Reaction of Dialkylaminogermanes and Dialkylaminosilanes with Benzoyl Chloride.—Reflux of 0.5 g. of $Ge(NMe_2)_4$ and 1.5 g. of benzoyl chloride (which spontaneously warmed on mixing) furnished a hydrolyzable compound boiling at about 86°, thus $GeCl_4$; this amino derivative also reacted vigorously with pure $CHCl_2COOH$. Similarly, $Si(NMe_2)_4$ and benzoyl chloride yielded $SiCl_4$, b.p. 57°; also the amine reacted with $CHCl_2COOH$ or even 40% aqueous formic acid; however, the amino compound apparently did not react readily with pure water, no heat being given off. Six grams of benzoyl chloride and 1.3 g. of $MeSi(NMe_2)_3$ upon gentle reflux in a distilling unit yielded 0.8 g. of $MeSiCl_3$, a hydrolyzable chloride boiling at 67°. Likewise, $Me_2Si(NMe_2)_2$ gave Me_2SiCl_2, boiling at 72°. When shaken with a twofold volume of water $Me_2Si(NMe_2)_2$ furnished a temperature rise of at least 20°, while free Me_2NH was detected easily by the odor.

Conversion of $EtGe(NEt_2)_3$ into $EtGeI_3$.—Twenty grams of the amino compound and an excess of anhydrous HI, in benzene solution, gave 35 g. of crude $EtGeI_3$ in the general Ruff method[6]; since the Et_2NH_2I precipitated a little slowly, the solution containing $EtGeI_3$ and excess HI was allowed to stand closed overnight in the dark before filtering the precipitate. $EtGe(NEt_2)_3 + 6HI \rightarrow EtGeI_3 + 3EtNH_2I$.

Unsuccessful Reactions.—Me_2SnCl_2 and aniline did not react in benzene solution; $EtGeCl_3$ or $GeCl_4$ and aniline in benzene solution gave anilinium chloride, but no definite germanium compound. Likewise $Si(NEt_2)_4$, $Et_2Si(NEt_2)_2$ and $Me_2Si(NEt_2)_2$ could not be isolated in the pure state, since halogen persisted in the filtrate obtained; Et_2NH_2Cl sublimed while the desired product distilled, even at low pressure. In a single try Me_3SiCl and Me_2NH gave no constant-boiling product.

CAMBRIDGE 38, MASSACHUSETTS RECEIVED JUNE 29, 1951

[CONTRIBUTION FROM THE DEPARTMENTS OF PREVENTIVE MEDICINE, PHYSIOLOGICAL CHEMISTRY, PATHOLOGY AND MEDICINE, OF THE JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDICINE]

Metachromasy of Toluidine Blue Induced by Nucleic Acids[1]

BY N. WEISSMAN, WM. H. CARNES, P. S. RUBIN[2] AND JEAN FISHER

It has been shown that aqueous solutions of pentose and desoxypentose nucleic acid react metachromatically with the thiazine dye toluidine blue. Two types of linkage, polar and non-polar, are involved in the process. The parameters of hydrogen ion concentration and ionic strength affect the formation of the polar bonds. The parameters of concentration of reactants, type of solvent and temperature affect the formation of non-polar bonds. The conditions necessary for nucleic acids to induce metachromasy in toluidine blue solutions are: *p*H between 6 and 7; temperature less than 30°; ionic strength of solution less than 0.03; ratio of dye to nucleic acid phosphorus between 0.4 and 1.4.

Metachromasy is a term applied to that variation in the absorption spectrum of certain dyestuffs, which depends on the nature of the substrate with which the dye interacts.[3] Since Ehrlich first described metachromasy, it has been used as a valuable cytological tool for the identification of mast cells, cartilage and mucins.[4] Lison thought that the metachromatic reaction was specific for the sulfate esters of polysaccharides. Later work showed that, in general, polymerized molecules containing negatively charged groups such as sulfate, carboxylate, phosphate, oleate, metaphosphate and silicate could react with cationic dyes to yield metachromatic products.[5-8]

Although Bank and Bungenberg de Jong[5] had reported that yeast nucleic acid was a metachromatic substrate, both Michaelis[3] and Wiame[7] failed to confirm this finding. Michaelis chose nucleic acid as a model for a "normally" staining substrate while Wiame reported that nucleic acid (variety not specified) inhibited metachromasy.

