Henry C. Lee,[1] Ph.D.; R. E. Gaensslen,[2] Ph.D.;
Elaine M. Pagliaro,[1] M.S.; and Beryl Novitch,[1] M.S.

Two-Dimensional Absorption-Inhibition

REFERENCE: Lee, H. C., Gaensslen, R. E., Pagliaro, E. M., and Novitch, B., **"Two-Dimensional Absorption-Inhibition,"** *Journal of Forensic Sciences*, Vol. 33, No. 5, Sept. 1988, pp. 1127-1138.

ABSTRACT: A novel inhibition procedure, called two-dimensional absorption-inhibition, is described. The theory underlying this technique is developed based on a review of and comparison with existing inhibition methods. Two-dimensional inhibition takes advantage of the best features of inhibition-titration and titration-inhibition, and is shown to be more sensitive than either of them. Results obtained using all the inhibition methods on secretor saliva, semen, urine, urine stain, and perspiration stain specimens show that the new technique is especially powerful in correctly determining the ABH antigens in secretor body fluids having lower concentrations of soluble blood group antigens. A two-stage version of the two-dimensional procedure that makes it a practical casework method is described as well.

KEYWORDS: pathology and biology, antigen systems, body fluids, genetic typing, ABO blood group system, blood grouping, absorption-inhibition, soluble ABH blood group antigens, forensic body fluid grouping, saliva, semen, urine, ABO secretors

Absorption-inhibition procedures have been used at least since the 1920s for determining the ABH antigens of dried blood [*1*], although the absorption-elution technique originally devised by Siracusa [*2*] and refined by Kind [*3,4*] is now employed almost exclusively for this purpose in most laboratories [*5,6*]. However, absorption-inhibition remains the method of choice for determining the ABH antigens in body fluids and body fluid stains, and numerous variations of the technique have been described [*5,6*].

Three categories of inhibition procedures are in common use: one-step (or all or none); inhibition-titration (I-T) [*7*]; and titration-inhibition (T-I) [*8,9*]. In this paper, we review the existing categories of inhibition procedures and introduce a new, more sensitive technique called two-dimension (2-D) absorption-inhibition. The new procedure takes advantage of the best features of I-T and T-I. Its underlying theory is described, along with results obtained on a representative specimen of body fluids and body fluid stains using all four inhibition techniques.

Various aspects of this work were presented in part at the 38th Annual Meeting of the American Academy of Forensic Sciences, New Orleans, LA, 10-15 Feb. 1986; 39th Annual Meeting of the American Academy of Forensic Sciences, San Diego, CA, 16-21 Feb. 1987; International Symposium on Forensic Immunology, FBI Academy, Quantico, VA, June 1986; 12th Annual Meeting of the Northeastern Association of Forensic Scientists, Peabody, MA, October 1986; and the 12th International Congress of the Society for Forensic Haemogenetics, Vienna, Austria, Aug. 1987. Received for publication 14 Jan. 1988; accepted for publication 19 Jan. 1988.

[1]Chief criminalist and director, supervisory criminalist, and senior criminalist, respectively, Connecticut State Police Forensic Science Laboratory, Meriden, CT.

[2]Professor and director, Forensic Sciences Program, University of New Haven, West Haven, CT.

Existing Inhibition Procedures and the Theory of the Two-Dimensional Inhibition Procedure

One-step inhibition is the oldest and simplest procedure; the one-step technique and its results are illustrated diagramatically in Fig. 1. The sensitivity of one-step inhibition is inversely proportional to the titers of the antisera employed. This procedure tests a single antigen concentration against a single antiserum concentration. It is designed to give an "all-or-none" result, that is, the specimen either shows complete inhibition or no inhibition. The one-step technique is expected to detect the antigens present in a secretor body fluid specimen or stain extract by showing complete inhibition in specimens containing significant concentrations of the corresponding antigen or antigens. False negative results are possible, however, with test specimens containing low concentrations of antigen/antigens, especially at higher antiserum titers.

It has been clear for some time that the concentrations of soluble ABH blood group substances in human body fluids and organ extracts varies significantly among different fluids and tissues as well as among different individuals [*10–12*]. More recent studies have focused on the distribution of group substance concentrations among different individuals and on the distribution of A : H, B : H, and A : B : H ratios in several body fluids and secretions [*13–16*].[3] The results of these works indicate that there is significant variation in the levels of A, B, and H as well as in the A : H, B : H, and A : B : H ratios among different individuals and in different body fluids and secretions of the same individual. One-step inhibition testing is not designed to take these variations into account, and for this reason some workers prefer one of the two inhibition procedures involving titrations.

