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Procedures for the Study of Purine and Pyrimidine Deaminases in Small Amounts[1]

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Procedures employing filter paper chromatography for the study of enzymes deaminating adenine, guanine and cytosine and for the determination of the reaction products are described. Enzyme preparations or suspensions of bacterial cells can be assayed by the direct mixing on the paper of exactly measured quantities (0.01 to 0.02 ml.) of enzyme and substrate, followed by incubation and chromatography; or the reactions are performed *in vitro* and the products then separated chromatographically. These methods make possible the simultaneous estimation of both the remaining substrates and the formed reaction products and the establishment of complete balances. The formation of hypoxanthine, xanthine and uracil by adenase, guanase and cytosine deaminase, respectively, have thus been followed. The enzymatic deamination of 8-azaguanine to 8-azaxanthine also is described. Micro methods for the quantitative determination of hypoxanthine and xanthine by chromatographic separation and spectrophotometry in the ultraviolet have been developed.

The micro-procedures for the separation by filter paper chromatography and the quantitative determination by spectrophotometry of purines and pyrimidines, developed in this Laboratory[2,3] and later applied, adapted, modified or improved by many workers, have made possible a novel approach to the study, in minute amounts, of enzymatic systems capable of degrading these nitrogenous nucleic acid constituents. A preliminary account of some of this work has appeared.[4] The present communication is limited to the enzymatic deamination of purines and pyrimidines; but the principles involved could doubtless be adapted to other enzymatic systems.

When only small quantities of enzyme or substrate (10–20 μl) are available, exactly measured amounts may be mixed directly on the filter paper.[5] The mixture is incubated in a moist atmosphere, the reaction is stopped by heating to 100°, and the chromatographic separation is performed. Bacterial suspensions may also be studied in this manner. When larger amounts are available, the incubation of the substrate with the enzyme is preferably carried out *in vitro*, in a total volume of 0.1 ml. or more, and aliquots are dispensed and subjected to chromatography. In the latter case the supplementary estimation of the liberated ammonia also is possible. The main advantage of the procedures, apart from their ease and adaptability to minute amounts, may be seen in the fact that dependence upon indirect criteria of enzymatic action (liberation of NH_3, etc.) is replaced by the direct estimation of both substrate and reaction product at any stage and even with crude enzyme preparations.

The enzyme systems examined were adenase from *E. coli*,[6] which deaminates adenine to hypoxanthine; guanase from liver,[7–9] which converts guanine to xanthine; and cytosine deaminase of *E. coli*[4] and baker's yeast,[4,10,11] which brings about the formation of uracil from cytosine. The last mentioned enzyme will be considered in detail in a paper to be published shortly. The enzymatic deamination[12] of 8-azaguanine, the triazolo analog of guanine,[13] to the corresponding 8-azaxanthine also was followed by a similar method. In addition, attention may be drawn to the estimation of hypoxanthine and xanthine after separation on paper chromatograms which supplements the quantitative procedures for other purines described previously.[3]

Experimental

Material.—With the exceptions noted below, the purines and pyrimidines were the standard preparations described previously.[3] Xanthine (Eastman Kodak Co.) was precipitated by the addition of concd. ammonia to its solution in hot 2 *N* HCl, after treatment with norite, and recrystallized from water several times. 8-Azaguanine and 8-azaxanthine were synthetic preparations obtained through the courtesy of Dr. A. Gellhorn of this College. 2,6-Diaminopurine[14] was kindly supplied as the sulfate by Dr. A. Bendich, Sloan–Kettering Institute for Cancer Research, New York. The solvents were rectified by distillation; the filter paper was Schleicher and Schüll, No. 597.

Quantitative Determination of Hypoxanthine and Xanthine.—The methods used closely resembled those applied to the separation and estimation of adenine and guanine.[3] Hypoxanthine was separated from adenine in a solvent system consisting of *n*-butanol, diethylene glycol and water (volume proportions 4:1:1) in an ammonia atmosphere. For the separation of xanthine and guanine *n*-butanol–diethylene glycol–0.1 *N* HCl (4:1:1) were employed. Following chromatography (about 20 hr.) the paper sheets were dried in air, the spots located by means of a suitable ultraviolet lamp,[15] and the separated zones and corresponding blank zones extracted, as described before[3]: adenine and hypoxanthine with 0.1 *N* HCl, guanine and xanthine with *N* HCl.

