[CONTRIBUTION FROM THE DEPARTMENT OF SURGERY, HARVARD MEDICAL SCHOOL AND THE YAMINS LABORATORY FOR SURGICAL RESEARCH, BETH ISRAEL HOSPITAL]

The Preparation of 2-(2-Naphthoxy)-propionic Acid and 2-(6-Bromo-2-naphthoxy)-propionic Acid as Possible Chromogenic Substrates for Fatty Acid Oxidases[1]

BY MORRIS N. GREEN, LEONARD J. FRIEDMAN AND ARNOLD M. SELIGMAN

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Of the various methods for the preparation of 2-(2-naphthoxy)-propionic acid which were explored, the best results were obtained with β-propiolactone. The 6-bromo derivative was prepared by bromination. These compounds when fed are oxidized to naphthols which appear in the urine. No consistent oxidation of these substrates could be demonstrated *in vitro* with tissue homogenates, slices or sections. Both 2-(2-naphthoxy)- and 2-phenoxypropionates inhibited oxygen uptake when octanoate was used as the substrate.

Compounds of the 2-(2-naphthoxy)-propionic acid type should be useful in the histochemical demonstration of the enzyme system which oxidizes fatty acids. If β-oxidation occurred, the resulting 2-naphthoxycarbonic acid would spontaneously lose CO_2 to form β-naphthol. Coupling with a suitable diazonium salt would produce an azo dye at the site of enzymatic activity. Similar methods for a number of enzyme systems have been reported.[2-13] Since it has been found that 6-bromo-2-naphthol binds more tightly to protein and is less soluble than β-naphthol itself,[11] a substrate which releases 6-bromo-2-naphthol is preferable for enzyme localization if coupling is performed after the enzymatic reaction is complete.

The procedure of Gresham, *et al.*,[14] using sodium naphthoxide and β-propiolactone[15] in boiling aqueous solution gave 26% of 2-(2-naphthoxy)-propionic acid, m.p. 136–139° uncor. One recrystallization from benzene–petroleum ether gave a product melting sharply at 142° uncor. Chakravarti and Dutta[16] reported the melting point as 144–145°, and Gresham and Shaver[17] reported 140–143°.

An experiment under identical conditions using 6-bromo-2-naphthol gave only traces of the desired product. The relative unreactivity of this compound is shown also by the fact that in the condensation with acrylonitrile reported by Bachman and Levine,[18] 6-bromo-2-naphthol gave only a 10% yield.

Dibromination of the 2-(2-naphthoxy)-propionic acid followed by reduction of the bromine atom from the 1-position in the intermediate with tin, essentially according to the procedure of Koelsch[19] for the preparation of 6-bromo-2-naphthol, gave 2-(6-bromo-2-naphthoxy)-propionic acid, m.p. 179–181° uncor., in 53% to 60% yield.

Chakravarti and Dutta[16] report the preparation of 2-(2-naphthoxy)-propionic acid by a Williamson synthesis from β-naphthol and β-chloropropionic acid in aqueous solution with excess potassium hydroxide. No yield was reported. Attempts to repeat their work failed to give consistent results and the usual yield was less than 1%. Rapid cleavage of the product even by dilute base as well as the ease with which the haloacid undergoes base-catalyzed elimination may explain our results.[20]

Preliminary experiments with rats and rabbits after feeding both 2-(2-naphthoxy)-propionic acid and 2-(6-bromo-2-naphthoxy)-propionic acid by stomach tube, showed that both compounds were oxidized, since free naphthol appeared in the urine (colorimetric assay). Similar experiments with 2-naphthoxy acetic acid (Eastman Kodak Co.) failed to yield urinary naphthol, as the theory of β-oxidation would predict.

Fatty acid oxidase occurs in rat liver mitochon-

(1) This investigation was aided by a research grant from the National Cancer Institute, National Institutes of Health, United States Public Health Service, The Lasker Fund and the Slosberg Fund for Research in Diabetes.

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(13) A. M. Rutenburg, R. B. Cohen and A. M. Seligman, *Science*, **116**, 539 (1952).

(14) T. L. Gresham, J. E. Jansen, F. W. Shaver, R. A. Bankert, W. L. Beears and M. G. Prendergast, THIS JOURNAL, **71**, 662 (1949).

(15) β-Propiolactone was given to us by the B. F. Goodrich Company through the courtesy of Dr. Gresham, and we acknowledge their kindness.