The inhibition-titration technique was introduced by Holzer in 1937 [*7*]. A five-tube test is illustrated diagrammatically in Fig. 2. Here, equal volumes of test solution and antisera adjusted to a titer of 32 are combined, incubated to allow inhibition to occur, and then titrated by doubling dilutions before test cells are added. One way of looking at the expected results and the sensitivity of the titration-type inhibition tests is to compare agglutination results for the saline control row (no inhibition) and then for those cases in which a specimen contained sufficient antigen to remove (inhibit) a defined fraction ($^1/_2$, $^1/_4$, $^1/_8$, and so forth)

[3]M. J. Davie, M. J. Dorrill, and P. H. Whithead, "A Quantitative Survey of ABH Blood Group Substances in Semen," HOCRE Report 318, Home Office Central Research Establishment, Aldermaston, Reading, Berkshire, U.K., Aug. 1979.

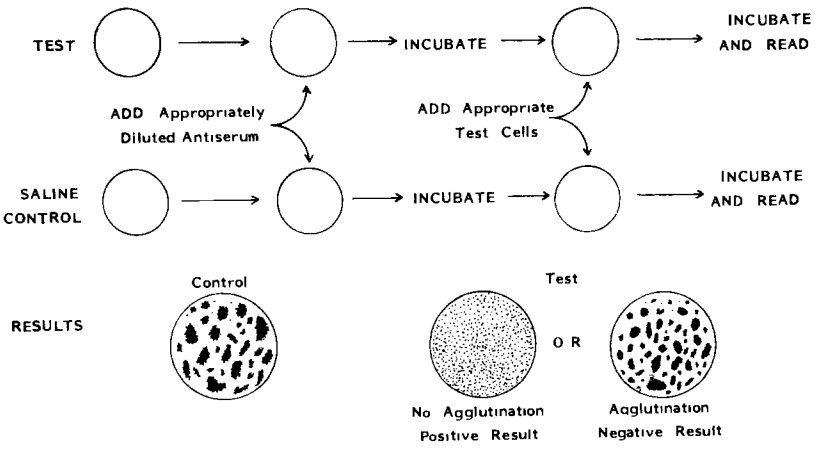

FIG. 1—*One-step inhibition test scheme and results.*

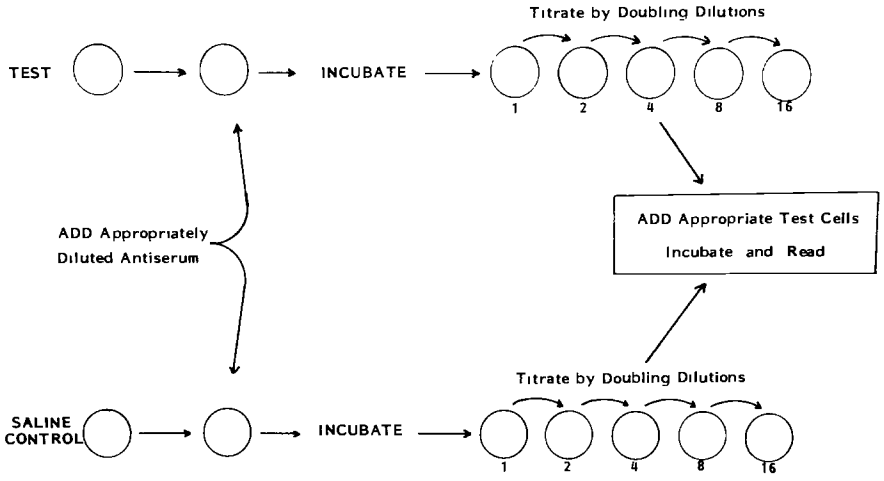

FIG. 2—*Inhibition-titration five-tube test scheme.*

of the antibody present. Figure 3 shows this analysis for the I-T test as constructed according to Fig. 2. A number of workers do not regard a test result as conclusive unless the specimen gives at least a three-tube reduction relative to the saline control. Following this criterion, only the "complete inhibition" row in Fig. 3 would be regarded as conclusive, and specimens having antigen sufficient to remove one half or less the antibody present would be interpreted as "inconclusive" or as "no antigens detected."

A more sensitive titration type inhibition procedure, titration-inhibition, introduced by Hirszfeld and Amzel in 1932 [*8*], is shown diagrammatically in Fig. 4 for a five tube test. Here, an antiserum having a titer of 32 is titrated out five tubes in saline. A volume of specimen equal to that of the antiserum is then added to each tube, and the tubes incubated to allow inhibition to occur before adding test cells. Figure 5 shows the results expected from a T-I test using the same logic as was described above for the I-T test and in Fig. 3. The increased sensitivity of T-I as against I-T is obvious in comparing Figs. 3 and 5. A specimen containing sufficient antigen to remove only one quarter of the antibody present yields a

