New and Improved Antigen Suspension for Rapid Reagin Tests for Syphilis

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THE RAPID REAGIN tests for syphilis using unheated plasma or serum (*1–3*) make use of an antigen suspension prepared by resuspending centrifuged VDRL slide antigen suspension in choline chloride. In an early publication (*1*) it was noted that the antigen suspension was stable for a period of at least 1 week. Subsequent observations indicated that some antigen suspensions retained their reactivity for as long as 18 months. However, it was evident that there was no uniformity to this stability; and, indeed, it was found that the antigen suspension might become subreactive even after 1 day of storage. This erratic behavior, although not vitiating the usefulness of the rapid reagin tests, constituted a disadvantage.

It was soon realized that the ultimate solution to the development of a more uniformly stable antigen suspension would depend upon the elucidation of the mechanism by which suspensions of antigen underwent altered reactivity. The investigations to be reported indicated that loss in antigen reactivity was mediated by an oxidative process which is catalyzed by cations. By use of a chelating agent to bind these cations a uniformly stable antigen suspension was obtained.

Materials and Methods

The RPR test was conducted according to the Manual of Serologic Tests for Syphilis (*4*).

Variations from standard procedures for preparing antigen suspension will be noted under particular experiments.

Stock solutions of cations were prepared from reagent grade chemical dissolved in distilled water to a concentration of 10^{-2} M. These were further subdiluted in water as indicated.

Antigen suspensions were stored in screw-capped test tubes at indicated temperatures and were brought to room temperature before testing. The stability of antigen suspensions was determined by use of serial twofold dilutions of pooled reactive human serum in saline.

Effect of Cations and Peroxide

RPR antigen suspensions were prepared to contain 10^{-5} M concentrations of the cations indicated and 0.5 percent hydrogen peroxide. This was accomplished by preparing a concentrated RPR suspension which was then dispensed in suitable aliquots and adding the required cations and peroxide. Following the addition of these reagents the antigen suspensions contained the usual concentrations of choline chloride (10 percent), sodium chloride (0.85 percent), and merthiolate (0.01 percent).

The capacity of the various cations to flocculate antigen suspension, independent of reagin, was determined on the day the antigens were originally prepared, by testing the indicated solutions against regular RPR antigen. Table 1

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shows that Cu^{++}, Fe^{++}, Fe^{+++}, and Zn^{++} produced clumping at a dilution of 10^{-3} M, whereas Ag^{+} caused clumping at a concentration of 10^{-4} M. The other cations did not flocculate the antigen at 10^{-3} M or lower concentrations. It was further determined that on the day they were prepared the antigens incorporating cations alone or in combination with peroxide gave results with test serums equal to that of a control antigen.

On storage at room temperature, Cu^{++} produced a loss in reactivity in 1 week; Fe^{+++}, Ca^{++}, and Zn^{++} caused a loss in reactivity in 2 weeks; the other cations showed no effect greater than the control antigen (table 1). In the presence of peroxide a shortening of the reactivity loss period was noted with Mg^{++} and Co^{++}.

Table 1. Effect of various cations and peroxide on the stability of RPR antigen

Salt used	Cation valence	Stability in presence of—		Flocculating action
		Cation alone [1]	Cation and peroxide [1]	
Cupric sulfate	2	1	1	10^{-3}M.
Ferrous chloride	2	3	2	10^{-3}M.
Ferric chloride	3	2	2	10^{-3}M.
Magnesium sulfate	2	3	1	Negative.
Manganese chloride	2	3	2	Do.
Nickel chloride	2	3	2	Do.
Cadmium chloride	2	2	2	Do.
Cobalt chloride	2	3	1	Do.
Zinc acetate	2	2	2	10^{-3}M.
Mercuric chloride	2	3	2	Negative.
Silver nitrate	1	3	2	10^{-4}M.
Controls:				
No cation, no peroxide	------	3	------	----------
Peroxide alone	------	2	------	----------

[1] Number of weeks at which time reactivity less than standard was observed.

