crystals so obtained showed a m.p. 238–240° dec. (in bath 230°) $[\alpha]_D$ +20.7°, *c*, 1.0 in water; yield approximately 200 mg.

Anal. Calcd. for $C_9H_{14}O_8N_3P$: N, 13.0; P, 9.6. Found: N, 12.9; P, 9.75.

The combined mother liquors on standing gave a second crop of crystals of $[\alpha]$ +40.5° consisting evidently of a mixture of the cytidylic acid isomers. The small amount of oil from the recrystallization procedures (soluble in water, insoluble in alcohol) finally solidified to yield a white powdery material which darkened in the m.p. at 245° but did not show the characteristic decomposition melting point of cytidylic acid.

The original mother liquor containing cytidylic acid phosphotungstates, on standing in the cold, produced a second crystalline fraction which was treated as above to obtain free cytidylic acid. When the acid was recrystallized, there was collected a first crop of crystals, 0.23 g., $[\alpha]_D$ +38.2°, and a second crop, $[\alpha]_D$ +29°. The latter upon recrystallization gave an additional yield of 81 mg. of the $[\alpha]_D$ +20.7° isomer.

Optical Properties.—A sample of the cytidylic acid $[\alpha]_D$ +20.7° was compared with cytidylic acid $[\alpha]_D$ +49.4° between crossed nicols of a polarizing microscope. Both sets of crystals were anisotropic showing oblique extinction for the views most often encountered (see Table I and Figs. 1 and 2). Determination of the indices of refraction by the Becke-line immersion method[20] gave for cytidylic acid $[\alpha]_D$ +20.7°, *n*, 1.600, 1.495; cytidylic acid $[\alpha]_D$ +49.6, *n*, 1.585, 1.512.

Deamination of Cytidylic Acid $[\alpha]_D$ +48°.—The high rotating isomer (0.1 g.) was suspended in 1.5 ml. of water to which 0.41 g. of sodium nitrite had been added.[10] Upon the gradual addition of 0.41 ml. of glacial acetic acid, there was considerable evolution of gas from the surface of the crystals. As the cytidylic acid was deaminated to form the highly water-soluble uridylic acid, the mixture became clear, indicating completion of the reaction. Although the solution had cleared in about two hours, it was permitted to stand 15 hours at 5°, after which it was taken to dryness twice at reduced pressure to remove excess acetic acid. Two moles brucine (0.245 g.) in 4 ml. of warm alcohol were added. A dense white crystalline precipitate formed on standing in ice. After filtering, washing with several small portions of 95% alcohol, and drying, the crude dibrucine uridylate, 0.32 g., 87% theory, was recrystallized and its optical rotation was found to be $[\alpha]_D$ −58.9°, *c*, 1.0 in pyridine. The rotation of dibrucine uridylate isolated from hydrolysates of yeast nucleic acid has ranged from $[\alpha]_D$ −55.0° to −57.7°.[11,12]

Deamination of Cytidylic Acid $[\alpha]_D$ +20.7°.—The same procedure was followed as in the preceding experiment; 50 mg. of this isomer was treated with 0.205 g. of sodium nitrite and 0.2 ml. of glacial acetic acid. The reaction proceeded more slowly than in the previous experiment and a clear solution was effected only after four hours at room temperature. After standing 12 hours at 5°, the solution was taken to dryness twice in a vacuum desiccator, and two moles brucine (0.123 g.) in 2 ml. of warm alcohol were added. This time, however, a precipitate did not form immediately, and after 48 hours in refrigeration, only a small amount of material was isolated. From its solubility in chloroform and crystalline behavior it was evidently unreacted brucine. In two other experiments in which 100-mg. and 180-mg. samples and sodium nitrite and glacial acetic or hydrochloric acid were used as deaminating agents, only small amounts of products ranging in rotation from $[\alpha]_D$ −59° to −37.6° could be isolated. These could not be definitely characterized.

(20) Chamot and Mason, "Handbook of Chemical Microscopy," Vol. I, 2nd Ed., John Wiley and Sons, Inc., New York, N. Y., 1939, p. 362.

