TABLE IV. Difference of Average Agglutination Index Due to Adsorption of SIII Immune Serum by Type I and Type III Pneumococci.

Significance between	Difference ± S.E.	P
Saline and nonadsorbed serum	6.2 ± .8	<.01
Saline and Type III adsorbed serum	1.1 ± 1.0	.30
Nonadsorbed serum and Type III adsorbed serum	7.3 ± 1.2	<.01
Saline and Type I adsorbed serum	8.4 ± 1.2	"
Type I adsorbed serum and Type III adsorbed serum	9.5 ± 1.4	"

SIII, .1 μg/ml.

pneumococcal sera was adsorbed by type III pneumococci but not by type I. From the work of Borduas and Grabar(9) it may be questioned whether the EA tanned cells really adsorbed anti-EA antibody, since these workers claim that tanned cells preferentially adsorb conalbumin rather than EA. Stravitsky has shown that EA is adsorbed about as well as conalbumin(10). The fact that this issue may be controversial does not invalidate the results of our study. Whether EA or one of its impurities was the principal antigenic unit. LAA was formed and adsorbed from serum by antigenic unit fixed to tanned red cells.

Waksman reported that rabbit anti-ovalbumin sera produced a decrease in leukocyte count when added to normal blood in presence of specific antigen(11). He found no correlation between white cell lytic activity of such antisera and precipitating antibody but did find some correlation with skin sensitizing antibody. It is likely that the antibody involved in decreased leukocyte count observed by Waksman and the LAA are identical. However, the nature of LAA and its exact relation to antibody of known type remain to be elucidated.

Summary. A humoral agent produced by single injection of antigen and responsible for leukocyte agglutination, which occurs in presence of specific antigen has been demonstrated in anti-EA and anti-type III pneumococcal sera. The factor is heat labile and, due to its specific affinity for EA treated tanned red cells and type III pneumococci respectively, it is thought to be antibody.

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Histochemical Demonstration of Erythrocyte Esterases.* (24842)

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Although the histochemical demonstration of esterases in tissues can be performed by means of azo and indoxyl technics, such technics have not been applied successfully to erythrocyte esterases(1). The demonstration of acetylcholinesterase, based on the Koelle method, was noted by Zajicek *et al.*(2); the precipitate, however, is crystalline and localization of enzyme activity within the erythrocyte has not yet been reported. During preliminary studies on development of high resolution histochemical technics for intracellular localization of enzymes, erythrocyte esterase activity could be demonstrated with excellent localization in ordinary air-dried blood smears.

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The salient characteristics of the technic are, (1) low temperature anhydrous osmium tetroxide fixation and, (2) use of "hexaazotized" pararosanilin in an azo coupling reaction(3,4).

Method. In our procedure, blood smears are first fixed in anhydrous osmium tetroxide, then incubated in a medium containing alpha naphthyl acetate and "hexaazotized" pararosanilin. Esterase enzymes present in the erythrocyte split the alpha naphthyl acetate to alpha naphthol and acetate ion. The "hexaazotized" pararosanilin then couples with alpha naphthol to produce a colored deposit at sites of enzyme activity. Discrete intracellular erythrocyte esterase activity has been demonstrated in blood smears from garter snake, frog, mouse, rat, rabbit and man.

Materials. (1) Fixative. 1% osmium tetroxide in dimethylformamide (DMF)†. DMF available as reagent grade chemical contains trace quantities of substances which reduce enzyme activity and which react with osmium tetroxide. Purification and dehydration are readily achieved by passage of DMF through freshly activated charcoal and then through Linde Molecular Sieve Powder, Type 4A.‡ The treated DMF is colorless and has a faint sweet odor. 1% solution of osmium tetroxide in DMF is prepared (in a hood). The deep yellow solution is stored in screw cap Coplin jar in freezing compartment of refrigerator at temperature of about –15°C. The solution is stable indefinitely at this temperature. Repeated use eventually introduces contaminants which react with osmium tetroxide as indicated by gradual appearance of a brown-black color. (2) Buffer solutions. 1/15 M Na_2HPO_4, 1/15 M KH_2PO_4. (3) Substrate. Alpha naphthyl acetate, reagent grade.§ (4) The "hexaazonium" salt is made prior to incubation by mixing hydrochloric acid solution of pararosanilin with sodium nitrite solution. (A) One gram of pararosanilin hydrochloride (C.I. 676) is added to 25 ml of 2N HCl, warmed to dissolve the dye, filtered and stored in dropper bottle at room temperature. (B) One gram of sodium nitrite is dissolved in 25 ml of distilled water and stored in dropper bottle at room temperature. (5) Slides. Thin blood smears are made. If smears are not used the same day they should be stored in refrigerator. Activity can be preserved for several months at 5°C. *Procedure.* (1) Fixation. Dry blood smears are placed in Coplin jar containing osmium tetroxide in DMF for 2 to 3 minutes at –15°C. At completion of fixation the slides are removed one at a time and *immediately* placed in stream of cold, briskly running tap water for 15 seconds to remove fixative. (If a refrigerated slide is to be fixed, care must be taken to prevent water vapor condensation on the slide since the introduction of a moist smear into the fixative produces, in effect, aqueous osmic acid fixation which inactivates erythrocyte esterases. Care also must be taken when the slide is removed from the fixative; unless the fixative is washed off without delay, water vapor condensation on the slide in the presence of osmium tetroxide causes rapid inactivation of the enzymes.) (2) Incubation medium. 25 ml of 1/15 M Na_2HPO_4 are added to Coplin jar. About 15 mg of alpha naphthyl acetate are placed in test tube, heated under hot water tap until the ester begins to melt (about 45°C). 2 to 3 ml of hot water are added to tube and the tube shaken. The resultant turbid mixture is transferred to the Coplin jar with stirring to give a saturated solution. "Hexaazonium" salt is made by adding 4 drops of sodium nitrite solution to 4 drops of pararosanilin-hydrochloric acid solution in a separate test tube and the tube agitated. "Hexaazotization" is complete in about 30 seconds and the amber solution is transferred to Coplin jar with stirring. pH of medium is now adjusted by pH meter to 7.3 by addition of 1/15 M KH_2PO_4.‖ (3)

