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Naturally Occurring Triglycerides Possessing Optical Activity in the Glycerol Moiety*

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Received September 9, 1963

Seed oils of the Chinese tallow tree, *Sapium sebiferum*, and of *Sebastiana lingustrina* were found to be optically active. By column chromatography, the light-absorbing and optically active triglyceride components of *Sapium sebiferum* were isolated and were found to have $[\alpha]_D^{20°} = -21.5°$ in 20% solution in chloroform. The comparable fraction from *Sebastiana lingustrina* exhibited $[\alpha]_D^{20°} = -17.5°$. The $E_{1cm}^{1\%}$ of the optically active fraction was 900 at 260 mμ. The optically active fraction was separated into four subfractions by countercurrent distribution and these had rotations similar to the optically active fraction, suggesting that all the triglycerides in the optically active fraction are optically active. The unsaponifiable portion of the optically active fraction was demonstrated by thin-layer and gas-liquid chromatography to be glycerol. Fatty acid composition of the four subfractions, measured by gas-liquid chromatography, indicated the ratio of 1 mole unsaturated carboxyl-conjugated fatty acid per mole triglyceride. The unsaturated carboxyl-conjugated acids are considered to be on the α position of the glycerol.

Naturally occurring compounds which possess potentially asymmetric centers usually are found in only one of the optically active enantiomorphic forms in a single source. Racemic mixtures of enantiomorphs are rarely found in nature. The principal components of proteins and carbohydrates exhibit definite optical rotation, whereas the most abundant components of fats, the triglycerides, rarely exhibit measurable optical activity despite the fact that the β-carbon of the glycerol moiety of a triacid triglyceride is potentially asymmetric. The measurable optical activity found in a few natural oils has been traceable to unsaponifiable matter, such as sterols, or to optically active fatty acids within the triglyceride.

Considerable effort has been expended to synthesize asymmetric triglycerides of fatty acids with possible optical rotatory power, but without success, whereas with substituents other than fatty acids glycerol derivatives with high optical activity have been obtained (Fischer and Baer, 1941; Baer and Fischer, 1937). Recently W. Schlenk, Jr. (1962) synthesized 1-trimethylacetyl-2,3-di-*n*-valerin and found it to have a specific rotation of $[\alpha]_D = +1.6°$. He also reported that triglycerides having one (or two) short-chain and two (or one) long-chain normal fatty acids exhibit small but distinct optical rotation in the ultraviolet. Triglycerides such as 1-palmito-2-oleo-3-stearin, containing three long-chain acids, showed no rotation in the visible or ultraviolet spectrum. In these reports the authors show that the naturally occurring triglycerides having mostly long-chain acids may occur in asymmetric forms, but that the structural differences in the three fatty acid radicals are not sufficient to exhibit measurable optical rotation in the visible or near-ultraviolet spectra. To our knowledge, an optically active naturally occurring triglyceride whose center of asymmetry resides in the glycerol moiety has not previously been isolated. We wish to report the isolation and characterization of mixtures of such triglycerides from the seed oil of two species of the botanical family *Euphorbiaceae*.

* Supported in part by a grant (HE 03559) from the National Institutes of Health, U. S. Public Health Service.

EXPERIMENTAL

Seeds of the Chinese tallow tree, *Sapium sebiferum* (otherwise known as *Stillingia sebiferum*), were collected in Houston, Texas, in 1954. The seeds were freed of the external tallow by steam treatment and the dried seeds were ground and pressed. The press cake was extracted with light petroleum hydrocarbon (bp 30–45°) to give a second crop of oil. The seeds of *Sebastiana lingustrina* were collected in eastern Texas in 1962. These were ground in an Omni-mixer and extracted with petroleum hydrocarbon–diethyl ether (1:1) and with chloroform.

In a preliminary study of the oil of *Sapium sebiferum* (Huang *et al.*, 1949), its high optical activity had been noted. In an attempt to identify the component responsible for this activity, a preliminary analysis of the oil was made by thin-layer adsorption chromatography. In this analysis Silica Gel G (W. R. Grace Co.) (Stahl, 1958) was the adsorbent and petroleum hydrocarbon (bp 30–45°)–diethyl ether–acetic acid, 80:20:2, was the solvent. Components were made visible by exposure to iodine vapor or by charring with sulfuric acid. Such thin-layer chromatographic analysis indicated two components near the area normally occupied by triglycerides.

