amine oxidase exhibits a high degree of physical as well as chemical homogeneity and that it contains at least one *b*- and one *c*-type cytochrome. Electron microscopic examination reveals the enzyme to be a spherical particle, and a diameter of 122 Å is calculated from the experimentally determined values of its molecular weight (200,000 g/mole) and sedimentation coefficient ($s_{20,w}$ = 10 S).

The molecular properties of hydroxylamine oxidase which have been elucidated in this paper are summarized in Table II. Since considerable information is now available with regard to other components of the *Nitrosomonas* electron-transport system, these data have also been included in the table. To the author's knowledge, this is the first report of the existence of an electron-transport system which is entirely soluble in the absence of prior treatment with detergents or physical fragmentation procedures such as sonic oscillation.

References

Bonner, W. D., Jr. (1961), *in* Haematin Enzymes, Falk, J. E., Lemberg, R., and Morton, R. K., Ed., Oxford, Pergamon.

Edelstein, S. J., and Schachman, H. K. (1967), *J. Biol. Chem. 242*, 2.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Rees, M. K. (1968), *Biochemistry 7*, 353 (this issue, preceding paper).

Rees, M. K., and Nason, A. (1965), *Biochem. Biophys. Res. Commun. 21*, 249.

Schachman, H. K., and Edelstein, S. J. (1966), *Biochemistry 5*, 2681.

Schachman, H. K., Gropper, L., Hanlon, S., and Putney, F. (1962), *Arch. Biochem. Biophys. 99*, 175.

Wilson, D. F. (1967), *Arch. Biochem. Biophys.* (in press).

Yphantis, D. A. (1964), *Biochemistry 3*, 297.

Glutamate Biosynthesis in Anaerobic Bacteria. III. The Origin of the Carboxyl Groups of Glutamic and Aspartic Acids Isolated from *Clostridium kluyveri* Grown on [1-^{14}C]Acetate*

Joseph R. Stern and R. W. O'Brien

ABSTRACT: Radioactive glutamic and aspartic acids were isolated by acid hydrolysis of the protein from whole cells of *Clostridium kluyveri* (Worthington strain) grown on [1-^{14}C]acetate and the distribution of ^{14}C was determined in each compound. With glutamic acid, 25.5% of the ^{14}C was in C-1, 36.7% in C-2 to -4, and 38.8% in C-5. With aspartic acid, 23.9% was in C-1, 49.1% in C-2 and C-3, and 27.0% in C-4. The glutamate results were consistent with those previously obtained with cell-free extracts of the same strain (Stern, J. R., Bambers, G., and Hegre, C. S. (1966), *Biochemistry 5*, 1119) and demonstrated that the stereospecificity of the citrate synthetase enzyme of the glutamate biosynthetic pathway was essentially the same in the intact cell as in extracts of this strain. It was shown that some of the [1-^{14}C]acetate was converted to $^{14}CO_2$, about 4% of the acetate radioactivity being recovered as $^{14}CO_2$ in the spent medium. This conversion accounted for the finding that both carboxyls of aspartate were approximately equally labeled. The radioactivity found in C-1 of glutamate was shown to have arisen mostly from $^{14}CO_2$ *via* [4-^{14}C]oxalacetate (aspartate) and the remainder (16%) by carbon translocation involving unknown reactions. The nature of the reactions leading from [1-^{14}C]acetate to $^{14}CO_2$ was not identified but may involve formation and decarboxylation of acetoacetate.

The pioneering work of Tomlinson (1954a) on the distribution of ^{14}C in the glutamic acid isolated from cells of *Clostridium kluyveri* grown in a synthetic medium containing $^{14}CO_2$ or [1-^{14}C]acetate demonstrated that the label was found mostly but not exclusively in the opposite carboxyl of glutamate to that predicted by the operation of the Krebs citric acid cycle. It has now been clearly established that glutamic acid biosynthesis in this microorganism does occur mostly, if not entirely, by a pathway involving the enzymes of the upper half of the citric acid cycle (Stern and Bambers, 1966; Gottschalk and Barker, 1966; Ilse and O'Brien, 1967). Gottschalk and Barker, using cell-free extracts, found a labeling pattern of glutamic acid

* From the Department of Pharmacology, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106. *Received July 24, 1967.* This work was supported by grants from the National Science Foundation (GB-4716) and the U. S. Public Health Service (AM00739).