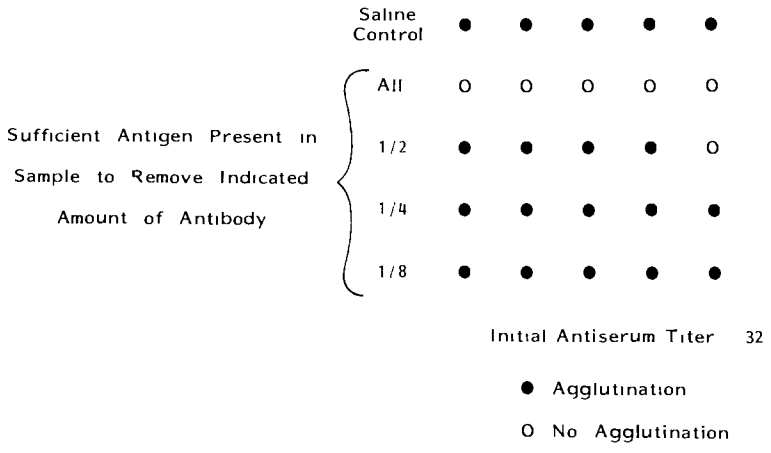

FIG. 3—*Results of a five-tube inhibition-titration test.*

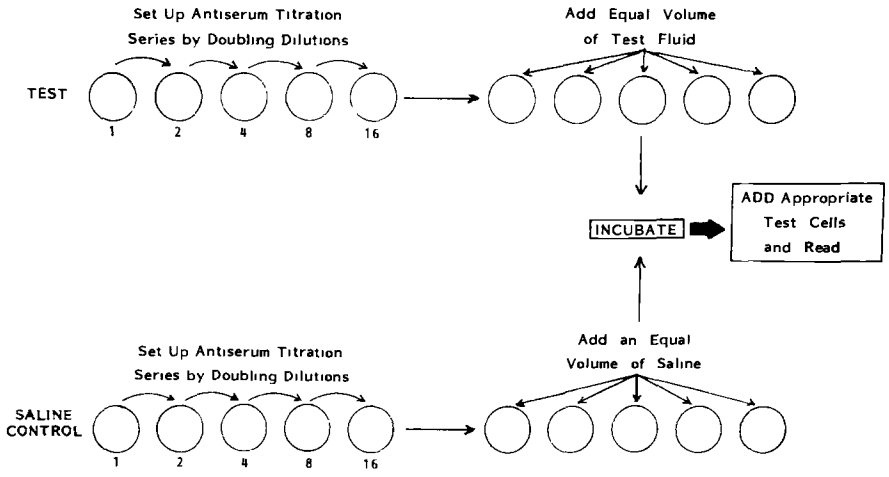

FIG. 4—*Titration-inhibition five-tube test scheme.*

conclusive (three tube reduction) result with T-I, whereas the same specimen would give a false negative result with I-T.

Two-dimensional inhibition takes advantage of the features of both T-I and I-T. A complete 2-D protocol is illustrated in Fig. 6. First, an antiserum is titrated by doubling dilutions in saline. Next a volume of specimen equal to that of antiserum is added to each tube, and the test is incubated to allow inhibition to occur. This setup is identical to that for a T-I test. Then, after inhibition has reached the equilibrium stage, each tube in the row is titrated in the second dimension, treating each tube in effect as if it were the first tube in a I-T test. In Fig. 6, the procedure is illustrated with antiserum titers up to 1:128. In practice, titers this high are not used for inhibition tests. A complete 2-D test in practice would use six tubes in

