm.