The Biosynthesis of Penicillin

2. THE INCORPORATION OF CYSTINE INTO PENICILLIN

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In the preceding paper (Arnstein & Grant, 1954) it was shown that [β-^{14}C]cystine is utilized by the mould *Penicillium chrysogenum* for penicillin formation. It was found that the radioactivity in the penicillin molecule was located almost entirely at position 5, which is the carbon atom common to the thiazolidine and β-lactam rings. Furthermore, L-cystine, which has the same configuration as the relevant carbon atom (6) of penicillin was a far better precursor than the D enantiomorph. It seemed likely, therefore, that cystine, after reduction to cysteine, is used directly for penicillin biosynthesis. However, the possibility of a less direct utilization of cystine, involving the formation of other intermediates, could not be excluded. In particular, since the above experiments were carried out with cystine labelled only with isotopic carbon, the results are also compatible with an initial conversion of cysteine into β, β-dimethyllanthionine, cleavage of this intermediate to penicillamine and serine and subsequent condensation of these two amino acids to give the thiazolidine-β-lactam ring structure of penicillin.

It was expected that further information about the pathway of penicillin biosynthesis might be obtained by using cystine labelled with isotopic nitrogen and sulphur as well as carbon. The present experiments with D- and L-[β-^{14}C, ^{35}S, ^{15}N]cystine, some of which have been briefly described in a preliminary communication (Arnstein & Grant, 1953), indicate that cysteine is used intact for penicillin formation.

EXPERIMENTAL

Fermentations

Organism and preparation of spore inoculum. Details of the strain of *Penicillium chrysogenum*, the preparation and storage of the master cultures have been described (Arnstein & Grant, 1954). Subcultures were prepared by inoculating molasses agar with spores from the master cultures (Perret, 1953). After 10 days at 24°, the spores growing on the mycelial felt (36 sq.in. area) were washed off with 40 ml. of 1% Teepol (Shell Chemicals Ltd., London) in 0·1 M potassium phosphate buffer (pH 6·9). After centrifuging, the spores were resuspended in fresh buffer (25 ml.) and used as inoculum of the fermentation medium.

Fermentation apparatus. The design was essentially that described by Brown & Peterson (1950), but the apparatus was scaled down to hold 1–1·5 l. of medium. In order to avoid leakage of effluent air, which contained $^{14}CO_2$, into the atmosphere, a seal running in liquid paraffin was attached to the propeller drive shaft. Aeration of the medium was carried out through a ring sparger. The incoming air was sterilized by passage through a cotton-wool filter (23 × 4·5 cm. diameter). The effluent air was passed through a second cotton-wool filter (10 × 4 cm. diameter). CO_2 was absorbed by 40% (w/v) NaOH (2 × 50 ml./day) contained in two gas-scrubbing bottles.

Details of medium and fermentations. The basal medium used was the synthetic medium of Jarvis & Johnson (1950), supplemented with 0·05% Bacto yeast extract (Difco Laboratories Inc., Detroit, U.S.A.). The inorganic salts were dissolved in water and steam-sterilized in the fermentation vessel, which had been connected to the air filters, at 20 lb./sq.in. for approx. 15 min. The organic constituents were Seitz-filtered and added aseptically followed by the inoculum. Ammonium phenylacetate (10·5% (w/v) in water) was added to a final concentration in the fermentation medium of 0·2% (w/v) every 24 hr. starting 36 hr. after inoculation. The fermentation was carried out at 24 ± 1°. The solution was stirred at 540 rev./min. and aerated with sterile air at the rate of 1 vol. air/vol. medium/min. Foaming was controlled by adding a steam-sterilized aqueous silicone emulsion (compound F4003, Midland Silicones Ltd., London) when required.

