[CONTRIBUTION FROM THE SQUIBB INSTITUTE FOR MEDICAL RESEARCH]

Biosynthesis of Streptomycin. I. Studies with C-14-Labeled Glycine and Acetate[1]

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In a fermentation medium comprising glucose, acetate and glycine as the sole carbon sources, *Streptomyces griseus* utilized only the glucose carbons for the synthesis of streptomycin even though both acetate and glycine had to be present for efficient streptomycin production. Less than the equivalent of one of the carbon atoms in the streptomycin molecule could be accounted for by all the four carbons of glycine and acetate. During the course of the fermentation, more than half of the acetate and glycine carbon appears as carbon dioxide. Despite the highly inefficient incorporation of labeled carbon from acetate and glycine into streptomycin, it was still possible to demonstrate localization in the guanidine carbons of the molecule.

The metabolism and antibiotic production of different strains of *Streptomyces griseus* have been the subjects of a number of investigations. In a study of the metabolism of *Streptomyces griseus* using a peptone, meat extract, glucose and sodium chloride medium, Waksman, Schatz and Reilly[2] showed that cells of *S. griseus*, when added to a fermentation medium, could replace the meat extract but could not replace both the peptone and the meat extract. This observation led to the postulation that an "activity factor," presumably a natural precursor or part of an enzyme system, must be supplied to the medium for the biosynthesis of streptomycin. Production of streptomycin on a medium composed of soybean meal, glucose, and salts,[3] and the development of purely synthetic media[4,5] made unnecessary the absolute requirement of an "activity factor" for biosynthesis of moderate amounts of streptomycin. Later studies by Falconer, *et al.*,[6] showed that cultures grown on a glycine-containing medium produced more streptomycin than when glycine was absent. O'Brien, Wagman and Perlman[7] have also described a simple glycine-containing medium in which the antibiotic yields are equal to those obtained with any available medium. Elimination or replacement of the glycine from their synthetic medium resulted in poor growth, low antibiotic yields and unsatisfactory fermentations.

These authors suggested that further study on the role of glycine in these fermentations would be desirable in order to demonstrate whether or not glycine is a direct precursor for streptomycin. In an effort to clarify the roles of glycine and other substrates in the production of increased streptomycin titers, we have undertaken an investigation on the extent of incorporation of glycine and acetate carbons into carbon dioxide, broth, cells and streptomycin.

Experimental

Aeration Train.—The aeration train used for supplying sterile air for the fermentations consisted of the following, in order: A compressed air cylinder, KOH scrubber for removal of CO_2 in the air, water scrubber for removal of entrained KOH and saturation of the air with water vapor, fermentation flask, KOH absorber for collection of CO_2 from the fermentation, and a gas flowmeter. The fermentation flask consisted of a 500-ml. erlenmeyer flask with the following glass attachments: An air-inlet tube permitting passage of air *over* the medium, a gas outlet tube, and a ₸ 29/42 male joint attached to the neck of the flask to permit use of a bacteriological filter.

Fermentation Procedure.—These studies were carried out using a strain of *Streptomyces griseus* designated as Squibb Strain N. All fermentations were conducted in the 500-ml. erlenmeyer flasks, described above, each flask containing 100 ml. of medium. The flasks were placed on a reciprocating shaker operating at 120 cycles per minute with a horizontal displacement of 1.5 inches. The shaker was located in a constant-temperature room maintained at 25 ± 1°.