Effect of EDTA on RPR Antigen Suspensions

Because of the pronounced activity of Cu^{++}, experiments were designed to determine the effect of varying concentrations of this cation alone or in combination with peroxide. The ability of ethylene dinitrilo tetra-acetic acid, disodium salt (EDTA) to overcome the deteriorative effects of copper and peroxide was determined. The experimental design was similar to that reported above for study of the different cations. EDTA was prepared as a stock 0.2 M solution in water, adjusted to pH 7.0 (potentiometric) with NaOH and incorporated into the antigen suspensions. Storage was at room temperature.

The deteriorative influence of Cu^{++} alone and the accelerated change produced by both the cation and peroxide can be observed in table 2.

Table 2. Effect of peroxide, copper ions, and EDTA on the stability of RPR antigen

Antigen	Peroxide	Concentration copper (molar)	Concentration EDTA (molar)	Days storage at room temperature				
				1	7	14	22	31
1	(+)	10^{-4}	0	L	L	M	M	M
2	(+)	10^{-5}	0	S	L	L	M	M
3	(+)	10^{-6}	0	S	L	L	L	L
4	(+)	0	0	S	S	L	L	L
5	0	10^{-4}	0	S	S	S	L	L
6	0	10^{-5}	0	S	L	L	L	L
7	0	10^{-6}	0	S	S	L	L	L
8	0	0	0	S	S	S	L	L
9	(+)	10^{-4}	1.25×10^{-2}	S	S	S	S	L
10	(+)	10^{-4}	1.25×10^{-4}	S	S	S	S	L
11	(+)	10^{-4}	1.25×10^{-6}	S	L	M	M	M
12	(+)	10^{-5}	1.25×10^{-2}	S	S	S	S	L
13	(+)	10^{-5}	1.25×10^{-4}	S	S	S	S	L
14	(+)	10^{-5}	1.25×10^{-6}	S	L	L	L	L

S—Reactivity equal to standard.
M—Reactivity greater than standard.
L—Reactivity less than standard.
+—Present in concentration of 0.5 percent.
0—Absent.

EDTA in a concentration as low as 1.25×10^{-4} M inhibited these deteriorative changes for 3 weeks.

Old and Improved RPR Suspensions

The sediments from a common pool of VDRL slide antigen emulsion were resuspended in the usual way to yield the "old" or regular RPR suspension and in the following solution to produce the "improved" RPR suspension:

	Milliliters
0.1 M EDTA in distilled water	2.5
40 percent choline chloride in distilled water	5.0
0.02 M phosphate buffer, 0.2 percent merthiolate [1]	10.0
Distilled water	2.5

[1] Na_2HPO_4 1.42 gm., KH_2PO_4 1.36 gm., merthiolate 1.00 gm., distilled water to 500 ml., pH of solution 6.9.

The volume of the resuspending solution was in each instance equal to the volume of the antigen emulsion centrifuged.

Duplicate preparations of antigen suspensions were stored at refrigerator, room, and incubator (35° C.) temperatures. Table 3 indicates the superiority of the improved suspension. Whereas the old type varied in stability from 1 to 17 weeks, the improved suspension, particularly when stored in the refrigerator, was good for at least 8 months. Lesser stability was observed at room and incubator storage conditions.

Table 4 presents the results of comparative tests with unheated plasma and unheated serum samples. The improved suspension was only slightly less reactive than the old type.

Discussion

The possible role of cations in producing unstable characteristics in lipid antigens was suggested by the work of Ray, Davisson, and Crespi (*5*) who studied the degradative changes of the lipoproteins of rabbit and human serums undergoing dialysis. When all traces of cupric ions were removed the lipoprotein was stable on dialysis. Numerous other metal ions were without effect. Changes similar to those occurring during dialysis could be experimentally produced by the addition of hydrogen peroxide and a trace of Cu^{++}. The presence of a chelating agent inhibited the reaction. Ray and coworkers suggested that the degradation was oxidative in nature and catalyzed by copper.

