[CONTRIBUTION FROM THE LABORATORIES OF PHYSICAL AND PHYSIOLOGICAL CHEMISTRY, UNIVERSITY OF WISCONSIN]

Biophysical Studies of Blood Plasma Proteins. VIII. Separation and Properties of the γ-Globulins of the Sera of Normal Cows[1]

BY E. L. HESS AND H. F. DEUTSCH

By applying the latest methods of Cohn, *et al.*,[2] for the separation of γ-globulins from human plasma, two immune globulins have been fractionated from the plasma of hyperimmunized cows by Smith.[3] Prior to this time Cohn and associates[4] had described the fractionation of the bovine plasma proteins by equilibration across membranes with ethanol–water mixtures of controlled *p*H, ionic strength and temperature. Work in this Laboratory on the recovery of the γ-globulins of animal sera indicates that increased yields and sharper fractionations may be obtained by determining methods more or less specific for the removal of a given component in the plasma of a particular species of animal.[5] There has been evolved what is believed to be the optimum *combination* of conditions for the separation and recovery of the γ-globulins of normal bovine sera. Under these conditions two closely related γ-globulin fractions differing in electrophoretic mobility and in solubility have been separated in good yield from normal bovine serum.[5a] The conditions of separation, yield, and some of the physical properties of these normal bovine serum γ-globulins constitute the subject of this report.

Fractionation Studies

Pooled sera of Holstein cows served as a source of starting material. The experimental conditions used to separate the γ-globulins in crude form are consistent with those previously reported.[5] In this way there was obtained a fraction of composition 85% γ-globulins and 15% β-globulins. The electrophoretic patterns in Figure 1 follow the course of the fractionation. Figures 1A and 1B represent the serum and the first crude fraction (hereafter called precipitate A), respectively. The conditions necessary to precipitate the beta globulins from precipitate A, while maintaining the major portion of the γ-globulins in solution, were then studied in detail. These experiments were carried out on 1-g. samples of precipitate A. As shown in Fig. 2, the conditions *p*H 4.95 (±0.05), μ = 0.01, and ethanol concentration = zero, represented suitable conditions for this separation. The γ-globulin resulting after the removal of β-globulin is indicated by the electrophoretic pattern shown in Fig. 1C.

This γ-globulin fraction (Fig. 1C) which is heterogeneous on electrophoresis, was separated into two sub-fractions in a manner similar to that employed previously in the analogous system of human γ-globulin.[6] The effect of variation of ethanol concentration at *p*H 5.65 (±0.05) was first studied on 2-g. samples of precipitate C since the γ-globulins of higher electrophoretic mobility should be isoelectric near this *p*H. The effect of *p*H variation in the sub-fractionation of the γ-globulins was likewise investigated, starting with 2-g. samples of precipitate C, with the results shown in Fig. 3. A *p*H of 5.8 and an ethanol concentration of 10% was selected as suitable for a separation of the γ_1- and γ_2-globulins.

The effect of variation of ionic strength, at *p*H 5.80 (±0.05) and 10% ethanol concentration was then studied, with the result shown in Fig. 4. The electrophoretic patterns of the γ_1- and γ_2-globulin sub-fractions obtained by precipitating the γ_1 fraction at *p*H 5.8 (±0.05) ethanol concentration of 10%, and μ = 0.01, under which conditions approximately equal amounts of γ_1- and γ_2-globulins result, are shown in Fig. 1 for precipitates C-1 and C-2, respectively.

The complete fractionation procedure, with yield data for the gamma globulins, is indicated schematically in Fig. 5.

Characterization Studies

Diffusion and Sedimentation.—Diffusion studies of Kahn and Polson,[7] which were carried out in this Laboratory, indicate the same diffusion constant for both γ_1- and γ_2 fractions (precipitates C-1 and C-2), giving $D_{20w} = 4.1 \times 10^{-7}$ sq. cm./sec. at a protein concentration of 1%. The normalized curves indicate traces of polydispersity.

The two fractions were analyzed in the standard Svedberg oil turbine ultracentrifuge for their molecular mass spectra. Observations of the change of position of the boundaries were made by using a schlieren optical system. Within the limits of experimental error, the same sedimentation content is observed for the γ_1- and γ_2-globulin sub-fractions. At a protein concentration of 1%

(1) This work was supported in part by grants from the Wisconsin Alumni Research Foundation, and the U. S. Public Health Service.