To accumulate these two components in sufficient quantity, 25-g portions of *Stillingia* oil were chromatographed on a column 2 × 140 cm, packed under vibration with silica gel, and cooled by a water jacket. The column was prewashed with 2 liters of petroleum hydrocarbon, and the sample was added and eluted with 4 liters petroleum hydrocarbon containing 5% dry diethyl ether. This eluted the first component, called fraction 1, the uppermost spot on the thin-layer chromatography plate. The second component was eluted with 3 liters petroleum hydrocarbon containing 10% dry diethyl ether. The fractionation was monitored by thin-layer chromatography and stopped when the second component had emerged. The column was then purged with diethyl ether–methanol (1:1) to recover the remaining more polar components as fraction 3.

Gas-liquid chromatography was performed on methyl esters obtained from the several fractions by interesterification using 5% HCl in methanol. A Barber-Coleman Model 10 instrument equipped with an argon ionization detector was used. The column, 8 ft × 0.25-in. glass, was packed with 20% ethylene glycol succinate polyester on Gas Chrom P (Applied Science Laboratories). The chromatographs were operated at 155° under an argon flow of 60 ml/min.

Specific rotations were measured at 589 mμ (sodium D) in a Bellingham & Stanley polarimeter using a 2-dm tube and 20% solutions in chloroform. The customary second measurement in an alcohol was precluded because of the insolubility of our triglycerides in these solvents.

Countercurrent distribution was effected upon the components of fraction 2 in a 200-tube Post apparatus holding 40 ml of lower phase in each tube. The lower phase was furfural-nitroethane (1:1) and petroleum hydrocarbon (bp 30–45°) served as upper phase (Scholfield and Hicks, 1957). Three series were performed, and each was repeated twice to provide 600 transfers. To start each operation, 5 g of fraction 2 was distributed equally over the first ten tubes. At the end of 600 transfers the upper phases were collected from each tube, and the concentration of substance in the upper phase of every fifth tube was determined gravimetrically. The center portion of each major maximum was collected for further use.

The unsaponifiable matter of fractions 1 and 2 was isolated as follows: Three grams of each fraction was saponified with 30% methanolic KOH at room temperature overnight. The mixture was extracted serially with diethyl ether and chloroform. The extract was evaporated, weighed, and found to be essentially zero, thus precluding significant amounts of lipid-soluble unsaponifiable components. The alkaline phase was acidified and the liberated fatty acids were removed by extraction with petroleum hydrocarbon. The aqueous phase, containing salts, and the unsaponifiable residue were lyophilized and taken up in a few ml of ethanol. This procedure was repeated twice to remove most of the inorganic contaminants. The crude product was finally distilled in high vacuum in a microstill to yield a yellow highly viscous liquid.

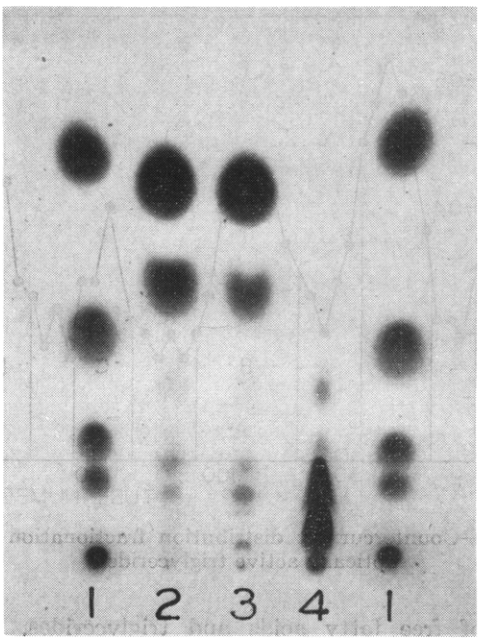

FIG. 1.—Thin-layer chromatography of the seed oils of *Sapium sebiferum* and *Sebastiana lingustrina*. (1) Standard containing from bottom to top: mono-olein, 1,2- and 1,3-diolein, oleic acid, and triolein. (2) Whole oil of the seeds of *Sapium sebiferum* (*Stillingia* oil). (3) Whole oil of *Sebastiana lingustrina*. (4) Castor oil, containing optically active triglycerides containing hydroxy fatty acids, demonstrating the absence of such glycerides in the oils of *Sapium sebiferum* and *Sebastiana lingustrina*.

