MIXED AGGLUTINATION WITH TISSUE SECTIONS*

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Mixed agglutination has been used for several years as a valuable research tool in studies on tissue antigens. In addition to cell suspensions (*cf.* reference 1), mixed agglutination has also been applied to monolayer cell cultures (2–4).

The major advantage of mixed agglutination procedures lies in the possibility of testing practically all cells. This is in sharp contrast to the plain agglutination technique which is applicable to only some selected cells, such as erythrocytes, that may be obtained in stable suspensions. On the other hand, all known mixed agglutination procedures, similarly as plain agglutination tests, can detect only the antigens localized on the cell surface but not those present inside the cell.

The major purpose of this study was to apply the principle of the mixed agglutination technique for the serological examination of tissue sections. It was anticipated that by using tissue sections, not only the cell surface antigens, but also the intracellular antigens would be detected.

Materials and Methods

Organs.—Organs of healthy laboratory animals were removed after exsanguination. Organs of other animals were obtained from a slaughter house. Suitable pieces of the organs were quickly frozen on dry ice or in liquid nitrogen. If not used immediately they were stored at −20°C.

Immune Sera.—Immune sera which had been prepared for earlier studies on tissue antigens were employed (4–7). All antisera were of rabbit origin; they had been previously investigated by conventional serological methods. Glycerinated rabbit anti-sheep erythrocyte serum (amboceptor) was purchased from Baltimore Biological Laboratories and stored at 4° to 8°C. Antiserum against rabbit serum was produced in this laboratory by the intramuscular immunization of a goat with rabbit serum precipitated by potassium alum. All antisera were inactivated for 30 minutes at 56°C.

Tissue Sections.—Microtome sections were cut in a cryostat following the usual procedure (8). They were 2 to 6 μ thick and had a diameter of about 6 mm. The tissue section was placed in the center of a coverglass 40 mm long, 20 mm wide, and approximately 0.2 mm thick (Text-fig. 1 *a*). The tissue was thawed by placing the underside of the coverglass on the fingertip for

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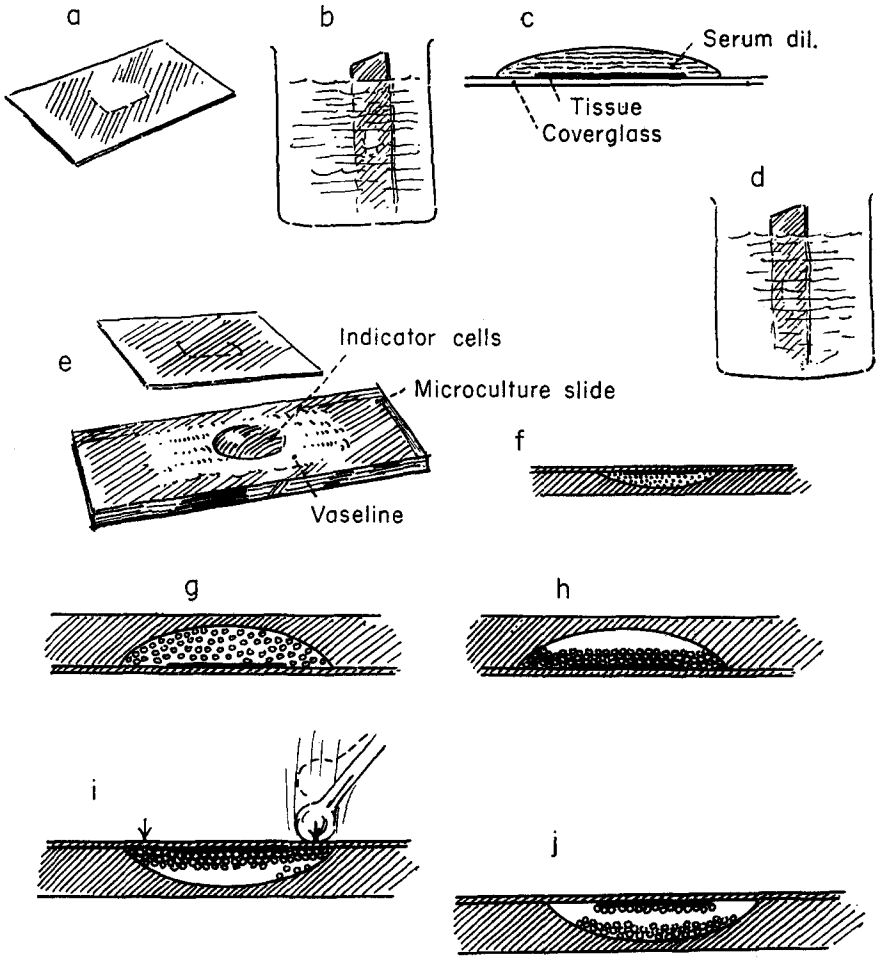
a few seconds. On melting, the tissue adhered to the glass. If not used immediately, the sections were stored at −20°C in tightly closed boxes.