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[CONTRIBUTION FROM THE LABORATORY OF PLANT PHYSIOLOGICAL CHEMISTRY, DEPARTMENT OF BOTANY, COLUMBIA UNIVERSITY]

Alkaloid Biogenesis. III. Specificity of the Nicotine–Nornicotine Conversion

BY R. F. DAWSON

The specificity of the biological conversion of nicotine to nornicotine has been examined by feeding homologs and analogs of nicotine to excised leaves of *Nicotiana glutinosa* rendered initially alkaloid free by grafting to tomato roots. Nornicotine was produced from *l*-nicotine, *d,l*-nicotine and *d,l*-N′-ethylnornicotine. Anabasine was produced from *d,l*-N′-methylanabasine and *d,l*-N′-ethylanabasine. The specificity of the process is thus so low that the formation of nornicotine in the green tobacco leaf can be considered as a specific case of a more general N-dealkylating activity of the leaf tissues.

It has been shown[1,2] that nornicotine, a principal Nicotiana alkaloid, is derived from nicotine by N′-demethylation in the plant leaf. The overall chemical nature of the transformation, its inherited character and restriction to leaf tissues suggest the possibility of a coupling with some non-alkaloidal phase of plant metabolism. Since alkaloid biosynthetic pathways and their metabolic couplings in plants are at present almost wholly unknown, the nicotine–nornicotine conversion has been subjected to closer examination in this Laboratory. It has been found that excised leaves of *Nicotiana glutinosa* can remove N-methyl and N-ethyl groups from both pyrrolidine and piperidine ring moieties of the Nicotiana alkaloids. The process may also lack stereochemical specificity. Thus, the demethylation of nicotine in certain strains of cigarette tobaccos and in many wild species of Nicotiana emerges as a single instance, albeit the naturally occurring one, of a more general N-dealkylating activity of the leaf tissues of these plants.

Completion of this phase of the inquiry depended upon the availability of two simple but highly propitious botanical techniques. One of these, the grafting of tobacco scions to tomato rootstocks,[3] permitted the production of virtually alkaloid-free leaves for use in the feeding experiments. Secondly, by the use of ice-cooled culture solutions, bacterial degradation of the test substances as well as clogging of the vascular tissues of the leaf petioles were avoided. Absorption of large volumes of the culture solutions by the leaf blades was thus facilitated. By these devices it was possible to feed substantial amounts of the various homologs and analogs of nicotine and to determine their metabolic fate without encountering the exceedingly difficult problem of separating such substances from pre-existing quantities of nicotine and nornicotine.

(1) R. F. Dawson, THIS JOURNAL, **67**, 503 (1945); *Am. J. Bot.*, **32**, 416 (1945).

(2) G. S. Il'in, *Biokhimya*, **13**, 193 (1948).

(3) R. F. Dawson, *Am. J. Bot.*, **29**, 66 (1942).

Experimental

Materials.—Pure *l*-nicotine was prepared by distilling *in vacuo* the highest grade offered by the Eastman Kodak Company. The ultraviolet absorption spectrum, molecular extinction coefficient at 259.5 mμ and melting point of the dipicrate compared well with published constants. *d,l*-Nicotine was secured by methylation of *d,l*-nornicotine with formic acid and formalin.[4] The *d,l*-nornicotine used in this preparation was kindly provided by Dr. C. F. Woodward of the Eastern Regional Research Laboratory in Philadelphia. The sample contained 3% nicotine as impurity by assay and by partition between ether and phosphate buffer at *p*H 7.2.

d,l-Anabasine isolated from *Nicotiana glauca*[1] was methylated by the same procedure used for methylating nornicotine. The melting point of the picrate (238–240°) was a little higher than that reported for N′-methylanabasine.[4] This preparation was homogeneous according to distribution measurements employing 0.5 *M* phosphate buffer at *p*H 6.6 and ethyl ether by the method of Evans and Partridge.[5]

Samples of *d,l*-N′-ethylnornicotine (picrate m.p. 179.5–182°) and of *d,l*-N′-ethylanabasine were obtained from Dr. Abner Eisner of the Eastern Regional Research Laboratory. Dr. Eisner kindly provided an analysis of the former.

Anal. Calcd. for $C_{11}H_{16}N_2$: C, 74.89; H, 9.14; N, 15.96. Found: C, 74.80; H, 8.96; N, 15.84.

