toms consisted of unreacted alcohols and probably some polymerization products. The properties of the obtained olefins, together with literature data, are presented in Table I. Raman spectra are listed in Table II.

Both olefins were hydrogenated to corresponding paraffins over nickel–alumina catalyst at 180°. The properties of the obtained paraffins were as follows: *n*-hexane, b. p. 68.7°, $n^{20}D$ 1.3750; *n*-octane, b. p. 125.6°, $n^{20}D$ 1.3976.

Discussion

The Raman spectrum of the dehydration product of 1-hexanol agrees well, in general, with the literature[2] values for 1-hexene. The concurrence is much closer, however, with the more complete spectrum obtained on a sample of 1-hexene, b. p. 63.3–64.0°, synthesized in this Laboratory.[3] The strong line at 1643 cm.$^{-1}$ in the spectrum of the dehydration product is the only one in the olefinic region and this arises from the R—CH=CH$_2$ group.

The Raman spectrum of the dehydration product of 1-octanol is in excellent agreement with the spectrum reported by Cleveland[4] for an A. P. I. sample of 1-octene. Besides the strong olefinic line at 1644 cm.$^{-1}$ which arises from the R—CH=CH$_2$ group, the spectrum of the dehydration product has also a very weak line at 1671 cm.$^{-1}$. This line is not found on a comparable spectrogram[5] obtained from the A. P. I. sample and hence shows the presence of a small amount (of the order of 2%) of an impurity in the product.

The position of the line of the impurity permits one to eliminate as possibilities *cis*-RCH=CHR′ (1658 cm.$^{-1}$), RR′C=CH$_2$ (1650 cm.$^{-1}$), and with less certainty RR′C=CCHR″ (1678 cm.$^{-1}$) and RR′C=CR″R‴ (1678 cm.$^{-1}$). In each case the approximate frequency expected is given in parentheses. For the *trans*-octenes (2-, 3- or 4-) one should find a line at 1674, and this is within experimental error of the 1671 cm.$^{-1}$ value which was observed. The origin of the small amount of this olefin is uncertain.

Regardless of the status of the weak line discussed above, the Raman spectrum gives unambiguous evidence that the vapor phase dehydration by aluminum oxide of 1-hexanol and 1-octanol produces mainly the olefin expected in each case.

Summary

The vapor phase dehydration of 1-hexanol and 1-octanol over alumina catalyst results in the production of the expected 1-olefin as the main product. The purity of the product in each case was checked not only by the usual physical constants but also by the Raman effect.

(2) Hibben, "The Raman Effect and Its Chemical Applications," Reinhold Publishing Corp., New York, N. Y., 1939, p. 162.

(3) Prepared by H. J. Taufen through the action of allyl bromide on *n*-propylmagnesium bromide: Taufen, Murray and Cleveland, THIS JOURNAL, **63**, 3500 (1941).

(4) Forrest F. Cleveland, *J. Chem. Phys.*, **11**, 1 (1943).

(5) Kindly lent by Dr. Cleveland.

CHICAGO, ILLINOIS RECEIVED JANUARY 8, 1945

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

The Mode of Action of Sulfonamides. Dissociation Constants of the Enzyme-Drug Complex

BY IRVING M. KLOTZ AND HELMUT R. GUTMANN[1]

Abundant evidence has been accumulated to show that the action of sulfonamides is to be attributed to their competition with various substrates associated with particular enzyme systems.[2,3,4] Recently it has been shown also that the inhibition of bacterial growth by sulfonamides may be accounted for quantitatively on the assumption that the action is due to a reversible combination between the basic form of the drug and an enzyme and that the law of mass action is applicable.[5] In this quantitative treatment, dissociation constants for the enzyme–drug complex were unavailable and had to be evaluated by indirect methods. A direct measurement of these equilibrium constants was considered desirable, both for their intrinsic value in describing the system as well as to substantiate some of the assumptions made in the theoretical approach.