synthesized from radioactive precursors essentially similar to that described by Tomlinson. They presented concrete evidence that this labeling pattern resulted from an unusual or opposite stereospecificity of the citrate synthetase of *C. kluyveri* compared to the usual stereospecificity demonstrated for the pig heart citrate synthetase (Hanson and Rose, 1963). On the other hand, Stern *et al.* (1966) demonstrated that the citrate synthetase present in extracts of a commercially grown Barker strain of *C. kluyveri* was the same as the pig heart enzyme, the labeling pattern of glutamic acid synthesized being essentially the reverse of that observed by Tomlinson and by Gottschalk and Barker.

However, it should be noted that although the experiments of Stern, *et al.* and of Gottschalk and Barker yielded opposite results, in each case some 5–8% of the radioactivity of the labeled precursors, [4-^{14}C]oxalacetate and [1-^{14}C]acetate, did translocate to the opposite carboxyl of glutamate to that predicted from an absolutely stereospecific course of citrate synthesis. The fact that pure [1-^{14}C]citrate on conversion to glutamate by *C. kluyveri* extract (Stern *et al.*, 1966) also gave a similar amount of translocation suggested that this translocation of carbon did not occur at the citrate synthetase step but either beyond it (*e.g.*, aconitase) or as a result of unknown interfering reactions. The data of Ilse and O'Brien (1967) with *C. kluyveri* extract show an even greater degree of carbon translocation during glutamate biosynthesis from labeled [4-^{14}C]oxalacetate and [1-^{14}C]acetate. In *C. thermoaceticum*, which possesses the same citrate pathway of glutamate biosynthesis, no translocation of radioactivity of labeled precursors was seen (Stern, 1966) and only one carboxyl of glutamate became labeled, permitting the conclusion that its citrate synthetase had the usual stereospecificity and was 100% stereospecific.

The work reported in this paper demonstrates that the labeling pattern of glutamate synthesized by whole *C. kluyveri* (Worthington strain) cells is consistent with that found previously with extracts prepared from the same strain (Stern *et al.*, 1966) and that the stereospecificity of citrate synthetase is essentially the same in the intact cells as in the cell extracts.

Experimental Procedures

Materials. The Worthington strain of *C. kluyveri* used in previous experiments (Stern *et al.*, 1966) was grown on 1 l. of the synthetic medium described by Stadtman and Burton (1955) to which was added 114.4 μmoles (500 μc) of sodium [1-^{14}C]acetate and 2574 μmoles of bicarbonate. The inoculum consisted of 20 ml of a 3-day culture grown on the same nonradioactive medium. After 7 days of growth at 37°, the cells were harvested by centrifugation at 12,000*g* for 20 min at 0° and were washed with distilled water. All enzymes used, with the exception of L-glutamate decarboxylase (Worthington Biochemical Corp.) and oxalacetate decarboxylase (prepared in this laboratory by Dr. G. M. Frost), were products of Boehringer & Co. *Clostridium tetanomorphum*, kindly supplied by Dr. H. A. Barker, was grown on the medium of Barker *et al.* (1959).

Hydrolysis of Cells and Recovery of [^{14}C]*Glutamic and* [^{14}C]*Aspartic Acids.* Wet cells (221 mg) were dried *in vacuo* over P_2O_5 and then hydrolyzed in 1.8 ml of 6 N HCl at 110° for 21 hr. Excess hydrochloride was removed by repeated evaporation *in vacuo* over NaOH at approximately 45°. The residue was taken up in water and insoluble material was removed by centrifugation. The solution was applied as a band 10 in. long on a sheet of Whatman 3MM chromatography paper (21 × 14 in.) and was subjected to electrophoresis for 3 hr at 2700 v in pyridine–acetic acid (pH 3.5). Radioactive glutamic and aspartic acids were located by standard markers and radioscanning and were eluted with 10 ml of water. Glutamic acid was further purified by chromatography in butanol–acetic acid–water (4:1:1) while aspartic acid was purified by chromatography in butanol–acetic acid–water (4:1:5). Both acids were assayed by the ninhydrin method of Troll and Cannan (1953).