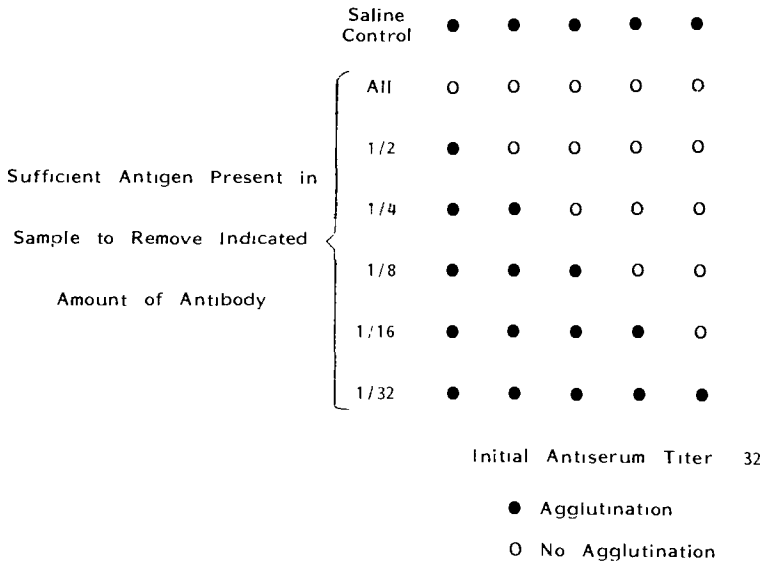

FIG. 5—*Results of a five-tube titration-inhibition test.*

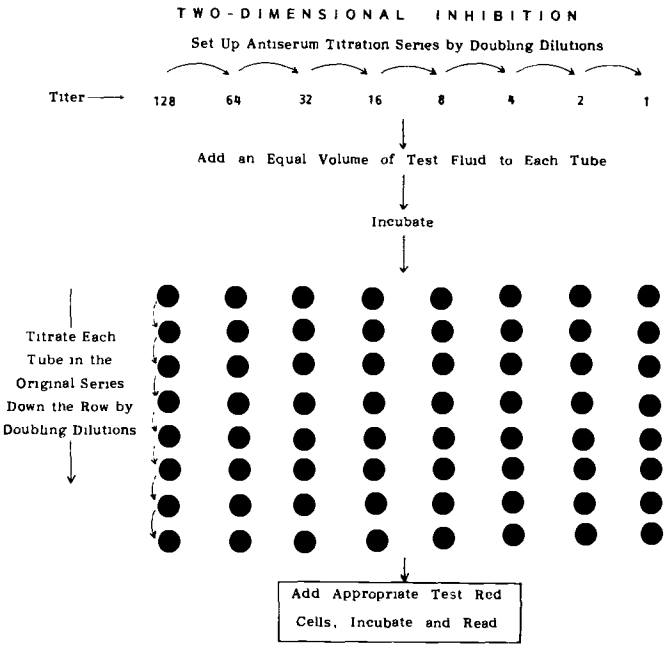

FIG. 6—*Complete two-dimensional inhibition test scheme.*

the first row, with antiserum titers adjusted to 64, 32, 16, 8, 4, and 2. After the addition of an equal volume of specimen, the titers of the antiserum become 32, 16, 8, 4, 2, and 1. The results of a 2-D test that was set up in this manner are shown in Fig. 7, following the same logic that was used in illustrating the results of I-T (Fig. 3) and T-I (Fig. 5). It can be seen that the 2-D is some eightfold more sensitive than T-I. Even a specimen containing antigen sufficient to remove only 1/32 of the antibody present is distinguishable from the saline control, whereas specimens containing antigen sufficient to remove less than 1/4 of the antibody present would be regarded as inconclusive or negative in a T-I test (Fig. 5).

Figure 8 illustrates the clear differences in sensitivity among the four inhibition procedures, one-step, I-T, T-I, and 2-D. The results that would be obtained with all four procedures are shown for a specimen that contained sufficient antigen to remove one eighth the antibody present in a test protocol where the initial antiserum titer was 32. Both the one-step and I-T tests give false negative results. T-I gives some inhibition, but the result would be regarded as "inconclusive" if the "three-tube reduction" criterion were followed. The 2-D procedure, however, allows a clear and unequivocal diagnosis of inhibition relative to the saline control.

The 2-D test protocols represented in Figs. 7 and 8 are relatively complicated in comparison with the other titration type procedures, and this procedural complexity might represent a drawback in busy casework laboratories in which a significant number of specimens are routinely analyzed. The procedure can be simplified, however, without sacrificing the sensitivity afforded by the use of this method. Figure 9 shows a two-dimensional inhibition protocol in which the first row (the T-I component of the test) is set up with three selected dilutions of antiserum, corresponding to antiserum titers of 32, 8, and 2. Figure 10 shows the agglutination results that would be obtained in such a test scheme with sufficient antigen present in the specimen to remove selected fractions of the antibody present from 1/2 to 1/32. The specimen containing only sufficient antigen to remove 1/32 of the antibody present is still clearly distinguishable from the saline control.

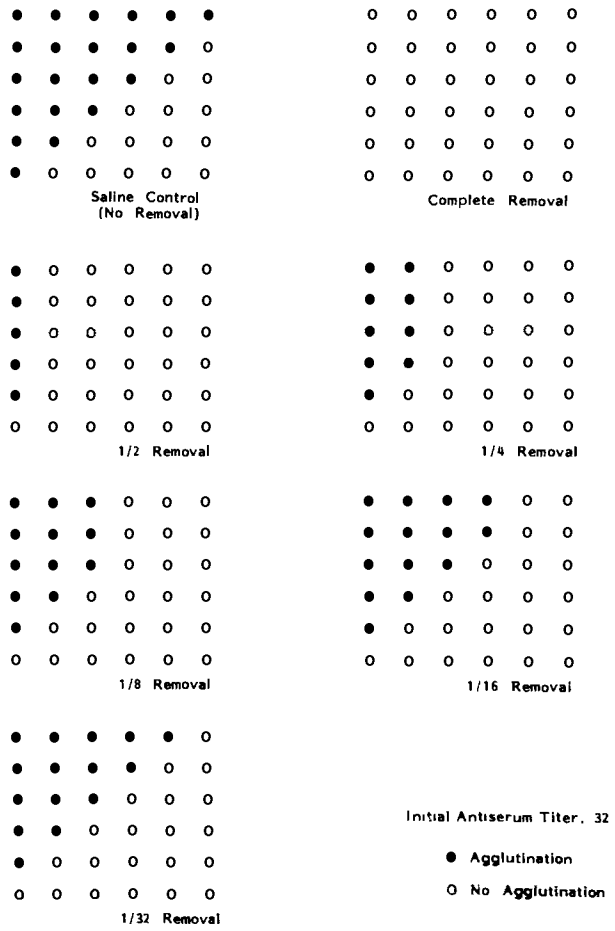

FIG. 7—*Results of a six-tube two-dimensional test.*

The increased sensitivity of the 2-D procedure is obviously desirable, especially for the analysis of specimens containing relatively small quantities of antigen. Many specimens submitted for analysis contain comparatively large quantities of antigen and can be successfully analyzed using I-T or T-I. An additional slight modification of the 2-D procedure allows an examiner to take advantage of this situation and thus simplify the technique further for busy casework laboratories. Figure 11 illustrates this modification.