Since we had observed that the nuclei of cells could be stained metachromatically,[9] we decided to investigate the *in vitro* relationships of the basic dye toluidine blue and both types of nucleic acid. In this paper, we will demonstrate that, under the proper physicochemical conditions, both pentose and desoxypentose nucleic acids react metachromatically with toluidine blue.

Materials and Methods

Desoxyribonucleic Acid.—The two samples $7W_7$ and $11W_{12}$ were prepared from calf thymus nucleoprotein according to the method of Mirsky.[10] Both were highly polymerized, yielding very viscous solutions at low concentrations. The nitrogen to phosphorus ratios were 1.70 and 1.89, respectively. Preparation $7W_7$ contained 13.7% N and 8.1% P; $11W_{12}$ contained 14.2% N and 7.5% P.

Ribosenucleic Acid.—A commercial preparation of yeast sodium nucleate (Schwarz) was used. This contained 8.5% P.

Toluidine Blue O (C.I. No. 925).—Two samples were used. One was a commercial certified stain (certification No. NU9) manufactured by the National Aniline Division of Allied Chemical and Dye Corp. and said to contain 67% dye. Kjeldahl analysis showed a dye content of 64% in the undried sample; after drying to constant weight *in vacuo* over P_2O_5 at 100°, there was a weight loss of 5.2%. The second sample was a specially purified preparation from the National Aniline Division said to contain 85% dye. Kjeldahl analysis showed a dye content of 76%.[11]

Spectrophotometric measurements were made at 10-mμ intervals in a Beckman model DU spectrophotometer. The cuvette chamber temperature was brought to a definite value by flowing tap water through a jacket on either side of the chamber. No effort was made to achieve constancy, the only consideration being the avoidance of over-heating. Corex cuvettes of 1-cm. light path were used for the dilute dye solutions. The most concentrated dye solutions were measured with 9-mm. quartz inserts which reduced the light path to 1 mm. All molar absorption coefficients are calculated on the basis of the Kjeldahl nitrogen determinations.[12]

(1) Presented before the Division of Biological Chemistry at the 119th Meeting of the American Chemical Society, Boston, Massachusetts, April 5, 1951.

(2) National Research Council Senior Fellow in Orthopedic Surgery, aided by a grant from the National Foundation of Infantile Paralysis.

(3) L. Michaelis and S. Granick, THIS JOURNAL, **67**, 1212 (1945).

(4) L. Lison, "Histochimie Animale," Gauthier, Paris, 1936, p. 236.

(5) O. Bank and H. G. Bungenberg de Jong, *Protoplasma*, **32**, 489 (1939).

(6) L. Michaelis, *Cold Spring Harbor Symposia Quant. Biol.*, **12**, 131 (1947).

(7) J. M. Wiame, THIS JOURNAL, **69**, 3146 (1947).

(8) R. C. Merrill, R. W. Spencer and R. Getty, *ibid.*, **70**, 2460 (1948).

(9) W. H. Carnes, N. Weissman and P. S. Rubin, paper read March 21, 1951, before the second annual meeting of the Histochemical Society at Detroit, Michigan.

(10) A. E. Mirsky and A. W. Pollister, *J. Gen. Physiol.*, **30**, 117 (1946).

(11) W. M. Clark, B. Cohen and H. D. Gibbs, *Pub. Health Rep.*, **40**, 1131 (1925).

(12) That the Kjeldahl method is a valid one for the determination of dye content will be shown elsewhere in a study of toluidine blue perchlorate.

We have used Beer's law in the form $-\log_{10}T = \epsilon_{mol}\ C\ d$ where T is transmittancy, C is the concentration of dye in mole per liter, d is the light path in cm. and ϵ_{mol} is the molar absorption coefficient.

Relative viscosities were determined in Ostwald viscosimeters at 30°. The *p*H of all solutions was measured by a Beckman Model G glass electrode.