This unsaponifiable component, suspected to be glycerol, was converted to its $LiAlH_4$ complex, which was treated with acetic anhydride to yield the triacetate. The acetylation with acetic anhydride gives better yields of acetate when the $LiAlH_4$ is used than when the free alcohol is treated. The acetate was identified by comparison with known substances using thin-layer and gas-liquid chromatography. The identification of the glycerol component was verified on other samples of fractions 1 and 2 by the procedure of Horrocks and Cornwell (1962) in which esters are reduced and converted to acetates, followed by the gas-liquid chromatographic analysis of the acetates of the fatty alcohols and of glycerol.

RESULTS

The total *Stillingia* oil was found to have a specific rotation of $[\alpha]_D^{20°} = -6.0°$ for both expressed and extracted samples, in fair agreement with the −5.0° found by Huang *et al.* (1949). The oil had an ultraviolet spectrum showing a smooth maximum at 260 mμ and a significant shoulder at 310 mμ, similar to that previously reported (Huang *et al.*, 1949). The fatty acid composition of the oil is given in Table I, and agrees reasonably with data gathered by fractional distillation methods (Crossley and Hilditch, 1949). The methyl esters of fraction 2, chromatographed to remove tri-, di-, and monoglycerides, were found to have a specific rotation of zero.

Thin-layer chromatography of the total oil (Fig. 1) revealed two major components and a series of very minor ones. The fastest-migrating major component moved at a rate equal to that of authentic triglycerides. The second component had an R_F value between

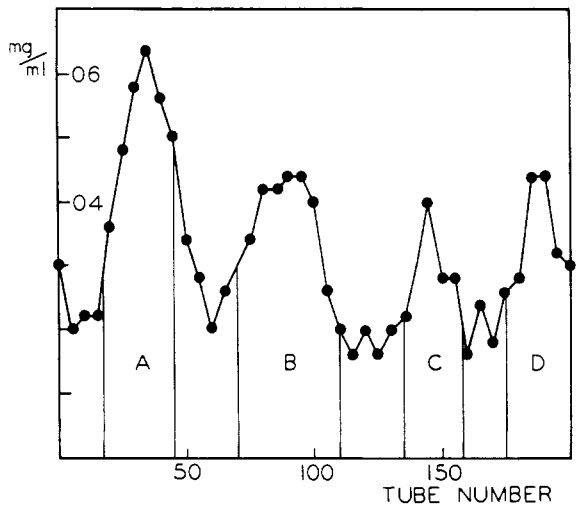

FIG. 2.—Countercurrent distribution fractionation of the optically active triglycerides.

those of free fatty acids and triglycerides, but it did not correspond to any known lipid component.

Fraction 1 from the column chromatography (71%) had no optical activity, and it exhibited no maximum in its ultraviolet spectrum. Its fatty acid composition, given in Table I, indicates the absence of short-chain fatty acids. The major fatty acids are oleic, linoleic, and linolenic. Therefore this fraction consisted of "normal" triglycerides, and no further investigation was required. The polar components were collected together as fraction 3 (2.5%) in the column chromatography. Fraction 3 contained at least six components but, because its specific rotation was only −0.5°, no further study was made of this fraction.

Fraction 2 (26%) was found to have a specific rotation of $[\alpha]_D^{20°} = -21.9°$ in good agreement with the value −22.5° calculated from the rotation of the whole oil and the content of fraction 2. The ultraviolet absorption spectrum of fraction 2 indicated the presence of the 2,4-decadienoate, having an extinction coefficient, $E_{1cm}^{1\%}$, at 260 mμ equal to 900. The fatty acid composition (Table I) showed that 31.5 mole % of the fatty acids were short-chain carboxyl conjugated acids. Of these, 2,4-decadienoic acid was by far the major acid. Because of the atypical R_F value of fraction 2 and because of its unique optical rotation, it was suspected not to be a true triglyceride. Therefore the unsaponifiable moieties were isolated from fractions 1 and 2 and identified. After lyophilization and conversion to the acetate, both the compounds showed the same R_F value as a standard of authentic triacetin run on the thin-layer chromatography plate. Gas-liquid chromatographic analysis of these samples revealed that each consisted of one component having retention time identical to that of the triacetin standard which had a carbon number of 17.7 on the ethylene glycol succinate column. Thus the components of both fractions 1 and 2 are triglycerides.