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(20) Shifting to non-aqueous media only increased the rate of elimination from the haloacid, and attempts with 2 moles of potassium in *t*-butyl alcohol and in methanol–dioxane systems gave only a trace of the desired product. To avoid the unfavorable effect of the negatively charged carboxylate ion, ethyl β-bromopropionate and sodium naphthoxide were refluxed in absolute alcohol. After 10 minutes the *p*H of the solution had dropped to 6 and much salt had separated. Elimination from the haloester was the predominant reaction, since only 5% of a brown, neutral solid, m.p. 88–110° uncor., was recovered. The product was not further characterized because of the poor yield. The corresponding nitrile was obtained in 95% yield by the method of Bachman and Levine [THIS JOURNAL, **69**, 2343 (1947)] from β-naphthol and acrylonitrile, m.p. 104–105° uncor. (reported 105.7–107°). However, all attempts to hydrolyze the nitrile without cleaving the ether failed. Sixty per cent. sulfuric acid gave tars. Concentrated hydrochloric acid, alone and with varying amounts of glacial acetic acid to dissolve the nitrile, gave negligible amounts of the carboxylic acid. The main product was β-naphthol. This is in striking contrast to the 91% yield Wiley [THIS JOURNAL, **73**, 4205 (1951)] obtained in the hydrolysis of 2-(*p*-methoxyphenoxy)-propionitrile with concentrated hydrochloric acid. Treatment of our nitrile with base produced only β-naphthol.

dria in a highly labile form[21] and in a stable form in soluble extracts of acetone powders of rat liver mitochondria[22] or similar extracts of pig heart muscle.[23] The former oxidase is inhibited by fluoride, calcium ion, 2,4-dinitrophenol, methylene blue, arsenate, arsenite[21] and phenyl substituted fatty acids such as cinnamic acid and phenyl butyrate.[24] The oxidase is also sensitive to changes in osmotic pressure and temperature above 0°.[21] The soluble oxidase, however, is not inhibited by fluoride, 2,4-dinitrophenol[22] or malonate which is inhibitory to some of the mitochondrial preparations.[21,22] Adenosine triphosphate (ATP) is required for the action of the soluble enzyme[22] but not for the mitochondrial preparation.[21] A variety of substances involved in oxidative phosphorylations will prime the mitochondrial preparations.[21]

Several *in vitro* methods were tried with tissue homogenate, tissue sections and slices in an attempt to demonstrate oxidation of 2-(2-naphthoxy)-propionic acid and the 6-bromo derivative. We were unable to obtain consistent results with any of the methods tried. In general a small amount of oxidation was irregularly observed when large amounts of tissue homogenate remained standing overnight in contact with the substrate. In order to analyze these results, oxygen uptake was determined with both octanoate and the synthetic compounds as substrates. It was observed that both the phenoxy[16] and naphthoxy derivatives completely or strongly inhibited both octanoate oxidation and endogenous respiration when the compounds were tested in equimolar concentrations.

The preceding observations may be explained possibly as follows: The phenoxy and naphthoxy derivatives inhibit fatty acid oxidation in a manner similar to that already described for the phenyl substituted fatty acids by Graflin and Green.[24] Overnight incubation of the homogenates with the synthetic substrates may permit the elaboration of a small amount of the soluble fatty acid oxidase[22] to produce some oxidation or this enzyme may be destroyed by the proteolytic enzymes in the tissue preparations which would account for the failure to produce any oxidation to naphthol in many of the experiments.

Experimental[25,26]

2-(2-Naphthoxy)-propionic Acid.—The general procedure of Gresham, *et al.*,[16] for phenols was followed exactly, using 72 g. (1 mole) of β-propiolactone, 144 g. (1 mole) of β-naphthol recrystallized from toluene, and 40 g. (1 mole) of sodium hydroxide. The precipitated solid finally obtained weighed 56 g. (26%), m.p. 136–139°. The same yield was obtained on a 0.5-mole scale. One crystallization from benzene–petroleum ether gave a product melting sharply at 142°.

2-(6-Bromo-2-naphthoxy)-propionic Acid.—The procedure was essentially that of Koelsch[20] for the preparation of 6-bromo-2-naphthol. 2-(2-Naphthoxy)-propionic acid (5 g., 0.023 mole), m.p. 142°, was suspended in 70 cc. of glacial acetic acid in a 500-cc. round-bottom standard taper flask. A solution of 7.42 g. (0.046 mole) of bromine in 10 cc. of glacial acetic acid was added intermittently in 0.5-cc. portions, over a period of 30 minutes. The mixture was gradually heated and kept just under boiling for the last 15 minutes. Water (3 cc.) was added, the mixture was heated to boiling, mossy tin (3.8 g.) was added and the mixture was refluxed for 2 hours. It was allowed to cool to 50–60°, and white needles of the tin salt were removed by filtration. The pale yellow filtrate was poured into 80 cc. of water, stirred for 2 minutes, and the mixture made up to 1 liter with water. This was allowed to stand at room temperature overnight. The precipitated solid was filtered, washed with water and dried at 100°. The yellow solid was extracted for a few minutes with 150 cc. of boiling absolute alcohol and filtered. A second extraction with 100 cc. of boiling acetone was made. Both filtrates were evaporated to dryness on the steam-bath; yield 4.7 g. (69%), m.p. 159–178°, of which 4.2 g. came from the alcohol extract. The solid was crystallized from methanol after treatment with norite; yield 4.1 g. (60%) of fine white needles, m.p. 179–181°. The analytical sample was recrystallized from ethanol and then from methanol, without change in the melting point.