Experimental

Toluidine blue may show absorption peaks in three regions of the spectrum, depending on its concentration, the solvent used, and the presence of other solutes. In organic solvents or dilute aqueous solution, the dye shows an absorption maximum at 620–630 mμ (Fig. 1, curve 3). This is known as the α or monomer peak. In concentrated aqueous solution, the α peak is depressed and a new β-peak appears at 590 mμ (Fig. 1, curve 1). Both of these solutions appear blue to the eye. When toluidine blue reacts metachromatically with a substrate, the absorption maximum falls somewhere below 590 mμ, yielding broad γ-peaks (Fig. 1, curves 1A, 2A, 3A).[6] The solution then appears violet or purple.

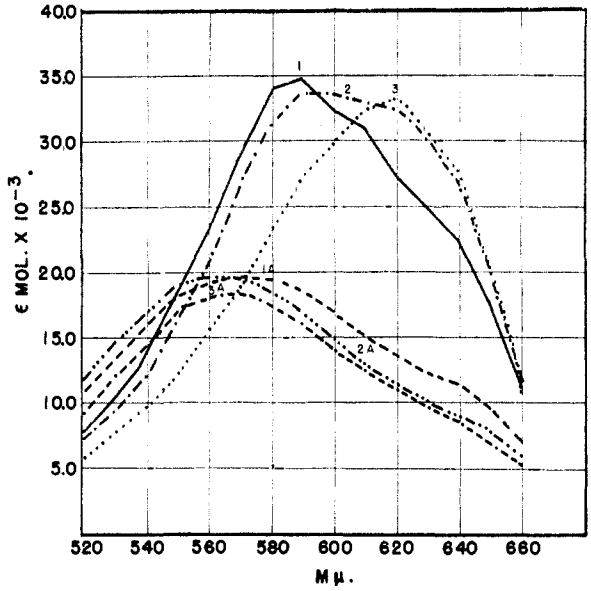

Fig. 1.—Absorption spectra of toluidine blue solutions: curves 1, 2 and 3 are for toluidine blue in water: —, 4.06 × 10^{-5} M; -·-·-, 2.03 × 10^{-5} M; ...., 5.10 × 10^{-6} M, curves 1A, 2A and 3A are for solutions containing the same amount of dye plus sufficient DNA to give a dye:nucleic acid phosphorus ratio of 1.35; temperature, 11°.

Effect of Dye Concentration.—Figure 1 shows three absorption curves (1, 2, 3) for toluidine blue solutions of varying concentrations. These solutions contain the limiting concentrations which can be used with 1-cm. cuvettes. It can be seen that when nucleic acid is added (1A, 2A, 3A) the absorption maximum shifts to the γ-region, regardless of the peaks existing in the original dye solution. The molar absorption coefficient of 18,000 to 20,000 is quite characteristic for metachromasy induced in toluidine blue by such substrates as agar, sodium oleate, metaphosphate, silicates, etc.

Dye–Nucleic Acid Ratio.—Figure 2 shows the results obtained when toluidine blue and nucleic acid are allowed to react at different molar ratios of dye to nucleic acid phosphorus. The ordinate shows the location of maximum absorption peak for each of the varying ratios. With dye alone, the peak is at 590 mμ. The highly polymerized DNA shows a greater shift in the absorption maximum than the commercial RNA preparation. In general, both nucleic acids exhibit metachromasy between the ratio limits of 0.4 to 1.4. Michaelis[6] did not report experiments within these limits, although he employed both larger and smaller ratios.

Effect of State of Polymerization.—Two samples of DNA were dissolved in water to form solutions containing approximately 0.6 mg. per ml. Five-ml. samples were sealed in glass ampules and placed in a vigorously boiling water-bath. Ampules were withdrawn at 5, 15, 30 and 60 minutes and cooled to room temperature by immersion in a water-bath. Viscosity determinations were made on the controls and heated samples. These were then diluted 1:100 and made to react with an equal volume of the proper amount of dye to give ratios of 1.3 ($7W_7$) and 1.4 ($11W_{12}$) for dye:DNA phosphorus. The results are shown in Fig. 3. It can be seen that although the viscosity, and hence the degree of polymerization, changed sharply, the molar absorption coefficient remained practically constant. Other preparations of DNA and a preparation of PNA have yielded the same molar absorption coefficient, without regard to their state of aggregation. The increase in viscosity of $11W_{12}$ at five minutes is unexplained. We have noticed this increase in one other sample of DNA. There might have been a slight amount of nucleoprotein present which hydrolyzed to give the more viscous free nucleic acid.[13]