Lineweaver and Burk[6] have modified and extended the original method of Michaelis and Menten[7] for the determination of dissociation constants of enzyme complexes and have derived convenient expressions for the evaluation of these constants. These equations have been applied by Wyss[3] to the inhibition of bacterial growth by sulfanilamide, but no attempt was made to evaluate the enzyme–drug dissociation constant. Dorfman and Koser,[4] on the other hand, have calculated equilibrium constants, but only for nicotinamide-stimulated respiration, and not for the growth-enzyme system associated with *p*-aminobenzoic acid. Data are presented in this paper which allow one to calculate the dissociation constants of the enzyme–drug complexes of sulf-

(1) At present, Lieutenant, Medical Corps, U. S. Army.

(2) Woods, *Brit. J. Exp. Path.*, **21**, 74 (1940); Kohn and Harris, *J. Pharmacol.*, **73**, 343, 383 (1941); Jensen and Schmith, *Z. Immunitäts*, **102**, 261 (1942); *C. A.*, **38**, 3308 (1944); Sevag, Shelburne and Mudd, *J. Gen. Physiol.*, **25**, 805 (1942).

(3) Wyss, *Proc. Soc. Exp. Biol. Med.*, **48**, 122 (1941).

(4) Dorfman and Koser, *J. Infectious Diseases*, **71**, 241 (1942). This article lists many other pertinent references.

(5) Klotz, THIS JOURNAL, **66**, 459 (1944).

(6) Lineweaver and Burk, *ibid.*, **56**, 658 (1934).

(7) Michaelis and Menten, *Biochem. Z.*, **49**, 1333 (1913).

anilamide, sulfapyridine, sulfathiazole and N[1]-benzoylsulfanilamide, respectively, in a medium containing growing *E. coli*.

Experimental

A synthetic medium was prepared similar to that used by MacLeod,[8] except that the iron salt was omitted. Glycerol was used in place of glucose in an effort to grow only the smooth form of *E. coli*. Test-tubes containing specified amounts of *p*-aminobenzoic acid and sulfonamide were inoculated with equal quantities of the culture of *E. coli* and set in a water-bath maintained at 37.00 ± 0.05°. Growth was followed photometrically by observing increases in turbidity and referring to a calibration curve of turbidity *versus* bacterial density.

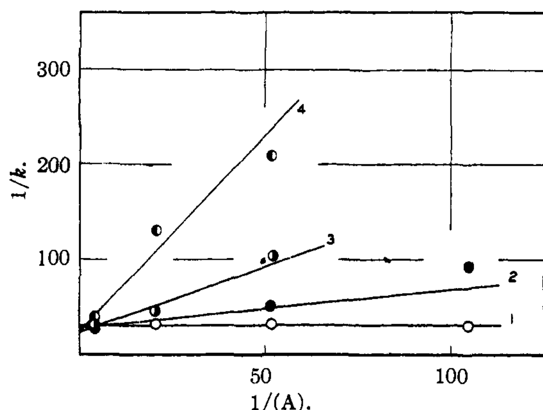


Fig. 1.—Reciprocals of growth rate constants as a function of the concentration of *p*-aminobenzoic acid [(A), in mg. %] in the presence of various amounts of N[1]-benzoylsulfanilamide: 1, 0 mg. %; 2, 2.25 mg. %; 3, 5.6 mg. %; 4, 9.5 mg. %.

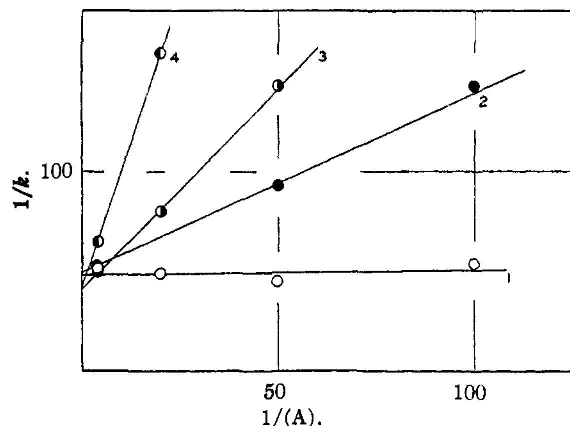


Fig. 2.—Reciprocals of growth rate constants as a function of the concentration of *p*-aminobenzoic acid [(A), in mg. %] in the presence of various amounts of sulfathiazole: 1, 0 mg. %; 2, 0.365 mg. %; 3, 0.91 mg. %; 4, 1.46 mg. %.