(2) (a) E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin and H. L. Taylor, THIS JOURNAL, **68**, 459 (1946); (b) J. L. Oncley, M. Melin, D. A. Richert, J. W. Cameron and P. M. Gross, Jr., in press.

(3) E. L. Smith, *J. Biol. Chem.*, **164**, 345 (1946).

(4) E. J. Cohn, J. A. Luetscher, J. L. Oncley, S. H. Armstrong, Jr., and B. D. Davis, THIS JOURNAL, **62**, 3396 (1940).

(5) J. C. Nichol and H. F. Deutsch, *ibid.*, **70**, 80 (1948).

(5a) In accordance with our terminology for the immune globulins of humans, we have called the fraction of higher electrophoretic mobility, γ_1-globulins, and the fraction of lower mobility, γ_2-globulins. Thus the γ_1-globulin fraction is comparable to the "T" component of Smith[3].

(6) H. F. Deutsch, R. A. Alberty and L. J. Gosting, *J. Biol. Chem.*, **165**, 21 (1946).

(7) D. S. Kahn and A. Polson, *J. Phys. Coll. Chem.*, **51**, 816 (1947).

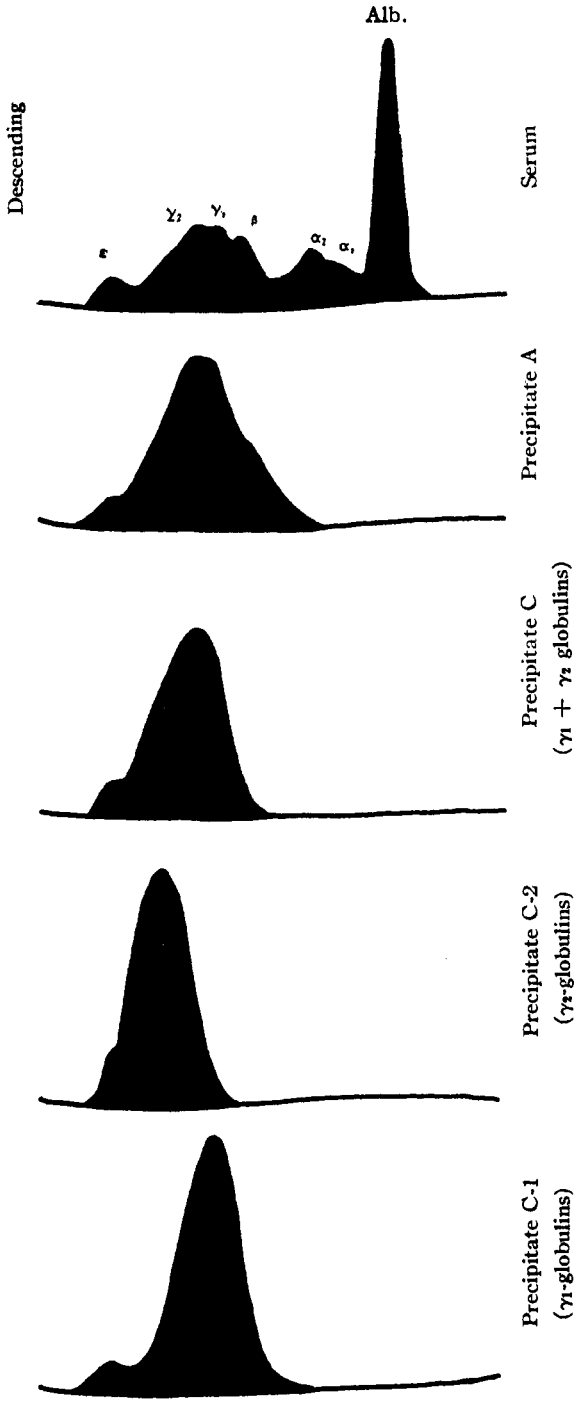


Fig. 1.—Electrophoretic patterns of bovine serum fractions in barbiturate–citrate buffer: μ, 0.088; pH, 8.6 after 120 minutes at 8.5 volts/cm.; concentration of protein 3%.