Determination of ^{14}C in the Carboxyl Groups of [^{14}C]*-Glutamic Acid.* A. C-1 CARBOXYL. Degradation was carried out in Warburg flasks using L-glutamate decarboxylase as previously described (Stern *et al.*, 1966). Samples of the supernatant from the decarboxylated residue were used for counting and paper chromatographic identification of the GABA.[1] The solvent used was butanol–acetic acid–water (4:1:1).

B. C-5 CARBOXYL. Degradation was carried out in Warburg flasks using a cell suspension of *Clostridium tentanomorphum* as described by Gottschalk and Barker (1966). Results were corrected for the 65% recovery obtained with authentic [5-^{14}C] glutamic acid.

Determination of ^{14}C in the Carboxyl Groups of [^{14}C]*-Aspartic Acid.* A. C-1 PLUS C-4 CARBOXYLS. Degradation of both carboxyls was carried out in Warburg flasks using chloramine T as described by Gottschalk and Barker (1966).

B. C-4 CARBOXYL. This carboxyl group was degraded by the coupling of glutamate–oxalacetate transaminase with oxalacetate decarboxylase. Warburg flasks contained 200 μmoles of Tris-HCl buffer (pH 7.5), 20 μmoles of potassium α-ketoglutarate, 10 μmoles of NaCl, and 1.6 μmoles of [^{14}C] aspartic acid in a total volume of 1.6 ml in the main compartment. Side arm A contained 0.02 mg of transaminase and 1.2 mg of oxalacetate decarboxylase (total volume, 0.3 ml) and side arm B contained 0.2 ml of 30% trichloroacetic acid. The center well contained 0.15 ml of Hyamine solution. The reaction was started by tipping the enzymes and terminated after 40 min at 30° by tipping the acid. Shaking was continued for 1 hr to allow absorption of CO_2. The method was tested with authentic [4-^{14}C]-aspartic acid and gave a recovery of 92%.

[1] Abbreviations used: GABA, γ-aminobutyric acid; ATP, adenosine triphosphate; FdH, reduced ferredoxin; TPN and TPNH, oxidized and reduced triphosphopyridine nucleotide; acetyl-CoA, acetyl coenzyme A.

Determination of $^{14}CO_2$ in the Spent Medium. The spent medium (1.5 ml) was introduced into the main compartment of a Warburg flask and 0.2 ml of 2 N H_2SO_4 into one side arm. After CO_2 evolution due to acid had ceased, 0.2 ml of Hyamine solution was introduced through a capillary stopper into the second side arm and the flask was shaken for 1 hr at 30° to absorb the evolved CO_2.

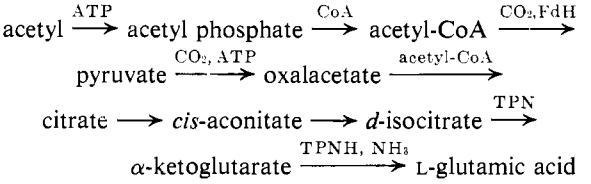
Determination of the Specific Activity of [^{14}C]Acetate in the Spent Medium. Acetate was converted to acetyl phosphate by incubating 0.4 ml of the spent medium with the following (in micromoles): Tris-HCl buffer (pH 7.4), 100; $MgCl_2$, 8; ATP, 10; acetokinase, 0.1 mg; and 0.2 ml of neutralized 2.5 M hydroxylamine in a total volume of 1.05 ml for 1 hr at 30°. Radioactive acetohydroxamate was recovered and chromatographed as described by Stadtman and Barker (1950) and then eluted with water. The quantity of hydroxamate in the eluate was determined by the colorimetric method of Lipmann and Tuttle (1945).

Counting of Radioactivity. The various radioactive materials were counted in a Nuclear-Chicago scintillation spectrometer either as aqueous solutions in Bray (1960) scintillation mixture or as Hyamine salts dissolved in toluene containing 0.4% 2,5-diphenyloxazole. Samples were counted long enough to ensure statistical errors of 3% or less. Efficiency of counting was measured by the channels ratio technique.