(8) MacLeod, *J. Exp. Med.*, **72**, 217 (1940).

Results and Calculations

Logarithms of the turbidity readings were plotted against time and the slope of the linear region, the time interval during which logarithmic growth was obtained, was used as a relative measure of the rate constant. (Slopes were not multiplied by 2.303 to convert to true first-order rate constants because this factor would cancel out in the calculation of the enzyme–drug dissociation constant.) The reciprocals of these rate constants (in hours) for various concentrations of sulfonamide are plotted against the reciprocals of the concentrations of *p*-aminobenzoic acid in Figs. 1, 2, 3 and 4.

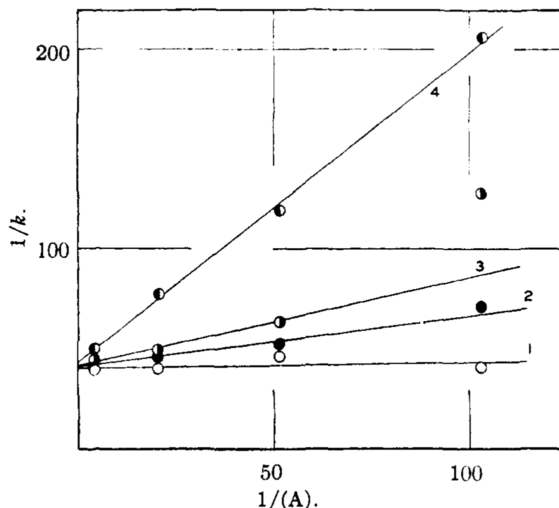


Fig. 3.—Reciprocals of growth rate constants as a function of the concentration of *p*-aminobenzoic acid [(A), in mg. %] in the presence of various amounts of sulfapyridine: 1, 0 mg. %; 2, 0.71 mg. %; 3, 1.78 mg. %; 4, 2.84 mg. %.

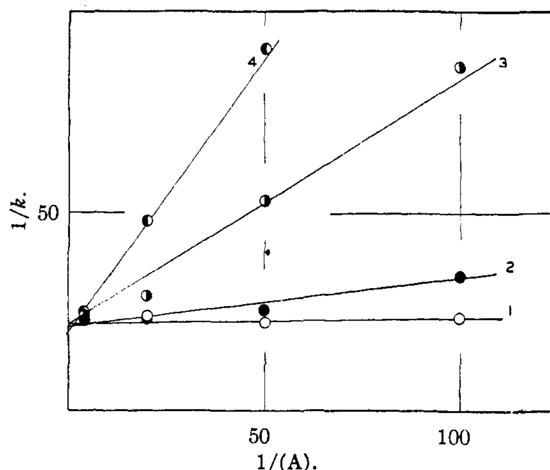


Fig. 4.—Reciprocals of growth rate constants as a function of the concentration of *p*-aminobenzoic acid [(A), in mg. %] in the presence of various amounts of sulfanilamide: 1, 0 mg. %; 2, 10 mg. %; 3, 25 mg. %; 4, 40 mg. %.

Inspection of these graphs shows that the rate constants for a given concentration of sulfonamide fit a straight line, and that for a particular drug, these lines all have the same intercept. Such behavior indicates competitive inhibition of the sulfonamide and *p*-aminobenzoic acid. Upon closer analysis, however, certain disconcerting features arise. For competitive inhibition, the following equation should apply.[6]

$$\frac{1}{k} = \frac{1}{k_\alpha}\left[K_{PA} + \frac{K_{PA}}{K_{PS}}(S)\right]\frac{1}{(A)} + \frac{1}{k_\alpha} \qquad (1)$$

where

k = velocity of growth
k_α = velocity of growth when the enzyme is saturated with *p*-aminobenzoic acid
K_{PA} = dissociation constant for enzyme–*p*-aminobenzoic acid complex
K_{PS} = dissociation constant for enzyme–sulfonamide complex
(S) = concentration of sulfonamide
(A) = concentration of *p*-aminobenzoic acid

