13. Jamison, R. and Mayor, H. D., J. Bacteriol. **90**, 1486 (1965).
14. Cheong, L., Fogh, J., and Barclay, R. K., Federation Proc. **24**, 596 (1965).
15. May, P., Niveleau, A., Berger, G., and Brailovsky, C., J. Mol. Biol. **27**, 603 (1967).
16. Bergs, V. V. and Bergs, M., Federation Proc. **27**, 681 (1968).

Received Dec. 20, 1968. P.S.E.B.M., 1969, Vol. 131.

Skin-Fixing Properties of Papain Digested Rhesus Antihapten Antibody* (33887)

A. Malley and B. Mackler
Oregon Regional Primate Research Center, Beaverton, Oregon 97005

Many investigators (1–3) have demonstrated that the F_c fragment of rabbit I_gG retains skin-fixing properties following papain digestion. However, attempts to demonstrate skin fixation with the F_c fragments of papain digested human I_gG or guinea pig γS_1-globulin have been unsuccessful (4–5). Hsiao and Putnam (6) demonstrated that the kinetic rate of digestion of I_gG with papain varied greatly within the animal species studied. Their studies also indicated that the 3.5S fraction B was susceptible to further breakdown upon prolonged digestion with papain. Poulik (7) and Grey and Abel (8) demonstrated that prolonged digestion of both human and nonhuman primates I_gG with papain results in the loss of at least one antigenic determinant of the F_c fragment. This new fragment, F'c, was shown to have slower sedimentation properties (2.1S), a greater electrophoretic mobility than the intact F_c fragment, and was detectable by immunoelectrophoresis after 2 hr of incubation with cysteine and papain.

With these studies in mind, we decided to examine the kinetic rate of digestion of specifically purified rhesus monkey (*Macaca mulatta*) antitrinitrophenol (TNP) antibody in an attempt to determine the optimum time of papain digestion that will retain the skin-fixing properties of the F_c fragment.

Materials and methods. Immunoelectrophoresis of specifically purified rhesus anti-TNP antibody before and after papain digestion was run in 0.05 *M* barbital buffer, pH 8.1, $\mu = 0.05$, for 45 min at 150 V and 4 mA/slide. Upon completion of electrophoretic migration upper and lower troughs were cut and filled, respectively, with rabbit antirhesus total globulins and rabbit antirhesus $7S\gamma_2$-globulin.

Rhesus γ-globulin purified by column chromatography on diethylaminoethyl-cellulose in 0. 1 *M* Tris–HCl buffer, pH 8.0, appeared homogenous in the analytical ultracentrifuge (7S peak only) and showed only a single precipitin band by immunoelectrophoresis against rabbit antirhesus total globulins. Rabbit antirhesus $7S\gamma_2$-globulin antisera was prepared by immunizing animals with 10 mg of the purified γ-globulin in complete Freund's adjuvant at weekly intervals for 3 weeks, and were bled 3–6 weeks after the last injection.

A group of eight rhesus monkeys (3–5 kg) were immunized by intravenous injection with 15 mg of protein of picryl-keyhole limpet hemocyanin (TNP-KLH). The TNP-KLH conjugate was prepared as previously described by Rittenberg and Amrkaut (9), and contains 750 moles of TNP/mole of hemocyanin (determined spectrophotometrically). The amount of antihapten antibody was determined by quantitative precipitation (10) using TNP_{28}-bovine serum albumin as the precipitating antigen. Antihapten antibody titers of the sera obtained from the eight immunized rhesus monkeys varied between 0.5–3.4 mg of antibody protein/ml.

* Supported by U. S. Public Health Grant AI-06274-04. Oregon Regional Primate Research Center Publ. No. 388.

TABLE I. Skin Sensitizing Activity of Native and Papain Digested Rhesus Anti-TNP Antibody in Reverse Passive Cutaneous Anaphylaxis.[a]

Digestion time[b] (hr)	Latent period (hr)	Antigen N (μg) injected intracutaneously					
		10	1	0.1	0.05	0.1	Control[c]
		Diam of skin reaction (mm)[d]					
0	0.5	10	7.5	3	—	—	—
	1	15	11	7	tr	—	—
	3	27.5	18.5	7.5	6	tr	—
2	0.5	10	3	tr	tr	—	—
	1	4	tr	—	—	—	—
	3	4	—	—	—	—	—
3.5	0.5	12	3	tr	tr	—	—
	1	6	—	—	—	—	—
	3	4	—	—	—	—	—
8	0.5	3	—	—	—	—	—
	1	4	—	—	—	—	—
	3	4	—	—	—	—	—

[a] Rabbit antirhesus IgG was injected intravenously with 0.3 ml of freshly prepared 1% Evans blue dye 0.5, 1, or 3 hr after the intracutaneous injection of antigen. Reactions were read 15 min after the intravenous challenge.

