Biosynthesis of Chloramphenicol. Origin and Degradation of the Aromatic Ring†

William P. O'Neill,‡ Robert F. Nystrom, Kenneth L. Rinehart, Jr.,* and David Gottlieb

ABSTRACT: A procedure has been developed for carbon-by-carbon degradation of the aromatic ring of the antibiotic chloramphenicol, whose biosynthesis by *Streptomyces venezuelae* has been studied by adding various ^{14}C-labeled precursors to glucose–tryptone and glycerol–tryptone media. Glucose-*6*-^{14}C was especially well incorporated and gave labeling in the side chain and ring of chloramphenicol consistent with the operation of the shikimate pathway of aromatic biosynthesis in the organism.

Chloramphenicol (**1**, Figure 1) is a clinically important antibiotic and its biosynthesis has been the subject of considerable interest in the past 20 years. Structural features of special biosynthetic interest are its relationship to the aromatic amino acids and the presence of the rare nitro and dichloroacetyl groups. Published results to 1968 have been reviewed elsewhere (Gottlieb, 1967; Vining *et al.*, 1968).

Early results were sometimes conflicting and generally difficult to evaluate and interpret, since a variety of experimental conditions were employed—different organisms and strains, different media, different times of addition, and both growing cells and resting cells. Moreover, interpretation has always been hampered by low antibiotic production as well as by low and nonspecific incorporation of precursors. In spite of these difficulties, the early studies demonstrated labeling of the dichloroacetyl group by a number of precursors (most notably the specific labeling of its carbonyl group by acetic acid-*1*-^{14}C) (Gottlieb *et al.*, 1962) and lack of labeling of that group by dichloroacetic acid-*2*-^{14}C (Smith, 1958) indicated that *p*-nitrophenylserinol (**2**, Figure 1) is not a precursor (Gottlieb *et al.*, 1955) and demonstrated that the three-carbon side chain is specifically labeled by glycerol-*1,3*-^{14}C, which along with propionic acid-*3*-^{14}C served as the most efficient precursor for the *p*-nitrophenyl portion of chloramphenicol (Robbins, 1955).

The high and specific incorporation of glycerol immediately suggested the shikimate pathway of aromatic biosynthesis. Although early attempts to demonstrate shikimic acid incorporation into chloramphenicol gave negative results (Gottlieb *et al.*, 1962), Vining and Westlake (1964) later reported a reasonable incorporation (0.38%) with labeling restricted to the aromatic ring, but with high dilution of activity (1:168). Those workers reported additional evidence favoring the shikimate pathway—a similar level of radioactivity in the aromatic amino acids phenylalanine and tyrosine produced by the microorganism grown in the presence of labeled shikimic acid, and a similar proportion of the radioactivity in each of the side-chain carbon atoms of *p*-nitrophenylserinol and phenylalanine when the organism was grown on specifically labeled glucose or glycine. The degradation scheme employed by Vining and Westlake (1964), as well as that described earlier (Gottlieb *et al.*, 1962), is shown in Figure 1.

A further test of the shikimate pathway would be provided by a specific labeling of the carbon atoms of the aromatic ring of chloramphenicol by specifically labeled glucose, which is very well incorporated into chloramphenicol (Vining and Westlake, 1964). However, none of the previously reported biosynthetic studies on chloramphenicol have described procedures for such a specific degradation scheme. Accordingly, we have undertaken an extensive study of procedures for carrying out the degradation of the chloramphenicol ring. Any method developed for determination of the incorporation of specifically labeled precursors into the individual carbons of the chloramphenicol ring required degrading approximately 1 mmol (323 mg) of chloramphenicol with a millimolar radioactivity of 0.5–1.0 μCi/mmol, due to the low production of antibiotic (less than 100 mg/l.) and the minimal dilution permitted by the low incorporation of labeled precursors (less than 1%).

Although we regard the principal import of the presently reported work to be its development of a new and superior route (shown in Figure 2) for the specific degradation of labeled para-disubstituted benzenes, our results with labeled glucose provide striking confirmation of the earlier proposed formation of chloramphenicol *via* the shikimate pathway.

Experimental Procedure

Melting points were determined on a Kofler micro hot stage, pH values with pHydrion paper or, where reported to two decimal places, with a Beckman pH meter. Reagents and solvents used were reagent grade and undistilled, unless otherwise specified. Microanalyses were obtained by Mr. J. Nemeth and his associates and all known compounds gave acceptable analyses.

Chromatography. Gas–liquid partition chromatography was performed on an F&M gas chromatograph, Model 500, employing helium and 8-ft Apiezon L and Lac 728 (on P 500) columns. Identifications were made by comparison of retention times with those of authentic samples under identical conditions. The purity of all organic products of the degradation was checked by thin layer chromatography (tlc) on silica gel G (E. Merck AG, Germany) and/or ascending chromatography on Whatman No. 1 paper. Identification was made by

† From the School of Chemical Sciences and the Department of Plant Pathology, University of Illinois, Urbana, Illinois 61801. *Received June 1, 1973.* This work was supported in part by Public Health Service Research Grant AI 01278 from the National Institute of Allergy and Infectious Diseases. This is paper V in the series Biosynthesis of Chloramphenicol.

‡ National Science Foundation Cooperative Fellow; National Institutes of Health Predoctoral Fellow.

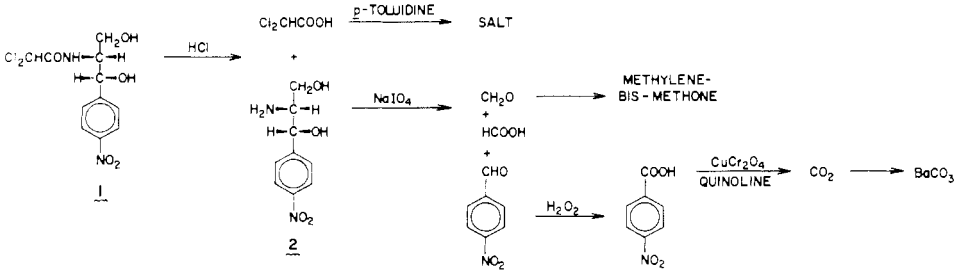

FIGURE 1: Degradation scheme employed previously for labeled chloramphenicol.

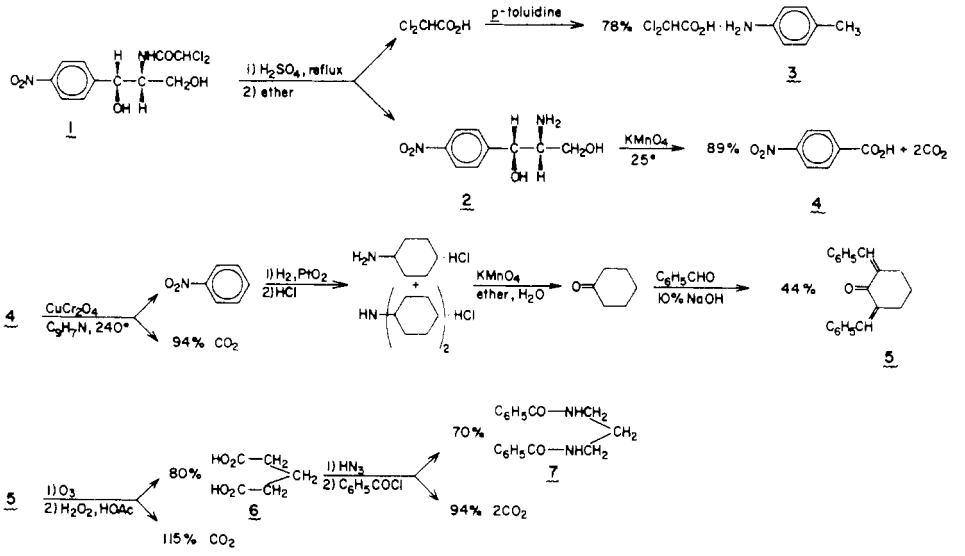

FIGURE 2: Degradation scheme employed for labeled chloramphenicol in the present study.

comparison with standards run at the same time. Neutral compounds were developed on tlc with chloroform containing 1–6% methanol and detected by exposure to iodine vapor. Carboxylic acids were spotted for tlc as the ammonium salts and developed with 95% ethanol–water–28% ammonium hydroxide (100:12:16, by volume) (Braun and Green, 1962) or benzene–methanol–acetic acid (45:8:4, by volume) (Petrowitz and Pastuska, 1962); they were separated on paper by use of the solvent systems phenol–water–98% formic acid (75:25:1, by volume) (Stork *et al.*, 1951) and water–95% ethanol–28% ammonium hydroxide (12:100:16, by volume) (Long *et al.*, 1951). Acids were identified with Bromocresol Green reagent. Primary amines were separated on paper by use of a one-phase solvent system of *n*-butyl alcohol–glacial acetic acid (3:1, by volume) (Bertelli, 1953) and identified with Bromophenol Blue reagent.