Any particular set of rate constants for a fixed sulfonamide concentration fits this equation in that a plot of $1/k$ *vs.* $1/(A)$ is a straight line. However, equation (1) also predicts that if the slopes of these lines are plotted against the sulfonamide concentration, they too should give a linear relation, and, in fact, in this particular case should also pass through the origin since the slopes for zero concentration of sulfonamide are practically zero, as can be seen in Figures 1–4. In no case was this linear relationship observed. A typical curve is shown in Fig. 5 for sulfanilamide. Only for very high concentrations does a linear relationship seem to be approached and that line would not pass through the origin.

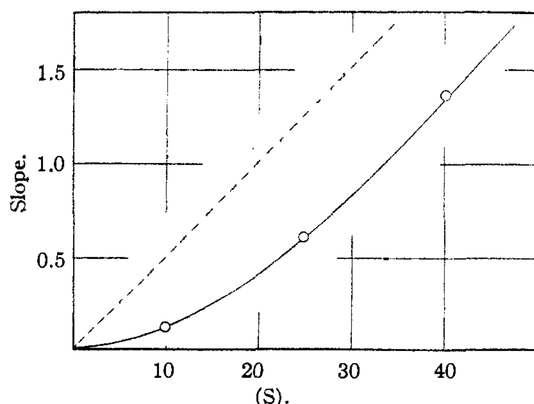


Fig. 5.—Slopes of plots of $1/k$ *versus* $1/(A)$ as a function of the concentration of sulfanilamide, (S), in mg. %. The dotted line represents the limiting slope reached at high concentrations.

It seems reasonable to attribute this behavior to the removal of some of the drug by proteins other than the growth enzymes. That sulfonamides combine readily with proteins has been shown repeatedly.[9,12] It seems obvious, and it has been proved by a rigorous analysis, that in the event of binding by extraneous proteins, the concentration (S) in equation (1) should not refer to the total sulfonamide present but should be corrected by the amount that is removed by the proteins. So corrected, the slopes of the plots of $1/k$ *vs.* $1/(A)$ will be related linearly to (S).

Since at high concentrations the slopes of the graphs of $1/k$ *vs.* $1/(A)$ seem to approach a linear relation with (S), it seems reasonable to estimate the amount of drug bound by extraneous proteins by taking the difference between the observed curve (solid line in Fig. 5) and a line with slope equal to the limiting slope of the curve but passing through the origin (dotted line in Fig. 5). Using this procedure one can determine a true value for the amount of sulfonamide available for competition with *p*-aminobenzoic acid in the growth-enzyme system.

It would be desirable to have a direct measurement of the amount of free sulfonamide. Unfortunately none of the common color tests would be suitable for they would all interfere with the equilibrium between bound and unbound drug, and hence give misleading results.

The calculation of the dissociation constants of the enzyme-drug complex has been carried out on the assumption that the anion combines with the enzyme, in line with the approach previously used.[5] These constants are listed in Table I, as relative values compared to that for the dissociation of the complex between enzyme and *p*-aminobenzoic acid. This procedure has been adopted because the precision in the determination of K_{PA} is extremely poor since the slopes of the lines in Figs. 1–4, corresponding to zero concentration of sulfonamide are all practically zero. This lack of precision is not carried over, however, into the ratio of K_{PS} to K_{PA}.