The observations made in the present study suggest that a similar mechanism underlies the loss in reactivity of lipid antigen suspensions. Of the cations studied, copper was most active in producing degradation even in the absence of added peroxide. Magnesium and cobalt were quite active in the presence of added peroxide. EDTA reversed the deteriorative changes produced by copper and peroxide. The subsequent incorporation of EDTA into RPR suspension produced uniform stability particularly when suspensions were stored in the refrigerator. The omission of sodium chloride from the improved suspension was prompted by the observation that a finer dispersion of particles was obtained with nonreactive specimens. The reactivity of the improved sus-

Table 3. Comparison of stability of old and new types of RPR antigen

Type of antigen	Number of lots	Expiration period (weeks) when stored in—		
		Refrigerator	Room	Incubator
Old	16	1–17	1–11	2–10.
New	16	Indefinitely.	8–25	22 or more.

Table 4. Comparison of reactivity of regular and improved RPR antigen suspension

Type of sample	Results with regular antigen	Number	Results with improved antigen		
			Reactive	Weakly reactive	Nonreactive
Plasma	Reactive	35	35	------	------
	Weakly reactive.	2	1	1	------
	Nonreactive	43	------	------	43
Serum	Reactive	50	47	3	------
	Weakly reactive.	6	------	3	3
	Nonreactive	81	------	------	81

pension was found to be essentially similar to regular RPR suspension.

Samples of the improved suspension exposed to a wide variation of temperature over a 10-day period have maintained a uniform stability. These samples varied in age from 1 to 7 months at the time they were exposed. Preliminary studies have likewise suggested that the principle of preservation by the addition of EDTA may be of value for other lipid antigen emulsions used in the serology of syphilis, but further experience is needed before a specific recommendation can be made for its broader use.

Summary and Conclusions

The loss of reactivity of stored antigen suspensions used in reagin tests for syphilis is mediated in part by an oxidative process and catalyzed by cations.

More uniformly stable antigen suspensions were obtained by the incorporation of a chelating agent ethylene dinitrilo tetra-acetic acid, disodium salt (EDTA) in antigen suspension used in the rapid reagin tests.

The use of this agent in the suspensions used for the rapid reagin tests is recommended.

REFERENCES

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Unexploited Breakthroughs in Cancer Research

Although the ultimate research goals in cancer are still in the future, Dr. Michael B. Shimkin of the National Cancer Institute, Public Health Service, directs attention to the following discoveries that, if fully applied, would have a major impact upon the occurrence, mortality, and tragedy of cancer.

In the prevention of cancer, the elimination of the cigarette habit would reduce the incidence of lung cancer by 60 percent, a saving of some 20,000 deaths from lung cancer per year. Additional reduction in the lung cancer incidence could be achieved by controlling major sources of air pollution, such as fumes from automobile exhausts. (Burney, L. E.: J.A.M.A. 171: 1829–1837, Nov. 28, 1959. Shimkin, M. B.: *In* Tumors of the Chest, edited by D. Spain, New York, Grune and Stratton, 1960, pp. 1–16.)

In the diagnosis of cancer, the application of cervical cytology to the total female population remains unrealized. Self-obtained smears, central laboratories to which smears could be mailed, and intensified research in methods of cytoanalysis need to be applied to solve the logistics of this problem. The full use of this discovery should reveal 10,000 cases of cervical cancer and precancer per year, at a stage when the disease is curable in almost 100 percent of the cases. (Brunschwig, A: Cancer 7: 1182–1184, 1954. Dunn, J. E., et al.: J. Nat. Cancer Inst. 23: 507–528, 1959.)

In the treatment of cancer, modern therapeutic trials must be undertaken to test the traditional concepts of operability. There is no convincing evidence that radical mastectomy yields better results than the simple mastectomy for breast cancer, and a comparison of the operations is overdue by a decade. If no significant difference can be demonstrated, 20,000 women each year would be managed more conservatively and gently. (Skimkin, M. B., et al.: Surg. Gynec. & Obst. 94: 645–661, 1952. Smith, S. S., and Meyer, A. C.: Am. J. Surg. 98: 653–656, 1959.)