Bioassay. The cup-plate method with *Bacillus subtilis*, I.C.I. strain (NCTC 8241), as test organism (Humphrey & Lightbown, 1952) was used. When penicillin *N*-ethylpiperidine salt was assayed, a sample of pure benzylpenicillin which had been converted into the *N*-ethylpiperidine salt and recrystallized three times from chloroform–acetone was used as standard.

Isolation and degradation methods

Isolation and purification of penicillin. At the end of the fermentation, usually about 130–140 hr., the broth was cooled in ice and the pH was adjusted to 7 with 50% (w/v) phosphoric acid. The mycelium was filtered off and washed thoroughly with 0·05 M potassium phosphate buffer (pH 6·9). The filtered broth and washings were adjusted to pH 2–2·5 with 50% (w/v) phosphoric acid, penicillin was extracted with pentyl acetate and concentrated by successive extractions between ether and buffer (Lester Smith & Hockenhull, 1952), all operations being carried out at 4°. Finally, *N*-ethylpiperidine was added to a solution of the free acid in dry ether (10 ml.) and the *N*-ethylpiperidine salt was recrystallized from chloroform–acetone to constant radio-

activity. Usually, the yield was 50–60% of the penicillin present in the broth as calculated by bioassay. The purity of the product was estimated by its biological potency and by the inverse isotopic dilution method (cf. Radin, 1947), i.e. by measuring its radioactivity after accurate dilution of a small portion with pure nonradioactive benzylpenicillin *N*-ethylpiperidine salt.

Degradation of penicillin. The numbering of the thiazolidine-β-lactam rings of penicillin and the essential details of the degradation method have been given in the preceding paper (Arnstein & Grant, 1954). However, some modifications in the degradation procedure were necessary in order to locate and estimate the radioactivity due to ^{35}S and the relative abundance of the ^{15}N.

To determine whether the radioactivity of the penicillamine portion was wholly associated with the sulphur atom, the radioactivity of isopropylidenepenicillamine hydrochloride was measured directly and compared with that of the sulphur, which was converted into benzidine sulphate as described below.

The relative abundance of ^{15}N was measured on the nitrogen of the isopropylidene derivative of penicillamine and the 2-naphthalenesulphonyl derivative of glycine. The glycine was obtained by further degradation of penilloaldehyde and is derived from $C_{(5)}$, $C_{(6)}$ and the side-chain N of penicillin. The 2-naphthalenesulphonyl derivative (Abraham, Baker, Chain & Robinson, 1943) was recrystallized from hot water to constant radioactivity and also used to estimate the ^{14}C in $(C_{(5)} + C_{(6)})$ of penicillin.

Cystine and serine from mycelium. In some of the present experiments cystine was isolated as cysteic acid and the β-carbon atom of serine as the formaldehyde dimedon by the following procedures. Dried mycelium (17 g.) was continously extracted with ether for 24 hr. The mycelium was then extracted three times with 6% (w/v) trichloroacetic acid at 100°; the remaining solid was extracted with boiling ethanol and ether and dried at 80°. The dried residue (8 g.) was hydrolysed with a mixture of equal volumes of 12 N-HCl and formic acid (50 ml.) for 24 hr. at 105°. Excess of acid was removed *in vacuo* and the amino acids were precipitated with $HgCl_2$ (Campbell & Work, 1952; cf. Neuberg & Kerb, 1912) and regenerated from the ppt. with H_2S. The solution was made normal with respect to HCl, warmed to 60° and treated with an excess of sat. aqueous Br_2. After being stirred for 20 min. cystine had been oxidized completely, as shown by a negative nitroprusside test for sulphydryl groups. Excess of bromine was removed by evaporating the solution *in vacuo*. The residue was dissolved in water and the filtered solution was percolated through a column of 60 g. (wet wt.) Zeo-Karb 225 (Permutit Co. Ltd., London) in the hydrogen form. The column was developed with glass-distilled water, the effluent being collected in 7 ml. fractions. Cysteic acid was identified in fractions 4–7 by paper chromatography. No other amino acids were present in these fractions. The appropriate fractions were bulked and in some cases the cysteic acid was estimated quantitatively by the ninhydrin method of Moore & Stein (1948). The yield was approx. 3 mg./g. dry mycelial residue. After addition of non-isotopic carrier the cysteic acid was isolated as the monohydrate by addition of ethanol to a concentrated aqueous solution of the amino acid. On drying at 100° *in vacuo*, anhydrous cysteic acid was obtained. The radioactivity of this material was unchanged by subsequent recrystallization.