It is impractical to attempt to determine K_{PA} precisely by using lower concentrations of *p*-aminobenzoic acid, for *E. coli* have been found to grow as rapidly in the absence of any added *p*-aminobenzoic acid as in its presence. Apparently the organisms are able to synthesize the minute

TABLE I

DISSOCIATION CONSTANTS OF THE ENZYME-SULFONAMIDE COMPLEXES

Sulfonamide	Log K_{HS}	Log K_{PS}/K_{PA}
N[1]-Benzoylsulfanilamide	− 4.57	1.48
Sulfathiazole	− 7.12	0.57
Sulfapyridine	− 8.43	.72
Sulfanilamide	−10.43	− .53
Sulfanilamide (Wyss)	−10.43	.08

(9) Schoenholzer, *Klin. Wochschr.*, **19**, 790 (1940); Kimmig and Weselmann, *ibid.*, **21**, 675 (1942); Davis and Wood, *Proc. Soc. Exp. Biol. Med.*, **51**, 283 (1942); Gilligan, *J. Pharmacol.*, **79**, 320 (1943); Fisher, Troast, Waterhouse and Shannon, *ibid.*, **79**, 373 (1943); Reinhold, Flippin, Domm and Pollack, *Am. J. Med. Sci.*, **207**, 413 (1944).

quantities necessary. Similar results have been reported by Wyss[3] who has also discussed the significance of the behavior more fully.

The experiments of Wyss[3] with sulfanilamide also have been analyzed by the methods outlined and the constant so calculated is listed in Table I. The acidity constants, K_{HS}, are those determined by Bell and Roblin.[10]

Discussion

The nature of the enzyme system inhibited by sulfonamides is unknown as yet though recent reports[11] indicate significant progress in this field. Nevertheless, the enzyme-sulfonamide dissociation constants are useful descriptions of the properties of the system. In particular the fact that such constants can be evaluated substantiates the approach used previously[5] in which the quantitative data on the bacteriostatic activity of various sulfonamides have been correlated in terms of two controlling equilibria, that between drug and enzyme and that of the drug as a base. Bacteriostatic activity of the drugs would be related directly to the value of K_{PS}, the measure of their strength of binding by the enzyme, were it not for the fact that the anions of the sulfonamides are bases of varying strength so that their effective concentration at a given pH is determined by K_{HS}, the acidity constant of the acid form. The presence of this latter equilibrium gives a parabolic shape to a curve relating activity to pK_{HS}.

The corroboration of the equilibrium description proposed is even more striking if one compares the two possible calculations of the value of the exponent f in the expression relating K_{PS} and K_{HS} (equation 23 in ref. 5)

$$K_{PS} = aK_{HS}^{f} \qquad (2)$$

where a is a constant. From activity data, a value of 0.37 was obtained. From the rate experiments described in this paper one can determine f from a plot of log K_{PS}/K_{PA} *vs.* log K_{HS} (Fig. 6). The slope of the line so obtained is 0.30. In view of the approximations necessary to evaluate K_{PS}, the agreement in values of f is quite satisfactory.

Deviations between predicted and observed bacteriostatic activities have been pointed out previously for drugs with pK's below 3.5. For such sulfonamides a much higher concentration is necessary to produce bacteriostasis that one would expect on the basis of the mass-law analysis of the enzyme-complex theory. In view of the evidence presented in this paper that the drugs are bound by extraneous proteins as well as by the growth enzyme, one would be inclined to attribute the deviations from the predicted activity to the same cause, for since it is the ion which is bound by the proteins, a large fraction

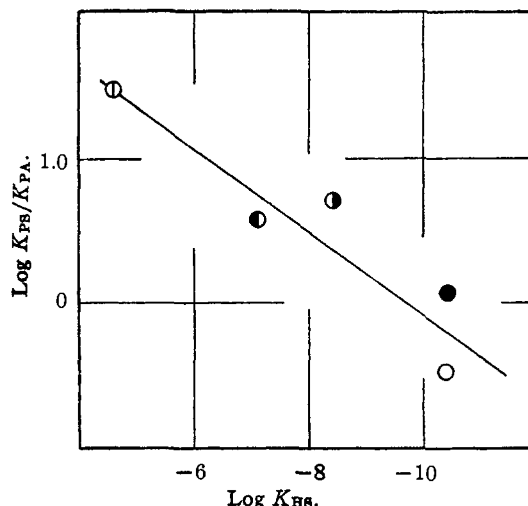


Fig. 6.—Relative dissociation constants of enzyme-sulfonamide complex as a function of the acidity constant of the sulfonamide: ⦶, N[1]-benzoylsulfanilamide; ◐, sulfathiazole; ◑, sulfapyridine; O, sulfanilamide; ●, sulfanilamide, calculated from data of Wyss.[3]