Radioactivity Measurements. The ^{14}C-labeled compounds were assayed by liquid scintillation counting using the Bush Channels Ratio method (Bush, 1964) to correct for quenching. All colorless radioactive samples were solubilized in suitable scintillator systems. Of the compounds obtained from the degradation of chloramphenicol, only dibenzylidenecyclohexanone was found to be too highly colored for efficient solution counting. This compound was burned to carbon dioxide by a modified Pregl method (Wilzbach and Van Dyken, 1950; Niederl and Niederl, 1942) and the gas was allowed to react with Hyamine hydroxide. The resulting salt was dissolved in a scintillator and counted. As a cross-check the millimolar radioactivity of samples of compounds in the degradation scheme was also determined by combustion to carbon dioxide and counting as Hyamine carbonate in a scintillator. These values were compared with the millimolar radioactivities determined by solution counting. Only dibenzylidenecyclohexanone showed greater than 2% error when compared with solution counting data.

Although no conclusions with regard to biosynthesis were based on the carbon dioxide obtained by decarboxylation of **4**, by ozonolysis of **5**, or by Schmidt degradation of **6**, it was converted in each case to barium carbonate and counted. These results were compared with the results based on the millimolar radioactivities of the purified organic derivatives. Samples of barium carbonate were counted by regeneration of carbon dioxide as described by Robbins (1955) or suspended in Cab-O-Sil gel (Ott *et al.*, 1959) and counted immediately.

Carbon Dioxide Collection. Carbon dioxide from a reaction was swept with argon or nitrogen into a gas washing cylinder, with a fritted glass dispersion tube, containing 50 ml of 1.0 N sodium hydroxide and handled as described by Calvin *et al.* (1949).

Production of Chloramphenicol. Streptomyces venezuelae, strain 1491a, was used in all of the present studies summarized in Table I (except that with acetate-2-^{14}C on glycerol–lactate medium (expt 8), for which strain 170 was used) because of its consistent production of chloramphenicol. Glycerol–tryptone was the basic medium (Legator and Gottlieb, 1953) and glucose was substituted for glycerol when the precursor to be added was other than glycerol. The streptomycete was cultured on an agar slant for 7 days; then 5 ml of a sterile 0.01% aqueous solution of Vatsol detergent was added to the slant and the spores were scraped off the mycelium to

TABLE I: Incorporation of Labeled Precursors.

Expt[a]	Precursor: Compd[d]	Precursor: Millimolar Radioact. (μCi/mmol)	Medium	Mycelial Wt (g)	Chloramphenicol: Broth Bioassay (μg/ml)	Chloramphenicol: Added Carrier, Dilution Factor	Chloramphenicol: Millimolar Radioact. (μCi/mmol): Isolated	Chloramphenicol: Millimolar Radioact. (μCi/mmol): Broth	Precursor–Chloramphenicol: Dilution[h]	Precursor–Chloramphenicol: Incorp, %[i]
1	Glucose-*1*-^{14}C	58.8	Glucose–tryptone	0.101	56	32.0	0.451	14.4	4.1	0.07
2	Glucose-*1*-^{14}C	58.8	Glucose–tryptone	0.633	60	27.0	0.200	5.4	10.9	0.03
V_2[b]	Glucose-*1*-^{14}C	13.6	Glycerol–lactate					0.460	29.6	0.58
3	Glucose-*2*-^{14}C[e]	27.6	Glucose–tryptone	1.078	67	24.7	0.839	20.7	1.33	0.28
V_3[b]	Glucose-*2*-^{14}C[e]	10.0	Glycerol–lactate					0.601	16.6	1.04
4	Glucose-*6*-^{14}C[e]	29.4	Glucose–tryptone	1.107	61	26.0	1.638	42.7	0.69	0.51
5	Glycerol-*1,3*-^{14}C	27.0	Glycerol–tryptone	1.871	47	32.6	1.431	46.5	0.58	0.23
BG_5[c]	Glycerol-*1,3*-^{14}C	57.8	Glycerol–lactate					109.9	0.54	0.14
6	Acetate-*1*-^{14}C[f]	4280	Glucose–tryptone	1.146	70	23.6	0.130	3.07	1395	0.02
GR_6[c]	Acetate-*1*-^{14}C[f]	71.3	Glycerol–lactate					1.86	38.3	0.02
7	Acetate-*2*-^{14}C	7400	Glucose–tryptone	1.179	61	28.3	0.800	22.7	327	0.14
8	Acetate-*2*-^{14}C	7400	Glycerol–lactate	0.366	40	45.0	0.036	1.62	4580	0.01
GR_8[c]	Acetate-*2*-^{14}C	24.6	Phosphate buffer					37	0.68	0.12
V_8[b]	Acetate-*2*-^{14}C	10.2	Glycerol–lactate		30			0.0455	222	0.09
9	Pyruvate-*1*-^{14}C[g]	4700	Glucose–tryptone	1.168	90	13.3	0.034	0.452	10,400	0.02
10	Pyruvate-*2*-^{14}C	1720	Glycerol–tryptone	1.641	52	32.1	0.400	12.8	134	0.07
11	Pyruvate-*3*-^{14}C[g]	2270	Glucose–tryptone	0.636	50	54.6	0.040	2.18	1042	0.03

[a] *S. venezuelae*, strain 1491a, was employed for all except expt 8, for which strain 170 was used. [b] Vining and Westlake (1964). [c] B = Burg (1958); G = Gottlieb *et al.* (1962); R = Robbins (1955). [d] Except as noted, 1000 μCi was added. [e] 500 μCi. [f] 1100 μCi. [g] 200 μCi. [h] Dilution of substrate radioactivity is the millimolar radioactivity of the substrate divided by the millimolar radioactivity of chloramphenicol or a derivative after correction for dilution by added carrier. In general, the smaller the dilution of substrate radioactivity, the closer the precursor–product relationship of the substrate and the antibiotic. [i] Incorporation refers to the per cent of the total activity of the substrate found in the antibiotic.

form a suspension. The suspension obtained from two slants was transferred aseptically to 100 ml of glycerol–lactate medium (Gottlieb *et al.*, 1954) in a 500-ml erlenmeyer flask. This culture was allowed to grow on a reciprocal shaker at 28° for 48 hr; then a 3-ml portion was used as the inoculum for 100 ml of medium in the biosynthesis experiments.

The radioactive precursors were added to 300 ml of the proper tryptone medium and the medium was then divided equally among three 500-ml erlenmeyer flasks, which were sterilized at 120° for 15 min before being inoculated. The one exception to this procedure was with pyruvate precursor, which was dissolved in water, sterilized through an ultrafine sintered glass filter, and then added to the autoclaved media. The antibiotic potencies of the media were determined periodically and the flasks were removed from the shaker at the time of maximum yield, usually 6 days. Small portions of culture fluid (pH 6.8–8.0) from a pooled culture from three flasks were taken for a final assay and the remainder was then stored at −70°.

Assay for Chloramphenicol. Biological assays for antibiotic production were made by the diffusion plate disk assay technique (Gottlieb *et al.*, 1954) with *Bacillus mycoides*. The production of antibiotic varied from 50 to 90 μg/ml depending on the experiment.