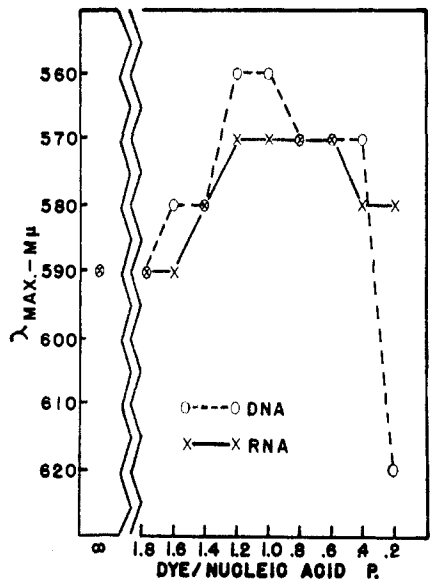

Fig. 2.—Absorption maxima for solutions with varying ratios of dye to nucleic acid phosphorus; temperature RNA, 22°; DNA, 18°.

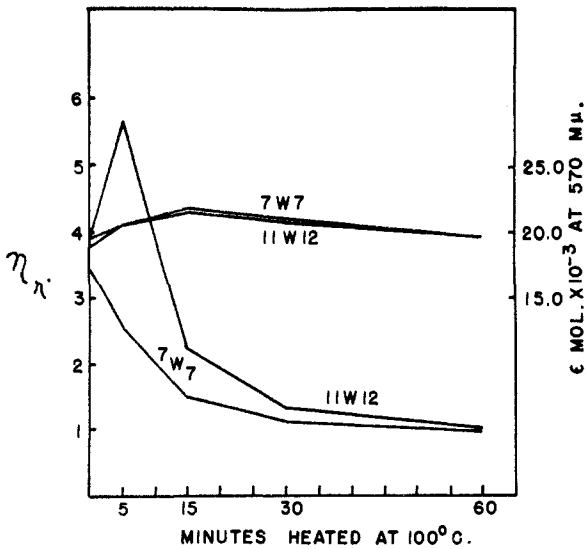

Fig. 3.—Viscosities and molecular absorption coefficients of DNA solutions after heating; η_r = relative viscosity, temperature, 12°.

(13) S. S. Cohen, *J. Biol. Chem.*, **158**, 255 (1945).

This sample had a N:P of 1.89 and gave repeated negative biuret tests.

Effect of Ionic Strength and *p*H.—Since the reaction we are interested in involves charged groups, we would expect ionic strength and *p*H to play a role in the metachromatic reaction. Figures 4 and 5 illustrate the results of an experiment designed to ascertain the relative importance of these two parameters. A series of toluidine blue solutions, 3.05×10^{-5} *M*, was made up in water, and in buffers of 0.1 and 0.02 ionic strength. A second set of solutions contained added DNA in a concentration of 3.09×10^{-5} *M*, which gave a dye:DNA phosphorus ratio of 0.99. The ordinates of the figures give the position of the maximum absorption. In Fig. 4, it can be seen that at each *p*H, in the presence of 0.1 μ buffer, the addition of nucleic acid moved the absorption maximum toward a longer wave length, away from metachromasy. The aqueous control exhibited the expected metachromasy. The influence of *p*H can be seen in the solutions of dye without nucleic acid, where the maximum is shifted toward the shorter wave lengths as the *p*H increases.

In Fig. 5, at low ionic strength, the situation is different. Here, all solutions move toward metachromasy upon the addition of nucleic acid. However, at *p*H 4 and 5, the absorption did not move below the β-peak. At *p*H 6, there is metachromasy, which becomes strongest at *p*H 7. If one goes to *p*H 8, the metachromasy begins to decline. It may be added that where Michaelis reported *p*H in his nucleic acid experiments, he used *p*H 4.6.