Here, three selected dilutions of antiserum are set up in the first row (the T-I component of the test). In the illustration, antiserum titers of 32, 8, and 2 are selected, but other dilutions could be selected, and protocols involving four tubes, for example, could easily be constructed. After addition of the specimen and incubation to allow inhibition to occur in the manner of a T-I test, one volume of the contents of each tube is removed and test cells are added. The results obtained from this one row, representing the results of a T-I test using selected dilutions of antisera, are then evaluated before proceeding to titration in the second dimension. If the results unequivocally demonstrate the presence of the antigen (that is, complete inhibition), the test need not be carried out in the second dimension. If, however, the results from evaluating the T-I component row of the test are negative or equivocal, the titration in the second dimension is carried out to completion. This two-stage approach

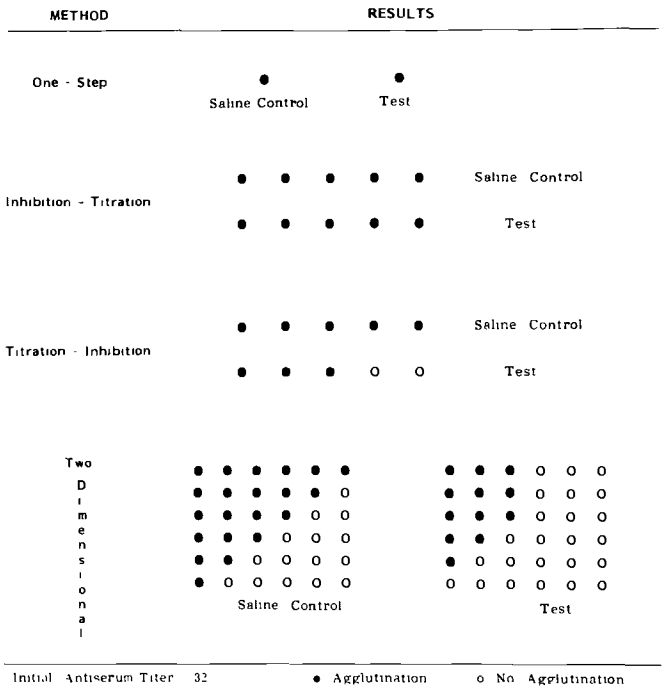

FIG. 8—*Results of one-step, I-T, T-I, and 2-D inhibition tests with initial antiserum titer of 32 and sufficient antigen present in the test specimen to remove 1/8 of the antibody present.*

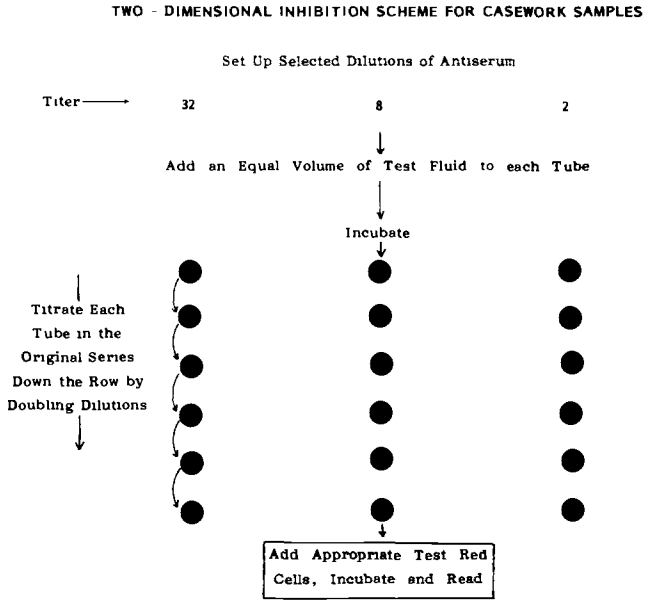

FIG. 9—*Two-dimensional inhibition test scheme with selected dilutions of antiserum (titers = 32, 8, and 2).*