Results

Radioactivity of Glutamic Acid. The distribution of radioactivity in the glutamic acid isolated from whole cells is shown in Table I. After treatment with the specific decarboxylase, 25.5% of the total radioactivity was liberated as $^{14}CO_2$ and the remainder of the radioactivity was recovered in the GABA formed. GABA was the sole residual radioactive product of the degradation and was identified by cochromatography with authentic GABA. Of the radioactivity present in the GABA fragment (representing C-2 to C-5 of glutamate) 38.8% was located in the C-5 position by the specific degradation of this carbon atom of glutamic acid to $^{14}CO_2$ by *C. tetanomorphum* cell suspension. The pathway of [1-^{14}C]acetate conversion to glutamic acid in *C. kluyveri* extracts has been shown (Stern, 1963, 1965; Stern and Bambers, 1966) to proceed *via* the following enzymatic steps:

Pathway A



Assuming the usual stereospecificity for citrate synthetase as found by Stern *et al.* (1966), then the glutamic acid formed should be equally labeled in the C-3 and C-5 positions (*cf.* Figure 1 of Stern *et al.*, 1966). The fact that almost exactly half the radioactivity of the GABA fragment was located in C-5 is consistent with the above pathway of glutamic acid biosynthesis in the intact cell and one may safely infer that 36.7% of the total radioactivity was located in C-3 of glutamic acid.

The proportion of radioactivity found in C-1 of glutamic acid, 25.5%, was greater than that observed by Stern *et al.* (1966) when [1-^{14}C]acetyl-CoA plus [^{12}C]-oxalacetate was converted to glutamic acid by cell extracts, namely 8.6%. The finding of radioactivity in C-1 of glutamic acid with [1-^{14}C]acetate as precursor in whole cells indicated: (a) that some of the labeled acetate was converted to $^{14}CO_2$, (b) that some citrate arose *via* the unusual stereospecific route, or (c) some alternate translocation mechanism occurs in the cell.

Analysis of the spent medium (Table II) demonstrated unequivocally that $^{14}CO_2$ had been formed from [1-^{14}C]-acetate during growth and this was further confirmed by demonstrating that the carboxyl groups of aspartate had also become labeled (see below) as required by pathway A. From the specific activity of $^{14}CO_2$ in the spent medium and of [1-^{14}C]acetate in the original medium (Table II), one may calculate that at least 3.74% of the [1-^{14}C]acetate was converted to $^{14}CO_2$ which was subsequently excreted into the medium. Considerable dilution of the [1-^{14}C]acetate occurred, almost certainly as a result of the oxidation of ethanol to acetate. Tomlinson and Barker (1954) observed

TABLE I: Distribution of Radioactivity in Carbon Skeleton of Glutamate Formed from [1-^{14}C]Acetate.

Carbon Atoms	Sp Radioactivity (dpm/μmole)	Radioactivity (%)
Total	18,418	100
C-1	4,697	25.5
C-2 to C-5 (GABA)	13,904	75.6
C-5	7,151	38.8
C-3 (calcd)	6,753	36.7[a]

[a] Calculated as GABA less C-5. About 2% of this could be in C-2 (Tomlinson, 1954a).

TABLE II: Specific Radioactivities of Acetate and CO_2 of Medium before and after Growth.

Compound	Sp Radioactivity (dpm/μmole) Initial	Final
[1-^{14}C]Acetate	25,093	5,350
$^{14}CO_2$	0	940

TABLE III: Distribution of Radioactivity in Carbon Skeleton of Aspartate Formed from [1-^{14}C]Acetate.

Carbon Atoms	Sp Radioactivity (dpm/μmole)	Radioactivity (%)
Total	20,610	100
C-1 + C-4	10,480	50.9
C-4	5,560	27.0
C-1 (calcd)	4,920	23.9[a]
C-2 + C-3 (calcd)	10,130	49.1[b]

[a] C-1 + C-4 less C-4. [b] Total less C-1 + C-4. Actually must all be in C-2 since no mechanism for conversion of $^{14}CO_2$ to [2-^{14}C]acetate exists in *C. kluyveri* which requires added acetate as well as CO_2 for growth.

about 0.82% conversion of the acetate carboxyl to CO_2 and a similar dilution of added [1-^{14}C]acetate by whole cells.

Radioactivity of Aspartic Acid. The distribution of radioactivity in aspartic acid isolated from whole cells is shown in Table III. After treatment with chloramine T, half the total radioactivity in aspartic acid was recovered as $^{14}CO_2$, showing that 50.9% of the label was in one or both carboxyls and the remainder in C-2 plus C-3. C-4 of aspartic acid, as determined by transamination plus decarboxylation to $^{14}CO_2$, contained 27.0% of the total radioactivity, so that both carboxyls were labeled and, by difference, C-1 of aspartic acid contained 23.9% of the total radioactivity.