Partition studies utilizing buffers at *p*H 7.2 and 6.6, respectively, showed no more than 0.5% impurity, calculated as nicotine, in either preparation. N-Ethylanabasine prepared in this Laboratory by the method of von Braun and Weissbach[6] contained 2.5% unreacted anabasine; picrate m.p. 197°.

The various bases were dissolved in sufficient HCl to yield the dihydrochlorides and diluted to 0.015–0.018 *M* for use in the feeding experiments.

Method of Assay.—A sample (250 mg.) of each of the dried leaf collections was extracted with hot water for 1 hr. and made to 50 ml. Five-ml. aliquots were treated with 0.5 ml. each of 2.4 *M* citric acid and sodium nitrite for 5 min. with shaking. This treatment converts secondary amines into non-distillable substances. An equivalent amount of sodium sulfamate was added and the residual tertiary amines steam distilled from an excess of MgO. Similar aliquots of the water extract were heated 30 minutes in a boiling water-bath with 0.50 ml. of formalin and 0.10 ml. of formic acid. By this means, secondary amines are converted into more readily distillable tertiary amines. Steam distillation from excess MgO followed. The distillates were conducted through cartridges of Amberlite resin IRC-50, hydrogen form. These were subsequently washed well with distilled water and eluted with 25 ml. of *N* HCl. Optical densities were determined in the Beckman spectrophotometer, model DU, at 259.5 mμ and compared with calibration curves for the different alkaloids. Calculations of the extent of dealkylation that occurred as a result of passage of the alkaloids through leaf tissues were, of course, based upon the recovery of the secondary amines as either nicotine or N-methylanabasine.

Separation of Metabolic Products.—The alkaloidal components of the various tissue samples were extracted in hot water and re-extracted from the cooled, alkalinized aqueous solutions with ether. Three-ml. portions of the ethereal solutions, each containing 3–5 mg. of total alkaloid, were passed through columns containing Johns-Manville Hyflo-SuperCel moistened with 0.50 *M* phosphate buffer.[5] Ether saturated with buffer was used to develop the columns, and the effluent was collected in consecutive 5-ml. portions. These were shaken out with 5 ml. each of *N* HCl and the optical densities of the resulting solutions determined as described above. Nornicotine could best be separated from its N-alkylated homologs at *p*H 7.2 and anabasine from its homologs at *p*H 6.6. The positions of the various curves on the collection ordinate were characteristic and readily reproducible. While tertiary amines gave smooth separation curves with relatively little skewness, the secondary amines tended to emerge from the columns more slowly. This difficulty could be overcome by the use of ether–chloroform mixtures following initial elution of the tertiary amines.

Nornicotine was isolated from one nicotine-fed sample as the dipicrate after removal of nicotine by azeotropic distillation.[7] Anabasine was isolated also as the dipicrate from another tissue sample which had been fed N-methyl anabasine. This procedure was not well adapted to the characterization of samples that had been fed N-ethylated homologs due to the lower volatility of the latter and to the somewhat less nicely crystalline nature of their picrates.

Feeding the Tertiary Amines.—Single leaves and leafy twigs of *Nicotiana glutinosa* from tomato rootstocks were detached and cultured for 2 days with their cut ends in ice-cold solutions of the different tertiary amines. These leafy tissues contained 0.04% nicotine and 0.01% nornicotine, dry weight basis, at the outset by assay. The upper portions of the twigs and the leaf blades were exposed to dim light at room temperature during the culture period. Sufficient plant material was employed for each experimental treatment to yield from 4 to 6 g. of dried tissue for analysis. At the end of the culture period the stems and petioles were slit lengthwise and all tissues dried in a forced-draft, hot-air oven at 70° for two hours. The dried material was ground through a Wiley mill and the powder stored in tight jars in the refrigerator.