The presence of radioactive chloramphenicol was determined on a small sample of culture which was adjusted to pH

TABLE II: Millimolar Radioactivities of Compounds Obtained from Degradation of Labeled Chloramphenicol (**1**).[a]

Expt	*p*-Nitrophenylserinol (**2**)[b] μCi/mmol	% of **1**	*p*-Nitrobenzoic Acid (**4**) μCi/mmol	% of **2**	$BaCO_3$[c] (μCi/mmol)	Dibenzylidenecyclohexanone (**5**) μCi/mmol	% of **2**	Dilution Factor	$BaCO_3$[d] (μCi/mmol × Dilution Factor)	Glutaric Acid (**6**) μCi/mmol × Dilution Factor	% of **2**	$BaCO_3$[e] (μCi/mmol × Dilution Factor)	Trimethylenedibenzamide (**7**) μCi/mmol × Dilution Factor	% of **2**
1	0.303	67	0.299	99	0.086	0.254	84	3.95	0.048	0.201	66	0.0264	0.152	50
2	0.128	64	0.129	100	0.0179	0.109	85							
3	0.715	85	0.540	76	0.051	0.491	69	2.95	0.032	0.426	60	0.141	0.189	26
4	1.200	73	1.159	97	0.328	0.847	71	11.35	0.002	0.786	66	0.002	0.812	68
5	1.136	79	0.913	80		0.808	71	1.00		0.550	48		0.368	32
6	0.060	46	0.020	33										
7	0.533	67	0.267	50		0.232	44	1.00		0.202	38		0.130	24
8	0.014	39	0.008	36										
9	0.020	59	0.007	35										
10	0.289	72	0.156	54		0.146	51							
11	0.032	80	0.016	50										

[a] Compounds **1**–**7** are given in Figure 2 in the text. [b] Calculated as the difference between the millimolar radioactivities of chloramphenicol (**1**) and *p*-toluidinium dichloroacetate (**3**). [c] From decarboxylation of *p*-nitrobenzoic acid (**4**). [d] From ozonolysis of dibenzylidenecyclohexanone (**5**). [e] From Schmidt degradation of glutaric acid (**6**).

10 and extracted with ethyl acetate. The extract was concentrated under vacuum and the chloramphenicol was chromatographed on Whatman No. 1 paper using two descending solvent systems—the organic phase of methyl isobutyl ketone–0.1 N ammonium hydroxide (1:1) and the chloroform phase of chloroform–acetic acid–water (50:1:3). Authentic chloramphenicol and the ^{14}C spot were compared by measuring the ^{14}C R_F value on a strip counter, inverting the same strip over a glass tray seeded with *B. mycoides* for 7 min, and incubating the plate for 16 hr before reading the R_F value of the zone of inhibition. The similarity in R_F between biological activity and radioactivity showed that radioactive chloramphenicol had been produced by *S. venezuelae* from the various precursors.

Degradation of Chloramphenicol Derived from Glucose-1-^{14}C. EXPERIMENT 1 (TABLE I). The degradation, which was typical, will be given in detail. Most reactions represent optimum conditions observed during ten or more runs. Results are given in Table I; results of the degradation experiments with chloramphenicol from labeled glucose and other precursors are summarized in Table II.

A. Isolation of Chloramphenicol (**1**). *S. venezuelae* was grown for 6 days; then the contents of the three flasks were pooled and frozen. The broth was thawed, the mycelium was filtered and washed, and unlabeled chloramphenicol carrier (424.4 mg, dilution factor[1] 32.0) was added to the filtered broth.

Chloramphenicol was extracted from the broth (after adjustment to pH 9 with sodium hydroxide) with ethyl acetate. The extracts were washed with 0.01 N sodium hydroxide and water and concentrated. The crude chloramphenicol was dried *in vacuo* and then recrystallized from water; 364.7 mg (84%); mp 150–151° (authentic sample mp 151–152°).

B. Isolation of *p*-Toluidinium Dichloroacetate (**3**). A solution of 308.6 mg (0.955 mmol) of chloramphenicol, 2 ml of concentrated sulfuric acid, and 10 ml of water was heated at reflux (145–150°) for 24 hr, cooled, and extracted with ether. The ether extract was washed with water (with the water washings being added to the sulfuric acid solution) and then dried and concentrated. The residue of dichloroacetic acid was dissolved in 2 ml of benzene and treated with 2 ml of benzene containing 206 mg of *p*-toluidine to give a precipitate of **3**, which was filtered, washed with benzene, and dried *in vacuo*: 172.0 mg (80%); mp 135–138° (Gottlieb *et al.* (1962), 134.5–138°).

C. Isolation of *p*-Nitrobenzoic Acid (**4**). The sulfuric acid solution containing *p*-nitrophenylserinol (**2**) was freed from residual ether and a procedure modified from that previously employed (Fieser, 1955) for oxidizing mandelic acid to benzoic acid was followed. The solution was stirred in an ice bath and 613.0 mg (3.85 mmol) of solid potassium permanganate was added. After 20 min the ice bath was removed[2] and the mixture was stirred at room temperature for 48 hr. Sufficient sodium bisulfite was added to reduce all of the manganese dioxide formed and about 5 ml of ether was added to dissolve the *p*-nitrobenzoic acid, thus permitting entrapped manganese dioxide to react. The ether was removed by gentle heating on the steam bath, the mixture was cooled in an ice bath and filtered, and the precipitate was washed repeatedly with cold water. Drying *in vacuo* gave 151.2 mg (95%) of *p*-nitrobenzoic acid, which sublimed below 242°.[3] A sample sublimed at 180° (0.4 mm) gave a single spot on tlc. In ten runs, yields of **4** varied from 86 to 96%.

D. Preparation of Dibenzylidenecyclohexanone (**5**). De-

[1] The dilution factor is defined as the amount of chloramphenicol in the broth plus the amount of carrier divided by the amount of chloramphenicol in the broth, *i.e.*, (broth + carrier)/broth.

[2] It is important to cool the reaction in an ice bath for the first 20 min after adding the permanganate, else the violence of the exothermic reaction lowers the yield by as much as 25%.

[3] A melting range of 238–242° with much sublimation was observed for all experimental and standard samples of *p*-nitrobenzoic acid. The Merck Index, 8th ed, p 737, lists mp 242.4°, with sublimation.

carboxylation of *p*-nitrobenzoic acid (**4**) was effected according to a standard method (Dauben *et al.*, 1950). A mixture of 134.8 mg (0.809 mmol) of **4**, 138 mg of copper chromite, and 20 ml of synthetic quinoline,[4] freshly distilled at 1 mm from sodium hydroxide pellets, was heated for 3 hr in an oil bath at 250° while a vigorous stream of argon bubbled through it and swept the carbon dioxide through a cold trap (0°) into 1 N carbonate-free sodium hydroxide. The yield of barium carbonate, filtered and dried at 120°, was 149.3 mg (94%) and the blank was 17.4 mg.

The reflux condenser and cold trap from the decarboxylation were washed with ether and the washings were added to the quinoline solution. The condenser and flask were arranged for steam distillation, the quinoline was neutralized with hydrochloric acid, and the ether was distilled into a 500-ml erlenmeyer flask in an ice bath. Then nitrobenzene was steam distilled. When about 50 ml of aqueous distillate had collected in the erlenmeyer flask, about 50 ml of ether was added to the hot quinoline hydrochloride mixture and allowed to distill as a chaser for the nitrobenzene. The condenser was also washed with ether, and the washings were added to the distillate. The distillate was saturated with sodium chloride and extracted with ether. The ether extracts were dried over sodium sulfate, the ether was removed on the rotary evaporator at 25°, and 104 mg of platinum oxide and 30 ml of glacial acetic acid were added. The mixture was stirred under hydrogen at 25° for 24 hr. A total of 4.20 mmol of hydrogen was absorbed (5.2 mmol of hydrogen/mmol of *p*-nitrobenzoic acid). The catalyst was filtered and washed with acetic acid and the solution was saturated with anhydrous hydrogen chloride and concentrated at 60° *in vacuo*. After it had been dried *in vacuo* the amine hydrochloride mixture weighed 66.8 mg; mp 175–183°.