Fixation.—All tissue sections were air-dried by leaving them for 10 minutes at room temperature. In some instances they were tested without any further fixation. For most experi-



TEXT-FIG. 1. Illustration of the procedure of mixed agglutination with tissue sections. For explanation see text.

ments, however, fixatives were used: air-dried tissue sections were submerged in acetone or 4 per cent neutral formaldehyde for 1 to 2 minutes (Text-fig. 1 *b*), and then they were dried at room temperature for about 10 minutes.

Some experiments were performed with heated sections. Acetone- or formalin-fixed sections were placed in a wet chamber which was then left in a boiling water bath for 1 hour.

Treatment with Antiserum.—Serial dilutions of heat-inactivated serum were made in phos-

phate-buffered saline, pH 7.2. One drop of diluted serum was placed on each tissue section (Text-fig. 1 *c*). The sections were placed in a wet chamber and incubated at 37°C for 30 minutes. After incubation the excess serum was removed and the sections were washed by submerging the coverglasses vertically in beakers with buffered saline for 5 to 20 minutes (Text-fig. 1 *d*).

Indicator System for Mixed Agglutination.—The indicator system was designed to demonstrate the binding of rabbit gamma globulin to the tissue sections; it was prepared in a similar way as the indicator system used in previous studies on mixed agglutination with cell culture monolayers (4). A 4 per cent suspension of washed sheep erythrocytes was mixed with an equal volume of amboceptor at a subagglutinating dilution corresponding to ½ or ¼ of the agglutination titer. The amboceptor used in most experiments had an agglutination titer of 1:1500, and accordingly the sensitizing dilutions used were 1:3000 or 1:6000. The erythrocyte-amboceptor mixture was left at room temperature for about 10 minutes, and then an equal volume of goat antiserum against rabbit serum was added. The latter antiserum was employed at a dilution of 1:20, which was a considerable antibody excess inasmuch as this serum agglutinated sensitized sheep erythrocytes up to a dilution of 1:1000. The mixture was left at room temperature for 15 minutes. During this time the erythrocytes were agglutinated in very small clumps. The erythrocytes were washed twice with saline; after each centrifugation they were thoroughly resuspended. After the second washing the cells were suspended to a concentration of 1 per cent.

Technique of Mixed Agglutination.—Microculture slides with a single concavity were used (Text-fig. 1 *e*). A thin layer of vaseline was spread on the slide surface around the concavity which was then filled with the indicator system using a Pasteur pipette. The indicator cells were thoroughly resuspended before application. The coverglass with the tissue section was removed from the saline and placed on the microculture slide in such a way that the tissue section was submerged in the indicator system in the center of the concavity. The coverglass was pressed so that it would adhere tightly to the microculture slide (Text-fig. 1 *f*). Care was taken to avoid any air bubbles which would later interfere with reading. The microculture slide was inverted, coverglass down (Text-fig. 1 *g*), placed in a wet chamber and left for 2 hours at 37°C. During this time the indicator cells settled down, covering the tissue (Text-fig. 1 *h*). Occasionally the incubation period was extended for an additional 3 to 16 hours at 4°C. This did not seem to influence the results to any significant degree.