Effect of Temperature.—The experiments with buffers were done at room temperature. If the solutions whose absorptions are shown in Figs. 4 and 5 are placed in a cold room at 2°, the metachromasy appears at *p*H 6 and 7, $\mu = 0.1$, and also at *p*H 5, $\mu = 0.02$. In fact, it is sufficient to cool solutions below 10° with running tap water to achieve the same effect.

Discussion

The production of metachromasy is dependent on the physicochemical status of both dye and substrate. Two types of bond are involved, polar and non-polar. The linkage between the substrate (PO_4^-) and the thiazine dye ($\equiv N(CH_3)_2^+$) is a polar bond. The primary formation of this polar bond does not necessarily lead to metachromasy (γ-peaks). Unless the dye molecules are subsequently linked to each other by other forces (see below), the orthochromatic color (α- or β-peak) is produced.

The parameters of *p*H and ionic strength determine the strength of the polar bond by virtue of their effect on the dissociation of the ionized groups. Thus, below *p*H 4 it is not possible to produce metachromasy with nucleic acids since the phosphoric acid groups are largely undissociated (see Figs. 4 and 5). By contrast, the highly dissociated sulfate groups of heparin allow metachromasy to occur at *p*H's below 2.[4]

When the dye and substrate have been linked by polar bonds, the non-polar bonds come into play. The basic dyes which exhibit metachromasy are characterized by their failure to obey Beer's law in aqueous solution. Sheppard and Geddes stated that in those dyes whose aqueous solutions exhibited marked aberrations from Beer's law, "The evidence from the general survey points to the formation of optically coupled plane-parallel molecules or ions, separated by an interval of about 4 Å., as responsible for the typical aberration in aqueous solution on increase of concentration."[14] Rabinowitch and Epstein calculated that 3.12 Å. is the closest equilibrium distance of approach for two

(14) S. E. Sheppard and A. L. Geddes, THIS JOURNAL, **66**, 1995 (1944).

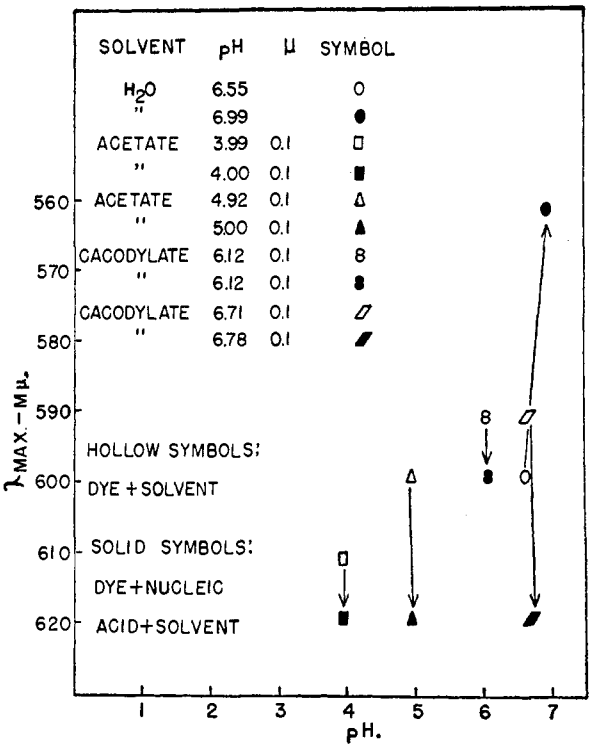

Fig. 4.—Effect of high ionic strength and *p*H on absorption maxima; temperature, 23°.