After the elution of cysteic acid from the Zeo-Karb column a further 150 ml. water was passed through in order to elute any remaining carbohydrate. The retained amino acids were then eluted with 0·2 M aqueous NH_3 (300 ml.) and the eluate concentrated to dryness *in vacuo*. This mixture, which contained no detectable reducing sugar (such as glucosamine, which might have remained on the column together with the amino acids), was oxidized with periodate (Rees, 1946). The formaldehyde arising from the β-carbon atom of serine was isolated as the dimedon derivative (cf. Arnstein & Neuberger, 1953).

Estimation of the ^{14}C, ^{35}S *and* ^{15}N *isotopes*

Radioactivity measurements. All measurements were carried out with a helium-filled, bell-shaped Geiger-Müller counter, using 'infinite thickness' samples (Popják, 1950). The substances were mounted on 1 sq.cm. Polythene disks or in the case of benzidine sulphate on filter paper (Whatman no. 50) on a 1 sq.cm. perforated stainless steel disk. All counts were compared with those of a ^{14}C-Perspex reference standard obtained from the Radiochemical Centre, Amersham. Radioactivity measurements of ^{35}S were corrected for decay by direct comparison with a subsidiary ^{35}S-standard.

For the assay of ^{35}S as benzidine sulphate enough material to give at least 25 mg. benzidine sulphate (3 mg. S) was digested with Pirie's reagent (Pirie, 1932). The subsequent procedure was essentially similar to that described by Simpson & Tarver (1950), except that the precipitation of benzidine sulphate was carried out in a much smaller volume (2–3 ml.).

Determination of ^{15}N. For ^{15}N assay, samples containing not less than 1 mg. N were digested by the micro-Kjeldahl procedure. The NH_3 was distilled, absorbed in 0·02 N-HCl (5 ml.) and converted into N_2 with alkaline hypobromite (Bentley, 1952; Sprinson & Rittenberg, 1949). Excess of ^{15}N was estimated in a 60° mass spectrometer.

Synthesis of labelled compounds

(All m.p.'s were determined on a micro block and are uncorrected.)

S-*Benzyl*-DL-[^{15}N]*cysteine.* Potassium phthalimide (5 g., 30 atom % ^{15}N excess), freshly distilled ethyl bromomalonate (7·1 g.) and dry xylene (50 ml.) were refluxed at 110–120° for 2 hr. and then heated at 140–145° for 1·5 hr. After 12 hr. at 4°, the solution was filtered and the residue was extracted with cold benzene (2 × 5 ml.). The washed residue was discarded and the filtrate and washings were combined. The solution was evaporated to dryness yielding a yellow oil which crystallized on standing. After addition of non-isotopic ethyl phthalimidomalonate (6·27 g.), the product was recrystallized from ether by adding light petroleum (b.p. 60–80°). The diluted ethyl [^{15}N]phthalimidomalonate (11·38 g.) was dissolved in dry ethanol, the solution was cooled quickly by immersion of the flask in liquid air and a solution of sodium (0·92 g.) in dry ethanol (20 ml.) was added simultaneously. The solvent was removed *in vacuo* giving the sodium derivative of ethyl [^{15}N]phthalimidomalonate, which was condensed with benzylthiomethyl chloride by the method of Wood & du Vigneaud (1939*a*). The product, ethyl benzylthiomethyl-[^{15}N]phthalimidomalonate, on hydrolysis and decarboxylation gave *S*-benzyl-DL-[^{15}N]cysteine (3·13 g., 32% based on the

potassium [^{15}N]phthalimide used; m.p. 217–218°, 14·2 atom % ^{15}N excess). From the mother liquors ^{15}N was recovered as $^{15}NH_3$ by Kjeldahl digestion and distillation.