After incubation the slides were cleaned of moisture that had accumulated on their surface. They were again turned over, coverglass up. In order to accelerate the detachment of erythrocytes, the coverglass was tapped gently around the tissue section (Text-figs. 1 *i* and 1 *j*). If there was no reaction between the indicator cells and the tissue, the cells detached first from the tissue and then from the glass around the tissue. In positive reactions the cells detached only from the glass. Obviously, the purpose of tapping was to accelerate the detachment of loosely adhering erythrocytes without causing the detachment of serologically bound erythrocytes. With some experience this can be achieved quite easily.

Reading.—The preparations were examined microscopically at 100-fold magnification. The coverglass around the tissue section was first examined in order to find out whether all erythrocytes were detached from the glass; if this was not the case, additional tapping on the coverglass was applied. Then the tissue section was examined for the attachment of erythrocytes. Care was taken to have the microscope exactly focused on the tissue. Otherwise the erythrocytes which sedimented on the bottom of the concavity might be mistaken for erythrocytes attached to the tissue. With some experience such a mistake can hardly be made. Before this experience was gained it was found convenient to use 400-fold magnification to clearly distinguish the attachment of erythrocytes to the tissue layer. Positive reactions were graded according to how heavily the tissue was covered by erythrocytes. A strongly positive reaction (3+) was characterized by complete covering of the tissue by erythrocytes (Fig. 1 *a*). In

moderately (2+) and weakly (1+) positive reactions partial covering of the tissue was observed (Figs. 1 *b* and 1 *c*). In negative tests all erythrocytes were sedimented, and no attachment to the tissue was noticed (Fig. 1 *d*). The titers were expressed as the reciprocals of the highest dilution of antiserum that gave at least 1+ reaction.

Absorption of Antisera.—The serum was diluted 1:10 and mixed with an equal volume of 20 per cent organ extract (5). The tubes were left for 1 hour at 37°C with occasional shaking, and centrifuged at high speed in a refrigerated centrifuge. Absorption was repeated until there was no further decrease in titer; usually the sera were exhausted after the second absorption.

RESULTS

Acetone-fixed tissue sections of bovine adrenal and kidney were tested with 5 antisera against antigenic preparations of bovine organs. The results of this experiment are presented in Table I. Positive reactions were obtained with antisera against both organ suspensions and extracts. No indication for organ

TABLE I

Mixed Agglutination with Acetone-Fixed Tissue Sections of Bovine Adrenal (A) and Kidney (B) and Antisera against Antigenic Preparations of Bovine and Rat Organs

		Antiserum diluted 1:					
		100	500	2500	12,500	62,500	312,500
Antisera against:							
Bovine adrenal suspension 1603	A	3+	3+	3+	2+	1+	—
	B	1+	3+	3+	1+	1+	—
Bovine spleen suspension 1612	A	3+	3+	2+	1+	±	—
	B	2+	2+	2+	1+	1+	—
Bovine brain suspension 1671	A	3+	3+	3+	1+	±	—
	B	2+	2+	2+	±	—	—
Bovine liver suspension 1946	A	3+	3+	3+	3+	2+	1+
	B	1+	2+	2+	2+	1+	±
Bovine liver extract 1942	A	1+	2+	2+	2+	1+	1+
	B	1+	2+	2+	1+	±	—
Rat liver suspension 1204	A	±	1+	—	—	—	—
	B	1+	1+	—	—	—	—
Rat kidney suspension 1205	A	1+	2+	—	—	—	—
	B	1+	2+	—	—	—	—
Normal rabbit serum	A	1+	—	—	—	—	—
	B	1+	±	—	—	—	—

specificity can be noticed in Table I. An antiserum against adrenal suspension gave equal reaction titers on adrenal and on kidney sections. Similarly, no significant difference was observed between the reaction titers obtained on adrenal sections with adrenal antiserum on one hand and with antisera against liver, brain, and spleen on the other hand. However, the observed reactions were species-specific inasmuch as the antisera against rat organs gave considerably weaker results than antisera against bovine organs. Normal rabbit serum gave weak positive reactions. Heating of this serum for 30 minutes at 65°C resulted in the complete disappearance of its activity, whereas similar treatment of immune sera against bovine organs hardly affected their titers.

