Inhibitory Action of α-(4-Chlorophenoxy)-α-methylpropionic Acid Analogs on Cholesterol Biosynthesis and Lipolysis *in Vitro*

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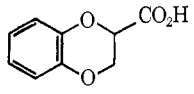
The antagonist activity of the title compound **2** and some open chain and cyclic analogs on glycerol release from adipose tissue and incorporation of mevalonate-2-^{14}C into nonsaponifiable products of rat liver homogenate *in vitro* is discussed. Greater structural specificity was observed for the inhibition of cholesterol biosynthesis than for inhibition of lipolysis. A proposed mechanism for the antilipolytic effect of **2** is described.

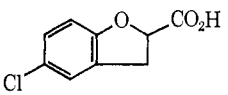
The hypocholesterolemic and hypolipidemic properties of ethyl α-(4-chlorophenoxy)-α-methylpropionate (**1**) are well established.[2] Since **1** undergoes rapid hydrolysis *in vivo* and *in vitro*, the resulting acid **2** is presumed to be the active drug.[3] Studies *in vivo* and *in vitro* with **1** or **2** indicate that the latter may exert its effect by multiple modes of action.[2,4] In this regard only a few investigators have reported the ability of **2** to inhibit free fatty acid (FFA) mobilization from adipose tissue.[5-7] In this paper we discuss some recent findings on the ability of certain analogs (**2**–**6**) to inhibit the release of glycerol from adipose tissue and to inhibit cholesterol biosynthesis in rat liver homogenate preparations *in vitro*. Drugs prepared represent minimum structural modifications of **2** and were designed for the ultimate purpose of having available optically pure analogs of the parent molecule **2** which is symmetric.[2,3a,b] Through the use of these chemical probes we hope to differentiate and classify biological receptors which are either blocked or stimulated by **2**.

p-$ClC_6H_4OC(R)(R')CO_2R''$

1, R = R′ = Me; R″ = Et
2, R = R′ = Me; R″ = H
3, L(*S*) or D(*R*), R or R′ = H or Me; R″ = H
4, racemic, R = Me; R′ = Et; R″ = H
5, racemic, R = R″ = H; R′ = Et

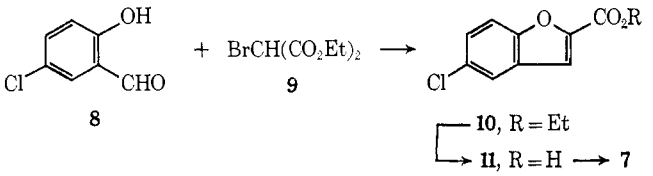


6, racemic



7, racemic

Synthetic Aspects.—The synthesis for **2** and L(*S*)- or D(*R*)-**3** have been previously described.[2] (*RS*)-α-(4-Chlorophenoxy)-α-methylbutyric acid (**4**) was prepared by condensation of 4-ClC_6H_4OH with 2-butanone and $CHCl_3$ in the presence of NaOH; acidification affords **4**.[8] Compound **5** was prepared according to a published method.[9] 1,4-Benzodioxane-2-carboxylic acid (**6**) was prepared according to a method described by Koo and coworkers.[10] 5-Chloro-2,3-dihydro-2-benzofurancarboxylic acid (**7**), the cyclic analog of **3**, was prepared from 5-chlorosalicylaldehyde (**8**) and diethyl bromomalonate (**9**). Reaction in the presence of K_2CO_3 afforded ethyl 5-chloro-2-benzofurancarboxylate (**10**). Hydrolysis afforded the free acid **11**[11] which upon reduction with NaHg yielded the dihydro derivative **7**.



Biological Results. Inhibition of Lipolysis *in Vitro*.—The results obtained in these studies using **2** at a concn range of 10^{-3} to 10^{-2} *M* show that this drug significantly inhibits the basal release of glycerol from adipose tissue (Table I). At the same concentration levels, **2** was also found to inhibit theophylline- and norepinephrine (NE)-induced lipolysis *in vitro* (Table II).