of the highly acid sulfonamides, which exist primarily as ions at a pH of 7, would be unavailable for combination with the enzyme. Studies have been made[12] of the extent of binding of sulfanilamide, sulfapyridine, sulfathiazole and N[1]-benzoylsulfanilamide by serum albumin and indicate, as expected, that large fractions of the total drug are combined with the protein if the drug is appreciably in the ionic form at a pH of 7. Hence it is necessary to recognize that in *in vitro* experiments with bacterial cultures, large portions of the added drug may be removed from solution by combination with bacterial proteins. Similarly, *in vivo*, large fractions of the sulfonamide in the blood stream will be combined with the serum proteins, and probably even with tissue proteins, and hence be unavailable for bacteriostatic action.

Though the description of the nature of sulfonamide activity in terms of a single enzyme-drug complex is probably incomplete, particularly in view of the evidence for sulfonamide antagonists besides p-aminobenzoic acid, it is apparent that, at least to a first approximation, both the equilibrium and kinetic behaviors can be correlated quantitatively on the basis of the same concepts of combination between enzyme and drug.

Acknowledgments.—We are indebted to Dr. A. W. Walker of the Northwestern Medical School for furnishing the cultures and to Dr. P. H. Bell and the American Cyanamid Company for supplying a number of the sulfonamides. This investigation was supported by a grant from the Abbott fund of Northwestern University.

Summary

1. The rates of growth of *E. coli* have been

(10) Bell and Roblin, THIS JOURNAL, 64, 2905 (1942).

(11) Sevag and Green, *Am. J. Med. Sci.*, 207, 686 (1944); Baumgärtel, *Deut. Med. Wochschr.*, 69, 748 (1943); *C. A.*, 38, 4002 (1944).

(12) Unpublished work in this Laboratory.

followed in the presence of sulfanilamide, sulfathiazole, sulfapyridine and N^1-benzoylsulfanilamide.

2. An analysis of the growth curves indicates that competitive inhibition is obtained in each case.

3. Dissociation constants for the enzyme-drug complexes have been calculated and their relation to bacteriostatic activity discussed.

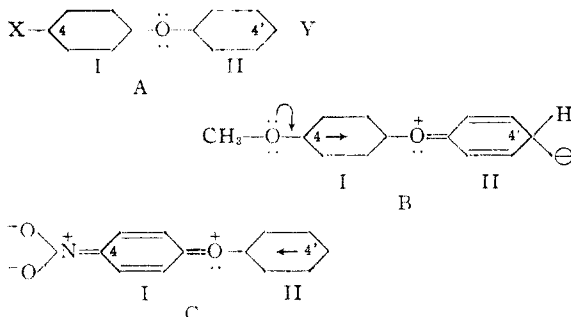
EVANSTON, ILLINOIS RECEIVED DECEMBER 4, 1944

[CONTRIBUTION FROM THE BAILEY CHEMICAL LABORATORY OF THE UNIVERSITY OF KANSAS]

Polarization Effects in Aromatic Ethers[1]

BY RAY Q. BREWSTER AND ROBERT SLOCOMBE[2]

Several observers[3] have reported the fact that a substituent in one ring of an aromatic ether, such as diphenyl ether, has a marked effect in both rings. Thus in 4-nitrophenyl phenyl ether the substitution of bromine in the 4-nitrophenyl radical is almost precluded and occurs only slowly in the unnitrated nucleus. On the other hand 4-methoxyphenyl phenyl ether undergoes bromination in the unsubstituted phenyl group at a rate which is even faster than that of diphenyl ether itself. Inasmuch as substituting agents are electrophilic, substitution would be expected to occur at position 4′ because of the increased electron density at that position, as shown in resonance form B, and a group X at position 4 would favor or hinder this resonance form in proportion to its ability to donate electrons or accept electrons. Thus, if X is a nitro group, which has a strong attraction for electrons (form C) substitution still occurs at position 4′ but at a reduced rate. Conversely, when X is an electron donor, such as the methoxy group, the electron release in ring I increases resonance form B with the result that the rate of substitution at position 4′ is greatly enhanced. We have found also that the presence of a nitro group at position 4 ($X = NO_2$) facilitates the oxidation of a methyl radical at position 4′ ($Y = CH_3$) almost as much as if these two substituents were on the same ring as in *p*-nitrotoluene.