the sedimentation constant is $s_{20w} = 7.4S$. The sedimentation diagrams themselves are shown in Figs. 6B and 6D. An analysis of Fig. 6B indicates the presence of approximately 4% of heavier constituents with $s_{20w} \cong 9S$ (but no component

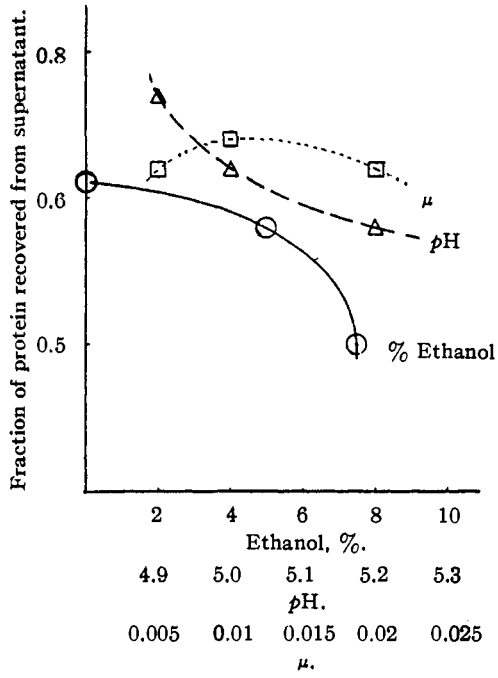


Fig. 2.—The effect of variation of per cent. ethanol, pH and ionic strength in the separation of β- and γ-globulins: ⊙, % ethanol at pH 5.1, μ = 0.0065; △, pH at μ = 0.0065, ethanol none; ⊡, ionic strength at pH 5.1, ethanol none. Protein concentration in all cases is 0.25%.

with $s_{20w} = 20S$) in the γ_1-globulin sub-fraction. An examination of Fig. 6D shows that all the γ_2-

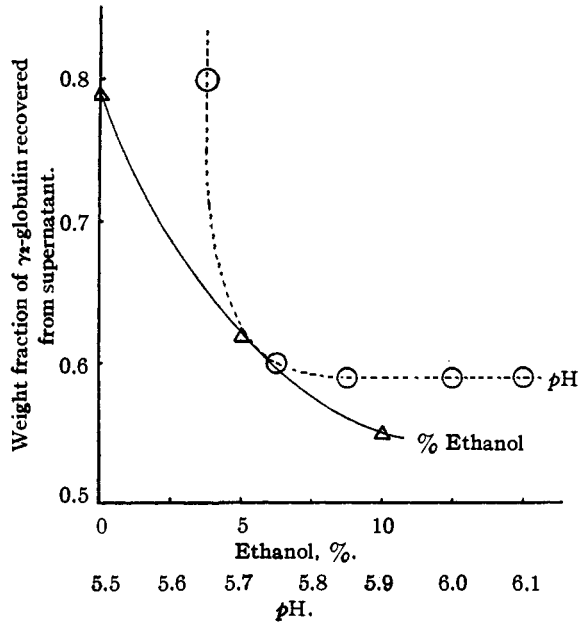


Fig. 3.—The effect of variation of ethanol concentration and pH in the subfractionation of γ-globulins: ⊙, pH variable with % ethanol = 0, μ = 0.008; △, ethanol variable, pH 5.65 ± 0.05, μ = 0.008. Concentration of protein is 0.5% in all cases.

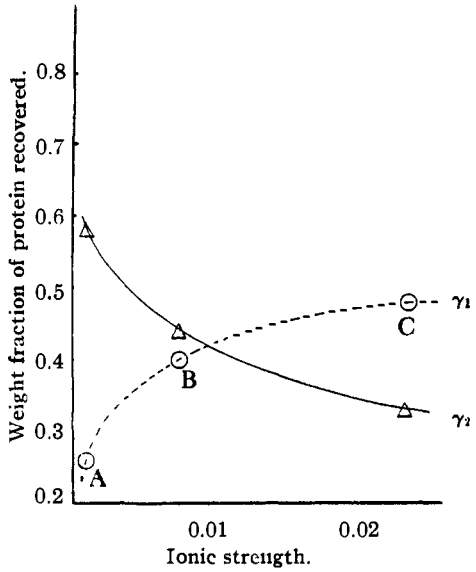


Fig. 4.—The effect of ionic strength on the yield of gamma one and gamma two globulins at *p*H 5.80 ± 0.05 and ethanol concentration = 10%; ⊙, weight fraction of γ_1-globulin precipitated; △, weight fraction of γ_2-globulin that can be obtained from the supernatant.

globulins have sedimentation constant $s_{20w} = 7.4S$ within the limits of experimental error.