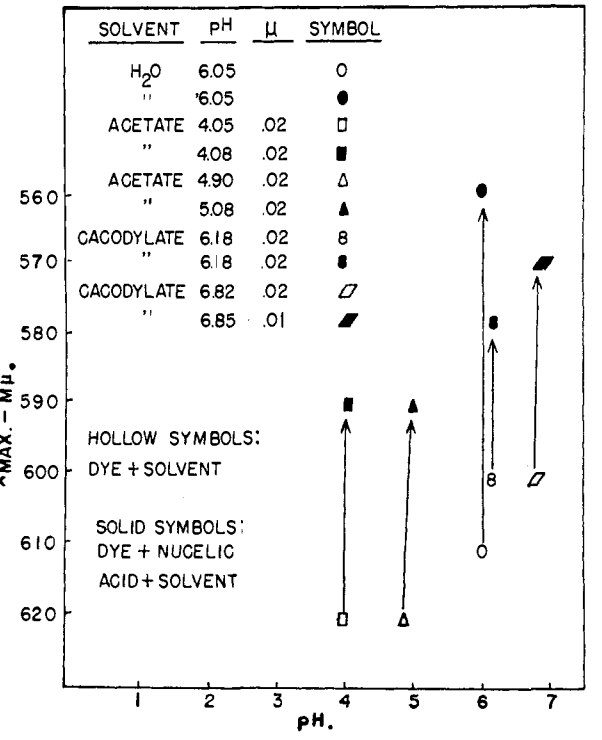

Fig. 5.—Effect of low ionic strength and *p*H on absorption maxima; temperature, 23°.

ions of methylene blue (MB^+) or thionine (TH^+), if the two ring systems lie flat on top of one another like stacked coins. It is the van der Waals force operating between the parallel rings of the dye molecules which leads to the formation of polymers.[15]

(15) E. Rabinowitch and L. F. Epstein, *ibid.*, **63**, 69 (1941).

These bonds are weak, being of the order of 5 kcal. per mole.[16] Thermal agitation, *i.e.*, increased temperature, disrupts them leading to the disappearance of metachromasy. Conversely, lowering the temperature to near 0° induces metachromasy in solutions which are orthochromatic at room temperature.

In order for the dye "non-polar" residues to polymerize, it is necessary to secure the correct ratio of dye to substrate concentration. In the presence of a large excess of substrate, a small number of dye molecules may form the usual polar link at random, but, being widely separated, the aromatic rings of the dye cannot polymerize to yield metachromasy. In view of the findings of Rabinowitch and Sheppard[15,14] with regard to the spacing requirement for aggregation of thiazine dyes, it should be noted that nucleic acids have been reported to have a spatial unit of 3.5 Å.[17]

(16) L. Pauling in K. Landsteiner, "The Specificity of Serological Reactions," Harvard University Press, Cambridge, Mass., 1945, p. 275.

(17) W. T. Astbury, "Symposia of the Society for Experimental Biology," Vol. I, Cambridge, 1947, p. 66.

The effect of solvents on the van der Waals binding also has been shown. Alcohol abolished metachromasy of toluidine blue induced by gum arabic.[5] We have found that alcohol also abolishes metachromasy induced by nucleic acids.[9] These findings supplement the observations of Sheppard that aggregation of thiazine dyes may involve the bonding of a molecule of water between neighboring resonating dye ions[18] and those of Rabinowitch and Epstein[15] that alcohol prevents the dimerization of the thiazine dyes methylene blue and thionine.

Acknowledgment.—This work was aided by a grant from the American Cancer Society to the Department of Preventive Medicine, the Johns Hopkins University School of Medicine, recommended by the Committee on Growth of the National Research Council.

(18) S. E. Sheppard and A. L. Geddes, THIS JOURNAL, **66**, 2003 (1944).

BALTIMORE, MARYLAND RECEIVED MAY 26, 1951

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, THE OHIO STATE UNIVERSITY]

Acidity of Trifluorinated Alcohols and Saponification Rates of their Acetates

BY ALBERT L. HENNE AND RALPH L. PELLEY[1]

Measured by an electrometric procedure, the ionization constants for CF_3CH_2OH, $CF_3CH(CH_3)OH$ and $CF_3C(CH_3)_2OH$ were found to be not more than 10^{-12}. In 50% aqueous acetone 0.1 *N* in NaOH at 25°, the saponification of CF_3CH_2OAc and $CF_3CH(CH_3)OAc$ showed second order kinetics with *K* 0.6 and 0.15 l. mole^{-1} sec.$^{-1}$. The rate constants for 0.1 *N* acid-catalyzed reactions expressed as $K \times 10^6$ sec.$^{-1}$ were found to be: CHF_2CH_2OAc 2.0, CF_3CH_2OAc 1.4, $CF_3CH(CH_3)OAc$ 1.2, $CF_3CH_2CH_2OAc$ 2.6 and C_6H_5OAc 4.0. These values are discussed.