TABLE I

EFFECT OF **2** ON THE RELEASE OF GLYCEROL FROM RAT EPIDIDYMAL ADIPOSE TISSUE IN THE ABSENCE OF EXOGENOUS STIMULATION *in Vitro*

Concentration of 2 (*M*)	μmole of glycerol released/g of tissue[a]	% response as glycerol released
0	1.12 ± 0.23[b]	100.0 ± 20.1[b]
10^{-3}	0.43 ± 0.11[c]	40.6 ± 9.8[c]
2×10^{-3}	0.34 ± 0.11[c]	31.8 ± 9.9[c]
5×10^{-3}	0.21 ± 0.09[c]	21.0 ± 5.7[c]
10^{-2}	0.18 ± 0.06[c]	15.7 ± 5.5[c]

[a] Each value represents the average of 4 experiments. [b] Standard error of the mean. [c] Significantly different from the control ($p < 0.05$).

(1) (a) Abstracted in part from the dissertations presented by E. E. S. (1970) and R. E. H. (1970), to the graduate school of The Ohio State University; (b) U. S. Public Health Service Predoctoral Fellow (5-F)1-GM-37591, 1967–1970; (c) American Foundation for Pharmaceutical Education Fellow, 1967–1970; (d) National Science Foundation Undergraduate Research Participant, Summer 1970.

(2) For applicable references through 1968, see D. T. Witiak, T. C-L. Ho, and R. E. Hackney, *J. Med. Chem.*, **11**, 1086 (1968).

(3) (a) D. T. Witiak and M. W. Whitehouse, *Biochem. Pharmacol.*, **18**, 971 (1969); (b) D. T. Witiak, R. E. Hackney, and M. W. Whitehouse, *J. Med. Chem.*, **12**, 697 (1969); (c) J. M. Thorp, *Lancet*, **1**, 1323 (1962).

(4) (a) R. J. Cenedella, J. J. Jarrell, and L. H. Saxe, *J. Atheroscler. Res.*, **8**, 903 (1968); (b) D. C. Macmillan, M. F. Oliver, J. D. Simpson, and P. Tothill, *Lancet*, **2**, 924 (1965); (c) B. M. Rifkind, *Metab. Clin. Exp.*, **15**, 673 (1966); (d) J. M. Thorp and A. M. Barrett, *Progr. Biochem. Pharmacol.*, **2**, 337 (1967); (e) A. M. Barrett and J. M. Thorp, *Brit. J. Pharmacol.*, **32**, 381 (1968).

(5) A. M. Barrett, *ibid.*, **26**, 363 (1966).

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Table II

Effect of 2 on the Release of Glycerol from Rat Epididymal Adipose Tissue in Response to Theophylline (10^{-2} *M*) and NE (2.4×10^{-6} *M*) *in Vitro*

Concentration of 2 (*M*)	μmole of glycerol released/g of tissue[a]	
	Theophylline	NE
0	3.97 ± 0.31[b]	5.39 ± 1.21[b]
10^{-3}	4.00 ± 0.55	4.80 ± 1.22
2×10^{-3}	3.75 ± 0.50	4.10 ± 0.92
5×10^{-3}	2.59 ± 0.16[c]	2.33 ± 0.56[c]
10^{-2}	0.83 ± 0.10[c]	0.04 ± 0.12[c]

[a] Each value represents the average of 3 experiments. [b] Standard error of the mean. [c] Significantly different from the control ($p < 0.05$).

At concns less than 10^{-3} *M*, **2** did not alter the rate of glycerol release induced by NE or theophylline; *i.e.*, no inhibitory effect was observed. The data presented in Figure 1 show that the lipolytic activity induced by NE is also inhibited by **2** in an albumin-free medium. Moreover, the inhibition of this lipolysis by **2** is clearly dose dependent in the presence or absence of albumin. Further studies indicated that 5×10^{-3} and 10^{-2} *M* **2** inhibited NE-induced lipolysis in a noncompetitive manner, *i.e.*, a decrease in the maximum rate of glycerol release was observed in the presence of **2** (Figure 2).