[b] Indicates the time aliquot of anti-TNP antibody was removed from the reaction mixture, and enzymatic action of papain stopped.

[c] Control site was injected with 112 μg of N of rabbit antitimothy antisera; (—) = negative reaction sites; and tr = trace reactions (less than 3-mm diam).

[d] Reactions are the average of experiments performed on three guinea pigs.

The serum from one of these animals (1383) containing 2.7 mg of antibody protein/ml was utilized in the experiments described below.

Anti-TNP antibody was purified by a slight modification of the method of Farah *et al.* (11). Enzymatic digestion of the anti-TNP antibody (I_gG), 81 mg, was perfomed with crystalline papain in 0.01 *M* phosphate buffer, pH 6, containing 0.01 *M* cysteine and 0.002 *M* ethylenediaminetetraacetate (EDTA). The proportion of 1 mg of papain to 100 mg of γ-globulin was used, and the incubation temperature was 37°. Samples were removed from the reaction mixture at various intervals, and the enzymatic reaction stopped by the addition of iodoacetate to a final concentration of 0.01 *M*. The products of the enzymatic digestion were studied by ultracentrifugation, immunoelectrophoresis, and reverse passive cutaneous anaphylacis (RPCA) (4).

The Spinco model E analytical ultracentrifuge was employed to follow the kinetics of cleavage of the purified anti-TNP antibody resulting from the action of papain. Aliquots removed from the incubation mixture at 2, 4, and 8 hr were immediately run at 59,780 rpm in standard cell with rotor temperature maintained at 20°.

Results and discussion. The ultracentrifugation results (Fig. 1) indicate that under the reaction conditions employed, all of the native 6.6S anti-TNP antibody was cleaved to 3.5S fragments within 2 hr of incubation. Increased incubation times (3.5, 4, and 8 hr) led to an increasing amount of material with a sedimentation constant near 2S (presumably the F'_c fragment). Immunoelectrophoresis (Fig. 2) of the products of enzymatic digestion at 2, 3.5, 4, and 8 hr against rabbit antirhesus I_gG antisera reveal, in addition to the F_c precipitation line, a precipitin band that has a slightly higher electrophoretic mobility than the F_c fragment. This precipitin band forms a line of partial identity (spur) with the precipitin line of the F_c fragment, and becomes more apparent with prolonged