The inoculum consisted of vegetative cells grown through two transfers. The first transfer was grown in soybean-meal–glucose medium for two days. The second transfer was grown on the fermentation medium for three days. The fermentation medium consisted of the following in g./100 ml.: glucose, 2.0; glycine, 0.26; sodium acetate (anhydrous basis), 0.136; $(NH_4)_2SO_4$, 0.054; $FeSO_4 \cdot 7H_2O$, 0.003; $CuSO_4 \cdot 5H_2O$, 0.05; $K_2HPO_4 \cdot 3H_2O$, 0.05; $CaCl_2 \cdot 2H_2O$, 0.005. In each experiment the salts were dissolved in 40 ml. of water; the solution was then autoclaved and inoculated. Immediately after inoculation, the glucose–acetate–glycine[8] mixture was added by sterile filtration through the top of the flask. The sterile filter was washed with sufficient water to bring the total volume of the medium to 100 ml. The filter was removed and the flasks plugged with sterile cotton and then capped. The flasks were placed on the shaker, attached to the aeration train, and CO_2-free sterile air allowed to pass over the surface of the medium at a rate of 10–20 ml. per minute. After a growth cycle of six days, fermentations carried out in this manner gave yields of streptomycin essentially the same as those obtained in control cotton-plugged flasks placed on the same shaker. All fermentations in which labeled substrates were used were carried out in triplicate.

In the control fermentations in which non-radioactive substrates were used, determinations were made daily for CO_2, cell weight,[9] ammonia nitrogen,[10] acetate,[11] glycine,[11] glucose[12] and *p*H. All the CO_2 determinations were corrected for removal of samples of culture fluid on previous days. Since it was not considered desirable to take daily samples from the flasks containing labeled substrates, the similarities between daily CO_2 output and final streptomycin

(1) Presented before the Division of Agricultural and Food Chemistry, American Chemical Society, Atlantic City, N. J., September 14 to 19, 1952.

(2) S. A. Waksman, A. Schatz and H. C. Reilly, *J. Bact.*, **51**, 753 (1946).

(3) G. Rake and R. Donovick, *ibid.*, **52**, 223 (1946).

(4) Thornberry and Anderson, *Arch. Biochem.*, **16**, 309 (1948).

(5) Dulaney, *J. Bact.*, **56**, 305 (1948).

(6) R. Falconer, M. Lumb and G. Sykes, Boots Pure Drug, Ltd., British Patent 616,102 (Jan. 17, 1949).

(7) E. O'Brien, G. M. Wagman and D. Perlman, paper in preparation.

(8) Glycine-1-C^{14}, glycine-2-C^{14} and sodium acetate-2-C^{14} were obtained from Tracerlab, Inc., Boston, Mass., under authorization granted by the Isotopes Division, United States Atomic Energy Commission. Sodium acetate-1-C^{14} was prepared in the usual manner by the action of $C^{14}O_2$ on the Grignard reagent from methyl iodide.

(9) Cell weights were determined by taking an aliquot of culture fluid and separating the cells by centrifugation. The cells were resuspended on water, collected again by centrifugation, and then transferred to tared beakers and dried to constant weight in an oven kept at 80–90°.

(10) E. J. Conway, "Microdiffusion Analysis and Volumetric Error," 3rd edition, Crosby, Lockwood and Son, Ltd., London, p. 87.

(11) Biochemical Institute Studies, Vol. IV, University of Texas Publication No. 5109, May 1, 1951.

(12) A. C. Neish, "Analytical Methods for Bacterial Fermentations," National Research Council of Canada (1950).

titers of the labeled and unlabeled flasks were taken as criteria for the equivalence of the two fermentations. If the daily CO_2 output and final streptomycin activity of the fermentations containing labeled substrates were approximately the same, it was assumed on the basis of previous experiments that *p*H, glucose, cell weight and ammonia nitrogen were also comparable.

Isolation of "Streptomycin Fraction."—To free streptomycin from the cells[13] at the end of the incubation period sufficient sulfuric acid (6 *N*) was added to bring the *p*H of the culture fluid to about 2–2.5. After shaking the flasks for 30 minutes, the culture fluid was transferred to centrifuge bottles and the cells separated by centrifugation. The cells were washed by suspending them in three 50-ml. portions of CO_2-free water and then freeze-dried. The cell washes were combined with the original supernatant and then diluted to a known volume. The streptomycin activity was determined on an aliquot by the tube dilution assay of Donovick, *et al.*[14] Isolation of the antibiotic fraction was carried out by adsorption on and elution from a cation exchange resin.[15] Further treatment of the resin eluate was carried out according to the procedure described by Heuser, Dolliver and Stiller.[16]

