periments to isolate and characterize coprotein are in progress.

Summary. Data are presented to show that purified serum albumin enhances the lipemia clearing reaction, probably by binding the fatty acids produced which, if not removed, are capable of inhibiting the reaction. Evidence that albumin is not the only cofactor in the clearing reaction is presented.

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Biosynthesis of C^{14}-Labeled Benzylpenicillin. (20580)

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Although phenylacetic acid is one of the well-established precursors of benzylpenicillin, only limited attention has been given to the metabolic changes which occur when the acid is utilized by a penicillin-producing fungus. For this reason we investigated the problem in more detail. The incorporation of phenylacetic acid-l-C^{14} into benzylpenicillin has been achieved and is reported together with observations which indicate other possible pathways of phenylacetic acid metabolism.

Experimental and results. Phenylacetic acid-l-C^{14} was synthesized by the method of Dauben *et al.**(1). Two separate preparations had specific activities of 15.29 x 10^5 and 30.15 x 10^9 c.p.m. per mole.† The acid of lower activity was used in this investigation. One hundred and sixty ml of semisynthetic medium‡ in 500 ml baffled Erlenmeyer flasks were inoculated with the young (24-36 hour) vegetative mycelium of *Penicillium chrysogenum,* BC-65, Upjohn, and incubated on a rotary shaker at 25-27°C. After incubating for 24 hours, potassium phenylacetate-l-C^{14}, equivalent to 375 μg of the free acid/ml was added. At the end of the fermentation period (96 hours) the pH had risen from 5.5 to 8.4. The amount of penicillin produced was 867 μg/ml, while the control with no phenylacetic acid contained 311 μg of penicillin/ml. The harvested dry mycelium from the fermentation with the added precursor amounted to 11.2 mg/ml. A 200 ml portion of the fermented beer was acidified (pH 2.2) with 25% H_2SO_4 and extracted with amyl acetate. The combined amyl acetate extracts in turn were extracted with saturated phosphate buffer (pH 6.8). The buffer was acidified (pH 2.2) and extracted with ether. After the

* The $BaC^{14}O_3$ used was purchased from the Oak Ridge National Laboratory, Oak Ridge, Tenn.

† Measurements of radioactivity were made on the solid neutralized samples with a thin-window counting tube and autoscaler manufactured by Tracerlab, Inc., Boston, Mass. The values reported are corrected for infinitely thin samples.

‡ The medium consisted of: lactose 50 g, glucose 5 g, corn steep liquor solids 17 g, $CaCO_3$ 3 g, $FeSO_4 \cdot 7H_2O$ 0.004 g, Na_2SO_4 0.5 g; H_2O to 1000 ml; pH 5.5.

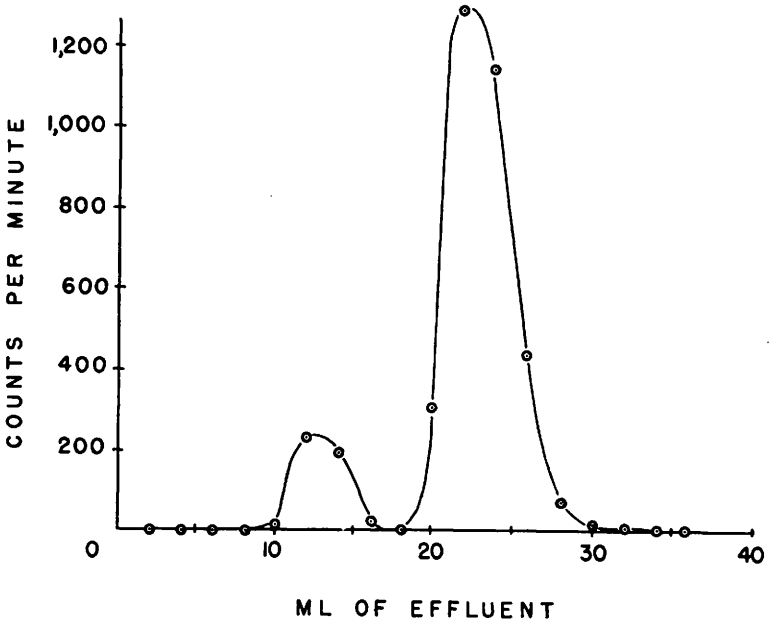

FIG. 1. Distribution of radioactivity in chromatographed fractions of crude penicillin. 1.7 mg of penicillin, 'Hyflo Supercel' column; values corrected for background.

ether was evaporated with a mild stream of air at room temperature, the resulting amorphous material was dried *in vacuo*. A portion was then dissolved in 0.2% phosphate buffer (pH 6.8) and separated by a modification of the paper chromatographic procedure of Karnovsky and Johnson(2), using water-saturated ether as the mobile phase. After development the paper was cut lengthwise into 2 strips, each 5 mm wide, in such a way that each strip contained an equal quantity of the separated material. One of these strips was used to identify the individual penicillins by bioassay on an agar layer seeded with *Micrococcus pyogenes* var. *aureus*. The other strip was used for radioautograms.