The effects of a number of compounds structurally related to **2** are shown in Table III. The results are

Table III

Effect of Compounds Structurally Related to 2 on the Release of Glycerol from Rat Epididymal Adipose Tissue in Response to Norepinephrine (2.4×10^{-6} *M*) *in Vitro*

Compd No.	Concentration, *M*	% inhibition of glycerol release[a]
2	5×10^{-3}	57.3 ± 3.1[b,c]
	10^{-2}	100.7 ± 1.1[c]
L(*S*)-**3**	10^{-3}	−2.3 ± 10.9
	2×10^{-3}	11.3 ± 14.0
	5×10^{-3}	54.7 ± 5.7[c]
	10^{-2}	84.3 ± 10.7[c]
D(*R*)-**3**	10^{-3}	9.1 ± 10.3
	2×10^{-3}	5.3 ± 13.4
	5×10^{-3}	45.3 ± 3.9[c]
	10^{-2}	73.6 ± 5.0[c]
5	5×10^{-3}	61.6 ± 7.2[c]
	10^{-2}	93.6 ± 5.8[c]
6	5×10^{-3}	37.7 ± 5.1[c]
	10^{-2}	82.4 ± 4.2[c]
7	5×10^{-3}	53.2 ± 8.5[c]
	10^{-2}	75.7 ± 8.5[c]

[a] Each value represents the average of at least 3 experiments. [b] Standard error of the mean. [c] Significantly different from the control ($p < 0.05$).

expressed in terms of the per cent inhibition of glycerol release by NE in the presence of each compound. Nearly all of the compounds examined exhibit a similar degree of inhibition to that observed with **2**. For example, a significant reduction in NE-induced lipolysis was observed for each compound at 5×10^{-3} and 10^{-2} *M*. Furthermore, the two enantiomorphs D(*R*)-**3** and L(*S*)-**3** exhibited a nearly identical antagonism.

Inhibition of Mevalonate-2-^{14}C Incorporation into Nonsaponifiable Products.—Thie inhibition of cholesterol biosynthesis by **2**, L(*S*)- and D(*R*)-**3**, and *p*-chlorophenoxyacetic acid in rat liver homogenate preparation

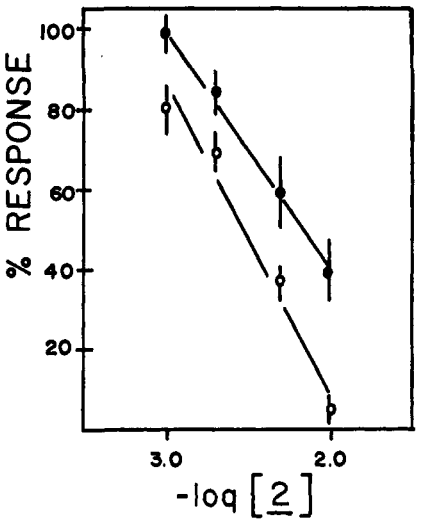

Figure 1.—The effect of **2** on the release of glycerol from rat epididymal fat tissue in response to NE (2.4×10^{-6} *M*) in the presence or absence of albumin. A maximum lipolytic rate of 1.96 μmoles/g per hr was employed to calculate the dose–response curve in the albumin-free medium: (○—○) = presence or (●—●) = absence of albumin. Values plotted represent the mean of 4 experiments ± the standard error as indicated.

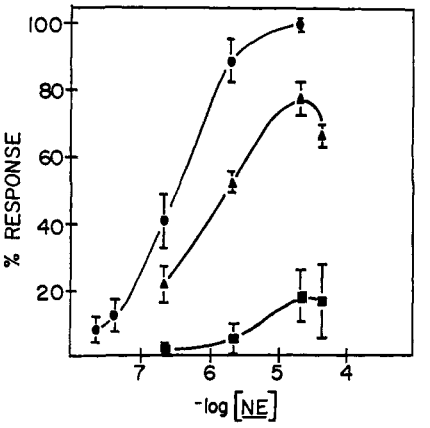

Figure 2.—Dose–response curves for NE on the mobilization of FFA (measured as glycerol released) from rat epididymal fat tissue *in vitro*: (●—●) = no inhibitor; (▲—▲) = 5×10^{-3} *M* **2**; (■—■) = 10^{-2} *M* **2**. Each value represents the average of 4 experiments with the standard error of the mean indicated.

has been previously described.[3b] As shown in Table IV, an inhibition of mevalonate-2-^{14}C incorporation