For the spectroscopy of the extracts in a Beckman quartz spectrophotometer the previously published principles[3] were applied. The standard preparation of hypoxanthine (in 0.1 *N* HCl) had its absorption maximum at 248 mμ with ϵ 10,900; xanthine (in *N* HCl) at 260 mμ with ϵ 9,230. For reasons outlined before,[3] the computations were based on the differences Δ between the extinction E at the maximum and that at 280 mμ for hypoxanthine or at 285 mμ for xan-

(1) Supported by a research grant from the National Institutes of Health, United States Public Health Service. This report is based on a dissertation submitted by Jacob Kream in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

(2) E. Vischer and E. Chargaff, *J. Biol. Chem.*, **168**, 781 (1947); *Federation Proc.*, **7**, 197 (1948).

(3) E. Vischer and E. Chargaff, *J. Biol. Chem.*, **176**, 703 (1948).

(4) E. Chargaff and J. Kream, *ibid.*, **175**, 993 (1948).

(5) Qualitative spot tests on paper for a number of enzymes were described by B. N. Sastri and M. Sreenivasaya, *Mikrochemie*, **14**, 159 (1933–1934).

(6) C. Lutwak-Mann, *Biochem. J.*, **30**, 1405 (1936).

(7) G. Schmidt, *Z. physiol. Chem.*, **179**, 243 (1928); **208**, 185 (1932); **219**, 191 (1933).

(8) Y. Wakabayasi, *J. Biochem. (Japan)*, **28**, 185 (1938).

(9) H. M. Kalckar, *J. Biol. Chem.*, **167**, 461 (1947).

(10) A. Hahn and W. Lintzel, *Z. Biol.*, **79**, 179 (1923).

(11) J. Kream and E. Chargaff, *Federation Proc.*, **9**, 192 (1950).

(12) A. Roush and E. R. Norris, *Arch. Biochem.*, **29**, 124 (1950).

(13) R. O. Roblin, Jr., J. O. Lampen, J. P. English, Q. P. Cole and J. R. Vaughan, Jr., THIS JOURNAL, **67**, 290 (1945).

(14) A. Bendich, J. F. Tinker and G. B. Brown, *ibid.*, **70**, 3109 (1948).

(15) The guanine and xanthine spots appear after separation in the acidic solvent system as vividly fluorescent areas on a less fluorescing paper background. These adsorption zones acquire the normal dark appearance, when the sheets are treated with gaseous NH_3 after chromatography.

thine. The corresponding values for 10 γ of purine per ml. were as follows: hypoxanthine, E_{248} 0.803; E_{280} 0.033; Δ =0.770; xanthine, E_{260} 0.606; E_{285} 0.042; Δ = 0.564.

The extracts were read at the specified wave lengths and also at 253 and 243 mμ for hypoxanthine and at 265 and 255 mμ for xanthine, in order to verify the shape of the spectra. In addition, readings were carried out at 300 mμ, and extracts with extinction values at this wave length outside the range of −0.01 and +0.04 were discarded.[3] The calculations were based on the proportion between the Δ_x values found for the unknown and the Δ values given above. The results of recovery and separation experiments are summarized in Table I. Since the acidic solvent system had not been applied previously to the estimation of guanine, some recovery experiments with this purine also are included. The R_F values of the purines in the solvents employed in the present study have been reported previously.[3]

TABLE I

QUANTITATIVE CHROMATOGRAPHY OF HYPOXANTHINE AND XANTHINE[a]

No. of experiments	Substance subjected to chromatography, γ: Hypoxanthine	Xanthine	Adenine	Guanine	Substance recovered as average % of initial weight: Hypoxanthine	Xanthine	Guanine
3	5.5				97		
6	11.0				92		
7	22.0				93		
6	5.7		58.5		98		
7		10.1				96	
4		5.1		12.6		95	97

[a] Average % of substance recovered in all experiments, with its standard deviation in parentheses: hypoxanthine, 95 (2); xanthine, 96 (4); guanine, 97 (1).

carried out in the usual manner. Extracts of the separated zones were read against extracts of the corresponding areas of the control lane as controls.[16] This method will be referred to in the following discussion as "on paper-moist."

It was observed in experiments of this type that the enzymatic activity tended to drop rapidly after the first 15 minutes of incubation (compare Fig. 1), and that the spots became almost dry, even in a moist atmosphere, after 30 minutes. In several experimental series the procedure therefore was modified: 20-μl. portions of distd. H_2O were placed on the spots containing the substrate–enzyme mixture at regular 15-minute intervals. This modification, which improved the regularity of enzymatic attack (compare Table II), is referred to as "on paper-wet."