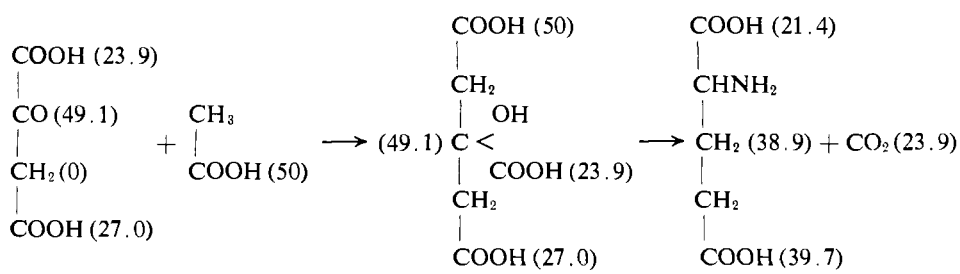
The only known pathway of aspartate biosynthesis from [1-^{14}C]acetate in *C. kluyveri* involves the successive carboxylation of acetyl-CoA to pyruvate and oxalacetate (pathway A) followed by transamination to yield aspartic acid. The label from acetate should then appear exclusively in C-2 while $^{14}CO_2$ would enter C-1 and C-4 of aspartic acid. The data of Table III are completely consistent with this pathway of the origin of the carbon skeleton of aspartic acid and confirm that [1-^{14}C]-acetate was converted to $^{14}CO_2$ which was then incorporated into both carboxyls of oxalacetate (aspartate) and consequently into C-1 of glutamate. Tomlinson (1954b) found that with [1-^{14}C]acetate as precursor, 12.5% of the total radioactivity of aspartate was located in either or both carboxyls, while with $^{14}CO_2$ as precursor, both carboxyls were about equally labeled, the C-4 carboxyl having a somewhat higher specific radioactivity. One can calculate from the greater conversion of [1-^{14}C]acetate to $^{14}CO_2$ in our experiment than in Tomlinson's (3.74 ÷ 0.82 = 4.56) that we might expect to find 4.56 × 12.5 = 57% of the total radioactivity in both aspartate carboxyls, in good agreement with the 50.9% found.

Discussion

The results of these experiments on incorporation of [1-^{14}C]acetate into the glutamic acid of protein of whole cells of *C. kluyveri* show that 25.5% of the label was found in C-1, 36.7% in C-2 to C-4 (most of which must be in C-3), and 38.8% in C-5. They are essentially the reverse of those reported originally by Tomlinson (1954a) who found 47% in C-1, 45% in C-3, and 4.5% in C-5. Just as Gottschalk and Barker (1966) found that the labeling pattern of glutamic acid synthesized from radioactive precursors by cell-free extracts of the Barker strain was essentially that reported by Tomlinson for whole cells, so we now find that whole cells of the Worthington strain of *C. kluyveri* show a pattern of glutamic acid labeling which is consistent with that established for cell-free extracts of this same strain (Stern *et al.*, 1966) and which is the opposite of that observed by Tomlinson and by Gottschalk and Barker. The over-all evidence suggests that we are dealing with two separate strains of *C. kluyveri* which differ from one another by manifesting a reverse stereospecificity of their respective citrate synthetases. Both show some translocation of carbon (5–8%) during glutamate biosynthesis which remains unexplained, as does the small incorporation of $^{14}CO_2$ into C-2 of glutamic acid observed by Tomlinson. Drs. I. Kennedy and J. G. Morris (private communication), using extracts of the Leicester strain of *C. kluyveri* (Andrew and Morris, 1965), have found that 95% of [1-^{14}C]acetate was incorporated into C-5 and 5% into C-1 of glutamic acid. Thus, extracts of the Leicester strain, like the Worthington strain, possess a citrate synthetase with the usual stereospecificity and show some 5% carbon translocation.