Table IV

Effect of Compounds **4**, **6**, and **7** Added *in Vitro* on the Incorporation of Mevalonate-2-^{14}C into Nonsaponifiable Material in Rat Liver Homogenate

Compd (1.5 m*M*)	% inhibition of ^{14}C incorporation[a]
4	29 ± 1[b]
6	3 ± 3
7	25 ± 3

[a] Average value from 4 detns. [b] Standard error of the average value.

into nonsaponifiable materials was also observed for **4**, **6**, and **7** at the 1.5 m*M* concn. Only **6** and D(*R*)-**3**[3b] are considerably less potent than other analogs tested in this series. The dose–response curves (Figure 3) for **4**, **6**, and **7** confirm this conclusion. Compounds **4** and **7** show more inhibitory activity at the **4.5**-m*M*

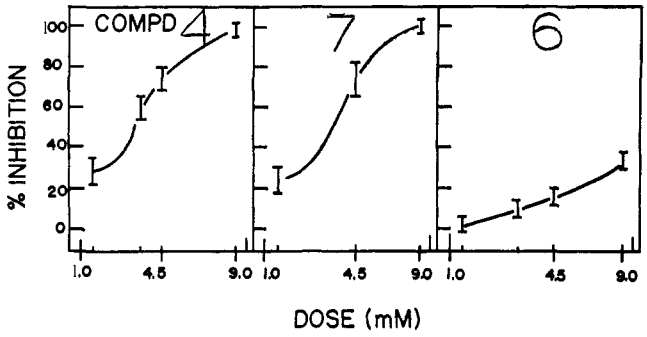

Figure 3.—Dose-response curves for the per cent inhibition of mevalonate-2-^{14}C incorporation into nonsaponifiable material in fortified rat liver homogenate. Compound **4** = α-(4-chlorophenoxy)-α-methylbutyric acid. Compound **6** = 1,4-benzodioxane-2-carboxylic acid. Compound **7** = 5-chloro-2,3-dihydro-2-benzofurancarboxylic acid. Each value represents the average of 3 experiments with the extreme values indicated.

dose than do **2**, L(*S*)-**3**, or *p*-chlorophenoxyacetic acid.[3b] At this concn both **4** and **7** exhibit about a 70% inhibition while **2**, L(*S*)-**3**, and *p*-chlorophenoxyacetic acid exhibit 35–40% inhibition.[3b]

Discussion

It has been suggested by numerous investigators[4-7] that the inhibition of FFA mobilization *in vivo* is responsible for at least a portion of the hypotriglyceridemic effect of **2**. Previous studies, however, have failed to establish a dose-dependent relationship for the effect, *in vitro*. The results presented in this paper demonstrate that **2** exerts a nonspecific dose-dependent inhibition of the hormone-sensitive lipase system and almost completely blocks NE- and theophylline-induced lipolysis at a concn of 10^{-2} *M* as well as the basal release of glycerol. We have also shown that the antipolytic effect of **2** is noncompetitive with NE (Figure 2). These observations differ quantitatively from an earlier report by Barrett[5] in which it was found that **2** inhibited epinephrine (E)-induced lipolysis only at the lowest of 3 concns (about 1.2–4.8 × 10^{-3} *M*), and that **2** did not modify the basal release rate of FFA. In another study, Blackard and Kokatnur[6] showed that the presence of **2** at 1000 ng/ml in the incubation medium resulted in a small (15%) but significant reduction in tissue FFA levels. The results of studies *in vivo* by Barrett and Thorp[4d,e] indicated that the resting level of plasma FFA was lower in rats treated with **1**. Even though the FFA levels were lower, pretreatment with **1** did not modify the increase in FFA induced by E. These investigators concluded that the reduction in plasma FFA was due to a decrease in the uptake and transport of FFA by plasma proteins. It was suggested that the decrease in uptake resulted from a competition between the hydrolysis product of **1**, namely **2**, and FFA for protein binding sites normally occupied by FFA released from adipose tissue depots. Barrett[5] also suggested that **2**'s ability to antagonize E-induced lipolysis *in vitro* was in part related to its occupancy of the anionic binding sites on albumin. In the present study, the inhibitory action of **2** on NE-induced lipolysis was only slightly reduced by the removal of bovine serum albumin (Figure 1). In view of these observations, it does not seem likely that the antilipolytic effect of **2** can be explained on the basis of the competition for plasma protein binding sites. More likely, the antilipolytic action of **2** *in vitro* is mediated at a step or steps in the lipolytic sequence within the adipose tissue cell.