The bioassay indicated that the isolated material consisted of 94.5% benzyl-, 4.3% heptyl-, 0.9% n-amyl-, 0.3% 2-pentenyl-, and traces of p-hydroxybenzylpenicillin. The radioautograms showed that the major portion of the radioactivity was located in the area coinciding with the benzylpenicillin spot as identified by bioassay. A second diffuse spot on the radioautogram was observed in the approximate area of phenylacetic acid, the identity of which was verified in a butanol-acetic acid-water (74:19:50 ratio) system.

Further purification of the antibiotic was performed chromatographically on a column of "Hyflo Supercel," according to the method of Perret(3). The effluent was collected in 18 two-ml portions, each portion neutralized with 0.006 M KOH and the radioactivity determined. With this method, 2 radioactive components were isolated (Fig. 1). The first (10th-16th ml) had no antibacterial activity, while the second (20th-28th ml) suppressed the growth of *M. pyogenes*. When known mixtures of non-radioactive phenylacetic acid and penicillin were separated by the same chromatographic method and the distribution followed titrimetrically(3), the phenylacetic acid peak corresponded to the first radioactive fraction, and the penicillin peak to the second. Evidently, the non-reacted phenylacetic acid was extracted simultaneously during the isolation of penicillin.

The amorphous material after column chromatography contained 68% purified antibiotic(3). The radioautograms of this material revealed a single spot which corresponded to benzylpenicillin. When the penicillin was hydrolyzed with H_2SO_4 as described(4) and counts were made on the resulting fractions (CO_2, phenylacetic acid and

TABLE I. Distribution of C^{14} in Fermented Beer (*Penicillium chrysogenum* BC-65, Upjohn). A 96-hour fermentation (25-27°C), shake flasks, initial pH 5.5, final 8.4, 375 μg phenylacetic acid/ml added.

	mg/160 ml	c.p.m./mg	c.p.m./mMol	c.p.m./160 ml	% recovery
Added phenylacetic acid	60.0	11.2×10^3	15.3×10^2	6.72×10^5	
Recovered penicillin	138.7	2.8×10^3	10.5×10^2	3.90×10^5	58.3
Unreacted phenylacetic acid	3.2	10.8×10^3	14.7×10^2	$.35 \times 10^5$	5.2
Harvested mycelium	1792	3.8	—	$.69 \times 10^5$	10.2
Beer residue (not extracted with amyl acetate)	—	—	—	$.03 \times 10^5$	.3
Total accounted for				4.97×10^5	74.0
Unaccounted for (CO_2, losses during the extraction, etc.)				1.75×10^5	26.0

the acidic ether-insoluble residue), only the resulting phenylacetic acid (m.p. 75-77°C) was found to be radioactive. These data indicate that the C^{14} in the phenylacetic acid used as precursor was incorporated only into the side-chain of benzylpenicillin and was not distributed throughout the whole molecule.

The specific activity of the purified penicillin (10.48×10^5 c.p.m. per mole) was lower than that of the phenylacetic acid used (15.29×10^5 c.p.m. per mole). This difference was found to be due to the dilution of the labeled antibiotic with penicillin produced from a precursor which was biosynthesized or preformed in the medium.

The distribution of radioactivity in a typical experiment, in which the described analytical methods were used is given in Table I.

The data in Table I indicate that the residual phenylacetic acid was not diluted during the fermentation, for it had practically the same specific activity as when initially added. If this acid is the normally occurring precursor of the side-chain of benzylpenicillin, it is either not released free into the medium, or is incorporated into the penicillin molecule at the same rate as it is biosynthesized.

The incorporation of phenylacetic acid into the penicillin, however, was not the only change observed when the acid was metabolized by *P. chrysogenum*. When, in similar experiments, the respiratory CO_2 was intercepted as $BaCO_3$ and counted, it was found to be radioactive, thus indicating that the acid was also decarboxylated. The data are in agreement with those recently published (5). In addition, the acid was found to be incorporated into the protein and non-saponifiable lipid fractions of the mycelium. Further work is being carried out to elaborate these diverse metabolic paths of phenylacetic acid brought about by *Penicillium*.

Summary. The biosynthesis of the C^{14}-labeled benzylpenicillin was achieved by preparing phenylacetic acid-1-C^{14} and incorporating the latter into the side-chain of benzylpenicillin. The antibiotic was successfully separated from the nonreacted labeled phenylacetic acid by column chromatography. The data obtained by radioautography and chemical degradation of the purified antibiotic indicated that the radioactivity resided in the side-chain of the benzylpenicillin molecule.

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