The amine hydrochloride was washed into a 100-ml three-necked flask with 0.6 ml of 1.010 N sodium hydroxide, 5 ml of water, and 25 ml of ether.[5] The mixture was cooled to 10° and stirred while 15 ml of 5% aqueous potassium permanganate was added at such a rate as to maintain the temperature below 20°. The solution was stirred at 15–20° for 2 hr, until all permanganate was consumed, and then extracted with ether. The ether extract was dried and then added to 207 mg of benzaldehyde and 100 ml of 10% aqueous sodium hydroxide, in a modification of the method for the preparation of dibenzalacetone (Conrad and Dolliver, 1943). The ether was removed on the rotary evaporator until the solution became opaque and the odor of ether no longer predominated over that of benzaldehyde; then 6 ml of absolute ethanol was added and the solution was stirred at room temperature for 18 hr. The precipitate was filtered, washed with water, and dried *in vacuo* to give 54.5 mg (25%) of dibenzylidenecyclohexanone, mp 112–114°. Recrystallization from absolute ethanol yielded 36.3 mg (17%) of **5**, which gave a single spot on tlc; mp 119–120° (McCasland (1951) 116–118°). Its radioactivity (84% of that of *p*-nitrophenylserinol) indicates that the carboxyl carbon contained 15% of the radioactivity of *p*-nitrophenylserinol.[6]

A portion (22.0 mg) of the labeled dibenzylidenecyclohexanone (0.254 μCi/mmol) was diluted with 64.9 mg of unlabeled recrystallized dibenzylidenecyclohexanone, mp 118–120°. The recrystallized, diluted material had a millimolar radioactivity of 0.0640 μCi/mmol, which corresponds to 0.252 μCi/mmol before dilution.

E. Preparation of Glutaric Acid (**6**). The diluted dibenzylidenecyclohexanone (**5**, 77.6 mg) dissolved in 50 ml of methylene chloride was saturated with ozone at −70° and then allowed to warm to room temperature. The solution was added in portions to 10 ml of glacial acetic acid, methylene chloride was removed, and 2 ml of 2% hydrochloric acid and 1.5 ml of 30% hydrogen peroxide were added. Argon bubbled through the solution for 12 hr at room temperature swept the carbon dioxide through a cold trap into 1 N sodium hydroxide. A total of 83.5 mg of barium carbonate was collected. Corrected for the blank this corresponded to a 115% yield, and the corrected millimolar radioactivity, 0.048 μCi/mmol, was 16% of that of *p*-nitrophenylserinol. The solution was then heated on the steam bath for 1.5 hr until all peroxide was decomposed (potassium iodide test), evaporated to dryness on the rotary evaporator, and dried *in vacuo*. The mixture of benzoic and glutaric acids, 83.3 mg, was separated by chromatography on silicic acid, employing gradient elution with chloroform and *n*-butyl alcohol. The chromatographed glutaric acid, after recrystallization from benzene, weighed 33.9 mg (90%). After recrystallization from tetrachloroethane it had mp 96–98° (the Merck Index, 8th ed, p 497, gives 97.5–98°), weighed 20.5 mg, and gave a single spot on paper chromatography.[7] Its radioactivity (Table II), indicating that the para carbon in *p*-nitrophenylserinol contains 18% of the label, compares well with 16% determined from the carbon dioxide collected.

F. Preparation of Trimethylenedibenzamide (**7**). Activated sodium azide was prepared by the method of Smith (1946) employing freshly precipitated sodium azide for each reaction. (Yields with activated azide are 10–15% better than with stock sodium azide.) The recrystallized glutaric acid, 14.6 mg, was combined with 12.5 mg from the mother liquors and treated with 0.1 g of activated sodium azide in 1 ml of 100% sulfuric acid (1 part fuming, 3 parts concentrated) for 80 min at 40° and 40 min at 65° with a nitrogen sweep. The reaction was cooled in an ice bath, diluted with 2 ml of water, and made basic with 10 ml of 10% sodium hydroxide solution. Treatment with 5 ml of chloroform containing 0.5 ml of benzoyl chloride for 30 min, followed by extraction with chloroform, gave trimethylenedibenzamide. Recrystallization from ethyl acetate in cyclohexane gave 21.4 mg (37%); mp 152–153° (Roberts *et al.* (1953), 150°); single spot on tlc. Its radioactivity indicates, by difference, that the two meta carbons together contain 16% of the *p*-nitrophenylserinol label. The carbon dioxide generated by this reaction was swept through 5% potassium permanganate in 1 N sulfuric acid and collected as

[4] Natural quinoline, distilled from sodium hydroxide pellets, contains about 1% of an ether-soluble, neutral fraction which inhibits seriously the hydrogenation of nitrobenzene in acetic acid in the presence of platinum oxide. Redistilled synthetic quinoline causes no inhibition of hydrogenation.

[5] It was found that diethyl ether is oxidized in this system to ethyl acetate, which was identified by the infrared spectrum of the ether layer after exposure to the potassium permanganate. In the absence of cyclohexylamine hydrochloride, all of the permanganate (4.8 mmol) in the above system was reduced in 30 min at room temperature.

[6] This conflicts with the 28% determined by counting the carbon dioxide evolved in the decarboxylation. The cause of this discrepancy was contamination by radioactive dichloroacetic acid of the *p*-nitrobenzoic acid which was decarboxylated. This was shown in a second run with glucose-*1*-^{14}C by purification of the *p*-nitrobenzoic acid by silicic acid chromatography and sublimation.

[7] In expt 5, employing glycerol-*1,3*-^{14}C, the glutaric acid was diluted 3.795 to 1.0; an identical observed dilution of radioactivity established the radiochemical purity of the glutaric acid.

usual. The barium carbonate collected weighed 76.4 mg (69% yield after correction for the blank). Its activity when multiplied by 2 was 17% of the *p*-nitrophenylserinol activity, compared to the 16% determined by difference.

EXPERIMENT 2. Because of the abnormally small mycelial production in expt 1 (Table I) and to clarify the difference in the values obtained for C-3′ of the side chain on the basis of the carbon dioxide generated and the dibenzylidenecyclohexanone isolated, a second experiment employing glucose-*1*-^{14}C was carried out. The data are summarized in Table II. Oxidation of the *p*-nitrophenylserinol as before gave a 90% yield of *p*-nitrobenzoic acid. This material gave a spot on paper chromatography corresponding to dichloroacetic acid. The *p*-nitrobenzoic acid was purified by silicic acid chromatography and sublimation (62% recovery overall). Its millimolar radioactivity was 100% of the *p*-nitrophenylserinol. When the purified *p*-nitrobenzoic acid was decarboxylated, the barium carbonate collected contained 14% and the dibenzylidenecyclohexanone isolated 85% of the radioactivity of the *p*-nitrophenylserinol. However, the low activity and small amount of dibenzylidenecyclohexanone obtainable by this route did not permit further degradation, and in all other runs the *p*-nitrobenzoic acid was decarboxylated without purification; the carbon dioxide, derived from dichloroacetic acid as well as *p*-nitrobenzoic acid, was ignored.

Degradation of Aniline-1-^{14}C. Hydrogenation of 129.3 mg (0.997 mmol) of aniline-*1*-^{14}C hydrochloride (mp 198–199°, 53.5 ± 0.1 μCi/mmol) over platinum oxide gave 140.9 mg of white solid amine hydrochloride, mp 180–190°. Oxidation of 131.0 mg of the amine hydrochloride with 5% potassium permanganate and condensation of the product with benzaldehyde as before gave 129.3 mg of crude product, which was recrystallized twice from absolute ethanol to give 100.0 mg (37%) of dibenzylidenecyclohexanone, mp 118–120°, 54.1 ± 0.5 μCi/mmol.