Analytical and Counting Procedures.—The absorbers for the collection of CO_2 each contained 57 meq. of KOH in 50 ml. of water, and were changed daily during the fermentation period. The absorber solutions were transferred under nitrogen to 250-ml. volumetric flasks and diluted to volume with CO_2-free water. Duplicate aliquots (50 ml.) were taken for analysis, and after precipitation of the carbonate with 10 ml. of 25% barium chloride solution, the excess potassium hydroxide was titrated to phenolphthalein endpoint with standard acid; the amount of CO_2 was determined as the difference between the amount of potassium hydroxide added and the amount consumed.[17] All samples were checked by weighing the barium carbonate precipitates.

The carbon content of broth samples was determined by the Weinhouse procedure.[18] Carbon determination on water-insoluble materials was carried out according to the procedure described by Steele and Sfortunato,[19] as modified by Anderson, *et al.*[20]

All radioactivity determinations were made on carbon as barium carbonate according to the procedures of Calvin, *et al.*[21] Correction for coincidence loss, background, self-absorption and counter efficiency were made as required. Radioactive water-soluble samples were checked by direct plating.[21]

The radioactivity level of the substrate, in microcuries/flask, for each of the fermentations was glycine-1-C^{14}, 14.5; glycine-2-C^{14}, 13.6; acetate-1-C^{14}, 16.1; and acetate-2-C^{14}, 15.2.

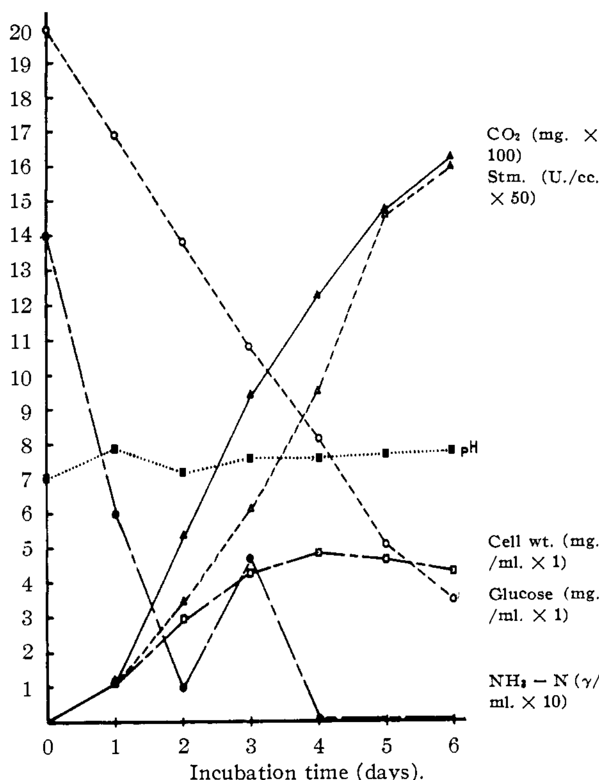

Fig. 1.—Changes in fermentation products with time. The ordinate is in arbitrary numbers with the units for each curve shown on the graph.

Results and Discussion

The use of a chemically defined synthetic medium in fermentation studies permits changes in any of the compounds in the medium to be followed without being complicated by contributions from natural substances of unknown composition and concentration. In the work reported here these changes in the medium as the fermentation progressed are summarized in Fig. 1. These data were taken from fermentations carried out in the same manner and at the same time as those in which radioactive substrates were used. Since the rates of utilization of glycine and of acetate were very rapid, data for these substrates were not included in the figure.