Anal. Calcd. for $C_{13}H_{11}O_3Br$: C, 52.90; H, 3.76; neut. equiv., 295. Found: C, 53.13; H, 3.73; neut. equiv., 300.

Experiments with Tissue Homogenates.—Rat liver, kidney, heart and pancreatic homogenates were incubated with coenzyme I and succinate in the presence of substrate. No oxidation, as evidenced by the formation of β-naphthol, was observed. In another experiment liver was homogenized in 0.9% sodium chloride; α-ketoglutaric acid (5 μmole) was used to spark the reaction; magnesium sulfate (7×10^{-3} *M*) and adenylic acid (10^{-2} *M*) were also added. The reaction was carried out at room temperature and at a *p*H of 7.3. Oxygen was bubbled through the reaction mixture. A slight amount of oxidation was observed after the reaction mixture remained standing overnight. These experiments were similar to those described by Green.[27]

Since the amount of oxidation by the methods described above was very slight, other methods were tried. A system similar to that described by Lehninger[28] using rat liver was investigated. In this system ATP and cytochrome C were used to activate the oxidation of the substrate. The liver was homogenized in a Krebs–Ringer solution with calcium omitted. Incubations were carried out at room temperature and 37°, in the absence of oxygen, or with oxygen, or with a 95% oxygen and 5% CO_2 mixture. The reaction mixture (10 cc.) was pipetted into a 50-cc. erlenmeyer flask, and the contents were vigorously shaken twice every hour for about three to four hours. No oxidation could be detected after six hours incubation. However, in some cases a slight amount of β-naphthol was formed after overnight incubation. Consistent results were not obtained with any of the homogenates. Usually no oxidation to naphthol was observed, but in rare instances the liver homogenates oxidized the substrate even in the absence of ATP. The minimum concentration of tissue to obtain any oxidation was about 100 mg. per cc. Cytochrome C did not increase the amount of β-naphthol formed in experiments in which oxidation was demonstrable. In each instance the controls were negative.

The tissues of the rat, rabbit and dog were compared to see which was most effective in oxidizing these substrates to naphthol. Liver and kidney were most frequently active. Skeletal muscle was inactive, whereas the heart muscle of the dog showed slight activity. Under the most favorable circumstances using 100 mg. of tissue per cc. and a 24-hour incubation the most active tissues showed 0.12 μmole of naphthol liberated in 24 hours.

Since the mitochondrial fatty acid oxidase is very labile, other methods of preparing the homogenates were investigated. Homogenization in sucrose as recommended by Hogeboom, *et al.*,[29] was tried as well as the salt mixture rec-

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(22) G. R. Drysdale and H. A. Lardy, *ibid.*, p. 281.

(23) H. R. Mahler, *ibid.*, p. 286.

(24) A. L. Graflin and D. E. Green, *J. Biol. Chem.*, **176**, 95 (1948).

(25) All melting points are uncorrected.

(26) The microanalyses were done by Dr. S. M. Nagy and associates, Microchemical Laboratory, Mass. Instit. Tech., Cambridge, Mass. The neutral equivalent was determined by Mrs. E. V. Rosenberg. Technical assistance in the animal experiments was provided by Mr. Thomas Barnett.

(27) D. E. Green, in "Respiratory Enzymes," ed. by H. A. Lardy, Burgess Publishing Co., Minneapolis, Minn., 1949, p. 209.

(28) A. L. Lehninger, *J. Biol. Chem.*, **157**, 363 (1945).

(29) G. H. Hogeboom, W. C. Schneider and G. G. Pallade, *ibid.*, **177**, 619 (1948).

ommended by Pressman and Lardy.[30] Suspensions prepared according to these methods were inactive.

Liver and kidney slices and frozen sections were incubated for varying periods of time up to 24 hours in the media described above with negative results.

Attempts were made to grow bacteria under conditions where the synthetic substrates constituted a large portion of the carbon source. *E. coli* was tested in a synthetic salt medium[31] containing only 0.01% glucose and 0.1 and 0.01 mg. of substrate per cc. of medium in order to force utilization of the substrate as a source of carbon. Neither phenol nor β-naphthol could be detected even though adequate growth occurred. Similarly, negative results were obtained with *B. subtilis* and *S. aureus*, using extract broth.

Experiments to Demonstrate Inhibitory Effects of the Substrates.—The procedure described by Kennedy and Lehninger[32] was followed exactly in studying the oxygen uptake of the synthetic substrates in the Warburg respirometer except that cytochrome C was omitted and a total volume of 3 cc. was used.