In order to measure the extent of these polarization effects we have conducted three series of experiments: (1) the speed of bromination of aromatic ethers, (2) the speed of benzylation in these ethers and (3) the yields of carboxylic acids obtained by the oxidation of a methyl radical in aromatic ethers.

Experimental

Materials.—All of the ethers used in this investigation were carefully purified by several crystallizations or fractional distillation. The melting points or boiling points of these ethers were: diphenyl ether, m. p. 26.8–27.0°; *p*-tolyl phenyl ether, b. p. 277° (745 mm.), 138–140° (9 mm.); *p*-bromophenyl phenyl ether, 165–167° (16 mm.); *p*-nitrophenyl phenyl ether, m. p. 58°; *p*-methoxyphenyl phenyl ether, 155–156° (6 mm.); *p*-bromophenyl *p*-nitrophenyl ether, m. p. 66°; *p*-nitrophenyl *p*-tolyl ether, m. p. 69°; *o*-nitrophenyl *p*-tolyl ether, m. p. 49°; 2,4-dinitrophenyl *p*-tolyl ether, m. p. 93°.

Bromination.—Molar solutions of diphenyl ether in glacial acetic acid and of bromine likewise in glacial acetic acid were prepared and placed in a thermostat at 44°. To a 100-ml. portion of the diphenyl ether solution was added 100 ml. of the bromine solution. The combined solution, still kept in the thermostat, was thus 0.5 molar with respect to each component. Immediately upon mixing 5 ml. of the solution was removed with a pipet, run into a solution of potassium iodide and the liberated iodine titrated with 0.097 *N* sodium thiosulfate solution. At intervals of ten to twenty minutes successive 5-ml. portions of the solution were withdrawn for titration and the rate at which the bromine concentration decreased was plotted. Similar experiments were performed with 4-methoxyphenyl phenyl ether, 4-nitrophenyl phenyl ether, 4-bromophenyl phenyl ether, and *p*-tolyl phenyl ether In all of these cases mono-bromination occurred para to the ether oxygen and all of the brominated ethers were identified by mixed melting points with known samples. The relative rates at which the bromine concentration decreased are shown in Fig. 1.

The rates at which these ethers undergo bromination are in the same order as the rates of bromination of anisole, benzene, bromobenzene and nitrobenzene and confirm the predictions as to rate of substitution that could be made from the resonance theory. The fact that substitution in toluene is more rapid than in benzene would lead to the expectation that *p*-tolyl phenyl ether should be brominated more rapidly than diphenyl ether. That the converse is true may be explained on the basis of there being two chances for bromination to occur para to the oxygen in diphenyl ether and only one in *p*-tolyl phenyl ether. In no case was the substitution of any bromine at positions 2 or 2′ detected. Under the conditions of this experiment 4-nitrophenyl 4-bromophenyl ether, which might be expected to undergo further substitution ortho to the ether oxygen, failed to react as there was no appreciable diminution of the bromine concentration.

Benzylation.—The benzyl chloride and aromatic ethers used in this experiment were carefully purified by frac-

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(1) Presented before the Division of Organic Chemistry at the 107th meeting of the American Chemical Society at Cleveland, Ohio, April 3–7, 1944.

(2) Present address: Monsanto Chemical Company, Anniston, Ala.

(3) Scarborough, *J. Chem. Soc.*, **132**, 2361 (1925); Scarborough and Sweeten, *ibid.*, 52 (1934); Brewster and Strain, THIS JOURNAL, **56**, 117 (1934); Brewster and Choguill, *ibid.*, **61**, 2702 (1939).