In order to apply a criterion of homogeneity to the sedimentation diagrams an apparent diffusion constant was calculated from the sedimentation diagram 6B. An apparent diffusion constant of $D_{20w} = 5.4 \times 10^{-7}$ sq. cm./sec. was obtained, indicating that, as regards sedimentation behavior, the γ_1 sub-fraction is essentially monodisperse. Similar analysis applied to the sedimentation diagram for the γ_2-globulin leads one to the same conclusion.

The γ_1- and γ_2-globulins have a molecular weight of approximately 175,000 based upon the sedimentation velocity and independent diffusion measurements. The frictional ratio $f/f_0 = 1.4$ may be computed from diffusion data. This molecular weight agrees well with values which have been previously reported for some animal serum globulins.[3,8,9,10]

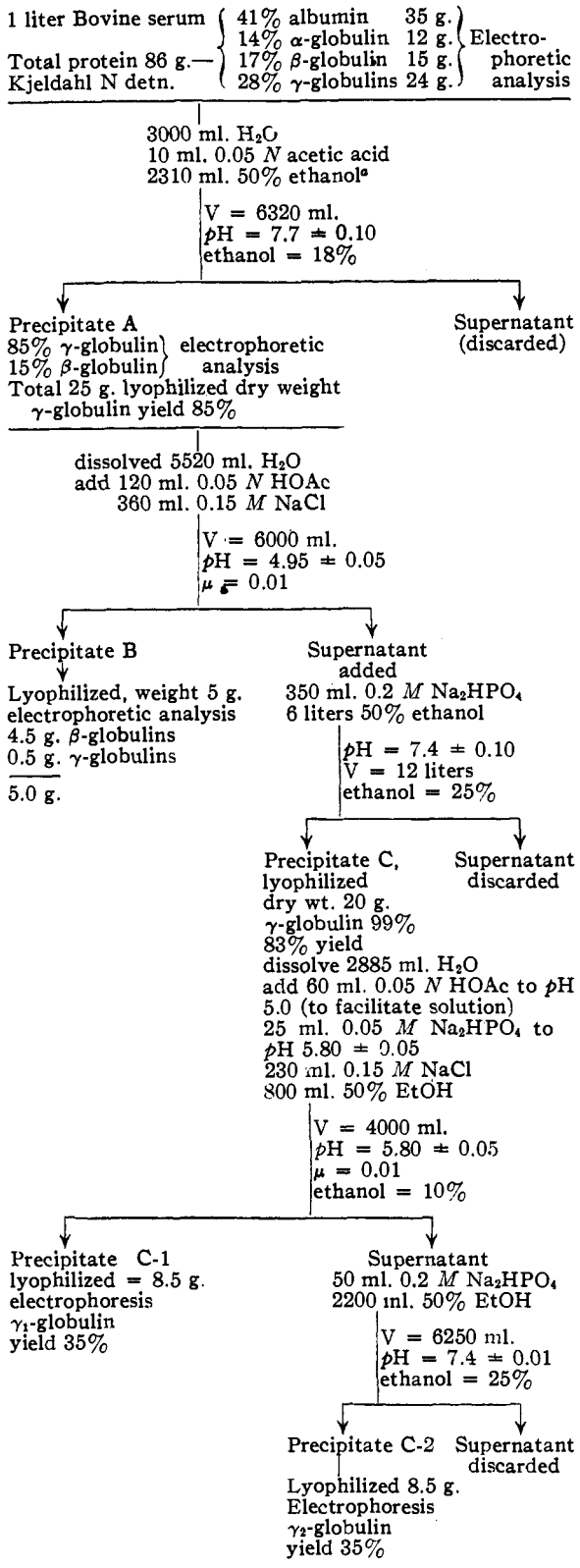
Electrophoresis.—The electrophoretic mobilities of the γ_1- and γ_2-globulin fractions were observed in a buffer system at a *p*H of 8.6 with the aid of the conventional Tiselius assembly. The medium was either a veronal buffer at $\mu = 0.1$ or a veronal–citrate buffer at $\mu = 0.088$. The veronal system was used when mobilities were measured. The patterns for the two gamma globulins are shown in Figs. 6A and 6C. The mobility of the γ_1-globulin fraction under these conditions is 2.1×10^{-5} sq. cm./sec./volt, the same value as that reported for the "T" component by Smith. The mobility of the γ_2-globulin fraction as observed, 1.25×10^{-5} sq. cm./sec./volt, is slightly higher than that reported by Smith for his component which corresponds. Mobilities were determined from the rate of movement of the maximum ordinate, which involves negligible error because of the essential symmetry of the refractive index gradient.[11]