Using adsorption chromatography, the optical activity had been separated in fraction 2. An effort was then made, using a partition method, to further concentrate the active component. Countercurrent distribution resolved fraction 2 into four distinct subfractions after 600 transfers. The center portions of each maximum (Fig. 2) were collected to assure minimum contamination by neighboring fractions. The optical activities and fatty acid compositions of the four subfractions are shown in Table II. The four had essentially equal optical activities, equal also to the starting material. However, the fatty acid compositions differed considerably. Therefore, although fraction 2 could not be completely resolved into single triglycerides, the optical activity was spread evenly in all subfractions, suggesting that all the triglycerides in fraction 2 are optically active and have approximately equal rotatory power. The optical activity is therefore due not to a single triglyceride but to all members of fraction 2.

From the approximate fatty acid composition (shown in Table II) it is evident that each triglyceride of fraction 2 contains one short-chain unsaturated carboxyl-conjugated fatty acid, whereas these acids (10:2, α, β, and γ) do not occur in the triglycerides of the optically inactive fraction 1. Hydrogenation of fraction 2 reduced the specific rotation to zero. The

TABLE I

FATTY ACID DISTRIBUTION IN THE OIL OF *Sapium sebiferum*

Fatty Acids	Gas-Liquid Chromatography Carbon Number of Methyl Ester	Total Oil (mole %)	Fraction 1 (mole %)	Fraction 2 (mole %)
10:2	12.9	8.7	—	25.5
α[a]	13.6	0.7	—	3.4
β[a]	14.5	1.3	—	1.1
γ[a]	15.4	1.1	—	1.5
16:0	16.0	6.4	6.0	4.8
18:0	18.0	2.0	1.1	7.6
18:1	18.4	11.8	11.6	9.0
18:2	19.1	25.8	27.2	22.1
18:3	19.9	42.3	54.1	25.1

[a] Unidentified unsaturated acids. Ultraviolet spectra indicate carboxyl conjugation.

unsaturated carboxyl conjugated acids therefore impart the optical activity to the triglycerides, although the ethyl esters of these short-chain acids were found to have no rotatory power. Therefore the center of asymmetry resides in the β-carbon of the glycerol moiety and not in the fatty acids.

As the wavelength at which optical rotation was measured approaches the absorption maximum of a substance, the observed specific rotation increases through a maximum, passes through zero at the absorption maximum, passes through a maximum of opposite sign, and decreases again as the wavelength moves from the absorption maximum. This is the Cotton effect sometimes seen in the measurement of optical rotatory dispersion (Djerassi, 1960). Asymmetric molecules which bear an absorbing chromophore have greater rotatory power at a given wavelength than their analogs without such chromophores. In Figure 3 the optical rotatory dispersion curves of native *Stillingia* oil and of the ethyl esters prepared from the oil indicated that the optical rotation of the triglyceride increased negatively as one approached the ultraviolet absorption maximum of the oil at 260 mμ. On the other hand, the esters of the oil exhibited a constant but negligible rotation. The phenomenon described in Figure 3 is ascribed to the triglycerides of fraction 2. We believe that in these triglycerides we have a strongly absorbing chromophore near the center of asymmetry, which accounts for the enhanced rotatory power.

TABLE II
AVERAGE FATTY ACID COMPOSITION OF FRACTIONS FROM THREE COUNTERCURRENT DISTRIBUTIONS

Fraction	Specific Activity	Fatty Acid (mole %)							
		10:2	$\alpha + \beta + \gamma$	16:0	16:1	18:0	18:1	18:2	18:3
A	18.5°	32.8	4.6	6.9	1.1	—	12.6	25.3	16.2
B	20.3°	24.1	4.0	16.6	0.6	3.1	16.7	30.4	4.7
C	19.8°	22.6	3.8	6.2	0.6	1.9	11.6	4.6	48.6
D	19.4°	17.4	3.2	2.1	0.8	0.9	2.8	38.7	34.1