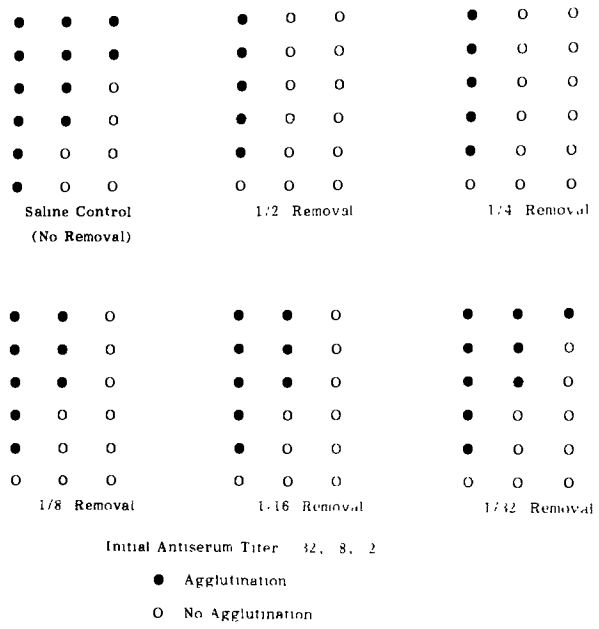

FIG. 10—*Results of two-dimensional inhibition test with selected dilutions of antiserum (titers = 32, 8, and 2).*

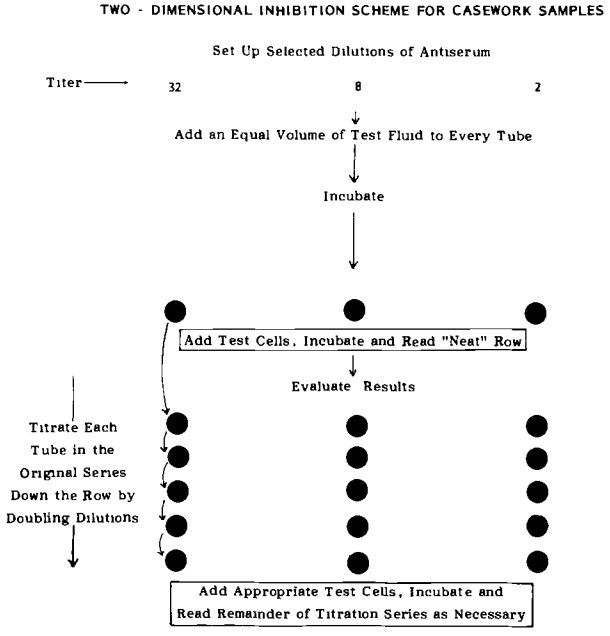

FIG. 11—*Two-stage, two-dimensional inhibition test scheme.*

makes the test more practical for a busy casework laboratory, as it allows for maximum efficiency in processing specimens without sacrificing the sensitivity afforded by using the method.

Materials and Methods

Anti-A and anti-B were standard, polyclonal reagents of human origin and were obtained from Ortho (Raritan, NJ) or Dade (through American Scientific Products, Boston, MA). Some anti-H lectin was prepared from *Ulex europaeus* seeds (F. W. Schumacher Co., Sandwich, MA) by the method of Kind [*17*]. Some anti-H lectin was obtained from Dade. Affinity-purified anti-H lectin (UEA-I from Miles Laboratories) was also employed in some experiments [*18*].

Red cells of groups A_2, B, and O were obtained from healthy volunteers, and washed three times in saline before use as test cells. Test red cell suspensions were adjusted to 0.1% in saline. Tests requiring titration were typically carried out in small 6- by 50-mm glass tubes, or in Boerner well slides, using 50 μL as one volume. Tests were also carried out in microtiter plates, then transferred to Boerner slides for rotation and reading. The Boerner well slides containing the complete tests were placed in a moisture chamber and rotated for 20 to 30 min before reading agglutination results microscopically. Inhibition tests were allowed to incubate 17 h at 4°C before the addition of test cells.

Saliva, semen, urine, and perspiration stain samples were collected from healthy donors of known red cell type and secretor status (as determined by classical inhibition procedures and red cell Lewis grouping). Known control urine stains were prepared on clean cotton cloth from urine specimens of known origin. Liquid saliva and semen samples were diluted 1:1 with saline, and the saliva samples subjected to a 100°C water bath for 5 min and centrifugation before use in inhibition tests. Some samples were frozen until they could be analyzed.

In experiments used to compare the four inhibition procedures, the I-T and T-I tests were constructed like the protocols illustrated in Figs. 3 and 5, respectively. 2-D tests were constructed like the protocol illustrated in Fig. 7. Results of I-T and T-I tests are reported as "inconclusive" if there was less than a three-tube reduction in antibody titer relative to the saline control. Results of any of the inhibition tests are reported as "incorrect" if the test failed to detect an antigen or antigens corresponding to the blood group known to be present. Thus, detection of "A" or "A" and "H" from a known A secretor specimen would be regarded as a correct result, but detection of "H" or no antigens would be regarded as incorrect, for example.

Results and Discussion

The one-step, I-T, T-I, and 2-D inhibition procedures were used to test 187 different known control specimens from individuals of known ABO blood group and secretor status, and the results were compared.