An apparent diffusion constant $D_{20w} = 245 \times 10^{-7}$ sq. cm./sec. was calculated from the electrophoretic pattern for γ_1-globulin (by using the diagram, Fig. 6A). A value $D_{20w} = 135 \times 10^{-7}$ sq. cm./sec. was obtained for γ_2-globulin by using Fig. 6C. These apparent diffusion constant data indicate boundary spreading of far greater magnitude than can be attributed to normal diffusion. In these studies, the ascending and descending patterns were virtually mirror images of one another. A reversal of the current and a calculation of the diffusion constant, after the spreading due to electrophoretic inhomogeneity had been eliminated, gave an apparent value for the diffusion constant of $D_{20w} = 11 \times 10^{-7}$ sq. cm./sec. for the γ_2-globulins. These data indicate that although there is some irreversible boundary spreading, the major part of the spreading is reversible and therefore most probably due to electrical inhomogeneity. The γ_1-globulin fraction is less homogeneous than the γ_2-globulin fraction.

The reversible boundary spread exhibited by the gamma globulin sub-fractions is similar to that observed by Sharp, Cooper and Neurath[7] for pseudoglobulin from horse serum. As a measure of heterogeneity Sharp, Taylor, Beard and Beard[12] have proposed a heterogeneity constant $H = \Delta\sigma/E\Delta t$ where $\Delta\sigma$ is the change in the standard deviation σ during the time interval Δt, and E is the potential gradient. An average value of $H = 5 \times 10^{-6}$ sq. cm./sec./volt was computed for γ_2-globulins from electrophoretic patterns obtained in veronal buffer at $\mu = 0.1$, *p*H = 8.6, which is slightly lower than the heterogeneity values obtained by Sharp, Hebb, Taylor and Beard[13] for pseudoglobulin from horse serum. Quite possibly a whole series of γ-globulins varying only slightly in their chemical and physical behavior is present in the serum of normal animals. In studies of hyperimmune bovine plasma, which are now in progress, we hope to obtain more information concerning this matter.

By reference to Fig. 1C it will be seen that there is no real resolution of the γ_1- and γ_2-globulins in veronal–citrate buffer of $\mu = 0.088$ and *p*H 8.6. Consequently, it is impossible to estimate from the pattern in the conventional way the relative amounts of the two families of γ-globulins which are present. However, a study was made of the electrophoretic behavior in the same buffer solution of synthetic mixtures of the γ_1- and γ_2-globu-

(8) Sharp, Cooper and Neurath, *J. Biol. Chem.*, **142**, 203 (1942).
(9) Tiselius, *Biochem. J.*, **31**, 1464 (1937).
(10) Kabat, *J. Exptl. Med.*, **69**, 103 (1939).
(11) Longsworth, *Ann. New York Acad. Sci.*, **41**, 267 (1941).
(12) Sharp, Taylor, Beard and Beard, *J. Biol. Chem.*, **142**, 193 (1942).
(13) Sharp, Hebb, Taylor and Beard, *ibid.*, **142**, 217(1942).



Total yield $\gamma_1 + \gamma_2$ = 17 g. = 70% of γ globulins in starting material.

