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The AB0 Grouping of Stains from Body Fluids

Angelo Fiori and Paolo Benciolini

Catholic University of S. Heart Institute of Legal Medicine, Rome,
and University Institute of Legal Medicine, Padua (Italy)

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Summary. The absorption-elution and absorption-inhibition methods have been used for AB0 grouping of stains from saliva and semen.

Absorption-elution requires an optimal antigen-antibody ratio and this explain the erroneous negative results which are frequently obtained. Absorption-inhibition is a reliable technique for grouping stains from secretor individuals. When this test is negative absorption-elution should be used as a complementary method is more likely to give positive results especially on stains containing large amounts of cells.

Zusammenfassung. Zwecks Bestimmung der AB0-Antigene in Spuren von Speichel und Sperma bedienten sich die Verfasser der Adsorptions-Elutions- und Adsorptions-Inhibitions-Methodiken.

Die Absorptions-Elutions-Methodik erfordert ein optimales Antigen-Antikörper-Verhältnis; so erklären sich die fälschlicherweise negativ ausfallenden Befunde, die öfters bei Untersuchung solcher Spuren unterlaufen. Die Adsorptions-Inhibitions-Methodik ist statt dessen die geeignete Technik zur Gruppenbestimmung an Materialspuren von Ausscheidern. Fällt diese Untersuchung negativ aus, so muß komplementär die Adsorptions-Elutions-Technik angewendet werden; diese kann positive Befunde liefern, besonders wenn die Spuren reichlich Zellmaterial enthalten.

Key words: Secretion stains, detection — Secretor individuals — AB0 bloodgroups, saliva, semen.

The AB0 grouping of stains from body fluids is usually carried out with the absorption-inhibition method. On the other hand the absorption-elution and the mixed agglutination techniques are now preferred for AB0 and also for MN grouping of blood stains (Kind, 1960; Yada, 1961; Coombs and Dodd, 1961; Outteridge, 1962; Nickolls and Pereira, 1962; Fiori *et al.*, 1963; Benciolini, 1964b).

Nickolls and Pereira (1962) and Pereira (1963) claimed positive results using the absorption-elution method also for grouping stains from saliva and semen. These findings were confirmed by Pappalardo (1964) on seminal fluid stains. Mixed agglutination was also successfully employed by Dodd and Hunter (1963), and Pereira and Dodd (1966) for grouping stains from semen and saliva, and the reliability of both techniques for this purpose was again confirmed by Pereira and Dodd (1966).

On the other hand Benciolini (1962a) and Fiori *et al.* (1963) reported negative results using the elution method for AB0 grouping of stains of different body fluids, especially saliva. Independently, Ueno (1963) also reported completely negative findings with the same method: in their experiments the stains pertaining to different groups of the AB0 system always behaved as 0 group stains. These results were confirmed by Fiori and Benciolini (1965, 1966) and by Marcinkowski (1966).

With the purpose of elucidating the cause or causes of these contrasting results, the experiments previously reported (1965, 1966) were repeated and new experimental procedures were worked out.

Material and Methods

1. *Saliva Stains.* 56 specimens of saliva were used for preparing stains on linen cloths and on Whatman No. 1 paper sheets. The secretor status of the donors was previously determined on each sample according to Boorman and Dodd. 24 samples of group A (17 secretors and 7 non-secretors), 22 of group B (16 secretors and 6 non-secretors), 10 of group AB (7 secretors and 3 non-secretors) were examined.

From each specimen of saliva stains were prepared in different amounts (from 5 μl to 1 ml) of (a) untreated whole saliva; (b) boiled and centrifuged saliva, using separately the supernatant and the cell-rich sediment previously washed 4—5 times with saline; (c) untreated saliva in various dilutions.

2. *Seminal Stains.* 33 specimens of seminal fluid were examined: 16 from group A subjects (13 secretors and 3 non-secretors), 13 from group B (10 secretors and 3 non-secretors) and 4 from group AB (3 secretors and 1 non-secretor).

The determination of the secretor status and the sperm count were performed on each sample before preparing the stains. These were made with different amounts (from 5 μl to 1 ml) of (a) untreated seminal fluid, (b) the supernatant of centrifuged seminal fluid and separately the several times washed sediment, (c) various dilutions of the cell-free supernatant.

3. *ABO Grouping.* Each type of stain was grouped by 3 modifications of the original absorption-elution method of Siracusa (1923): the slide technique of Kind, the slide method of Nickolls and Pereira, the test tube method of Fiori *et al.*

The absorption-inhibition method, according to the technique proposed by Holzer (1931), was used as a control.