Table 1 shows these results for 36 secretor saliva, 9 secretor semen, 60 secretor urine, 43 secretor urine stain, and 39 secretor perspiration stain specimens. No incorrect results were seen with either the T-I or 2-D methods. The majority of incorrect results with one-step and I-T procedures were observed in urine, urine stain, and perspiration stain rather than in saliva or semen specimens. These results are understandable in view of the fact that secretor saliva and semen typically contain significantly higher concentrations of soluble blood group substances than urine or perspiration. In the same way, the number of inconclusive results with the I-T procedure was higher in the urine and perspiration specimens. However, some inconclusive results were obtained on these specimens even with the T-I method, whereas the 2-D technique enabled these specimens to be typed correctly. The results confirmed our expectation that the increased sensitivity afforded by the 2-D method would be most valu-

TABLE 1—*Results with known control samples of secretor saliva, semen, urine, urine stains, and perspiration stains using all four inhibition procedures.*

Type of Sample	ABO Group	No. Tested	Number of Incorrect (X) or Inconclusive (INC) Results Obtained Using							
			One-Step		I-T		T-I		2-D	
			X	INC	X	INC	X	INC	X	INC
Saliva	A	13	1	N/A[a]	0	2	0	0	0	0
	B	7	1	N/A	0	1	0	0	0	0
	O	9	2	N/A	0	1	0	0	0	0
	AB	7	3	N/A	1	3	0	0	0	0
Semen	A	2	1	N/A	0	1	0	0	0	0
	B	2	0	N/A	0	0	0	0	0	0
	O	3	0	N/A	0	0	0	0	0	0
	AB	2	1	N/A	0	1	0	0	0	0
Urine	A	19	6	N/A	5	7	0	1	0	0
	B	15	2	N/A	0	5	0	1	0	0
	O	15	7	N/A	1	5	0	1	0	0
	AB	11	4	N/A	1	2	0	1	0	0
Urine stains	A	11	5	N/A	2	2	0	0	0	0
	B	16	4	N/A	0	3	0	1	0	0
	O	10	4	N/A	2	2	0	0	0	0
	AB	6	5	N/A	4	1	0	1	0	0
Sweat stains	A	12	8	N/A	3	4	0	0	0	0
	B	10	5	N/A	0	3	0	1	0	0
	O	10	6	N/A	2	2	0	0	0	0
	AB	7	3	N/A	2	0	0	0	0	0

[a]N/A = Not applicable.

able in the analysis of specimens containing relatively low concentrations of soluble blood group substances.

It is noteworthy that the 60 secretor urine specimens and 43 urine stains from secretor individuals were correctly grouped by the 2-D procedure without any concentration of the liquid urine or urine stains extract specimens. Our own and other previous investigations have indicated that unconcentrated urine specimens cannot always be grouped correctly using classical inhibition testing, although successful results were obtained with liquid urine specimens concentrated 20- to 25-fold [*19,20*]. Use of the 2-D procedure appears to obviate the necessity of concentrating the specimens. With the increased volume of drug screening tests being performed on urine specimens and the corresponding possibility of occasional sample mix-ups or questions being raised about whether the alleged donor of the sample was the true donor, the results of our studies indicate that urine grouping may be useful in the inclusion or exclusion of putative urine donors.

Although the majority of secretor semen and saliva specimens contain relatively large concentrations of soluble ABH substances, some specimens will not. As the data indicate, some incorrect and inconclusive results were seen in testing this type of specimen with the one-step of I-T procedures, but these specimens were correctly typed with I-T and 2-D techniques, suggesting that the incorrect and inconclusive results were obtained because of low concentrations of blood group substances in the specimens.

Table 2 shows the results obtained using all four inhibition techniques with several casework specimens that contained lower antigen concentrations. The one-step and I-T procedures did not yield accurate results with these specimens, and T-I resolved only three of them

TABLE 2—*Results with problematic case specimens from secretors using all four inhibition procedures.*

Type of Sample	ABO Group	Results Obtained Using			
		One-Step	I-T	T-I	2-D
Saliva	A	H	(A) (H)[a]	A,H	A,H
	AB	B	(B) (H)	(B) (H)	A,B,H
	AB	A	(A) (B)	A,B,H	A,B,H
Semen	AB	A,H	A, (B) (H)	A,B,H	A,B,H
	A	H	(A) (H)	(A) (H)	A,H

[a] ( ) = weak, inconclusive results for the antigen.

conclusively. The 2-D procedure enabled all the specimens to be diagnosed correctly, however.

The two-dimensional absorption-inhibition technique has been shown to be more sensitive than any of the classical techniques, and to yield reliable results on known control as well as casework specimens that could not be grouped conclusively or reliably by the classical techniques. Further, a two-stage protocol for use of the 2-D procedure that renders it suitable for routine casework analysis has been presented and discussed. The technique has provided a sensitive and reliable procedure for the grouping of urine, urine stains, perspiration stains, tissues, bone tissue, and other specimens with low concentrations of soluble blood group substances.