Positive results with antisera against organ extracts clearly indicated that the mixed agglutination detects saline-extractable antigens on tissue sections. The question arose as to whether saline–non-extractable antigens can also be detected. The experiments presented in Table I could not answer this question

TABLE II

Mixed Agglutination with Acetone-Fixed Tissue Sections of Bovine Adrenal. Effect of Absorption of Anti-Bovine Liver Sera with Liver Extract

	Titer in reaction with serum	
	Unabsorbed	Absorbed
Anti-bovine liver suspension serum 1946	51,200	12,800
Anti-bovine liver extract serum 1942	25,600	800

inasmuch as the results with antisera to organ suspensions could be due to the reaction with either of these two types of antigens. An experiment was performed in which antiliver suspension serum was extensively absorbed with liver extract. As is to be seen in Table II this absorption only slightly diminished the activity of the antiserum whereas similar absorption of an antiserum against liver extract resulted in a rather considerable decrease of its activity. From this and other similar experiments it could be concluded that the procedure under investigation is capable of detecting both saline-extractable and saline–non-extractable antigens.

Experiments were performed to compare the effect of acetone and formalin fixation. The effect of heating the sections was also investigated. As is to be seen in Table III, sections of adrenal exposed to acetone or formalin fixation combined with all antisera to bovine organs under study. Apparently the species-specific antigens detected by these antisera were not affected by either of these fixatives. After exposure to 100°C, adrenal sections ceased to combine with antisera to liver, spleen, and brain, but they still combined with the homologous antiserum even if its reaction titer decreased to a certain extent.

Apparently the species-specific antigens were destroyed by exposure to elevated temperature, whereas some organ-specific adrenal antigens were not affected by such treatment.

In most instances acetone fixation of brain sections resulted in the complete destruction of their serological activity (Table III). In some experiments the

TABLE III

Mixed Agglutination with Tissue Sections of Bovine Adrenal and Brain and Antisera against Bovine Organ Suspensions

	Titer in reaction with bovine					
	Adrenal			Brain		
	A	B	A + C	A	B	B + C
Antisera against bovine organ suspensions						
Adrenal 1603	62,500	62,500	2500	<20	12,800	<20
Liver 1946	312,500	62,500	<20	<20	12,800	<20
Spleen 1612	12,500	12,500	<20	<20	6400	<20
Brain 1671	12,500	12,500	<20	<20	25,600	12,800
Preimmune serum 1612	100	200	<20	<20	200	<20

A, fixation with acetone; B, fixation with formalin; C, heating at 100°C.

TABLE IV

Mixed Agglutination with Formalin-Fixed Tissue Sections of Rabbit Adrenal and Brain and Antisera against Bovine Organs

	Titer in reaction with rabbit	
	Adrenal	Brain
Antisera against bovine organ suspensions		
Adrenal 1603	<20	<20
Spleen 1612	<20	<20
Brain 1671	<20	6250

acetone-fixed brain sections retained some activity, however, the reaction titers were considerably lower than those obtained with formalin-fixed sections.

Formalin-fixed brain sections gave positive reactions with all tested antisera against bovine organs. Heated sections of brain were incapable of combining with antisera to adrenal, liver, and spleen, however, they still combined with the brain antiserum. Apparently, here again the species-specific antigens were destroyed by heating, whereas the organ-specific antigens were not affected by this treatment.

The results of experiments with tissue sections originating from the rabbit, *i.e.* the antibody-producing species, are presented in Table IV. It should be noted that adrenal sections did not combine with any antiserum under investigation, whereas the brain sections combined with the brain antiserum. This finding appears inconsistent with the above discussed experiment that demonstrated organ-specific antigens in both adrenal and brain sections of bovine origin. One should keep in mind, however, that the antisera used were obtained by immunization with bovine organs. It appears quite plausible that the organ-specific antigens detected in bovine adrenal sections is not shared by rabbit adrenal whereas the brain-specific antigens have similar serological structure in ox and rabbit and possibly also in many other species.

DISCUSSION

The experimental data presented above demonstrated that the mixed agglutination technique may be applied to tissue sections. The attachment of rabbit antibody molecules to the tissue sections was detected by an indicator system consisting of sheep erythrocytes sensitized by subagglutinating doses of rabbit anti-sheep erythrocyte serum and agglutinated by goat antiserum against rabbit serum.

The use of microculture slides for the present procedure provided a closed chamber preventing evaporation and drying out of the preparations. Therefore, the preparations could be preserved at 4°C for as long as 3 days without deterioration.