The most striking features of the results are the release of ammonia nitrogen into the medium between 48 and 72 hours and the relatively constant rate of utilization of glucose. As can be seen from the data, approximately 15 to 20% of the glucose initially present remains unconsumed. The parallelism between the total CO_2 and streptomycin production shown in Fig. 1 is somewhat illusory. A plot of the daily rate of CO_2 and streptomycin production as shown in Fig. 2 indicates that the maximum streptomycin production occurs during decreasing respiratory activity. Of the 350 mg. of carbon present in the medium at the end of the fermentation, glucose accounts for 140 mg., streptomycin for 34 mg.; the remaining 76 mg. is present in other products. This carbon is neither glycine nor acetate, for neither of these substrates could be detected after the fourth day of incubation. It is also unlikely that this carbon is largely representative of fragments released on acid treatment of the cells, since the difference in cell weights between their maximum weight on the fourth day and their weight at the end of the fermentation could account for only about 25 mg. of carbon.

Examination of the broth constituents using two-dimensional paper chromatography, revealed the

(13) G. Rake, W. Koerber and R. Donovick, U. S. Patent 2,461,922 (1949).

(14) R. Donovick, D. Hamre, F. Kavanagh and G. Rake, *J. Bact.*, **50**, 623 (1945).

(15) H. M. Dorey, E. C. Mason and D. E. Weiss, *Anal. Chem.*, **22**, 1038 (1950).

(16) L. Heuser, M. Dolliver and E. T. Stiller, THIS JOURNAL, **75**, 4013 (1953).

(17) H. H. Willard and N. H. Furman, "Elementary Quantitative Analysis," 3rd Edition, D. Van Nostrand Co., Inc., New York, N. Y., 1940.

(18) M. Calvin, C. Heidelberger, J. C. Reid, B. M. Tolbert and P. E. Yankwich, "Isotopic Carbon," John Wiley and Sons, Inc., New York, N. Y., 1949, p. 94.

(19) R. Steele and T. Sfortunato, "Techniques in the Use of C^{14}," Brookhaven National Laboratory, Bulletin BNL-T-6.

(20) R. C. Anderson, Y. Delabarre and A. K. Bothner-By, *Anal. Chem.*, **24**, 1298 (1952).

(21) See ref. 19, pp. 108–120.

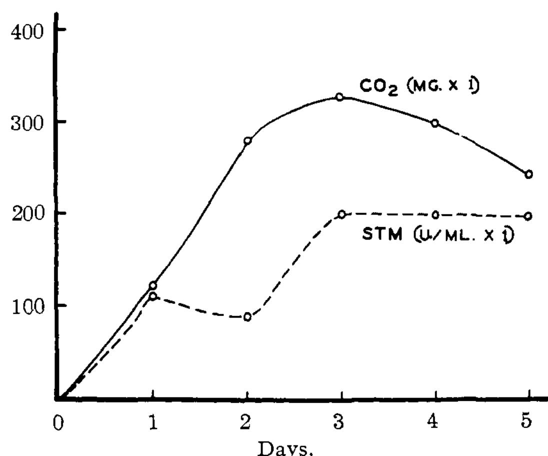

Fig. 2.—Comparison between daily CO_2 output and streptomycin production.

presence in trace amounts of three or four unidentified ninhydrin-reacting materials in addition to alanine and glutamic acid. The alanine appeared after 24 hours and glutamic acid after 48 hours; both were readily detectable during the remainder of the fermentation. In the fermentation with carboxyl-labeled acetate as the precursor, a small amount of radioactivity was associated with three of the ninhydrin spots. However, none of the other fermentations with labeled substrates disclosed any significant incorporation of radioactivity into the ninhydrin-reacting material. We have noticed in the fermentation with methyl-labeled acetate that a considerable amount of radioactivity is associated with an apparently neutral substance with R_f values similar to those of the ninhydrin-reacting material. Another chromatogram, carried out to test the possibility of incorporation of substrate carbon into glucose, revealed no detectable incorporation of either glycine or acetate carbon into glucose. This was confirmed by the absence of radioactivity in a sample of glucose isolated as the osazone. Thus the possibility of any net synthesis of glucose from glycine or acetate is eliminated.