*S-Benzyl-N-formyl-*DL-[*β*-^{14}C *or* ^{15}N]*cysteine.* DL-[*β*-^{14}C]-cystine, which had been synthesized by Dr J. C. Crawhall (Arnstein & Crawhall, 1953), and *S*-benzyl-DL-[^{15}N]cysteine were converted into the *S*-benzyl-*N*-formyl derivative as described by Wood & du Vigneaud (1939*b*).

N, S-*Diacetyl-*DL-[^{35}S]*cysteine.* [^{35}S]Thioacetic acid (ethanethiolic acid) (approx. 1·52 g., 20 m-moles, 71 mc; obtained from the Radiochemical Centre, Amersham) was distilled in high vacuum (0·001 mm. Hg) into a thick-walled Carius tube, which contained α-acetamidoacrylic acid (5·18 g., 40 m-moles; Bergmann & Grafe, 1930), cooled in liquid air (cf. Behringer, 1948). The tube was sealed and heated at 100° for 2 hr. and 120° for 1·5 hr. At the end of the reaction any volatile residue was removed by vacuum distillation at 0·001 mm. Hg and 100° into a trap cooled in liquid air. The solid product was repeatedly extracted with dry, boiling $CHCl_3$ (8 × 25 ml.), the $CHCl_3$ being removed through a filter stick and collected in a receiver to which two alkaline permanganate traps were attached in order to absorb any volatile material containing ^{35}S. The $CHCl_3$ extracts were evaporated in a stream of air to exactly 100 ml. To 5 ml. of this solution light petroleum (b.p. 60–80°) was added and the crystals formed after 48 hr. at 0° were filtered off. The material was recrystallized from ethyl acetate (yield, 169·1 mg.) and assayed for radioactivity after dilution with non-isotopic diacetyl-DL-cysteine and recrystallization from ethyl acetate to constant radioactivity. The calculated specific radioactivity of the undiluted material was 17·4 μc/mg.

DL-[^{35}S]*Cystine.* The main portion of the above $CHCl_3$ solution (95 ml. containing 55·8 mc, as calculated by assay of the diluted material) was evaporated to 30 ml. Light petroleum (10 ml.; b.p. 60–80°) was added slowly, and after 72 hr. the supernatant liquid was removed with a filter stick. After drying in a stream of air, the product was refluxed for 3 hr. with 40 ml. of a mixture of 4 N-HCl and formic acid (1:1, v/v). The acid was removed by repeated evaporation *in vacuo* at 100° and the solid residue dissolved in water (approx. 30 ml.). The pH was adjusted to 8 with conc. ammonia (sp.gr. 0·88), 2 drops of 1 % (w/v) aqueous $FeCl_3$ were added and a slow stream of air was passed through the solution for 8 hr. Acetic acid was then added to pH 4·5 and after 72 hr. at 4° the pale-buff solid was collected. It was purified by dissolving in 2 N-HCl (10 ml.), treating with charcoal and neutralizing the filtrate to pH 4·5 with 6 N aqueous NH_3 and 3 N sodium acetate. The white ppt. of DL-[^{35}S]cystine was centrifuged after 72 hr. at 4°, washed with water (2 × 5 ml.), ethanol (2 × 10 ml.) and ether (20 ml.) (yield: 0·98 g., 28·8 mc). The purity of this substance was checked by paper chromatography after conversion into cysteic acid, which showed the presence of a single radioactive peak coinciding with the ninhydrin-positive spot.