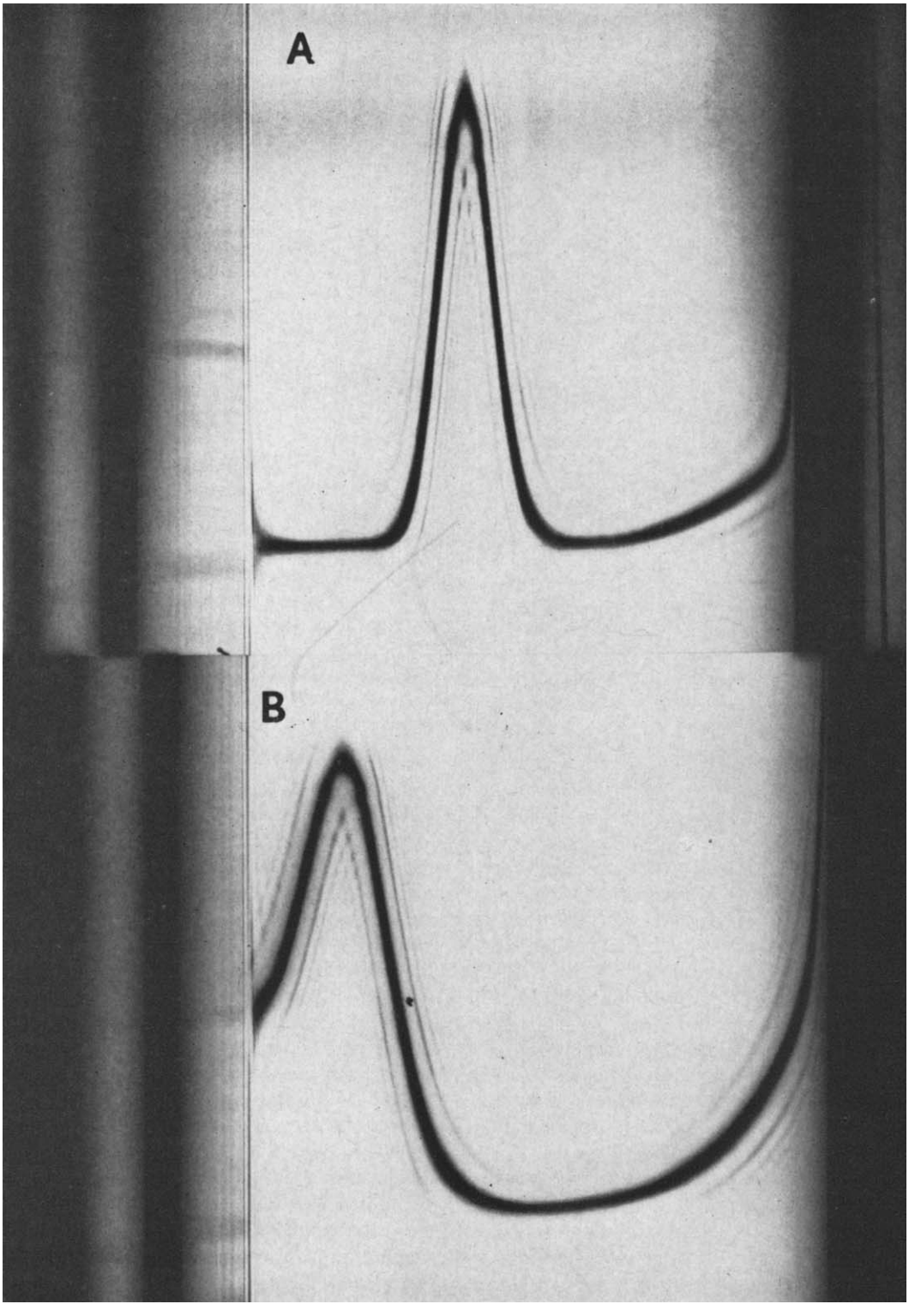

FIG. 1. Analytical ultracentrifugation of purified rhesus anti-TNP antibody (a) prior to papain digestion, and (b) after 2 hr of papain digestion. Samples were run in a standard cell (12 mm) at 20°, at 59,780 rpm, and a phase plate angle of 50°. Pictures were taken at 64 min.