DISCUSSION

Hirayama (1961) separated the oil of *Sapium sebiferum* into eleven fractions by countercurrent distribution. He did not observe the ultraviolet absorption or optical rotation of his fractions. He found that four of his fractions contained 2,4-decadienoic acid in the ratio of 1 mole per mole triglyceride. Our analyses of the four subfractions suggest the same ratio. That the proportions of short-chain fatty acids were found lower than theoretical is not surprising because of the volatility of the esters of these acids and the low response of short-chain esters in gas-liquid chromatographic analysis. Hilditch (1956) has postulated that the 2,4-decadienoic acid is esterified to the β-hydroxyl of glycerol, reasoning from melting points of hydrogenated preparations. His postulate was at first attractive because the conjugation would then lie at the closest possible position to the asymmetric carbon atom, and enhancement might be maximal. If, however, the 2,4-decadienoic acid were esterified with the α-hydroxyl of the glycerol moiety, the potential asymmetries would be greater. Even in the case where both long-chain acids are equal, asymmetry exists if the decadienoic acid is located in the α position. Subfraction C, obtained by countercurrent distribution, has considerably more than one mole linolenate per mole triglyceride, 1.5, 1.7, and 2.0, respectively, for the three countercurrent fractionations. Thus a large proportion of the triglycerides present must contain two moles linolenate per mole triglyceride, and would therefore be symmetrical according to Hilditch's postulate. Therefore we feel that the position of the conjugated acids in the triglyceride is most likely α.

To test our assumption that the unsaturated carboxyl-conjugated fatty acid was responsible for the rotatory power of the triglycerides isolated from *Stillingia* oil, we investigated the triglycerides from the seed oil of the closely related species *Sebastiana lingustrina*, which also has been reported to contain a 2,4-dienoic acid (Holman and Hanks, 1955). The thin-layer chromatogram of the total oil showed exactly the same pattern as that for *Stillingia* oil (Fig. 1). Therefore its fraction 2 was obtained by column chromatography. The total oil had a specific rotation of $[\alpha]_D^{20} = -2.8°$ and its fraction 2 had $[\alpha]_D^{20} = -17.5°$. Thus the optically active triglycerides of *Sebastiana lingustrina* are similar in rotatory power to those of *Sapium sebiferum*.

The results presented here indicate the existence of several optically active triglycerides in the seed oil of *Sapium sebiferum* and of a closely related species, *Sebastiana lingustrina*. The center of asymmetry has been located in the glycerol moiety. We believe this is the first demonstration of a naturally occurring optically active triglyceride whose center of asymmetry resides in the β-carbon of glycerol. These triglycerides exhibit strong optical rotation because of the enhancement afforded by carboxyl conjugation of one of the fatty acids which probably is esterified with the α-hydroxyl group of glycerol. The occurrence of such glycerides proves that, at least in the species examined, triglycerides are not racemic mixtures. If this be true in general, natural triacid triglycerides occur in one of the enantiomorphic forms. The common fatty acids in triglycerides differ only slightly in their chemical structures and physical properties, and the rotatory power of their asymmetrical triglycerides is too small to be measured at the sodium D line. Differences between fatty acids caused by a remote double bond or 2 carbon atoms are not sufficient to be expressed as measurable rotation in the visible spectrum. Common triglycerides have their absorption maxima near 190 mμ and their extinction coefficients are relatively low, $E^{1\%}_{1cm}$ = about 300 (Rusoff *et al.*, 1945). In our triglycerides containing unsaturated carboxyl-conjugated fatty acids, the absorption maximum is shifted to 260 mμ and $E^{1\%}_{1cm}$ = 900. The increased absorption at a wavelength closer to the visible spectrum contributes significant enhancement of the optical rotation measured at the sodium D line.

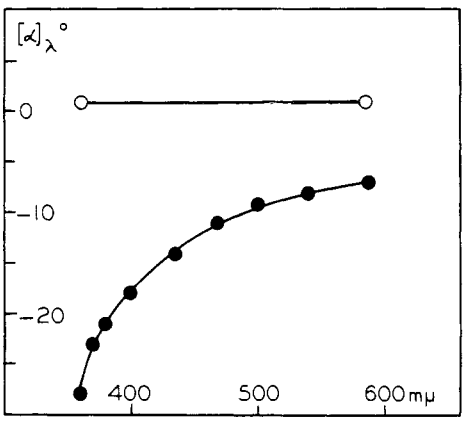

FIG. 3.—Optical rotatory dispersion of the oil of *Sapium sebiferum* and its ethyl esters.