With the fermentation train employed, it was possible to measure the rate at which each of the substrates, as well as the total carbon in the medium was burned to carbon dioxide. The results of these determinations are shown in Table I and Fig. 3.

TABLE I

PERCENTAGE OF DAILY CO_2 FROM LABELED SUBSTRATES[a]

Day	Total CO_2 produced (mg. carbon)	% of total CO_2 from acetate COOH	CH₃	% of total CO_2 from glycine COOH	-CH₂-	% of total daily CO_2 from labeled substrates
1	34	28.8	20.6	9.1	2.3	60.8
2	105	4.8	7.1	17.9	4.4	34.2
3	98	0.8	1.2	14.3	9.1	25.4
4	83	.6	0.8	6.7	6.4	14.5
5	75	.8	.8	0.7	3.1	5.4
6	62	.7	.7	0.5	2.8	4.7

[a] The total carbon in the CO_2 represents a conversion of 47% of the carbon initially present in the medium.

Qualitatively, the data of Table I and Fig. 3 show that the rate at which the carboxyl and methyl carbons of acetate are converted to carbon dioxide appear to be similar. This is not the case with the two carbon atoms of glycine, where the carboxyl carbon appears as carbon dioxide at a much more rapid rate than the methylene carbon. Of particular interest is the similarity in the curves for the conversion to CO_2 of total carbon and the methylene carbon of glycine.

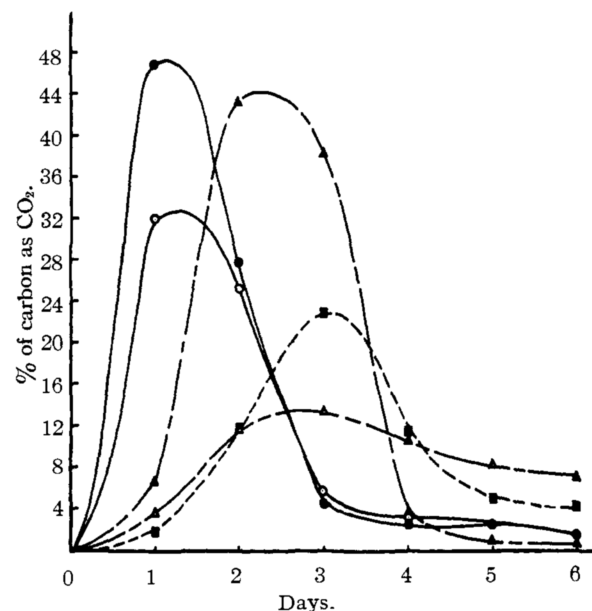

Fig. 3.—Rate of combustion of total carbon and of each of the labeled substrate carbons. The ordinate designates the percentage of substrate carbon evolved as carbon dioxide. △——△, total carbon in medium; ▲——▲, glycine: carboxyl carbon; ■-----■, glycine: methylene carbon; ●——●, acetate: carboxyl carbon; O——O, acetate: methyl carbon.

Incorporation of radioactivity into the cells is shown in the data in Table II. Of the four carbons of acetate and glycine, the major contribution of the methylene carbon of glycine to cell composition is striking.

TABLE II

INCORPORATION OF RADIOACTIVITY FROM LABELED SUBSTRATES INTO CELLS[a]

Substrate	% of starting substrate radioactivity in cells	% of total cell carbon from labeled substrates
CH_3*COOH	12.4	1.7
*CH_3COOH	17.5	2.3
H_2N-CH_2-*COOH	4.4	1.2
H_2N-*CH_2-COOH	31.0	8.6

[a] The cells represent 16% of the total carbon initially present in the medium.

Only with the methylene carbon of glycine is any significant degree of specificity for cell synthesis shown. Distribution of radioactivity among the cell constituents will be reported later.