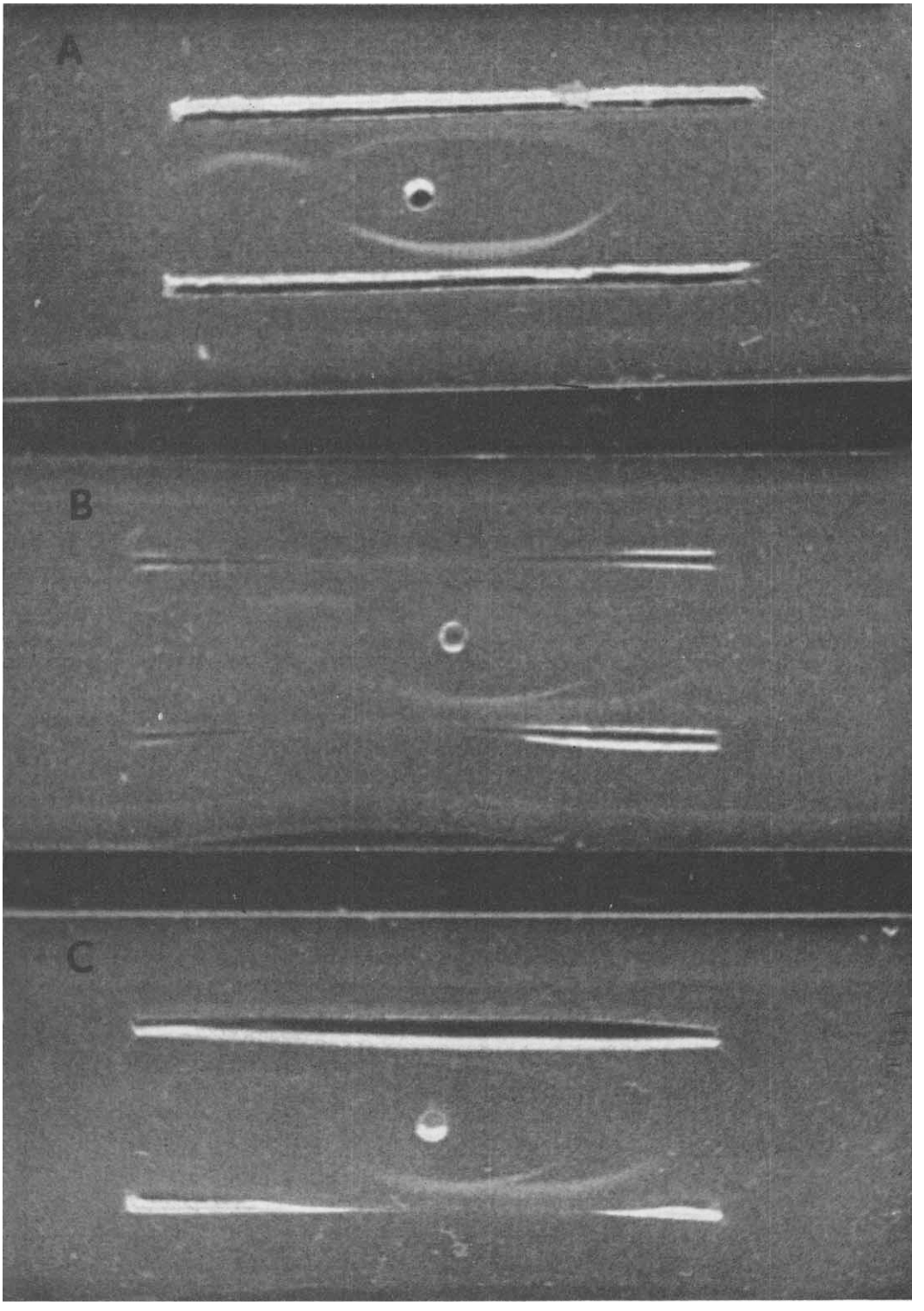

FIG. 2. Immunoelectrophoresis of specifically purified rhesus anti-TNP antibody in 0.05 *M* barbital buffer pH 8.1, $\mu = 0.05$. Center wells contain rhesus anti-TNP antibody (a) prior to papain digestion, (b) after 4 hr of papain digestion, and (c) after 8 hr of papain digestion. In each case the upper and lower troughs contain, respectively, rabbit antirhesus total globulins, and rabbit antirhesus 7Sγ_2-globulin.

digestion of the anti-TNP antibody. These immunoelectrophoresis observations made with purified rhesus anti-TNP antibody are similar to those obtained by Poulik (7) using human I_gG, and suggest that the additional precipitin line formed during enzymatic digestion is due to the formation of the F'_c component.

Table I shows the results of RPCA studies with the undigested and papain digested fragments of purified rhesus anti-TNP antibody. The data presented show that the time between intracutaneous injection of digested fragments and intravenous challenge with rabbit antirhesus I_gG, the latent period, is critical. With a short latent period (0.5 hr) we were able to demonstrate that both the 2- and 3.5-hr digests retained significant skin-fixing properties. In contrast, the RPCA reactions with undigested antihapten antibody increased significantly with longer latent periods (1 and 3 hr). Consequently, the observed positive reactions were not due to undigested antihapten antibody present in the 2- and 3.5-hr digested samples at a level below the limit of detection in the model E analytical ultracentrifuge. Control sites containing 112 μg of N of rabbit antitimothy sera were negative indicating that the positive reactions observed with a 0.5-hr latent period were not due to nonspecific dye leakage.

Immunoelectrophoresis and ultracentrifugation data indicate that papain digestion of rhesus antihapten antibody for periods greater than 2 hr results in increased amounts of F'_c fragments. The loss of skin-fixing properties of the F_c fragment associated with prolonged digestion may be due to the splitting off of critical peptides or changes in the three-dimensional structure of the remaining F_c fragment. The short latent periods necessary to demonstrate skin fixation of the F_c fragment suggest that as a consequence of prolonged papain digestion either the teritary structure of the skin fixation site has been altered limiting fixation or that peptides involved in secondary binding forces to tissues are lost. Employing the conditions described in these experiments it may be possible to extend these studies to further breakdown products of the F_c fragment in an attempt to elucidate the chemical structure of the skin-fixation site.

Summary. Digestion times less than 3.5 hr and a short latent period (0.5 hr) were found to be critical in demonstrating the skin-fixing properties of papain digested (3.5S) fragments of rhesus antitrinitrophenol antibody by reverse passive cutaneous anaphylaxis.

1. Ovary, Z., and Karush, F., J. Immunol. **86**, 146 (1961).

2. Ishizaka, K. and Ishizaka, T., J. Immunol. **93**, 59 (1964).

3. Paraskeras, F. and Doodman, J. W., Immunochemistry **2**, 391 (1965).

4. Franklin, E. C. and Ovary, Z., Immunology **6**, 434 (1963).

5. Nussenzweig, V. and Benacerraf, B., J. Immunol. **93**, 1008 (1964).

6. Hsiao, S. and Putnam, F. W., J. Biol. Chem. **236**, 122 (1961).

7. Poulik, M. D., Nature **210**, 133 (1966).

8. Grey, H. M. and Abel, C. A., Immunochemistry **4**, 315 (1967).

9. Rittenberg, M. B. and Amkraut, A. A., J. Immunol. **97**, 421 (1966).

10. Campbell, D. H., Garvey, J. S., Cremer, N. E., and Sussdorf, D., "Methods in Immunology," p. 136. Benjamin, New York (1963).

11. Farah, F. S., Kern, M., and Eisen, H. N., J. Exptl. Med. **122**, 1195 (1960).

Received Jan. 23, 1969. P.S.E.B.M., 1969, Vol. 131.