If one condenses oxalacetate having the distribution of radioactivity shown for aspartate in Table III with [1-^{14}C]acetate to yield citrate which is converted to glutamate *via* the citric acid cycle as illustrated in pathway B, then one can calculate that 21.4% of the radioactivity will be in C-1 of glutamate, 38.9% in C-3, and 39.7% in C-5. This agrees very well with the experimen-

Pathway B



tally determined value for the C-5 position and with the value for C-3 estimated by difference (*cf.* Table I). Of the 25.5% of the total radioactivity found in C-1, 21.4% can be accounted for by fixation of $^{14}CO_2$ (formed from [1-^{14}C]acetate) into the C-4 carboxyl of oxalacetate. The remaining 4.1% of the radioactivity in C-1 must have entered by another route. This excess approximates the amount of carbon translocation observed in cell extracts (5-8%) and suggests that the latter phenomenon also occurs in the intact cell.

It is interesting to note that the specific activity of C-1 of glutamic acid was only slightly less than C-4 of aspartic acid indicating that little or no dilution occurred in conversion of [4-^{14}C]oxalacetate to [1-^{14}C]glutamate. Furthermore, the specific activities of these carbon atoms were approximately the same as the final specific activity of the [1-^{14}C]acetate which had undergone a fivefold dilution during the growth experiment. This strongly suggests that a large oxidation of ethanol to acetate (which accounts for the dilution) had occurred before amino acid biosynthesis commenced. The specific activity of C-2 of aspartate which is derived from C-1 of acetate also supports this conclusion, which is consistent with the fact that ethanol oxidation is the ultimate source of energy for growth of *C. kluyveri* (Barker, 1957). It is clear that the specific activity of the $^{14}CO_2$ in the spent medium does not reflect that of the internal CO_2 metabolic pool since it is only 940 ÷ 5560 = 16.9% of C-4 of oxalacetate (aspartate) and 19.1% of C-1 of oxalacetate which replenishes the pool by being lost as $^{14}CO_2$ on conversion of *d*-isocitrate to α-ketoglutarate.

The pathway of conversion of [1-^{14}C]acetate to $^{14}CO_2$ is obscure. It cannot occur by acetate oxidation since a *complete* citric acid cycle is lacking in *C. kluyveri*, and so are the enzymes of the dicarboxylic acid cycle (Stern and Bambers, 1966). It is possible that $^{14}CO_2$ is formed from [1-^{14}C]acetate *via* synthesis of acetoacetate followed by decarboxylation of the latter to acetone and CO_2 as follows (*cf.* Barker, 1957): 2[1-^{14}C]acetyl-CoA→[1,3-^{14}C]-acetoacetyl-CoA→[1,3-^{14}C]acetoacetate→[2-^{14}C]acetone + $^{14}CO_2$.

References

Andrew, I. G., and Morris, J. G. (1965), *Biochim. Biophys. Acta 97*, 176.

Barker, H. A. (1957), Bacterial Fermentations, New York, N. Y., Wiley, Chapter 2.

Barker, H. A., Smyth, R. D., Wilson, R. M., and Weissbach, H. (1959), *J. Biol. Chem. 234*, 320.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Gottschalk, G., and Barker, H. A. (1966), *Biochemistry 5*, 1125.

Hanson, K. R., and Rose, I. A. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 981.

Ilse, D., and O'Brien, R. W. (1967), *Biochim. Biophys. Acta 141*, 454.

Lipmann, F., and Tuttle, C. (1945), *J. Biol. Chem. 159*, 21.

Stadtman, E. R., and Barker, H. A. (1950), *J. Biol. Chem. 184*, 769.

Stadtman, E. R., and Burton, R. M. (1955), *Methods Enzymol. 1*, 521.

Stern, J. R. (1963), *Federation Proc. 22*, 355.

Stern, J. R. (1965), in Non-Heme Iron Proteins, San Pietro, A., Ed., Yellow Springs, Ohio, Antioch, p 199.

Stern, J. R. (1966), *Bacteriol. Proc.*, 72.

Stern, J. R., and Bambers, G. (1966), *Biochemistry 5*, 1113.

Stern, J. R., Bambers, G., and Hegre, C. S. (1966), *Biochemistry 5*, 1119.

Tomlinson, N. (1954a), *J. Biol. Chem. 209*, 605.

Tomlinson, N. (1954b), *J. Biol. Chem. 209*, 597.

Tomlinson, N., and Barker, H. A. (1954), *J. Biol. Chem. 209*, 585.

Troll, W., and Cannan, R. K. (1953), *J. Biol. Chem. 200*, 803.