† Osmium tetroxide purchased from Engelhard Industries, Newark, N. Y., Dimethylformamide purchased from Matheson Coleman and Bell, East Rutherford, N. J. and from Distillation Products, Rochester, N. Y.

‡ Linde Molecular Sieve Powder, Type 4A purchased from Linde Air Products, N. Y.

§ Alpha naphthyl acetate purchased from Distillation Products, Rochester, N. Y.

‖ Selection of pH 7.3 was based on qualitative observations and represents a necessary but satisfactory compromise. It was found that, within limits, as pH of incubating medium was raised, erythrocyte esterase activity increased but rate of enzyme inacti-

Incubation. The slides (up to 5) are placed in Coplin jar and incubation performed at room temperature (approximately 25°C). Incubation time varies with species of organism. Erythrocytes of garter snake are incubated for about 5 minutes; rabbit for about 10 minutes; frog, mouse, rat and man for about 60 to 120 minutes.‖ At end of incubation the slides are washed in water, passed through acetone to remove naphthyl acetate which may have deposited on the preparation, and allowed to dry.

Results. Sites of erythrocyte esterase activity are demonstrated by presence of azo dye formed by coupling of "hexaazotized" pararosanilin and alpha naphthol, the latter component formed by enzymatic hydrolysis of alpha naphthyl acetate.¶ The final dye product is red-brown and insoluble in water, acetone, alcohol, benzene and all other solvents tested. It is stable and it does not diffuse in permanent mounting media. The dye is amorphous and clearly delineated against the background of erythrocyte cytoplasm.

No dye deposit is found if enzymes are inactivated, *e.g.*, by flaming the smear, or if either substrate or "hexaazonium" salt is omitted from incubating medium.

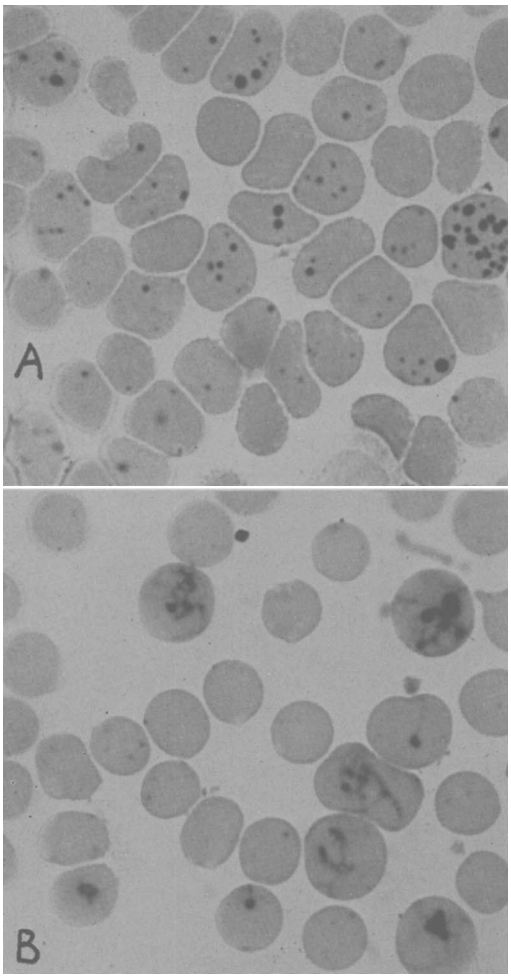

FIG. 1. Erythrocyte esterase in peripheral blood smears (×1134). A. Human with polycythemia vera. B. Rabbit during phenylhydrazine induced anemia.