Results

Table I illustrates the nature and extent of the changes that occurred as a consequence of feeding nicotine, its homologs and analogs. It is seen that methyl groups were removed from *l*- and *d,l*-nicotine with equal facility. That the secondary amine measured in the analytical procedure was actually nornicotine is proved by the isolation of the dipicrate (m.p. 191.7°) from the tissue extracts and by the results of partition experiments given in Fig. 1. Methyl groups were likewise removed from the piperidine ring of anabasine. The excellent yield of anabasine in this case could no doubt be traced to the use of a different crop of leaves grown in a different year. Analytical data indicated an 82% conversion of N-methylanabasine to anabasine based upon total alkaloid recovered. The melting point of the picrate isolated from the leaf tissues was a little low (207–209°) due to the presence of some remaining tertiary amine.

TABLE I

DEALKYLATION OF TERTIARY AMINES BY EXCISED LEAVES OF *Nicotiana glutinosa*[a]

Compound	Amount absorbed, mg.	Amount recovered, mg. Tertiary amine	Secondary amine
l-Nicotine	109	55	24
d,l-Nicotine	158	89	44
d,l-N′-Ethylnornicotine	175	119	41
d,l-N′-Methylanabasine[b]	207	17	78
d,l-N′-Ethylanabasine[b]	62	11	34
d,l-N′-Ethylanabasine	220	135	22

[a] Each compound was absorbed by the equivalent of 4–6 g. of dry leaf or shoot tissue. Prior to such feeding, each collection of plant material contained 2–3 mg. of nicotine and less than 1 mg. of nornicotine by assay. [b] These experiments were conducted in a different year and with a different crop of grafted plants.

Dealkylation of *d,l*-N′-ethylnornicotine occurred to the extent of about 25% of absorbed alkaloid. Partition of the tissue extracts yielded nornicotine (Fig. 1).

The first experiment involving the feeding of N-ethylanabasine was performed in the same year and with the same crop of grafted plants as had been used for the feeding of N-methylanabasine. The sample of N-ethylanabasine employed was that

(4) A. Orechoff and S. Norkina, *Ber.*, **65**, 1126 (1932).

(5) W. C. Evans and M. W. Partridge, *Quart. J. Pharm. Pharmacol.*, **21**, 126 (1948).

(6) J. von Braun and K. Weissbach, *Ber.*, **63**, 2018 (1930).

(7) H. H. Smith and C. R. Smith, *J. Agric. Res.*, **65**, 347 (1942).