In each of the four experiments, the streptomycin was purified to a potency not less than 300–400 units/mg. The incorporation of the radioactivity of each substrate into streptomycin, as well as a

summation of the gross distribution of radioactivity of each substrate, is shown in Table III.

TABLE III

PERCENTAGE OF DISTRIBUTION OF RADIOACTIVITY OF SUBSTRATES

Substrate	Cells	Broth	CO_2	% of substrate carbon in streptomycin	% of streptomycin from labeled substrate
CH_3–*COOH	12.4	6.4	86	0.49	0.31
*CH_3–COOH	17.5	11.9	71	.21	.20
H_2N–CH_2–*COOH	4.4	2.5	93	.48	.75
H_2N–*CH_2–COOH	31.2	13.4	56	.90	1.15
					2.41

It is clear from the data in the last column of Table III that the incorporation of glycine and acetate carbon into the streptomycin molecule is very low. These four carbon atoms together represent less than 3% of the total carbon in streptomycin. Additional information on the incorporation of acetate and glycine carbon into streptomycin can be obtained by a comparison of the molar specific activities of the substrates with that of the streptomycin. If one assumes that one position in the streptomycin molecule were derived exclusively from either the carboxyl carbon of acetate, the methyl carbon of acetate, the carboxyl carbon of glycine or the methylene carbon of glycine, then the respective specific activities of the streptomycin should be 37,000 dis./min./mg., 35,000 dis./min./mg., 16,000 dis./min./mg. and 15,000 dis./min./mg. The corresponding values calculated for streptomycin activity from the actual counting data on streptidine and streptobiosamine are 2600 dis./min./mg., 1400 dis./min./mg., 2560 dis./min./mg. and 3600 dis./min./mg. These data show only 7%, 4%, 16% and 24% of the activity expected in the streptomycin if only one position of the streptomycin molecule was derived exclusively from any of the substrate carbons. Therefore, under the conditions of our experiment, all the carbon atoms of acetate and glycine contributed less than the equivalent of one carbon atom to the biosynthesis of the streptomycin molecule.

Although all the above evidence indicates minor incorporation of acetate and glycine carbon into streptomycin, it was still of interest to determine if any specific positions in the antibiotic were labeled. The results of the degradation[22] of the labeled streptomycin, as indicated in Table IV, show that acetate carbon was incorporated in significant amounts only into the guanidine carbons of the streptidine fraction. This was also the site of incorporation of most of the activity from the glycine fermentations. However, in contrast to acetate, glycine carbon was also incorporated into streptobiosamine. This streptobiosamine activity must reside in the N-methylglucosamine fraction, since distillation of the streptose moiety as maltol[23] disclosed no detectable radioactivity in the maltol fractions.

TABLE IV

DISTRIBUTION OF SUBSTRATE CARBON IN STREPTOMYCIN

Substrate	Streptidine, dis./min./mg.	Streptamine	Streptose	Streptobiosamine, dis./min./mg.[a]
CH_3–*COOH	4070	0	0	130
*CH_3–COOH	2130	0	0	130
H_2N–CH_2–*COOH	3410	0	0	770
H_2N–*CH_2–COOH	4070	0	0	1850

[a] The radioactivity in the streptobiosamine fraction may be due to incomplete separation of the labeled streptidine.

The evidence obtained in this work indicates that the function of glycine in the production of higher streptomycin titers, under the fermentation conditions employed, appears to be concerned with some phase of cell metabolism, rather than as a precursor for part of the streptomycin molecule itself. In view of the dilution of radioactivity at the labeled position, any incorporation of glycine and acetate carbons into streptomycin can be presumed to be incidental to the principal synthetic sequence starting from glucose. Under the fermentation conditions employed, *this strain is capable of utilizing glucose as the sole source of streptomycin carbon.*

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(22) J. Fried, G. A. Boyack and O. Wintersteiner, *J. Biol. Chem.*, **162**, 391 (1946).

(23) G. E. Boxer, *et al.*, *ibid.*, **169**, 153 (1947).