The preliminary experiments presented in this paper showed that the selection of proper fixatives constitutes a crucial problem in employing this procedure. It might appear that a most convenient technique would be to use tissue sections that were only air-dried. However, this was technically difficult since sections prepared in this way very frequently detached from the slide. It is also probable that saline extractable antigens would be eluted from such sections. It seems therefore likely that one or another fixative has to be used, depending on the physicochemical properties of the antigens under investigation.

In titrating antisera by the described procedure very high titers up to and above 1:100,000 were observed proving that this method similarly to all agglutination tests, is very sensitive in detecting antibodies. In repeating the tests, very similar titers were obtained pointing to good reproducibility of the results.

Most experiments were performed using sections of bovine organs and antisera against suspensions and extracts of bovine organs. Positive results were obtained with antisera of both types. This was in contrast to the results obtained on monolayers of cell cultures where only the antisera against organ suspensions but not against organ extracts gave positive reactions (4). The monolayer procedure was capable of detecting only some saline–non-extractable antigens of

the cell surface. In the present procedure, in addition to these antigens, also the saline-extractable antigens were detected.

It was demonstrated that mixed agglutination on tissue sections detects some species-specific antigens, and in addition, organ-specific antigens. Here again the present procedure was superior to agglutination on tissue cultures by which no organ-specific antigens have thus far been detected (4). In interpreting this difference one may consider that the organ-specific antigens under investigation are intracellular components which cannot be detected by an antibody on the intact cell, but which were made available in tissue sections as a result of opening the cells. It is also possible that in culturing the cells, the organ-specific antigens disappear which obviously is not the case when sections of fresh tissue are examined.

On the basis of the results obtained, it appears that in the described reactions practically all tissue antigens may participate. Because of this feature, and of the very high sensitivity in detecting the antibodies, the described method might be a useful tool for studies on autosensitization to tissue antigens, which frequently is characterized by rather weak antibody formation. In addition, the observations on the effect of exposing the sections to fixatives and heat showed that the described procedure can be used as a relatively simple method for investigations on some physicochemical properties of tissue antigens.

Obviously, the principle of mixed agglutination with tissue sections is very similar to the principle of the indirect immunofluorescent staining technique. In both procedures the antibodies bound to the tissue sections are detected by the corresponding anti-gamma globulin antibody. Whereas in the latter technique the anti-gamma globulin antibody is labeled with fluorescent dye, in the former technique it is "labeled" by an erythrocyte. These two techniques, however, differ considerably in the applicability. The immunofluorescent technique cannot compete with mixed agglutination for sensitivity in detecting antibodies. On the other hand, the mixed agglutination technique cannot compete with the immunofluorescent technique in the accuracy of pinpointing the localization of the tissue antigen.

The experiments presented in the present paper were limited to rabbit antisera. The possibility of using a similar procedure for investigations on human antibodies is of particular interest. Preliminary experiments along these lines have been promising.

SUMMARY

The mixed agglutination procedure was applied to tissue sections. Microtome sections of bovine tissue were placed on coverglasses, fixed by acetone or formalin, and incubated with various dilutions of rabbit antisera. The binding of antibodies to the tissue sections was detected by the addition of an indicator system composed of sheep erythrocytes sensitized by subagglutinating doses of the

corresponding rabbit antiserum, and agglutinated by goat antiserum to rabbit serum. In positive reactions the indicator cells covered the tissue, whereas in negative tests, the erythrocytes detached and the tissue appeared uncovered.

It was demonstrated that the method is capable of detecting both saline-extractable and saline–non-extractable antigens. In addition to species-specific antigens, organ-specific antigens of adrenal and brain were detected. The method was characterized by a very high sensitivity in detecting antibodies.

Its possible application as a tool for investigations on tissue antigens and antibodies was discussed.