In this regard, the antilipolytic effect of **2** may be similar to that proposed for sodium salicylate,[12] that is an inhibition of lipolysis at a site beyond cyclic 3′,5′-AMP formation.[13] This nucleotide is proposed to mediate the lipolytic effects of a variety of agonists including NE, E, and ACTH.[14-17] The fact that high doses of sodium salicylate and **2** or related analogs were required to inhibit lipolysis *in vitro* may reflect a limited ability of these agents to penetrate tissue barriers. Alternatively, the high dose requirements might indicate that these drugs exert a block on a lipase system *not* controlled by 3′,5′-AMP; *i.e.*, the drugs may inhibit the monoglyceride or diglyceride lipase enzymes. This type of lipase inhibition could partially explain the lack of an appreciable effect on FFA release *in vitro* with **2**, since it is known that the triglyceride lipase is rate-controlling in the mobilization of FFA.[15b] While it is possible to point toward the lipase(s) as the site of inhibition for **2** and related analogs, the hypothesis requires an isolation and purification of a lipase(s) which is capable of being activated by cyclic 3′,5′-AMP.

The effects of a number of compounds structurally related to **2** are shown in Table III. All analogs evaluated exhibit an inhibitory potency on the lipase system which is equiv to **2**. From these data it is not possible to determine the structural features which give rise to antipolytic activity. Thus far it seems that antilipolytic activity of the type observed with phenoxyacetic acid analogs has a very low degree of structural specificity. This is supported by the results obtained using optically active desmethyl analogs **3**. Whereas little differences were noted in the antilipolytic activities for the D(*R*)- and L(*S*)-**3** desmethyl analogs of **2**, a considerable difference was observed between these isomers of α-(*p*-chlorophenoxy)propionic acid against cholesterol biosynthesis *in vitro*[3b] and *in vivo*.[2] Similarly, **4** and **7** are stronger blockers of cholesterol biosynthesis *in vitro* than is the benzodioxane analog **6**. For inhibition of lipolysis these 3 analogs were equally as potent. These observations are difficult to reconcile with a hypothesis that **2** exerts both its antilipolytic and hypocholesterolemic actions by competing with endogenous substances for acidic binding sites on plasma proteins. Therefore, our data are consistent with the hypothesis that the hypolipidemic and hypocholesteremic effects of **1** and **2** are dissociated.

Currently, considerable emphasis has been placed on the synthesis and biological evaluation of hypocholesterolemic and hypolipidemic agents related to

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(13) Unpublished observations in this laboratory also show that **2** and related analogs inhibit the increase in glycerol release mediated by glucagon, ACTH, and N^6,$O^{2'}$,-dibutyryl adenosine 3′,5′-cyclic monophosphoric acid.

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(15) (a) E. W. Sutherland and G. A. Robison, *Pharmacol. Rev.*, **18**, 145 (1966); (b) M. Vaughn, J. E. Berger, and D. Steinberg, *J. Biol. Chem.*, **239**, 401 (1964).

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1 and **2**. In light of the similarity of action *in vitro* of analogs L(*S*)-**3** and **7** to that of **2**, it would appear that these drugs might be useful therapeutic agents in the treatment of atherosclerosis; further research *in vivo* is indicated.

Experimental Section[18]

α-(4-Chlorophenoxy)-α-methylpropionic acid (2), L(−)-α-(4-chlorophenoxy)propionic acid (3) and D(+)-α-(4-chlorophenoxy)propionic acid (3) were synthesized according to methods previously published.[2] **α-(4-Chlorophenoxy)-α-methylbutyric acid (4)** was prepd according to ref 8 in 25% yield. Recrystn from 10% HOAc followed by petr ether (60–80°) afforded white crystals, mp 95–96°, lit.[8] mp 95–96°. Compd **5**, **α-(4-chlorophenoxy)butyric acid**, was prepd according to ref 9. **1,4-Benzodioxan-2-carboxylic acid (6)** was prepd according to ref 10. Recrystn from PhH–ligroin afforded white crystals, mp 118–119°, lit.[10] mp 119–120°.