Recrystallized dibenzylidenecyclohexanone was combined with 45.2 mg of material obtained from the mother liquor and the total, 117.1 mg (0.428 mmol), was ozonized as above to give 62.9 mg (75%) of barium carbonate, whose millimolar radioactivity after correction for the blank and the purity of the barium carbonate was 48.3 ± 5.0 μCi/mmol,[8] and 39.2 mg (70%) of crude glutaric acid. Two recrystallizations from tetrachloroethane gave 24.4 mg (43%) of glutaric acid, mp 96–97%, 0.358 ± 0.006 μCi/mmol (0.65% of the millimolar radioactivity of the dibenzylidenecyclohexanone).

Results

Incorporation of Precursors. Although the principal biosynthetic aim of the present study was to test the shikimate pathway by the incorporation of labeled glucose into chloramphenicol, other potential precursors were also studied, in part to compare our results with those of earlier investigators. Incorporation results are summarized in Table I. It is apparent that glucose and glycerol are well incorporated, as was noted in earlier studies (Gottlieb *et al.*, 1962; Vining and Westlake, 1964). The specific activities of the chloramphenicol isolated in our studies were much higher than those obtained by Vining and Westlake (1964), allowing us to carry through the desired ring degradation.

Degradation Scheme. The degradation scheme developed (Figure 2) has the following advantages: (1) that all intermediates isolated (all those numbered in Figure 2 except **2**) are crystalline solids which can be purified, by recrystallization or sublimation as well as by chromatography, for accurate determination of millimolar radioactivity; (2) that no material is wasted in preparing and purifying derivatives which cannot be used in subsequent steps of the degradation; (3) that the organic products, with the exception of dibenzylidenecyclohexanone, are colorless and can, therefore, be assayed in solution with a scintillation counter without further manipulations.

Since adequate information on the origin of the side chain was already available (Gottlieb *et al.*, 1962; Vining and Westlake, 1964), *p*-nitrophenylserinol (**2**) was not isolated but oxidized in solution to *p*-nitrobenzoic acid, giving an 89% yield from chloramphenicol *vs.* 35–67% in previous work (Vining and Westlake, 1964; Robbins, 1955).

Platinum oxide in glacial acetic acid at atmospheric pressure catalyzed uptake of the stoichiometric 6 mol of hydrogen/mol of nitrobenzene to give a mixture of amines shown by paper and gas–liquid chromatography to consist of approximately 60% cyclohexylamine, 30% dicyclohexylamine, and 10% aniline, a distribution comparable to that obtained from aniline reduced in the presence of rhodium (Greenfield, 1964) without solvents. The mixture of amines was oxidized with permanganate in a two-phase ether–water system designed to decrease the exposure of cyclohexanone to permanganate and, thus, to reduce the formation of adipic acid. Since diethyl ether is oxidized to ethyl acetate under these conditions,[9] a large excess of permanganate was required. To check on the specificity of the reduction and ring opening steps of the degradation, aniline-*1*-^{14}C was degraded to glutaric acid (43% yield) with only 0.65% of the millimolar radioactivity of the dibenzylidenecyclohexanone, which constitutes sound proof of the specificity of the degradation.

Numerous attempts made to cleave glutaric acid to a compound which could be further degraded, so that the activity of the carbon atom bearing the side chain in the *p*-nitrophenylserinol moiety could be separated from the activity of its ortho carbon atoms, proved neither efficient nor selective enough to allow degradation beyond that C_3 unit of the very small amount of glutaric acid available (0.25 mmol).

Position of Label in Chloramphenicol. Insofar as possible, the samples of labeled chloramphenicol prepared by the experiments summarized in Table I were degraded by the scheme of Figure 2. Results of these degradations are shown in Table II, with the positions of label summarized in Table III. All biosynthetic conclusions have been based on the relative millimolar radioactivities of the purified organic products of the degradation. However, the millimolar radioactivities of evolved carbon dioxide, corrected for blank, were also obtained and compared with these results. Insofar as they can be compared, the results agree with the labeling pattern of the side chain and the distribution of label between *p*-nitrophenylserinol and dichloroacetic acid found in earlier studies, as may be seen in Table III.

Discussion

Of the precursors studied, glucose-*6*-^{14}C gave the most definitive results, clearly substantiating the shikimate pathway of biosynthesis, as we shall see. Results with other precursors were less clear-cut but most could also be interpreted in terms of the shikimate pathway.

[8] This 10% error is probably due to the relatively large blank, the small amount of product, and its high activity. To keep the counter on scale counting time was very short (0.2 min) and sample size was small, which emphasized weighing errors.

TABLE III: Distribution of ^{14}C Label in Chloramphenicol (**1**) from Labeled Precursors.

			% of Label from *p*-Nitrophenylserinol (**2**) in						
			Side Chain			Ring			
Expt	% of Label From **1** in **2**[c]	% of Label From **1** in **3**[c]	C-1′	C-2′	C-3′	C-1 + C-2 + C-6	C-3 + C-5	C-4	All ring Positions
1	67	33		1[d]	15	50	16	18	84
2	64	36		0[d]	15				
V_2[a]	65	35	3	0	24				75
3	85	15		24[d]	7	26	34	9	69
V_3[a]	88	12	3	20	5				72
4	73	27		3[d]	26	68	0[e]	5	73
5	79	21		20[d]	9	32	16	23	71
BG_5[b]	77	22	14	1	14				71
6	46	54		67[d]					
GR_6[b]	2	96							
7	67	33		50[d]	6	24	14	6	44
8	39	61		64[d]					
GR_8[b]	62	38	9	13	13				65
V_8[a]	3	97							
9	59	41		65[d]					
10	72	28		46[d]	3				
11	80	20		50[d]					

[a] Vining and Westlake (1964). [b] B = Burg (1958); G = Gottlieb *et al.* (1962); R = Robbins (1955). [c] Compounds **2** and **3** in Figure 2. [d] C-1′ + C-2′. [e] Value by difference, −2%.

Glucose-6-^{14}C. The incorporation of glucose-*6*-^{14}C (expt 4, Table I) was the highest observed in the present experiments (0.51%), and the dilution of substrate radioactivity was the lowest, only 0.69. Within chloramphenicol, *p*-nitrophenylserinol contained 73% of the label. Only 3% of the label of *p*-nitrophenylserinol was in C-1′ and C-2′ of the side chain, and 26% was in C-3′. The para carbon (C-4) contained 5% or less of the label of *p*-nitrophenylserinol, and there was no label in the meta carbons (C-3 + C-5). Essentially all of the label in the ring, 68% of that in *p*-nitrophenylserinol, was in the ortho carbons (C-2 and C-6) and/or the carbon bearing the side chain (C-1).

This pattern is consistent with the shikimate–prephenate (Srinivasan *et al.*, 1956; Davis, 1955; Umbarger and Davis, 1962; Sprinson, 1960; Bohm, 1965; Gibson and Pittard, 1968) pathway of aromatic biosynthesis (Figure 3). If only the ring carbons are considered (as 100%), C-1 + C-2 + C-6 account for 93% of the label, C-3 + C-5 for none, and C-4 for 7%, *vs.* respective percentages of 96, none, and none found by Srinivasan *et al.* (1956). Moreover, the pattern does not agree with the acetate pathway (Bu'Lock, 1965; Richards and Hendrickson, 1964) in which the carbon atoms of the ring should alternate in their level of labeling.

The carbon atoms of shikimic acid are derived from 1 mol each of erythrose 4-phosphate and phosphoenolpyruvate (Figure 3). These in turn can arise from glucose *via* a number of reaction pathways (Figures 4 and 5). Since label from glucose-*1*-^{14}C is incorporated (Table I) a factor of ten more poorly than that for glucose-*6*-^{14}C, and the hexose monophosphate oxidative pathway (Magasanik, 1962) is known to be important in *S. venezuelae* (Pepin, 1959), that pathway is shown first (Figure 4). Indeed, the hexose monophosphate oxidative pathway alone is sufficient to account for the glucose-*6*-^{14}C results. When C-6 labeled glucose is used as a carbon source for the synthesis of shikimic acid, C-6 of glucose is incorporated by the reactions of Figure 4 into C-2 of shikimic acid and into C-6 of shikimic acid.