The experiments, in which the incubation was carried out in small test-tubes and the reaction mixture analyzed by paper chromatography, are designated as "*in vitro*." These assay mixtures frequently were analyzed for free ammonia formed in the course of the deamination of the substrates. A modification of a micro-procedure[17] was employed which permitted satisfactory analyses in the range of 10 to 600 γ of NH_3 nitrogen. Corrections based on the NH_3 given off by enzyme blanks were applied in all cases.

Determination of Adenase Activity.—The aqueous substrate solution (adjusted to *p*H 7.0) contained 2.09 mg. (15.5 micromoles) of adenine per ml. Resting cells of *E. coli* K 12, grown at 37° for 24 hr. on a 3% agar medium containing 2% Bacto-Tryptone, 0.5% NaCl and 0.5% yeast concentrate, served as enzyme source. The cells were harvested and washed in the cold in the usual manner and suspended in 0.15 *M* phosphate buffer of *p*H 7.3 in a concentration of 16 mg. dry weight per ml. In each assay on paper 10 μl. of substrate (0.155 micromole adenine) and 20 μl. of bacterial suspension (320 γ dry wt.) were employed. In experiments *in vitro*, larger proportionate quantities were incubated with agitation at 38° in small test-tubes, the enzymatic action was stopped by the addition of 1/20 volume of 10% H_2SO_4, and 30-μl. aliquots were subjected to chromatography after neutralization with gaseous NH_3. The separated purines were completely identical with authentic specimens, with respect both to their R_F values and absorption spectra. Several balance experiments are summarized in Table II. It will be seen that the extent of initial deamination is similar with all methods, but that for prolonged assays the "on paper-wet" procedure is preferable to the "on paper-moist" method. The efficacy of the procedures in permitting complete balances to be drawn will also be noticed.

TABLE II

DEAMINATION OF ADENINE BY RESTING *E. coli*[a]

Procedure	Incubation time, min.	Adenine remaining, Experiment No. 1, γ	2, γ	3, γ	Average, γ	Average, Micromole	%	Hypoxanthine formed, Experiment No. 1, γ	2, γ	3, γ	Average, γ	Average, Micromole	Total purine accounted for, %	Extent of deamination from estimation of Hypoxanthine, %	Ammonia, %
	15	16.2	16.1	15.8	16.0	0.119	77	3.2	3.1	3.1	3.1	0.023	92	15	
On paper, moist	30	14.4	14.2	15.4	14.7	.109	70	4.3	4.2	..	4.2	.031	90	20	
	60	13.5	13.5	13.8	13.6	.101	65	4.8	5.0	5.4	5.1	.037	89	24	
	120	13.7	14.3	..	14.0	.104	67	5.2	5.8	5.2	5.4	.040	93	26	
	15	16.1	15.6	16.1	15.9	.118	76	3.8	3.9	3.8	3.8	.028	94	18	
On paper, wet	30	13.8	13.3	12.9	13.3	.099	64	5.9	5.6	5.9	5.8	.043	92	28	
	60	9.5	10.1	9.5	9.7	.072	46	10.2	9.8	10.2	10.1	.074	94	48	
	120	6.9	6.7	6.8	6.8	.050	33	12.3	12.5	12.5	12.4	.091	92	59	
	15	14.7	15.8	15.6	15.4	.114	74	4.3	3.6	4.1	4.0	.029	93	19	15
In vitro	30	11.0	11.1	11.4	11.2	.083	54	7.3	7.4	7.8	7.5	.055	89	35	29
	60	7.0	6.7	7.2	7.0	.052	33	12.2	11.9	12.0	12.0	.088	90	57	49
	120	3.3	3.3	3.2	3.3	.024	16	15.8	15.3	16.3	15.8	.116	91	75	67

[a] In each experiment 20.9 γ (0.155 micromole) of adenine was subjected to deamination in 0.15 *M* phosphate buffer (*p*H 7.3) at 38°.