Resolution of the isotopically labelled cystines

The separation of the salts of a racemic acid with an optically active base by crystallization is usually incomplete, but the more insoluble diastereoisomer can be obtained in reasonable yield and purity by repeated recrystallization. The mother liquors then contain all the more soluble salt contaminated with a small amount of the insoluble salt. When the racemic compound is isotopically labelled, it is possible to replace the unwanted labelled diastereoisomer in the mother liquors by its non-isotopic counterpart by crystallization of the mixture after addition of a large excess of the required unlabelled diastereoisomer. By this method the total amount of the contaminating diastereoisomer in solution remains unchanged, but the amount of isotope which it contains can be reduced to any desired extent by repeating the above procedure. Thus, if M is the mass of labelled contaminant of isotope content x originally present and M' is the mass of unlabelled diastereoisomer added each time, then the isotope content, y, of the contaminant remaining after n crystallizations from a constant volume is given by the formula, $y = x\,[M/(M+M')]^n$.

Since x and y can be measured and M' is known, it is possible to use this equation, when $n = 1$, to calculate the mass, M, of contaminant originally present, i.e. $M = M'y/(x-y)$.

This method, which has been used for the resolution of labelled leucine (Schoenheimer, Ratner & Rittenberg, 1939), was applied to the resolution of cystine labelled with ^{14}C, ^{15}N or ^{35}S described below.

Resolution of [*β*-^{14}C]*cystine.* *S*-Benzyl-*N*-formyl-DL-[*β*-^{14}C]cystine (2·71 g., 63·1 μc) and anhydrous brucine (4·4 g.) were dissolved in *n*-butanol (20 ml.) at 70°. A small amount of insoluble residue was filtered off and discarded after washing with butanol (2 × 2·5 ml.). The crystals which had formed in the filtrate (25 ml.) after 72 hr. at 19° were isolated and the mother liquors kept for a further 72 hr. at 0°. The small second crop was combined with the first and recrystallized four times from butanol (20 ml.). All mother liquors except that from the final recrystallization were combined and retained. The yield of butanol-insoluble material, i.e. the brucine salt of *S*-benzyl-*N*-formyl-D-[*β*-^{14}C]cysteine, was 2·93 g. (26·4 μc; $[\alpha]_D^{19} = -24{\cdot}5°$ in water (*c*, 0·92); cf. Wood & du Vigneaud, 1939*b*). The conversion of this substance into D-[*β*-^{14}C]cystine was carried out essentially as described by Wood & du Vigneaud (1939*b*). The cystine thus obtained was dissolved in 2 N-HCl (3 ml.) and the solution was treated with charcoal (10 mg.), which was filtered off and washed with 2 N-HCl (2 × 1 ml.). The filtrate and washings were combined, 2 N aqueous NH_3 was added to pH 4·5 and the precipitated cystine was washed with water (2 ml.). Yield of D-[*β*-^{14}C]-cystine, 281 mg., 13·2 μc, $[\alpha]_D^{21} = +217{\cdot}3 \pm 1°$ in N-HCl (*c*, 0·549).

The combined mother liquors (approx. 80 ml.) containing the 'butanol-soluble' fraction, i.e. the brucine salt of *S*-benzyl-*N*-formyl-L-[*β*-^{14}C]cysteine contaminated with a small amount of the corresponding derivative of the D isomer, were evaporated to 25 ml. in a stream of air at 70°. The solution remained clear on keeping for 24 hr. at 0°. Non-isotopic *S*-benzyl-*N*-formyl-D-cysteine brucine salt (817·4 mg.) was then dissolved in the above solution by warming. After 72 hr. at 0°, the crystals were filtered off and recrystallized from butanol to constant radioactivity. The above procedure was repeated twice, using 761·5 and 892·2 mg. of non-isotopic *S*-benzyl-*N*-formyl-D-cysteine brucine salt. The radioactivity of the *S*-benzyl-*N*-formyl-D-[*β*-^{14}C]cysteine present as a contaminant of the corresponding derivative of the L-[*β*-^{14}C]cysteine in the butanol solution was thus reduced successively as shown in Table 1. The butanol solution remaining after the third dilution with