References

[1] Schütze, H., "Haemagglutination and its Medico-legal Bearing, with Observations upon the Theory of Isoagglutinins," *British Journal of Experimental Pathology*, Vol. 2, 1921, pp. 26–33.

[2] Siracusa, V., "La Sostanza Isoagglutinabile del Sangue e la sua Dimostrazione per la Diagnosi Individuale delle Macchie," *Archivio di Antropologia Criminale, Psichiatria e Medicina Legale*, Vol. 43 (Vol. 14, 4th Series), 1923, pp. 362–384.

[3] Kind, S. S., "Absorption Elution Grouping of Dried Blood Smears," *Nature*, Vol. 185, 1960, pp. 397–398.

[4] Kind, S. S., "Absorption Elution Grouping of Dried Bloodstains on Fabrics," *Nature*, Vol. 187, 1960, pp. 789–790.

[5] Gaensslen, R. E., *Sourcebook in Forensic Serology, Immunology and Biochemistry*, U.S. Government Printing Office, Washington, DC, 1983.

[6] Lee, H. C., "Identification and Grouping of Bloodstains," *Forensic Science Handbook*, R. Saferstein, Ed., Prentice Hall, Englewood Cliffs, NJ, 1982, pp. 267–337.

[7] Holzer, F. J., "Untersuchungen uber die gerichtlich-medizinische Verwertbarkheit der Ausscheidung Blutgruppensubstanzen," *Deutsche Zeitschrift fur die Gesamte Gerichtliche Medizin*, Vol. 28, 1937, pp. 234–248.

[8] Hirszfeld, L. and Amzel, R., "Beitrag zur Gerichtlich-medizinischen Verwertung der Blutgruppen," *Deutsche Zeitschrift für die Gesamte Gerichtliche Medizin*, Vol. 19, 1932, pp. 133–148.

[9] Kind, S. S., "A Modified Absorption Technique of Determining the ABO Group of Bloodstains," *Vox Sanguinis*, Vol. 5 (Old Series), 1955, pp. 15–19.

[10] Hartmann, G., *Group Antigens in Human Organs: A Discussion of the 'Secretor' 'Nonsecretor' Phenomenon*, Ejnar Munksgaard Forlag, Copenhagen, 1941.

[11] Wiener, A. S. and Kosofsky, I., "Quantitative Studies on the Group-specific Substances in Human Blood and Saliva. I. Group Specific Substance B," *Journal of Immunology*, Vol. 41, 1941, pp. 413–428.

[12] Wiener, A. S. and Kosofsky, I., "Quantitative Studies on the Group-Specific Substances in Human Blood and Saliva. II. Group-Specific Substance A, with Special Reference to the Subgroups," *Journal of Immunology*, Vol. 42, 1941, pp. 381–393.

[13] Sensabaugh, G. F., Blake, E. T., and Bashinski, J., "Titers of ABH Blood Group Substances in Human Semen," paper delivered at the 33rd Annual Meeting of the American Academy of Forensic Sciences, Los Angeles, Feb. 1981.

[14] Blake, E. T., Sensabaugh, G. F., and Bashinski, J., "Levels of Genetic Markers in Vaginal Fluids," presented at the 33rd Annual Meeting of the American Academy of Forensic Sciences, Los Angeles, Feb. 1981.

[15] Fowler, J. C. S. and Scott, A. C., "Examination of the Correlation of Groupings in Blood and Semen," *Journal of Forensic Sciences*, Vol. 30, No. 1, Jan. 1985, pp. 103–113.

[16] Baechtel, F. S., "Secreted Blood Group Substances: Distributions in Semen and Stabilities in Dried Semen Stains," *Journal of Forensic Sciences*, Vol. 30, No. 4, Oct. 1985, pp. 1119–1129.

[17] Kind, S. S., "The ABO Grouping of Bloodstains," *Journal of Criminal Law, Criminology and Police Science*, Vol. 53, 1962, pp. 367–374.

[18] Gaensslen, R. E., Lee, H. C., and Carroll, J. E., "Evaluation of Monoclonal Anti-A and Anti-B and Affinity-purified *Ulex europaeus* Lectin I for Forensic Blood Grouping," *Zeitschrift fur Rechtsmedizin*, Vol. 93, No. 4, Dec. 1984, pp. 259–268.

[19] Lee, H. C. and Gaensslen, R. E., "ABO Grouping of Urine and Urine Stains," *Forensic Serology News*, Vol. 6, No. 3, Sept. 1980, pp. 1–3.

[20] Chase, M. G., "ABO Typing Studies on Liquid Urines," *Journal of Forensic Sciences*, Vol. 31, No. 3, July 1986, pp. 881–885.

Address requests for reprints or additional information to
Dr. Henry C. Lee
Chief Criminalist and Director
Connecticut State Police Forensic Science Laboratory
294 Colony St.
Meriden, CT 06450