Fig. 5.—Schematic fractionation procedure bovine serum.

[a] Fifty per cent. by volume ethanol–water mixture prepared at room temperature. All ethanol additions were made at the freezing point of the solution to which the ethanol was added. The fifty per cent. ethanol was cooled to its freezing point before adding it to the solution.

lins. It was observed that in these mixtures a single peak (corresponding to Fig. 1C) appeared, and that a linear relationship was obtained when the average mobility was plotted against the weight fraction of the γ_1-globulin present in the mixture.

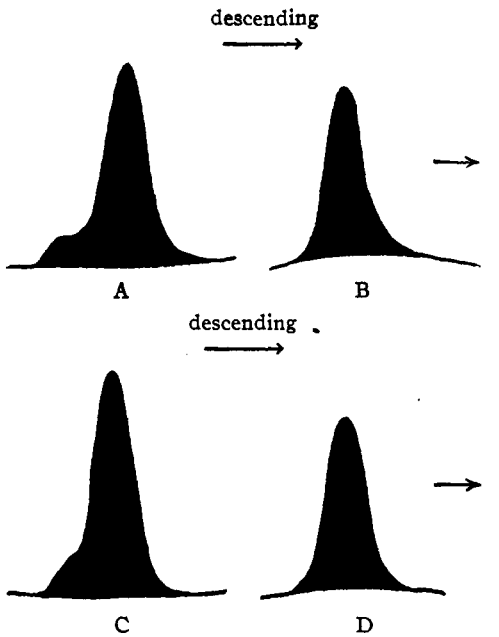


Fig. 6.—Electrophoretic and sedimentation patterns of γ_1- and γ_2-globulins: A, γ_1-globulins in μ = 0.1 veronal buffer, pH 8.6, 120 minutes at 6.25 volts/cm., conc. prot. 3%; B, sedimentation velocity diagram of γ_1-globulins 74 minutes at 50,400 r. p. m. in 0.15 M sodium chloride; C, γ_2-globulins μ = 0.1 veronal buffer, pH 8.6, 120 minutes at 6.45 volts/cm., 3% concn.; D, sedimentation velocity diagram of γ_2-globulins after 87 minutes at 50,400 r. p. m. in 0.15 M sodium chloride.

Discussion

There has been described a method for the recovery, with subsequent separation into two fractions, of the γ-globulin constituents of bovine serum. The application of these procedures is important in that a considerable increase in yield of the proteins concerned with antibody production results. At the same time, "purity" of the fractions, as judged by electrophoretic analysis has not been sacrificed. Comparison may be made with the experiments of Smith[3] whose yields of the γ-globulins were approximately one-half those obtained in our experiments, in spite of the fact that hyperimmune serum was available to him and not to us.

In the earlier publications[3,6] the starting material has been the Fraction II + III of the Cohn framework.[2a] In the present instance the γ-

globulins are precipitated directly from the blood serum for such later purification or separation into sub-fractions as may be desired. The important differences in conditions of separation are the immediate adjustment of the serum to *p*H 7.7 and the use of a considerably greater dilution of the serum as fractionation begins. The new conditions serve several very useful purposes. The sharpness of cleavage between β- and γ-globulins is markedly increased. The removal of the gamma globulin is simple and direct. Tendencies toward denaturation are reduced. Actual yields of γ-globulin are now in excess of 85%.

As a result of the work described in this report and in the companion article[5] it may be predicted that practical and economical ethanol fractionation methods for the removal of antibody from hyperimmune sera will be soon in operation.

Acknowledgments.—The authors wish to thank Dr. J. W. Williams and Dr. Gerson Kegeles for their many helpful suggestions during the course of this work. It is a pleasure as well to thank Mr. E. M. Hanson and Mrs. Alice McGilvery for their technical assistance.

Summary

A procedure for the fractionation of bovine serum, which recovers approximately 85% of the gamma globulins in normal serum, has been outlined. These γ-globulins can be further separated into fractions with varying electrophoretic mobilities. The γ_1 and γ_2 sub-fractions are essentially monodisperse as regards molecular kinetic behavior, but there are marked indications of electrophoretic inhomogeneity.