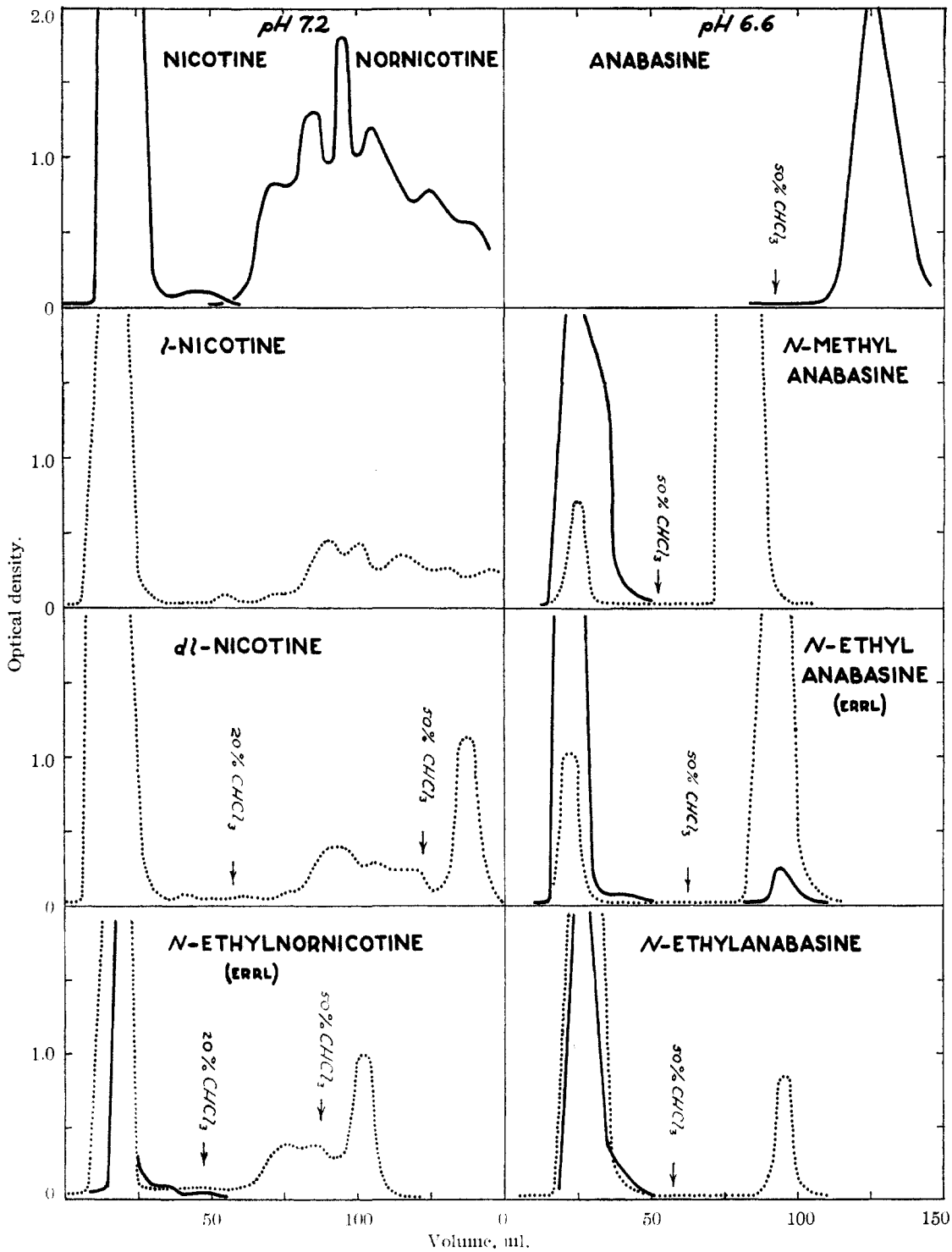


Fig. 1.—Partition of various secondary and tertiary amine preparations (solid lines) and tissue extracts (dotted lines) between phosphate buffers and ether on a column of Johns-Manville Hyflo-Supercel. Ether–chloroform mixtures were used to facilitate the emergence of the secondary amines from the column after residual tertiary amines had come through in ether. All nornicotine derivatives were partitioned at *p*H 7.2 and anabasine derivatives at *p*H 6.6. ERRL indicates the source of two preparations (*cf.* text).

prepared in this Laboratory. It is interesting to note that in this instance the extent of dealkylation was also greater than we have commonly encountered (*i.e.*, about 75%). With leaves from a different crop grown in a different year, the sample of N-ethylanabasine obtained from Dr. Eisner yielded only 14% anabasine by dealkylation based on total alkaloid absorbed. The partition data for this experiment leave no doubt, however, that some anabasine was formed.

Dealkylation is not the sole fate of tertiary amines in tobacco leaves. Utilizing leaves of a va-

riety of *Nicotiana tabacum* which does not possess the inherited capacity to convert nicotine to nornicotine, it has been shown[8] that about 25% of the nicotine absorbed during culture disappears as such and does not reappear in the steam-volatile alkaloid fraction or in the ether extracts of alkalinized aqueous digests. Twenty seven per cent. of the absorbed *l*-nicotine and 13% of the *d,l*-nicotine failed of recovery in the present experiments as the sum of tertiary and secondary amine. Three and four-tenths per cent. of the *d,l*-N-ethylnornicotine, 48% of the *d,l*-N-methylanabasine and 26–27% of the *d,l*-N-ethylanabasine were lost. It is not known whether this loss has any connection with the dealkylation reaction with which the present study has been concerned.

Discussion

The remarkable non-specificity of the N′-dealkylation of these natural and synthetic compounds in the Nicotiana leaf suggests that the process may depend more upon some general property of the cell contents such as oxidation–reduction potential than upon questions of molecular geometry such as would be expected to occur, *e.g.*, between nicotine and a specific dealkylating or alkyl transferring enzyme. Our current objective is, therefore, the attempt to prepare extracts or leaf macerates which will exhibit dealkylating activity and which can be used to settle the question of enzyme participation.