ACKNOWLEDGMENT

The authors are grateful to C. H. McLeod of Sam Houston State College, Huntsville, Texas, for the collection of the seeds of *Sebastiana lingustrina*, to James Hamilton for collection of seeds of *Sapium sebiferum*, to E. Horning of the National Heart Institute for the measurements of optical rotatory dispersion, and to H. K. Mangold and L. J. Morris for their many valuable suggestions.

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Differences among Antibodies Formed in Response to the *p*-Azobenzenephosphonate and *p*-Azobenzenearsonate Haptens*

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Received July 1, 1963

The relative binding constants of various substituted benzenephosphonates and benzenearsonates for the reaction with rabbit antibodies formed in response to the *p*-azobenzenephosphonate (P_p) haptenic group have been measured by hapten inhibition of specific precipitation. These values exhibit a variation with hapten structure that is distinctly different from that shown with antibodies formed in response to the *p*-azobenzenearsonate (R_p) haptenic group. Although anti-R_p antiserum contains two distinct antibodies, one formed in response to the doubly charged ion, the other to the singly charged ion, and anti-P_p antiserum apparently contains primarily antibody formed in response to the doubly charged ion, the difference between the two antisera in reaction patterns with various haptens appears to be attributable, for the most part, to a difference between antibodies formed in response to the doubly charged ions. The data are interpreted to mean that the combining site of the anti-P_p antibody fits more loosely about doubly charged benzenephosphonate than the combining site of anti-R_p antibody fits about doubly charged benzenearsonate.

Recent studies (Kreiter and Pressman, 1963) have indicated that anti-R_p serum contains two populations of antibodies formed in response to the two ionic forms of *p*-azobenzenearsonate. In contrast, anti-P_p serum contains antibody formed only in response to the doubly charged phosphonate ion. Such a difference between the two antisera suggests that their reaction patterns with various haptens should be different.

Although a large amount of information is available on the specificity of antiserum formed in response to the *para*-azobenzenearsonate group (R_p system) (Landsteiner, 1945; Pauling and Pressman, 1945; Erlenmeyer and Berger, 1932), very little information is available regarding the specificity of the antiserum formed in response to the structurally similar *para*-azobenzenephosphonate group (P_p system) except that it reacts strongly with benzenearsonate haptens (Kreiter and Pressman, 1963). The structural similarity between the benzenearsonate and the benzenephosphonate groups was found by Erlenmeyer and Berger (1932) to result in extensive cross reaction of these substances with anti-R_p antibody. On this basis it would appear that the specificity of anti-P_p antibodies should closely resemble the specificity of anti-R_p antibodies.

The relative binding constants for the reactions of several substituted benzenephosphonic acids and benzenearsonic acids with anti-P_p and anti-R_p rabbit γ-globulins have been measured by determining their ability to inhibit precipitation of antibody by the homologous hapten coupled to a foreign protein. A distinct difference in reaction patterns was found between the two systems that could not be attributed to the presence of more than one antibody in anti-R_p serum, but rather to a difference in specificity between antibody formed in response to the doubly charged *p*-azobenzenephosphonate ion and antibody formed in response to the doubly charged *p*-azobenzenearsonate ion.

* Supported in part by a grant (E-2342) from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, U. S. Public Health Service.

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EXPERIMENTAL

Antigens.—The antigens (R_p-bovine γ-globulin and P_p-bovine γ-globulin used for immunizing rabbits were prepared by coupling diazotized *p*-aminobenzenearsonic acid and *p*-aminobenzenephosphonic acid to bovine γ-globulin (fraction II) (Kreiter and Pressman, 1963). Test antigens were prepared by coupling the diazotized amines to ovalbumin. The ovalbumin test antigens were purified by acetone extraction (Nisonoff and Pressman, 1958).

Antisera.—Rabbits were injected intravenously with 1 ml of 1% R_p-bovine γ-globulin antigen three times a week for 3 weeks. The animals were bled 1 week after the last injection and weekly thereafter. One ml of antigen was injected immediately after each bleeding. Anti-P_p sera were obtained in a similar manner. The antisera from the first 4 months were pooled according to the titer obtained with the ovalbumin test antigens. A single pool of each antiserum was used in all the experiments. The pool of anti-P_p antiserum and the pool of anti-R_p antiserum used here were the same as those used in our previous study (Kreiter and Pressman, 1963). The anti-P_p pool was obtained from the sera of nine rabbits, three of which contributed equally to about two-thirds of the total