Procedures.—For the enzymatic assay directly on paper, the sheet (16 × 43 cm.), divided into four 4-cm. wide longitudinal lanes and with a transverse line 7 cm. below the top, to indicate the starting points, was rolled into a cylinder which was held in place by means of small paper clips; the outer surface, which included the starting line parallel to the axis of the cylinder, was so arranged as to protect it from contact with the rest of the sheet. Three of the lanes received aliquots of the substrate solution, usually 10 μl., the fourth lane, serving as control, the same volume of dist. water. Each of the wet spots then was rapidly overlaid with 20-μl. aliquots of the enzyme solution or bacterial suspension, the control spot being treated first. The paper cylinders were kept at 38° in a moist atmosphere in ground glass-covered jars for the desired period and then heated in an oven at 90–100° for 15 minutes, in order to stop the enzymatic action. Descending chromatography then was

(16) In all experiments, controls were run in which the substrates alone, with the omission of enzyme, were subjected to the various operations; they failed to show any spontaneous deamination. Enzyme control experiments, from which the substrate was omitted, also were carried out.

(17) A. E. Sobel, A. M. Mayer and S. P. Gottfried, *J. Biol. Chem.*, **156**, 355 (1944).

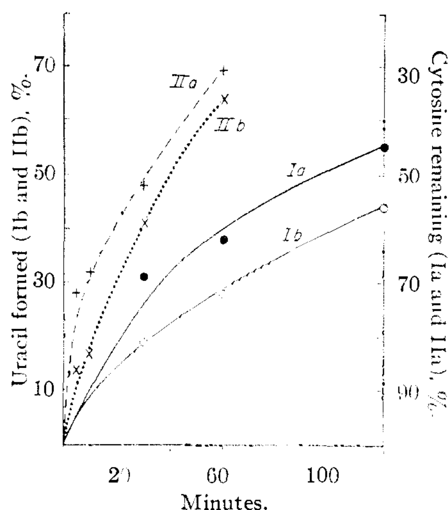

Fig. 1.—Formation of uracil from cytosine by *E. coli*, studied by incubation "on paper-moist": curves Ia and Ib, resting cells; curves IIa and IIb, cell-free extract.

Determination of Guanase Activity.—The scanty solubility of guanine around *p*H 7 has led to the use of gelatin as a dispersing agent.[7] In the present experiments gum acacia was found preferable. A few drops of 40% NaOH were added to 25 mg. of guanine in 5 ml. of 1% gum acacia, and the solution was adjusted to *p*H 7.5–8. The resulting stable suspension, diluted to exactly 10 ml. with 1% gum acacia, served as the substrate solution; it contained 2.5 mg. (16.6 micromoles) of guanine per ml. Acetone powder preparations of rabbit liver (stored at −15°) served as the enzyme source.[18] The powder was ground with 0.1 *M* borate buffer of *p*H 8.6 (100 mg. of liver powder per ml. buffer), extracted for 30 minutes at 40°, and the chilled mixture centrifuged at 1900 × g. The supernatant was used as the enzyme in the same proportion to the substrate solution as for the adenase determinations described above. The xanthine produced by deamination at 40°, and in some cases ammonia, was determined; the remaining guanine could not be estimated in these experiments, since gum acacia interfered with the chromatographic recovery of this purine, but not with that of xanthine. The position on the chromatograms and the complete absorption spectra of the xanthine produced enzymatically were identical with those of an authentic specimen. The customary control experiments were also performed. A selection of data is given in Table III.

It may be of interest to mention that a borate buffer extract (*p*H 8.6) of homogenized rabbit liver failed to deaminate 2,6-diaminopurine within 3 hr. at 38°.

Deamination of 8-Azaguanine.—This triazolo analog of guanine could be separated by paper chromatography from its deamination product 8-azaxanthine; the solvent system consisted of a mixture of 4 volumes of *n*-butanol and one volume each of diethylene glycol and 0.1 *N* HCl. Solutions containing these compounds were acidified prior to separation. The separated zones fluoresced beautifully in ultraviolet light. The R_F values were: 8-azaguanine 0.48; 8-azaxanthine 0.59. Complete absorption spectra of the separated substances, extracted with 0.1 *N* HCl, were identical with those of pure specimens.[19] The enzyme solutions were identical with those used in the guanase experiments described above. When a 0.03 *M* solution of 8-azaguanine was incubated with the enzyme at 38° in 0.1 *M* borate buffer of *p*H 8.6, the liberation of ammonia corresponded to the following percentage of deamination (with the incubation period in minutes given in parentheses): 21 (30); 35 (60); 42 (90); 50 (120). The usual controls were run for purposes of correction. The formation of 8-azaxanthine as the product of enzymatic action then was verified by chromatography and ultraviolet spectroscopy.

(18) Extracts of such powders were active in the deamination of guanine, 8-azaguanine and adenosine, but failed to deaminate adenine, cytosine and cytidine.