The results with glucose-*6*-^{14}C also agree with the intermediacy of prephenic acid, since that compound is formed from 1 mol of shikimic acid and 1 mol of phosphoenolpyruvate, in which C-1 to C-3 of phosphoenolpyruvate (from glucose C-3 to C-1 and C-4 to C-6) become C-1′ to C-3′ of the side chain. Thus, label at C-6 of glucose should appear at C-3′ of the chloramphenicol side chain, as is found.[9]

Glucose-1-^{14}C. Experiment 1 (Table I) with glucose-*1*-^{14}C resulted in the production of one-tenth as much mycelium as normal, but the bioassay indicated that the antibiotic production was of the normal level (56 μg/ml).[10] Only 0.07% of the label was incorporated into the chloramphenicol. When the experiment was repeated (expt 2), normal production of mycelium and antibiotic was observed, but the incorporation was even lower (0.03%). Both incorporations were much lower than that of glucose-*6*-^{14}C (or glucose-*2*-^{14}C). The dilution of substrate radioactivity was higher than that with glucose-*6*-^{14}C (or glucose-*2*-^{14}C), 4.1 and 10.9 in expt 1 and 2, respectively. These results are explained by the fact that *S. venezuelae* gets much of its energy through the hexose monophosphate oxidative pathway of glucose metabolism

[9] Vining *et al.* (1968) suggested that prephenic acid is not an intermediate but the argument was not convincing, apparently being based on the lack of incorporation of phenylalanine and tyrosine. On the other hand, the similarity of labeling patterns found by this group in the side chain of phenylalanine and *p*-nitrophenylserinol (Vining and Westlake, 1964) argues for the intermediacy of prephenic acid.

[10] The energy and carbon necessary for production of chloramphenicol are small with respect to those required for production of cellular materials. A feedback mechanism has been shown to limit production of the antibiotic to a maximum concentration in the medium (Gottlieb and Legator, 1953), a concentration which can apparently be produced as readily by a few cells as by many.

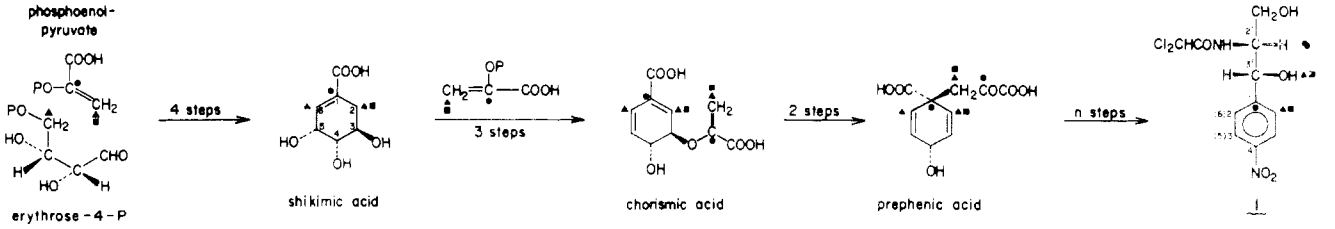

FIGURE 3: Biosynthetic route to chloramphenicol from erythrose 4-phosphate and phosphoenolpyruvate. Label from: (■) glucose-*1*-^{14}C; (●) glucose-*2*-^{14}C; (▲) glucose-*6*-^{14}C.

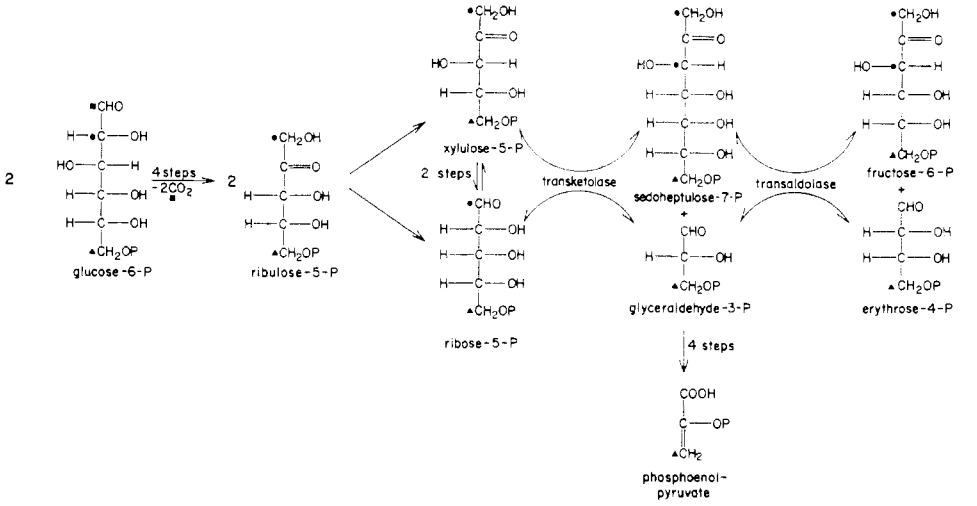

FIGURE 4: Hexose monophosphate oxidative pathway. Label from: (■) glucose-*1*-^{14}C; (●) glucose-*2*-^{14}C; (▲) glucose-*6*-^{14}C.

(Pepin, 1959), which results in the loss of C-1 of glucose as carbon dioxide. The portions of the chloramphenicol label in *p*-nitrophenylserinol were 67 and 64%, respectively, for the first and second experiments.

Although the level of incorporation was lower for glucose-*1*-^{14}C, the broad pattern of labeling was similar to that from the glucose-*6*-^{14}C experiment. Incorporation of label in the side chain was highly specific at C-3′ and the heaviest labeling of the ring was at C-1 + C-2 + C-6, as observed for glucose-*6*-^{14}C. The percentages of the ring label were 60% at C-1 + C-2 + C-6, 19% at C-3 + C-5, and 21% at C-4. Heaviest labeling at C-1 + C-2 + C-6 (92% of ring label) was observed earlier for shikimic acid by Srinivasan *et al.* (1956), with almost no label at C-3 + C-5.

In our studies C-1 of glucose is lost in the hexose monophosphate oxidative pathway; thus, that pathway cannot be the only (though it is presumably the major) route to erythrose 4-phosphate and phosphoenolpyruvate. The aldolase pathway (Figure 5) provides a reasonable alternative and that pathway was cited earlier for shikimic acid by Srinivasan *et al.* (1956). Label from glucose-*1*-^{14}C finds its way directly by this route into C-3 of phosphoenolpyruvate. A reversal of the aldolase reaction gives glucose-*1,6*-^{14}C and a turn of that compound through the hexose monophosphate pathway (Figure 4) introduces label at C-4 of erythrose phosphate as well as C-3 of phosphoenolpyruvate.

However, label in the ring of *p*-nitrophenylserinol was more widely distributed than expected from the pathways in Figures 4 and 5, 18% being found in the para carbon and 16% in the two meta carbons. These carbons require labeling of C-2 and C-1 (or C-3) of erythrose 4-phosphate.

The labeling pattern observed from glucose-*1*-^{14}C would be explained by prior conversion of a portion of glucose-*1*-^{14}C to glucose-*1,3,4,6*-^{14}C before its incorporation *via* the shikimate pathway into chloramphenicol. This could be effected *via* xylulose 5-phosphate, which can be formed in the transketolase reaction of Figure 5 and converted *via* the reactions of Figure 4 to fructose 6-phosphate. Initially glucose-*1*-^{14}C would produce xylulose 5-phosphate-*1,5*-^{14}C and fructose 1,6-diphosphate-*1,3,6*-^{14}C, but the aldolase reaction of Figure 5 would convert this to fructose 1,6-diphosphate-*1,3,4,6*-^{14}C and that would lead to erythrose 4-phosphate-*1,2,4*-^{14}C *via* the transketolase reaction of Figure 5.