Non-specific coupling of "hexaazonium" salt to proteins imparts a pale yellow color to proteins previously colorless and a light orange color to erythrocyte hemoglobin.

Differences in morphologic distribution of dye deposits within erythrocytes and in number of erythrocytes showing activity are readily observed. In general the morphologic distribution of enzyme activity is of 2 types: (1) [Fig. 1A] spherical granules which may vary both in number (from 1 to 20) and in size in a particular erythrocyte and (2) [Fig. 1B] an irregular mass with filamentous exten-

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vation by 'hexaazonium' salt, as with all azo couplers, also increased. At pH 7.3 at room temperature dye deposition from enzymatic hydrolysis proceeds at "adequate" rate for about 4 hours. At pH 6.9 rate of enzymatic hydrolysis is slower; however, 'hexaazonium' inactivation of the enzyme is further depressed: the net effect is an increase in detection sensitivity provided incubation is extended to 6 to 16 hours. Selection of pH and incubation time is, therefore, arbitrary and depends upon type of information required. Incubation at pH 7.3 has been satisfactory for all species of erythrocytes studied for unequivocal demonstration of positivity and for detection of differences between normal controls and abnormal specimens. If, on the other hand, greater sensitivity in enzyme detection is required it is necessary to perform the incubation at pH 6.9.

¶ Leucocytes and platelets are demonstrated by this technic. Optimum pH of incubating medium for leucocytes is 6.9. Human lymphocytes, when positive, contain a few distinct granules in the cytoplasm; monocytes, a fine diffusely distributed stippling of cytoplasm; neutrophils, a faint, diffuse activity throughout cytoplasm after at least 4 hours incubation. Of interest is the finding that rabbit pseudoeosinophil nuclei are positive.

sions, often forming an elaborate network. Spherical granules are often found attached to the filamentous extensions. Both types may be seen in the same blood smear.

Work in progress indicates that induced or naturally occurring diseases involving erythrocyte production are often associated with variations in enzyme activity. Thus, preliminary studies show an appreciable increase in number of cells containing both types of dye distribution in rabbits during the phase of reticulocytosis following phenylhydrazine induced anemia [Fig. 1B]. In humans with polycythemia vera the number of erythrocytes containing spherical granules and number of granules/cell are strikingly increased over normal controls [Fig. 1A]. Similar findings have been noted in some types of anemia in humans.

Discussion. Previous failures to demonstrate erythrocyte esterases by histochemical azo technics may have been due to inadequate fixation and/or inadequate detecting systems.

Anhydrous osmium tetroxide is an excellent fixative for blood smears(3). When used at low temperatures sufficient enzyme activity is preserved to keep incubation time within a reasonable range. Relative stability of "hexaazotized" pararosanilin and of alpha naphthyl acetate against spontaneous hydrolysis contribute to sensitivity of the system by reducing artifactual colored precipitates to a minimum. Rapidity of the coupling reaction, (and extreme insolubility) and high extinction coefficient of the coupling product result in precise localization and high sensitivity. Demonstration of enzyme activity in filaments so fine as to approach or possibly exceed the limit of resolution of the light microscope and absence of dye diffusion halos or crystalline deposits are evidence of high resolution of the technic.

Erythrocytes are known to contain at least 3 different types of esterases: acetylcholinesterase, cholinesterase and ali-esterase. Employing acetylcholine as substrate in biochemical assays, other workers have noted a correlation between erythrocyte esterase and reticulocytosis in rat and man(5,6). A similar correlation has been found here in rabbits by means of histochemical azo technic.

Histochemical observation of increased esterase activity in polycythemia vera erythrocytes requires confirmation by conventional quantitative methods.

Since the substrate, alpha naphthyl acetate, can be split by all 3 erythrocyte esterases(7) and since anhydrous osmium tetroxide may differentially preserve activity of different enzymes, further work is necessary to identify specific enzymes responsible for dye deposits seen in this histochemical technic.

Summary. A high resolution technic is described for localization of intracellular sites of erythrocyte esterases. Blood smears are fixed in 1% osmium tetroxide in dimethylformamide at −15°C and then incubated in medium containing alpha naphthyl acetate and "hexaazotized' pararosanilin. Alpha naphthol released by enzymatic hydrolysis of the substrate ester couples with "hexaazonium" salt to form a colored, insoluble, amorphous deposit at sites of esterase activity.

ADDENDUM. During the writing of this paper an article appeared in *J. Histochem. and Cytochem.,* 1958, v6, 457, by M. Wachstein and G. Wolf entitled: Histochemical Demonstration of Esterase Activity in Human Blood and Bone Marrow Smears.

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