(19) L. F. Cavalieri, A. Bendich, J. F. Tinker and G. B. Brown, THIS JOURNAL, **70**, 3875 (1948).

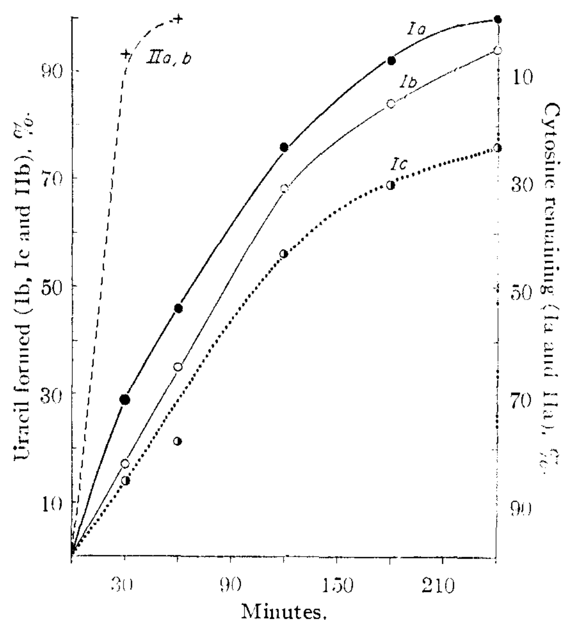

Fig. 2.—Formation of uracil from cytosine by *E. coli*, studied by incubation *in vitro:* curves Ia and Ib, resting cells; curves IIa and IIb, cell-free extract. In Curve Ic the production of ammonia by resting cells is plotted as % uracil produced.

Determination of Cytosine Deaminase Activity.—The substrate solution contained 5 mg. (45 micromoles) per ml. of 0.1 *M* phosphate buffer of *p*H 7.0. Two enzyme systems were employed, namely, resting cells or cell-free extracts of *E. coli, var. communior*, a strain obtained through the courtesy of Dr. T. Rosebury, Department of Bacteriology of this College, and cell-free extracts of baker's yeast.[4,11] Cultivation and assay techniques for the *E. coli* cells followed those described above in regard to adenase. The resting

TABLE III

DEAMINATION OF GUANINE BY RABBIT LIVER GUANASE[a]

Procedure	Incubation time, min.	Extent of deamination: Experiment 1[b] From xanthine formed, %	Experiment 1[b] From ammonia liberated, %	Experiment 2[c] From xanthine formed, %	Experiment 2[c] From ammonia liberated, %
On paper, moist	10			19	
	20	10		36	
	30	16			
	50			38	
	60	26			
	90			42	
	120	29			
	150			44	
	240			46	
In vitro	15	12	11		
	20			35	
	30	21	17		
	50			55	53
	90	34	33		
	120	34	34		
	150			70	68
	240			72	67

[a] In 0.1 *M* borate buffer of *p*H 8.6 at 40°. [b] The figures represent the average of 3 individual assays with the same enzyme preparation. In each experiment 25 γ of guanine was subjected to deamination. [c] The figures represent the average of 5 assays with an enzyme preparation different from that used in Expt. 1; 12–20 γ of guanine was subjected to deamination.

cells were used in a concentration of 24 mg. (dry weight) per ml. of dist. water.

For the preparation of a cell-free extract of crushed *E. coli* cells, 35 cc. of a chilled bacterial suspension (24 hr. growth, 3.3 g. dry weight) in distd. water was passed for 3 hr. through an ice-cooled wet crushing mill for bacteria,[20] constructed by Unicam Instruments, Ltd., Cambridge, England. All subsequent operations also were carried out in the cold. The opalescent supernatant from a centrifugation at 8000 × *g* for 30 minutes was divided into two fractions by centrifugation at 31,000 × *g* for 2 hours: a sediment, retaining a negligible deaminating activity for cytosine (about 2% deamination in 2 hr.), and a very active, clear yellowish supernatant (95% deamination in 2 hr. at 36°), containing 21 mg. of dry matter per ml. The latter was used in the enzyme experiments.

The preparation of a cell-free extract of baker's yeast (6 g. of fresh yeast per ml. of dist. water) followed similar procedures. Here again, the sediment obtained by centrifugation at 31,000 × *g*, similar to the lipoprotein particles described previously,[21] displayed little activity, in contrast to the very active supernatant, containing 62 mg. of dry matter per ml. (91% deamination in 60 minutes at 36°).