Table 1. *Purification of* S-*benzyl*-N-*formyl*-L-[β-^{14}C]*cysteine brucine salt by repeated dilution of the corresponding* D-[β-^{14}C]*cysteine derivative with non-isotopic carrier*

S-Benzyl-*N*-formyl-D-cysteine brucine salt was added to dilute the radioactivity of the corresponding ^{14}C-labelled compound, most of which was then removed by crystallization from *n*-butanol (25 ml.). By repeating this procedure, the radioactivity of the *S*-benzyl-*N*-formyl-D-[β-^{14}C]cysteine brucine salt remaining in solution after the third crystallization was only 5% of the original and 0·1% of that of the corresponding derivative of L-[β-^{14}C]cysteine. Details of the experimental procedure and the method for calculating the results are given in the text.

Amount of unlabelled *S*-benzyl-*N*-formyl-D-cysteine brucine salt added (g.) M'	Specific radioactivity of the D-[β-^{14}C]-cysteine derivative in solution before addition of unlabelled carrier (M') (μC/g.) x	Specific radioactivity of the D-[β-^{14}C]-cysteine derivative isolated by crystallization after addition of unlabelled carrier (μC/g.) y	Amount of brucine salt of D-[β-^{14}C]-cysteine derivative in solution (g.) $M=\frac{M'y}{x-y}$	Total radioactivity associated with the D-[β-^{14}C]cysteine derivative (M) in solution (μC) My
0·8174	9·00	2·17	0·260	0·564
0·7615	2·17	0·507	0·227	0·118
0·8922	0·507	0·114	0·260	0·030

Table 2. *Purification of* N, S-*diacetyl*-D-[^{35}S] *cysteine brucine salt by repeated dilution of the corresponding* L-[^{35}S]*cysteine derivative with non-isotopic carrier*

Experimental details, calculation of the results and definition of the symbols are given in the text. A similar experiment with *S*-benzyl-*N*-formyl-L-[β-^{14}C]cysteine is described in Table 1.

Amount of unlabelled *N*, *S*-diacetyl-L-cysteine brucine salt added (g.) M'	Volume of butanol solution (ml.)	Specific radioactivity of the L-[^{35}S]cysteine derivative in solution before addition of unlabelled carrier (M') (μC/g.) x	Specific radioactivity of the L-[^{35}S]cysteine derivative isolated by crystallization after addition of unlabelled carrier (μC/g.) y	Amount of L-[^{35}S]cysteine derivative in solution (g.) $M=\frac{M'y}{x-y}$	Total radioactivity associated with the L-[^{35}S]cysteine derivative (M) in solution (μC) My
1·468	25	230	48	0·387	18·6
0·528	25	48	20	0·377	7·5
0·689	20	20	5·5	0·261	1·4

non-isotopic brucine salt was evaporated to dryness in a stream of air at 70° and finally *in vacuo*. The yellow, glass-like residue (3·41 g.) contained 258 mg. of the brucine salt of *S*-benzyl-*N*-formyl-D-[β-^{14}C]cysteine (0·029 μC, Table 1) and hence the amount of brucine salt of the L-[β-^{14}C]cysteine derivative was 3·15 g. (28·3 μC, calculated from the specific radioactivity of this salt which must be identical with that of the original pure brucine salt of the D-[β-^{14}C]cysteine derivative, i.e. 9·0 μC/g., cf. Table 1). The contamination of the *S*-benzyl-*N*-formyl-L-[β-^{14}C]cysteine brucine salt by radioactivity due to corresponding D-[β-^{14}C]cysteine derivative was therefore approx. 0·1%.