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[CONTRIBUTION FROM THE EMERYVILLE LABORATORIES OF SHELL DEVELOPMENT COMPANY]

Decompositions of Di-*t*-Alkyl Peroxides. I. Kinetics

BY JOHN H. RALEY, FREDERICK F. RUST AND WILLIAM E. VAUGHAN

The di-*t*-alkyl peroxides have been reported, almost simultaneously by Milas and Surgenor[1] and George and Walsh,[2] to undergo clean-cut decompositions in the vapor phase to ketone and hydrocarbon which, for the di-*t*-butyl compound, can be represented by

$$(CH_3)_3COOC(CH_3)_3 \xrightarrow{k_1} 2(CH_3)_3CO \quad (1)$$

$$(CH_3)_3CO \longrightarrow (CH_3)_2CO + CH_3 \quad (2)$$

$$CH_3 + CH_3 \longrightarrow C_2H_6 \quad (3)$$

During the past few years we have investigated the preparation and properties of these compounds in some detail,[3] and in this paper we present the results of a kinetic study of the pyrolyses of the di-*t*-butyl and di-*t*-amyl derivatives. The reactions of the free radicals produced in these decompositions with various compounds are reported in a following paper.[4]

In the studies of Milas and co-workers the vaporous peroxides were decomposed in the presence of a large amount of glass surface. Under such conditions, reactions of the alkyl radicals are essentially limited to combinations with one another and, in the case of di-*t*-butyl peroxide, ethane and acetone are the sole products. However, if the decompositions are carried out in a large diameter, unpacked vessel, interaction of the alkyl radicals and the ketone becomes important. Thus, methyl, ethyl and higher ketones and methane can also be formed

$$CH_3 + CH_3COCH_3 \longrightarrow CH_4 + CH_3COCH_2 \quad (4)$$

$$CH_3 + CH_3COCH_2 \longrightarrow CH_3COCH_2CH_3 \quad (5)$$

In the kinetic experiments to be described the fraction of released methyl radicals which reacts substitutively (5–10%) is dependent on the acetone concentration as well as the surface. Somewhat higher values (20%) are observed if the decomposition proceeds to completion or if acetone is added.

The reality of the *t*-butoxy radical is demonstrated by the formation of *t*-butyl alcohol when the decomposition is carried out in isopropylbenzene solution. The solvent is converted to 2,3-dimethyl-2,3-diphenylbutane.[5]

Experimental

1. **Materials.**—The di-*t*-butyl peroxide[3] ($n^{20}D$ 1.3890), redistilled under high vacuum, assayed at least 98% (reduction by hydriodic acid[3]) and gave negative tests for *t*-butyl alcohol and hydroperoxide. Steam-distilled di-*t*-amyl peroxide[3] was treated with bromine and vacuum distilled to remove olefin. The resultant material ($n^{20}D$ 1.4086) was also redistilled on the high vacuum line. The nitrogen, oxygen and propylene were taken directly from commercial cylinders and the carbon dioxide sublimed from Dry Ice. Nitric oxide was prepared according to the method of Noyes.[6]

2. **Rate Measurements.**—The apparatus used for all rate measurements is shown in Figs. 1 and 2. The temperature of the oil-bath was measured by a thermometer which was calibrated in position by comparison with a platinum resistance thermometer. The accuracy of the temperature readings is estimated as 0.04°. The quartz spiral gage[7] and leads from the reaction vessel were heated electrically to prevent condensation. The degassed liquid

(1) Milas and Surgenor, THIS JOURNAL, **68**, 205, 643 (1946).

(2) George and Walsh, *Trans. Faraday Soc.*, **42**, 94 (1946); based on data supplied by Asiatic Petroleum Company.

(3) Vaughan and Rust, U. S. 2,403,771, July 9, 1946; *cf.* also earlier patents.

(4) Rust, Seubold and Vaughan, THIS JOURNAL, **70**, 95 (1948).

(5) Kharasch, McBay and Urry (*J. Org. Chem.*, **10**, 401 (1945)) have previously demonstrated this free radical coupling reaction using acetyl peroxide.

(6) Noyes, THIS JOURNAL, **47**, 2170 (1925).

(7) Vaughan, *Rev. Sci. Instruments*, **18**, 192 (1947).