Commercial sera, titred 1/128—1/256 were employed for absorption-elution, and antisera titred 1/16 for the absorption-inhibition test. The H antigen was not examined.

Results

1. The absorption-elution method gave variable results on stains from *untreated whole saliva of secretors.* Sometimes a completely negative (false positive) result was obtained, i.e. stains from A, B and AB subjects behaved as group 0 stains. Sometimes positive results according to the group of the subject were recorded. Finally, paradoxical data were sometimes obtained, i.e. a stain of group A was grouped as B and viceversa. Regular results were recorded in most cases with stains from non-secretors.

Some differences were observed with the 3 modifications of the absorption-elution test: the negative results were obtained especially with the test tube method while the paradoxical data were given by the slide tests.

Some typical experiments are reported in Table 1.

A regular and specific grouping was obtained with the 3 absorption-elution tests on stains made with *the washed sediment of boiled saliva.* On the other hand variable results were obtained on this material by means of the absorption-inhibition method, i.e. in many experiments inhibition was observed.

A stronger positivity of the absorption-elution test was clearly recorded on stains previously fixed by methanol or by heating. This has been confirmed by performing some control determinations by the Fiori *et al.* and Kind methods without previous fixation of the stains.

Table 1. *Stains of untreated saliva*

No.	Blood group	Absorption-elution methods						Blood group of the stain with the inhibition test
		fixed stains	anti-A	anti-B	unfixed stains	anti-A	anti-B	
1	A	Kind	+++	—	Nikolls	+	—	A
		Fiori *et al.*	++	—	Pereira			
2	A	K	—	+[a]	NP	++	—	A
		F	—	—				
3	A	K	++[b]	+[b]	NP	—	+[a]	A
		F	—	—				
4	B	K	+[b]	++[b]	NP	—	++	B
		F	—	+				
5	B	K	+[b]	+++[b]	NP	+[a]	—	B
		F	—	+				
6	B	K	—	+	NP	+[b]	++[b]	B
		F	—	—				
7	A non secr.	K	+++	—	NP	++	—	—
		F	++	—				
8	B non secr.	K	—	++	NP	++	—	—
		F	—	+				

[a] Paradoxical results. + agglutination
[b] Non specific results. — no agglutination

The *supernatant of boiled, undiluted saliva from secretors* gave in most cases *negative results* and the stains were therefore grouped as 0 stains. Some weak positive results were obtained in non-secretors.

The absorption-inhibition test was regularly positive in secretors, usually negative in non-secretors.

Some typical experiments on stains prepared with the supernatant and with the washed sediment are reported in Table 2.

2. The stains of *untreated whole seminal fluid* gave frequently regular positive reaction with the absorption-elution slide tests. Stronger positivity was obtained on stains rich in spermatozoa. On the other hand stains from azoospermic secretors behaved as group 0. Paradoxical results were sometimes obtained as in saliva stains.

The test tube method gave almost constantly negative results.

Constantly positive data were recorded by the 3 methods in the case of stain from normospermic non-secretors.

Some experiments are reported in Table 3.

The experiments performed on the stains from the supernatant of boiled or unboiled seminal fluid and from the washed sediment gave data very similar to that reported for the saliva stains prepared in the same manner.

3. To elucidate whether the contrasting results could be determined by the different experimental conditions, with special regard to the antigen-antibody ratio, experiments were carried out on stains from diluted saliva. Several 5 µl

Table 2. *Stains of boiled saliva*

	No.	Blood group	Absorption-elution methods						Blood group of the stain with the inhibition test
			fixed stains	anti-A	anti-B	unfixed stains	anti-A	anti-B	
Supernatant	1	A	K	—	—	NP	—	—	A
			F	—	—				
	2	A	K	—	—	NP	—	—	A
			F	—	—				
	3	A	K	—	—	NP	—	±[a]	A
			F	—	—				
	4	B	K	—	—	NP	—	—	B
			F	—	—				
	5	B	K	—	—	NP	—	—	B
			F	—	—				
	6	B	K	—	—	NP	—	—	B
			F	—	—				
	7	A non secr.	K	+	—	NP	—	—	—
			F	—	—				
	8	B non secr.	K	—	—	NP	—	—	—
			F	—	—				
Washed sediment	1	A	K	+++	—	NP	+	—	A
			F	++	—				
	2	A	K	++	—	NP	+	—	—
			F	++	—				
	4	B	K	—	++	NP	—	+	—
			F	—	++				
	5	B	K	—	+++	NP	—	+	—
			F	—	++				
	7	A non secr.	K	++	—	NP	+	—	—
			F	++	—				
	8	B non secr.	K	—	+++	NP	—	++	
			F	—	++				

[a] Paradoxical results.
[b] Non specific results.

stains from saliva of secretors of known titer were prepared with a microsyringe on white cloths, using the supernatant of centrifuged saliva undiluted and diluted 1/2, 1/5, 1/10, 1/25, 1/50.