Vining and Westlake (1964) have reported the incorporation of glucose-*1*-^{14}C on the glycerol–lactate medium (Gottlieb *et al.*, 1962), where glycerol was the primary source of carbon for chloramphenicol synthesis. Glucose-*1*-^{14}C was incorporated into chloramphenicol to a greater extent (0.58%) under those conditions than in the glucose medium, but the dilution of substrate radioactivity (29.6) was higher. The proportion of the label in the *p*-nitrophenylserinol moiety (65%) was the same as that observed in the glucose medium, and C-1′ and C-2′ of the side chain were unlabeled in both cases. The proportion of label in C-3′ of the side chain, determined from evolved carbon dioxide, was reported to be somewhat higher (24% *vs.* 15%) in the glycerol medium than in the glucose medium, but this may be the result of decreased randomization due to the later time of addition (48 hr) in their work.[11] On the other hand the 24% figure may be some-

[11] Vining and Westlake (1964) reported that their assay indicated chloramphenicol production is just beginning at 48 hr, but Robbins (1955) in our laboratories found that chloramphenicol production in this medium starts at about 20 hr and reaches a maximum at 60 hr.

what high, as our determination of radioactivity in C-3′ was higher based on barium carbonate than on crystallization of derivatives.

Glucose-2-^{14}*C*. Glucose-*2-*^{14}*C* (expt 3, Table I) was incorporated into chloramphenicol about half as well as glucose-*6-*^{14}*C* but much better than glucose-*1-*^{14}*C*. The incorporation was 0.28%, the dilution of substrate radioactivity was 1.33, and 85% of the label was in *p*-nitrophenylserinol. The side chain contained 24% of the *p*-nitrophenylserinol label in C-1′ and C-2′, and 7% in C-3′.

These data for glucose-*2-*^{14}*C* (both for the relative labeling of dichloroacetic acid and *p*-nitrophenylserinol and the position of labeling within *p*-nitrophenylserinol) are essentially identical (Table III) with those of Vining and Westlake (1964), although they reported higher incorporation (1.04%) despite much greater dilution of substrate radioactivity (16.6) when glucose-*2-*^{14}C was added at 48 hr to the glycerol–lactate medium. Both results for *p*-nitrophenylserinol agree well with the labeling pattern of phenylalanine isolated by Vining and Westlake (1964) from this organism.

Degradation of chloramphenicol derived from glucose-*2-*^{14}*C* indicated that 9% of the label of *p*-nitrophenylserinol was in the para carbon (C-4), 34% in the meta carbons (C-3 + C-5), 26% in the three remaining ring carbon atoms (C-1 + C-2 + C-6), 7% in C-3′, and 24% in C-1′ + C-2′.

The hexose monophosphate pathway (Figure 4) cannot account for these results with glucose-*2-*^{14}*C* directly but can if the glucose has equilibrated *via* the aldolase pathway of Figure 5 to give fructose 6-phosphate-*2,5-*^{14}*C*. The latter compound would give glyceraldehyde-*2-*^{14}*C* (hence phosphoenolpyruvate-*2-*^{14}*C*) and erythrose 4-phosphate-*3-*^{14}*C*, which in the shikimate pathway (Figure 3) would label C-2, C-1, and C-5 of chloramphenicol. The predominant labeling at C-2′, most clearly shown by Vining and Westlake (1964), and inferred by us (Table III), is in agreement with this pathway, as is the large amount of labeling in the present study at C-1 of the ring (counted together with C-2 and C-6) and at C-5 (counted with C-3). The results for the ring carbons in the present study (38% of ring label at C-1 + C-2 + C-6); 49% at C-3 + C-5; 13% at C-4) compare rather well with those of Srinivasan *et al.* (1956), who found labeling nearly exclusively at C-1 and C-5 of shikimate from glucose-*2-*^{14}*C* (corresponding to 49% of ring label at C-1 + C-2 + C-6; 46% at C-3 + C-5; 2% at C-4) and invoked the aldolase reaction (Figure 5) to explain their results.

The small amount of labeling at C-4 (9%) and C-3′ (7%) of *p*-nitrophenylserinol (Table III) can be explained by the pathways of Figures 4 and 5. Operation of the hexose monophosphate oxidative pathway (Figure 4) on glucose-*2,5-*^{14}*C* gives ribulose 5-phosphate labeled at C-1 and C-4, which would give fructose 6-phosphate labeled at C-1, C-3, and C-5. A turn of this hexose through the aldolase reaction of Figure 5 gives glucose labeled in all positions.

Glycerol-1,3-^{14}*C*. Incorporation studies with other substrates were not as revealing as those with glucose. In most cases incorporations were low (Table I) and, except for glycerol, label was concentrated in the side chain relative to the ring, in agreement with earlier studies (Table III).

When glycerol-*1,3-*^{14}*C* was added to the glycerol–tryptone medium the proportion of the chloramphenicol label in the *p*-nitrophenylserinol fragment was 79%. The fraction of the *p*-nitrophenylserinol label in C-1′ and C-2′ was 20% (presumably nearly all in C-1′) and that in C-3′ was 9% (based on purified dibenzylidenecyclohexanone).[12] Degradation of the ring indicated that 23% of the *p*-nitrophenylserinol label was in the para carbon, 16% in the meta carbons, and 32% in the remainder of the ring.

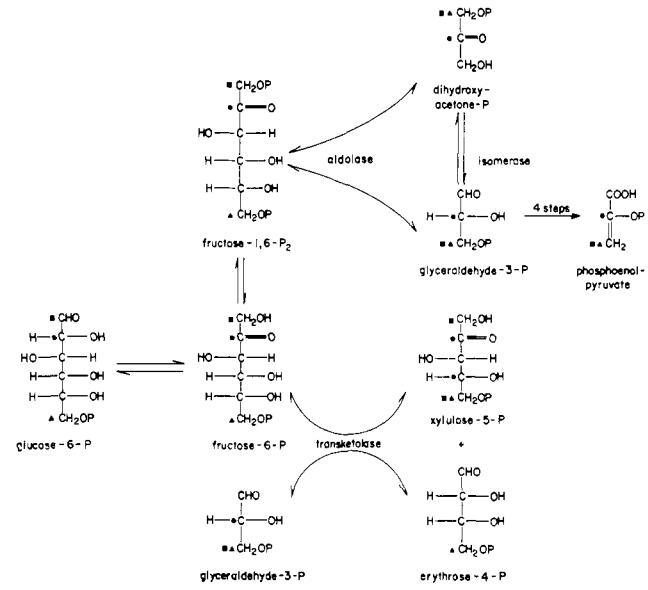

FIGURE 5: Aldolase and transketotolase pathways. Label from: (■) glucose-*1-*^{14}*C*; (●) glucose-*2-*^{14}*C*; (▲) glucose-*6-*^{14}*C*.

The pattern is consistent with the incorporation of glycerol into the ring of chloramphenicol through shikimate from glucose (labeled at C-1, C-3, C-4, and C-6) formed *via* the enzymes of the Embden–Meyerhof–Parnas pathway.

Glycerol-*1,3-*^{14}*C* is a better source of label in the para carbon than 1-, 2-, or 6-labeled glucose, because it can label glucose at C-4 without the randomization steps necessary for the incorporation of label from those compounds. The side chain probably arises from phosphoenolpyruvate formed both directly (C-1, C-3 labeled) from glycerol and, to some extent, indirectly (C-1 labeled) by carbon dioxide fixation. This unequal labeling of C-1′ and C-3′ probably results from the oxidation of glycerol to $^{14}CO_2$ and subsequent fixation of $^{14}CO_2$ into phosphoenolpyruvate-*1-*^{14}*C* (Ochoa, 1950; Kurahashi, 1957). This mode of incorporation was verified in the pyruvate studies described below.