All assays were carried out at 36° and *p*H 7.0; the proportions of substrate and enzyme were the same as for adenase and guanase. When incubation was performed *in vitro*, aliquots were acidified prior to their analysis for uracil, cytosine and ammonia. The separation of the reaction mixtures was achieved with *n*-butanol saturated with water as the chromatography solvent.[3] The paper strips were equilibrated with gaseous NH_3 before chromatography. The deamination of cytosine to uracil by *E. coli*, followed by the technique described as "on paper-moist," is illustrated in Fig. 1. Several *in vitro* experiments are shown in Fig. 2. Between 90 and 100% of the pyrimidine substrate could be accounted for in all experiments. The uracil formed by deamination was identified by chromatography and determination of the complete ultraviolet absorption spectrum. Experiments with cell-free yeast extracts, including some carried out in a nitrogen atmosphere, are assembled in Table IV. The yeast enzyme preparations were inactive toward adenine and guanine.

TABLE IV

DEAMINATION OF CYTOSINE BY CELL-FREE YEAST EXTRACTS[a]

Preparation	Incubation time, min.	Extent of deamination From uracil formed, %	From ammonia liberated, %
1900 × *g* supernatant	30	65	
	60	81	
31,000 × *g* supernatant, prepn. 1	60		91
31,000 × *g* supernatant, prepn. 2	120	98	
31,000 × *g* supernatant, prepn. 3	60	89	
	60[b]	100	
31,000 × *g* supernatant, prepn. 4	60		70
	60[c]		72

[a] The incubations were carried out *in vitro*. [b] Nitrogen atmosphere. [c] Evacuated Thunberg tube, after replacement of air by nitrogen.

NEW YORK, N. Y.

(20) V. H. Booth and D. E. Green, *Biochem. J.*, **32**, 855 (1938).

(21) M. A. Nyman and E. Chargaff, *J. Biol. Chem.*, **180**, 741 (1949).

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF DENISON UNIVERSITY]

Stereochemistry of Formation and Hydrolysis of Esters of Perfluoro Acids

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High yields of esters have been obtained by direct esterification of (−)2-octanol with trifluoroacetic acid and with perfluorobutyric acid. Both the formation and hydrolysis of the esters have been shown to proceed without inversion or racemization.

Esterification of secondary alcohols with acetic acid and saponification of the esters have been shown to occur with complete retention of configuration[1]; *i.e.*, only the acyl–oxygen bond is broken. The same course has been established even for the stronger carboxylic acid, trichloroacetic acid; formation and saponification of neopentyl trichloroacetate occurs without rearrangement of the neopentyl skeleton.[2] However, when small amounts of sulfuric acid are used as catalysts for the esterification reaction, esters are formed from acetic acid and optically active alcohols with a small but measurable degree of racemization.[1] It is well known that with the strong acids HCl and HBr, halide ion displaces –OH in the alcohol to give the inorganic ester with inversion of configuration.[3] The hydrolysis of esters of the strong acid *p*-toluenesulfonic acid also proceeds with inversion.[4]

It was of interest then to ascertain the course of similar reactions involving very strong carboxylic acids such as trifluoroacetic acid and perfluorobutyric acid. Each of the three paths, *i.e.*, retention of configuration (normal esterification with un-ionized acid), partial racemization (strong acid catalysis), or inversion (displacement by perfluoroalkanoate anion), while not equally probable, at least seemed plausible.

From both trifluoroacetic acid and perfluorobutyric acid, the esters were prepared in high yields by simply refluxing (−)2-octanol with a slight excess of the acid. The optical rotations of these esters were identical both in sign and magnitude with the corresponding esters prepared from the acid chlorides, whereby no attack is made on the asymmetric center and therefore no change in configuration can occur. Similarly hydrolysis of the esters with aqueous alkali led to recovery of the

(1) E. D. Hughes, C. K. Ingold and S. Masterman, *J. Chem. Soc.*, 840 (1939).

(2) O. R. Quayle and H. M. Norton, THIS JOURNAL, **62**, 1170 (1940).

(3) For summary see H. Gilman, ed., "Organic Chemistry: An Advanced Treatise," 2nd Ed., John Wiley and Sons, Inc., New York, N. Y., 1943, pp. 278, 279.

(4) F. C. Fuson, "Advanced Organic Chemistry," John Wiley and Sons, Inc., New York, N. Y., 1950, p. 584.