N′-Ethyl alkaloids are not known to occur in Nicotiana, and their metabolism to nornicotine and anabasine in these experiments can be considered as indicative solely of the lack of specificity of the dealkylation process. In the case of N-methylanabasine, however, a possible biosynthetic link between this minor Nicotiana alkaloid and the parent alkaloid anabasine is indicated.

Acknowledgment.—The author wishes to thank the Misses Mary Elizabeth Eichrodt and Hope Howeth Robson for technical assistance. It is a pleasure to record the participation of Mrs. Cynthia James, colleague of Loftus Hills from Australia, who carried out the partition studies recorded herein. The work was aided variously by grants from the Dean's Special Fund and the Higgins Fund of Columbia and by substantial support from a large philanthropic foundation which prefers anonymity.

NEW YORK 27, N. Y. RECEIVED MARCH 8, 1951

(8) R. F. Dawson, *Arch. Biochem.*, **21**, 279 (1949); *Am. J. Bot.*, **27**, 190 (1940).

[CONTRIBUTION FROM ABBOTT LABORATORIES]

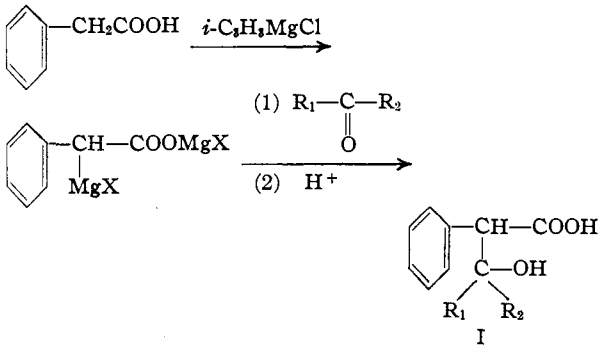
Antispasmodics. Esters of β-Alkyltropic Acids

BY ARTHUR W. WESTON AND ROBERT W. DENET

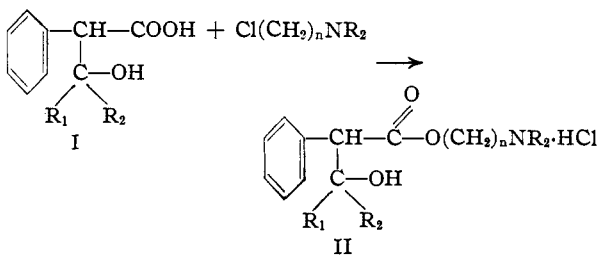
A series of β-alkyltropic acids was obtained by addition of the $C_6H_5CH(MgX)COOMgX$ or $C_6H_5CH(MgX)COONa$ Grignard complex to a number of aldehydes and ketones. Condensation of these acids with some basic alkyl halides in isopropyl alcohol solution gave the corresponding basic esters which have been found to possess antispasmodic action.

As part of a research program[1] directed toward the finding of a clinically useful antispasmodic, the investigation of a series of basic esters (II) derived from β-substituted tropic acids was undertaken.

The requisite acids (I) were obtained by treating phenylacetic acid or sodium phenylacetate with a slight excess of isopropylmagnesium halide and condensing the resulting Grignard complex with the appropriate aldehyde or ketone.[2] Phenylacetic acid is undoubtedly present as a contaminant in the reaction product and probably accounts for the difficulty experienced in purifying some of the simpler β-alkyl acids and the concurrent low yields. Although reaction with acetaldehyde and *n*-butyraldehyde introduced a second asymmetric carbon atom into the molecule, only one racemic modification was isolated in each case. Information pertaining to the acids prepared in this study is recorded in Table I.



The esters, listed in Table II, were prepared by condensing the acids and basic alkyl chlorides in boiling isopropyl alcohol solution.[3] Removal of the solvent, followed by addition of ether, was required to precipitate the basic ester hydrochlorides. Most of the products were obtained as oils which solidified after trituration with dry



(1) For related papers see A. W. Weston, THIS JOURNAL, **68**, 2345 (1946); A. W. Weston and W. B. Brownell, in press.

(2) This reaction has been extensively investigated by Ivanov and co-workers; see D. Ivanov and N. I. Nicolov, *Bull. soc. chim. France*, [4] **51**, 1325 (1932), and previous references.

(3) H. Horenstein and H. Pählicke, *Ber.*, **71**, 1644 (1938).