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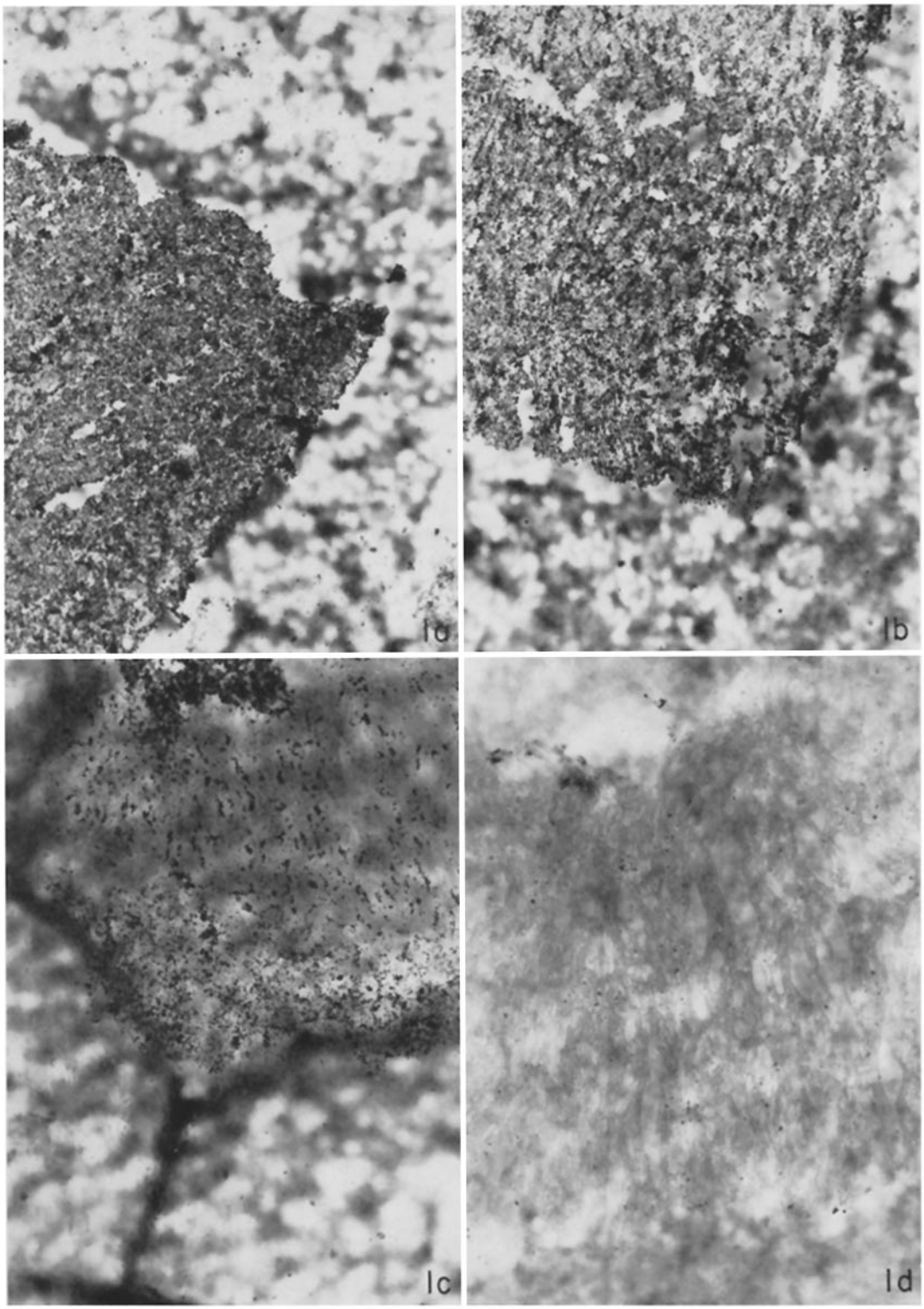
EXPLANATION OF PLATE 18

FIGS. 1 *a* to 1 *d*. Pictures of microscopical readings of mixed agglutination. ×30.

FIG. 1 *a*. Strong mixed agglutination. The tissue is so heavily covered with erythrocytes that it is difficult to see at lower magnification.

FIG. 1 *b*. Moderate mixed agglutination. The tissue can be distinguished between the adhering erythrocytes. Fig. 1 *c*. Weak mixed agglutination. Scattered distribution of erythrocytes attached to the tissue.

FIG. 1 *d*. No mixed agglutination. All erythrocytes detached from the tissue.



(Tönder *et al.*: Mixed agglutination)