A typical experiment consisted of octanoate in one cup, the naphthoxy or phenoxy[16] substrate in another cup and equimolar combinations of both octanoate and either the naphthoxy or phenoxy substrate in a third cup, and finally a control without substrate. In a typical experiment octanoate consumed 68 μl. of oxygen, the naphthoxy substrate 0 μl., naphthoxy plus octanoate 0 μl. and the endogenous control 12 μl. A similar demonstration of inhibition of oxidation of octanoate was obtained with the phenoxy derivative.

Feeding Experiments.—Aqueous suspensions of 100 mg. of 2-(2-naphthoxy)-propionic acid, the 6-bromo derivative, or naphthoxyacetic acid were given to two rats by stomach tube. Urine from the rats fed with the propionic acid derivatives gave a very strong qualitative reaction for β-naphthol on coupling with tetrazotized diorthoanisidine, while the urine from the rats fed the acetic acid derivative was negative. Similar results were obtained with rabbits.

BOSTON, MASS.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY]

The Friedel–Crafts Alkylation of Methyl 2-Furoate

BY CHARLES D. HURD AND GENE L. OLIVER[1]

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The products of the alkylation of methyl 2-furoate with *s*-butyl bromide and aluminum chloride in carbon disulfide were found by infrared analysis to contain about 43% of the rearranged tertiary and 57% of the secondary butyl groups. The principal absorption peaks of pure *s*- and *t*-butylfurans and 2-methylfuran are reported.

In the investigation of the Friedel–Crafts reaction in the furan series of compounds, Gilman and Calloway[2] reported that alkylation of methyl 2-furoate with the isomeric butyl halides in the presence of aluminum chloride yielded methyl 5-*t*-butyl-2-furoate with all four isomers. Specifically, these percentage yields of methyl *t*-butylfuroate were reported from these halides: *n*-butyl chloride, 45; isobutyl bromide, 66; *s*-butyl bromide, 1.6; and *t*-butyl bromide, 46. The yields were based on isolation of 5-*t*-butyl-2-furoic acid. No other isomeric butylfuroic acid was isolated.

Because alkylation in the benzene series with *s*-butyl halides under various conditions almost without exception has produced the corresponding *s*-butylarene in preponderant amounts,[3] reinvestigation of this irregular alkylation in the furan series was undertaken.

Methyl 2-furoate[2] also has been alkylated with *n*-propyl bromide and isopropyl bromide to give the expected methyl 5-isopropyl-2-furoate. Methyl chloride and ethyl chloride and bromide (with aluminum chloride and carbon disulfide) yielded compounds which were considered to be dithio esters. The products from alkylation with *n*-pentyl and *n*-hexyl halides were identified as branched alkyl substitution products by analogy with the results in the butyl series. Other alkylations in the α-position in the furan series have been performed. 2-Furyl phenyl ketone was alkylated with *t*-butyl chloride to produce 5-*t*-butyl-2-furyl phenyl ketone.[2] Although furfural was found anomalously to yield 4-isopropylfurfural with isopropyl chloride and aluminum chloride,[4] the *n*-butyl, *t*-butyl and isobutyl chlorides gave yields of the order of 10% of product which was identified as 5-*t*-butylfurfural. When the α-position was blocked, profound cleavage of alkyl groups occurred during the alkylation on the β-position[5,6] as witnessed by the formation of ethyl 4-*t*-butyl-5-bromo-2-furoate from ethyl 5-bromo-2-furoate, aluminum chloride and either *t*-butyl, *n*-hexyl or octadecyl bromide.[5]

In the present work it was desired to compare the properties of the alkylation products resulting from the reaction of *s*-butyl bromide and methyl 2-furoate in the presence of aluminum chloride in carbon disulfide solution with the known pure compounds. The most satisfactory procedure for this purpose was decarboxylation of the butylfuroic acids resulting from the alkylation into butylfurans, which then could be compared with pure *s*- or *t*-butylfuran. The last two compounds were prepared by unequivocal methods and their infrared spectra were obtained. The methyl furoate was alkylated according to the directions of Gilman and Calloway.[2] The product was distilled at 10 mm. and the fraction boiling from 107 to 125° was hydrolyzed by potassium hydroxide in methanol. As indicated by Gilman and Calloway, the acid appeared as a viscous oil. This oil was decarboxylated to yield a mixture of butylfurans boiling between 119 and

(1) Holder of a University Fellowship, 1951-1953.

(2) H. Gilman and N. O. Calloway, THIS JOURNAL, **55**, 4197 (1933).

(3) C. C. Price, "Organic Reactions," Vol. III, John Wiley and Sons, Inc., New York, N. Y., 1946, p. 31.

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(5) H. Gilman and R. R. Burtner, *ibid.*, **57**, 909 (1935).

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