The stains were fixed 5 min with methanol which was then allowed to evaporate to dryness. The stains were incubated overnight at +4°C with 0.1 ml antisera of various titers (1/64 to 1/256). The antiserum was recovered and retitred for the inhibition test. The stains were washed and eluted at 56°C for 10 min for a tube absorption-elution test.

Table 3. *Semtnal stains*

No.	Blood group	Sperm cells in seminal fluid	Absorption-elution method						Blood group of the stain with the inhibition test
			fixed stains	anti-A	anti-B	un-fixed stains	anti-A	anti-B	
11	A	XX	K	—	+[a]	NP	—	—	A
			F	—	—				
12	A	XXX	K	++	—	NP	+	—	A
			F	—	—				
13	A	XX	K	—	—	NP	—	—	A
			F	—	—				
14	A	XXXX	K	++	—	NP	+	—	A
			F	+	—				
15	B	XXX	K	—	++	NP	—	+	B
			F	—	—				
16	B	X	K	—	—	NP	—	—	B
			F	—	—				
17	B	=	K	—	—	NP	—	—	B
			F	—	—				
18	AB	XX	K	—	+	NP	—	—	AB
			F	—	—				
19	A non secr.	XXX	K	++	—	NP	++	—	A
			F	++	—				
20	A non secr.	XX	K	++	—	NP	++	—	A
			F	+	—				
21	B non secr.	XXX	K	—	+++	NP	—	++	B
			F	—	++				
22	B non secr.	XXX	K	—	++	NP	—	++	B
			F	—	++				

[a] Paradoxical results. X
[b] Non specific results.

Table 4
Influence of the antigen-antibody ratio on the absorption-inhibition and absorption-elution tests

Saliva stain (5 µl)	Absorption-inibition method				Absorption-elution method
	1/2	1/4	1/8	1/16	
Undiluted	w	—	—	—	(+)
1/2	(+)	w	—	—	(+)
1/5	+	(+)	w	—	(+)
1/10	++	+	(+)	w	++(+)
1/25	++	++	+	(+)	++(+)

w: very weak agglutination.

By means of these new experiments it has been shown that the saliva stains which have a sufficiently strong inhibitory activity, i.e. a high content of specific antigens, do not release the absorbed antibodies when the absorption-elution test is performed by the test tube method of Fiori *et al.* On the other hand, when inhibitory activity decreases, e.g. in minimal stain of diluted saliva, the elution test becomes clearly positive.

In Table 4 a dimostrative experiment is reported.

Discussion

The experiments reported here confirm that erroneous results can be obtained in AB0 grouping of stains from body fluids by means of the absorption-elution method and give, at the same time, an explanation of the contrasting experience reported by the workers in the field.

1. A quite different antigen-antibody ratio is apparently required for a good performance of the absorption-inhibition and of the absorption-elution test employed for AB0 grouping of blood and secretion stains.

For the absorption-inhibition method, a relative excess of antigens is needed to saturate all or most reacting antibodies and thus to make the inhibition reaction clearly interpretable. For this reason, low titred sera and relative large amounts of the stain are usually employed for grouping both blood and secretion stains.

The absorption-elution test, on the other hand, requires a relative excess of antibodies. In fact, in grouping blood stains the best results are obtained by using high titred sera and minimal size stain. We have frequently observed that the agglutinating activity of the eluate decreases when increasing amounts of dried blood are incubated with the same volume of a given antiserum (Fiori and Benciolini, 1966 — not publ.).