Other Substrates. The other substrates studied, acetate and pyruvate, gave low incorporations and very high dilutions (Table I), which limited the number of degradation steps possible. With these substrates both the dichloroacetic acid and the *p*-nitrophenylserinol fragments were reasonably well labeled. The striking result, however, was the heavy concentration of label in C-1′ and C-2′ of the *p*-nitrophenylserinol side chain (46–67% of the label in **2**) *regardless of the position of the label in the precursor*. With pyruvate especially this

[12] Per cent activity at C-1′ plus C-2′ is somewhat higher than the 15% reported by Burg (Table III) (Gottlieb *et al.*, 1962; Burg, 1958) based on the measured activities of formaldehyde and formic acid from the periodate oxidation of *p*-nitrophenylserinol. However, the specific activity of Burg's *p*-nitrobenzaldehyde and *p*-nitrobenzoic acid would indicate 18–24% of the label in C-1′ plus C-2′ and, as the aldehyde and acid were not purified by him, their activity could have been higher than reported (Burg, 1958). Moreover, in the present work, the carbon dioxide from decarboxylation of *p*-nitrobenzoic acid (probably contaminated with dichloroacetic acid as shown for glucose-*1-*^{14}*C*) indicated 17% of the label in C-3′ of the side chain, which would agree with the 14% obtained by Burg, whose carbon dioxide was also probably contaminated.

constitutes evidence for the lack of incorporation of pyruvate as a unit and implies the active operation of the citrate cycle enzymes, with phosphoenolpyruvate arising *via* oxaloacetate. Since the reaction leading to the formation of pyruvate from phosphoenolpyruvate is almost irreversible, the conversion of pyruvate-*1*-^{14}C to phosphoenolpyruvate and, thence, to hexose must proceed by carboxylation of pyruvate *via* pyruvate carboxylase (Utter and Keech, 1960) or, possibly, *via* malic enzyme (Wiame and Bourgeois, 1955) to oxaloacetate-*1*-^{14}C, which gives phosphoenolpyruvate-*1*-^{14}C by decarboxylation. The carbon dioxide fixation reaction to form oxaloacetate must be involved because oxaloacetate formed in the Krebs cycle would not be labeled by pyruvate-*1*-^{14}C, since the labeled carbon would be lost as carbon dioxide upon entry into the cycle. Acetate-*1*-^{14}C would give oxaloacetate labeled at C-1 by either route. In this connection it is significant that pyruvate-*1*-^{14}C gave much higher dilution than any of the other four acetate or pyruvate substrates, presumably due to its decarboxylation to acetate with loss of label.

In only one experiment with pyruvate or acetate was there sufficient radioactivity to allow degradation of the ring. Acetate *2*-^{14}C on glucose–tryptone medium gave chloramphenicol with 67% of the label in the *p*-nitrophenylserinol moiety. Degradation of *p*-nitrophenylserinol indicated that 50% of the label was in C-1′ and C-2′, 6% in C-3′ of the side chain, 6% in the para position, 14% in the meta carbons, and 24% in the remaining three carbons. Such randomization of label in the ring would strongly contradict an acetate pathway of biosynthesis, even if the positive evidence for the shikimate pathway were not available.

Comments

The results of the present study employing glucose as a precursor are in accord with the observation of Vining and Westlake (1964) that shikimic acid itself is incorporated—to a small extent and with high dilution—into chloramphenicol. The Vining group has more recently shown (McGrath *et al.*, 1968) that a number of compounds more closely related to chloramphenicol—L-*p*-aminophenylalanine, DL-*threo*-β-(*p*-aminophenyl)serine, and L-*threo*-β-(*p*-aminophenyl)-*N*-dichloroacetylserinol—were incorporated relatively well with rather low dilutions. Indeed, the structures of those compounds suggest the sequence in the preceding sentence as that followed in the biosynthesis of chloramphenicol itself.

Acknowledgment

We thank Dr. P. D. Shaw, University of Illinois, and Dr. R. B. Bates, University of Arizona, for helpful suggestions concerning portions of this work.

References

Bertelli, J. (1953), *Ann. Chim. (Paris) 43*, 361.
Bohm, B. A. (1965), *Chem. Rev. 65*, 435.
Braun, D., and Green, H. (1962), *J. Chromatogr. 7*, 56.
Bu'Lock, J. D. (1965), The Biosynthesis of Natural Products, London, McGraw-Hill, p 23.
Burg, R. W. (1958), Ph.D. Dissertation, University of Illinois, Urbana, Ill.
Bush, E. T. (1964), *Anal. Chem. 36*, 1082.
Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M., and Yankwich, P. E. (1949), Isotopic Carbon, New York, N. Y., Wiley, pp 83–84.
Conrad, C. R., and Dolliver, M. A. (1943), *in* Organic Syntheses, Collect. Vol. II, New York, N. Y., Wiley, p 167.
Dauben, W. G., Reid, J. C., Yankwich, P. E., and Calvin, M. (1950), *J. Amer. Chem. Soc. 72*, 121.
Davis, B. D. (1955), *Advan. Enzymol. 16*, 247.
Fieser, L. F. (1955), Experiments in Organic Chemistry, Boston, Mass., D. C. Heath and Co., p 202.
Gibson, F., and Pittard, J. (1968), *Bacteriol. Rev. 32*, 465.
Gottlieb, D. (1967), *Antibiotics 2*, 32.
Gottlieb, D., Carter, H. E., Legator, M., and Gallicchio, V. (1954), *J. Bacteriol. 68*, 243.
Gottlieb, D., Carter, H. E., Robbins, P. W., and Burg, R. W. (1962), *J. Bacteriol. 84*, 888.
Gottlieb, D., and Legator, M. (1953), *Mycologia 45*, 507.
Gottlieb, D., Robbins, P. W., and Carter, H. E. (1955), *J. Bacteriol. 72*, 153.
Greenfield, H. (1964), *J. Org. Chem. 29*, 3082.
Kurahashi, K. (1957), *J. Biol. Chem. 226*, 1059.
Legator, M., and Gottlieb, D. (1953), *Antibiot. Chemother. 3*, 809.
Long, A. G., Quayle, J. R., and Stedman, R. J. (1951), *J. Chem. Soc.* 2197.
Magasanik, B. (1962), *Bacteria 3*, 297.
McCasland, G. E. (1951), *J. Amer. Chem. Soc. 73*, 2295.
McGrath, R., Vining, L. C., Sala, F., and Westlake, D. W. S. (1968), *Can. J. Biochem. 46*, 587.
Niederl, J. B., and Niederl, V. (1942), *in* Micromethods of Quantitative Organic Analysis, New York, N. Y., Wiley, p 101.
Ochoa, S. (1950), *J. Biol. Chem. 187*, 891.
Ott, D. G., Richmond, C. R., Trujillo, T. T., and Foreman, H. (1959), *Nucleonics 17*, 106.
Pepin, H. S. (1959), Ph.D. Dissertation, University of Illinois, Urbana, Ill.
Petrowitz, H. J., and Pastuska, G. (1962), *J. Chromatogr. 7*, 128.
Richards, J. H., and Hendrickson, J. B. (1964), *in* The Biosynthesis of Steroids, Terpenes, and Acetogenins, New York, N. Y., W. A. Benjamin, p 40.
Robbins, P. W. (1955), Ph.D. Dissertation, University of Illinois, Urbana, Ill.
Roberts, J. D., Simmons, H. E., Carlsmith, L. A., and Vaughan, C. W. (1953), *J. Amer. Chem. Soc. 75*, 3290.
Smith, C. G. (1958), *J. Bacteriol. 75*, 577.
Smith, P. A. S. (1946), *Org. React. 3*, 382.
Sprinson, D. B. (1960), *Advan. Carbohyd. Chem. 15*, 235.
Srinivasan, P. R., Shigeura, H. T., Sprecher, M., Sprinson, D. B., and Davis, B. D. (1956), *J. Biol. Chem. 220*, 477.
Stork, J. B., Goodban, A. E., and Owens, H. S. (1951), *Anal. Chem. 23*, 413.
Umbarger, E., and Davis, B. D. (1962), *Bacteria 3*, 222.
Utter, M. F., and Keech, D. B. (1960), *J. Biol. Chem. 235*, PC 17.
Vining, L. C., Malik, V. S., and Westlake, D. W. S. (1968), *Lloydia 31*, 355.
Vining, L. C., and Westlake, D. W. S. (1964), *Can. J. Microbiol. 10*, 705.
Wiame, J. M., and Bourgeois, S. (1955), *Biochim. Biophys. Acta 18*, 269.
Wilzbach, K. E., and Van Dyken, A. R. (1950), *U. S. At. Energy Comm.* AECD-2998.