5-Chloro-2-benzofurancarboxylic acid (11) was prepd from **8** and **9** *via* **10** according to ref 11. Recrystn from EtOAc afforded 57% of **11**, mp 259–260°, lit.[11] mp 258°.

5-Chloro-2,3-dihydro-2-benzofurancarboxylic Acid (7).—Compd **11** (15.0 g, 0.08 mole) was added to a soln of NaOH (19 g) in H_2O (300 ml). A sparingly sol Na salt sepd. NaHg, prepd from Na (4.9 g) and Hg (195 g), was added with stirring during 20 min. The suspended Na salt went into soln after 45 min. The soln was stirred for another 45 min and allowed to stand overnight. The Hg was sepd and the soln was filtered. Acidification of the aq soln with 10% H_2SO_4 pptd the acid. Filtration and recrystn from petr ether (60–80°) afforded 11.8 g (75%) of **7**, mp 114.5–115°. Nmr (Me_2CO-d_6) δ for the calcd ABX spectrum[19] shows 8 lines (AB, 2 H, CH_2), δ_A 3.63, δ_B 3.42, and 4 lines (X, 1 H, CH), δ_X 5.32 with J_{AB} = 16.8 Hz, J_{AX} = 10.74 Hz, and J_{BX} = 6.06 Hz. The arom region shows the typical ABC pattern for those protons which are also long-range coupled to the AB CH_2 protons. *Anal.* ($C_9H_7ClO_3$) C, H, Cl.

Biological Studies. Inhibition of Lipolysis *in Vitro*.—The method employed for the isolation, prepn, and incubation of adipose tissue with the various compds is similar to the one described by Finger, *et al.*[20] In our studies, however, the production of glycerol rather than FFA was used as the index of lipolysis.

Nonfasted, white, male Harlan Wistar rats (180–250 g) were used. Epididymal fat pads collected from 4–6 animals per expt were minced with a scissors to yield adipose tissue fragments of 5–15 mg. For each test, 300 mg of adipose tissue was added to 2.5 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, contg 4% bovine albumin fraction V. Compds tested for inhibitory activity were dissolved in DMSO and a 0.1-ml aliquot was added to the appropriate flask to obtain the desired final concn. In the inhibition studies, all flasks were preincubated for 10 min before the addn of the agonist. Incubations were carried out under an atm of 95% O_2–5% CO_2 (1.5 l./min) for 1 hr at 37° in a metabolic shaker oscillating at 120 rpm. After 1 hr duplicate 1.0-ml aliquots were transferred to tubes contg 1 ml of 10% CCl_3CO_2H to stop the reaction. Glycerol was assayed in the supernatant by oxidn to CH_2O[21] followed by colorimetric anal.[22]

Rates of glycerol release (μmoles of glycerol/g of adipose tissue per hr of incubation) were calcd by subtracting the production of glycerol in the absence of an agonist from the amount released in its presence. A maximum rate of glycerol release was obtained in the presence of 2.4×10^{-6} *M* NE or 10^{-2} *M* theophylline. These maximal rates were then employed to calc the dose–response relationships. Data were analyzed for significance by Student's *t*-test; *p* values <0.05 were considered to represent significant differences between mean values.

Inhibition of mevalonate-2-^{14}C incorporation into nonsaponifiable products was studied according to the method described in ref 3b. Identical conditions were employed except that **4** was initially dissolved in 0.2 ml of DMSO prior to diln with phosphate buffer, pH 7.4. A similar concn of DMSO was employed in the control assay.

Acknowledgment.—We are grateful to the National Institutes of Health for support of this work through Grant HE 12740. This investigation was supported (in part) by National Institutes of Health Research Grant No. FR-00328 from the Division of Research Facilities and Resources.

(18) Nmr spectra were taken on a Varian A-60-A spectrometer. Mp were taken on a calibrated Thomas-Hoover mp apparatus. Analyses were determined by Clark Microanalytical Labs, Urbana, Ill.

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(21) M. Lambert and A. Neish, *Can. J. Res.*, **28**, 83 (1950).

(22) T. Nash, *Biochem. J.*, **55**, 416 (1953).