The different behaviour of the two serological tests could probably be explained by the Pauling hypothesis on the different antigen-antibody combination at the various antigen-antibody ratios. The excess of antigens is believed to give rise to full saturation of both the combining sites of each antibody molecule. In these conditions, it is probable that the antigen-antibody complex is not easily dissociable by heating. On the other hand if an excess of antibodies is employed, it is probable that part of them react with the antigen by one combining site only, thus realizing a weaker, heat dissociable bond. Goodman (1962) was able to observe that eluting anti-A antibodies from columns of formalinized red cells at a programmed temperature in a range of 0 to 100°C, only about 50% of the antibody activity was recoverable, probably a because a large part of the antibodies formed a firmer bond with the antigens.

In the light of these considerations, the different results of the absorption-inhibition and of the absorption-elution tests in grouping blood stains (and blood group antigens containing cells) and stains from body fluids could be explained.

In the cells, the AB0 blood group antigens are chiefly contained in the membrane, in combination with many other cellular compounds. It is therefore likely that only a part of their combining sites are a vailable for reacting with antibodies. In this way it is probably easier, under the usual experimental conditions of

grouping biological stains, to reach a antigen-antibody ratio more favorable for the absorption-elution test than for the absorption-inhibition one.

In the secretions, i.e. saliva and semen, the free antigens are prevalent so that a greater number of antigenic combining sites is available to combine with the antibodies. Therefore the antigen-antibody ratio is more readily favorable for the inhibition test.

Stains made with whole saliva or semen of secretors and non-secretors behave differently according to their titer in soluble group specific substances and to the amount of cells: when the soluble specific substance content is low or absent (non-secretors) a good antigen-antibody ratio is reached for the absorption-elution test, the positivity of which is then due to the cellular antigens. In the seminal stains of azoospermic non-secretors, of course, no positive reaction is obtained.

2. Some differences have been observed in the behaviour of the 3 modifications of the absorption-elution test used for grouping saliva and seminal stains. In our opinion, these differences are due mainly to the washing step.

In fact, in the slide methods no control of the washing fluid is made to ensure that all free antibodies have been washed off. Therefore non-specific or paradoxical reactions due to the non-specific absorption of the antibodies to the substrate may be observed. With regard to the so called paradoxical reactions, it should be noted that the non-reacting antibodies, e.g. anti-B incubated with a group A stain, require a more prolonged washing than the reacting ones (anti-A incubated with a group A stain), because the number of the free molecules to be washed off is greater.

The influence of fixation can be studied only in stains of untreated secretions or of washed sediment. The positive effect of methanol fixation on ABH substances has been already demonstrated by Fiori *et al.* (1963), Ueno (1963), Fiori and Benciolini (1969), Fiori *et al.* (1971a), and therefore the results on stains of washed sediment from saliva and seminal fluids are easily explained. On the other hand no effect of fixation can be observed on stains from the supernatant of the examined secretions. Finally some unexplained positive results have been observed on stains from untreated secretions.

In conclusion on points 1. and 2. the different results claimed by the various authors on the reliability of the absorption-elution method for grouping secretion stains should be ascribed both to some differences on the technique and to different experimental conditions with special regard to the titre of the antisera (Dodd and Hunter, 1963, e.g. reported to have used powerful 1/2000 antisera).

3. The practical value of the *absorption-elution* for AB0 grouping of stains from body fluids should be viewed with great caution, since erroneous negative results due to an unfavorable antigen-antibody ratio and, in some cases, paradoxical results, due to the technique employed, are obtained.

In fact, for routine determinations, high titred sera are not easily available and, on the other hand, the antigen concentration in the saliva and seminal stains is usually unknown. The latter depends on many variable factors such as the secretor status, and its degree, the concentration of the body fluids on the stained substrate.

In our opinion positive reactions only can be considered reliable, but this conclusion can be reached only if the absorption-elution test tube method is used, by which the causes of errors are minimized.

The *absorption-inhibition test* is the best way to group secretors' stains. In fact the concentration of antigens is usually so high that a fragment of stain is sufficient to give clearly interpretable results. When the inhibition is negative the possibility should be considered that the stain come from a non-secretor or from a very weak secretor. In such cases the absorption-elution method should be tried since it may give positive results especially when the stain contains a large amount of cells (buccal epithelium; spermatozoa).

Therefore, for grouping the stains from body fluids the absorption-elution method should be performed after the inhibition method, as an important complementary test.

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Prof. Dr. med. Angelo Fiori
Istituto di Medicina Legale
Università Cattolica del Sacro Cuore
I-X 00168 Roma
Italy

Prof. Dr. med. Paolo Benciolini
Istituto di Medicina Legale
Università di Padova
I-X 35100 Padova
Italy