Fluorinated alcohols are known to be acidic. They are reported to form alcoholates on standing with alkali carbonates, alkaline-earth carbonates, but not bicarbonates,[2] not to form complexes with calcium chloride,[3] tenaciously to resist dehydration,[4] and one of them, $CF_3CHOHCH_3$, is reported to have an ionization constant of $K = 10^{-7}$, intermediate between phenol and acetic acid.[5]

The following alcohols were synthesized: CF_3CH_2OH,[6] CHF_2CH_2OH by reduction of difluoroacetic acid obtained in much improved yield, $CF_3CHOHCH_3$ by reduction of CF_3COCH_3 obtained in improved yield, $CF_3C(CH_3)_2OH$[7] and $CF_3CH_2CH_2OH$.[8]

Ionization constants were determined by measuring the *p*H at the half-equivalence point, which is equal to the *pK* between the limits of 10 and 4[9] and, for acids with K_a in the range of 10^{-3} to 10^{-5}, a good approximation of it slightly beyond these limits.[10] The observed results at 25° were: CF_3CH_2OH 4.0, $CF_3CH(CH_3)OH$ 6.3 and $CF_3C(CH_3)_2OH$ 2.5×10^{-12}. The order of magnitude contradicts that given by Swarts,[5] 10^{-7}, but agrees with those obtained by Richter[11] for $HOCH_2CF_2CF_2CH_2OH$, 10^{-11} and 10^{-12} and by McBee[12] for $HOCH_2CF_2CF_2CH_2OH$ and $HOCH_2(CF_2)_4CH_2OH$, 10^{-12}. To test the results, U.S.P. phenol was measured in like fashion, and found to have $K = 1 \times 10^{-9}$, while the accepted value[13] for specially repurified phenol is 1×10^{-10}. Since glass electrode measurements tend to give values which are too low in solutions of *p*H > 10, and since the actual difference of *p*H before and after adding the alcohols to the sodium hydroxide was very small, it may be said that the ionization constants of these alcohols has been shown to be not more than 10^{-12}, and that a plausible value might be taken as 4×10^{-12}. This shows that the fluorinated alcohols are at least 10^4 times more acid than ethanol.[14]

The hydrolysis of esters by hydroxide ions in aqueous solvents is known to show second order kinetics. The accepted explanation is

$$R'\overset{\overset{O}{\|}}{C}—OR + OH^- \rightleftharpoons R'\overset{\overset{O^-}{\|}}{\underset{\underset{OR}{|}}{C}}—OH, \text{ slow}$$

(1) Socony Vacuum Fellow, 1949–1950.

(2) F. Swarts, *Bull. Classe sci., Acad. Roy. Belg.*, 731–760 (1902).

(3) F. Swarts, *Bull. soc. chim. Belg.*, **43**, 471 (1934).

(4) F. Swarts, *ibid.*, **36**, 191 (1927), and J. V. Schmitz, Ph.D. dissertation, The Ohio State University, 1949.

(5) F. Swarts, *ibid.*, **38**, 99 (1929).

(6) A. L. Henne, R. M. Alm and M. A. Smook, THIS JOURNAL, **70**, 1968 (1948).

(7) E. Gryszkiewicz-Trochimowski, *Rec. trav. chim.*, **66**, 427 (1947).

(8) E. T. McBee and A. Truchan, THIS JOURNAL, **70**, 2910 (1948).

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(10) A. L. Henne and C. J. Fox, THIS JOURNAL, **73**, 2323 (1951).

(11) S. B. Richter, PhD. dissertation, The Ohio State University, 1951.

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(13) M. Mizutani, *Z. phys. Chem.*, **118**, 318 (1925).

(14) J. H. Hildebrand and P. S. Denner, THIS JOURNAL, **44**, 2824 (1922).