L-[β-^{14}C]Cystine was isolated from the above derivative as previously described for the D enantiomorph. Yield: 310 mg., 13·2 μC, $[\alpha]_D^{21} = -161{\cdot}0 \pm 0{\cdot}70°$ in N-HCl (*c*, 0·7). The optical rotation indicated that this material was a mixture containing 87% L- and 13% D-cystine. However, from the composition of the brucine salt (see above and Table 1) the amount of D-cystine present was expected to be only 7·6%. The difference between the radioactivity of this substance and that of the pure D-[β-^{14}C]cystine was 8·4%, which agrees well with the latter figure, since the D-cystine in the mixture has an almost negligible radioactivity. No definite explanation for the small but significant discrepancy between the optical and isotopic measurements can at present be put forward, but it is possible that a certain amount of racemization occurred during the hydrolysis of the *S*-benzyl-*N*-formyl-cysteine. The presence of occluded salt, which seemed at first another possible explanation, was excluded by X-ray powder photographs. Furthermore, such an impurity would decrease the specific radioactivity as well as the optical rotation of the cystine.

Resolution of [^{15}N]*cystine*. *S*-Benzyl-*N*-formyl-DL-[^{15}N]-cysteine was resolved exactly as previously described for [β-^{14}C]cystine. The D-[^{15}N]cystine contained 14·2 atom % ^{15}N excess and had $[\alpha]_D^{21} = +218{\cdot}4 \pm 1°$ in N-HCl (*c*, 0·568). The 'L-cystine' fraction had $[\alpha]_D^{21} = -148{\cdot}1 \pm 0{\cdot}7°$ in N-HCl (*c*, 0·562). It was calculated to contain 16% of the D enantiomorph by measurement of the optical rotation or 13% by the isotope dilution procedure given above. The isotope content of this fraction was 12·3 atom % ^{15}N excess by direct analysis and the composition of the mixture was 0·083 g. D-[^{15}N]cystine (0·96 atom % excess) and 0·6226 g. L-[^{15}N]cystine (14·2 atom % excess).

Resolution of [^{35}S]*cystine*. In trial experiments with non-isotopic material it was found that a solution of *N*, *S*-diacetyl-DL-cysteine brucine salts in *n*-butanol (0·1 g. *N*, *S*-diacetyl-DL-cysteine/ml.) on cooling gave the brucine salt of *N*, *S*-diacetyl-L-cysteine in 95% yield. After recrystallization from *n*-butanol to constant rotation, this salt

had m.p. 171–172°, $[\alpha]_D^{22} = -28{\cdot}4°$ in water (*c*, 1·974). Its solubility in butanol was 0·460 g./100 ml. at 0°. Conversion into the free amino acid afforded pure L-cystine, $[\alpha]_D^{23} = -223{\cdot}1 \pm 1°$ in N-HCl (*c*, 0·674).

N, *S*-Diacetyl-DL-[^{35}S]cysteine (2·50 g., 1·57 mc) and anhydrous brucine (4·81 g.) were dissolved in warm butanol (25 ml.). After 72 hr. at 4°, the crystalline solid was separated and recrystallized twice from butanol (12 ml.). The combined mother liquors were concentrated to 25 ml. in a stream of air at 70° and kept at 0° for 104 hr., giving a small amount of crystalline material which was added to the first crop. After three recrystallizations from butanol (12 ml.), the yield of *N*, *S*-diacetyl-L-[^{35}S]cysteine brucine salt was 2·86 g. (0·635 mc). The salt was dissolved in N aqueous NH_3 (30 ml.) and the precipitated brucine washed with 0·5 N aqueous NH_3 (3 × 10 ml.). The solution and washings were evaporated *in vacuo* at 50° to remove the NH_3 and the crude *N*, *S*-diacetyl-L-[^{35}S]cysteine was hydrolysed to cystine, which was purified as described above. Yield: 353 mg., 288 μc; $[\alpha]_D^{20} = -221 \pm 1°$ in N-HCl (*c*, 0·340).

To the mother liquors remaining after removal of the second crop of the *N*, *S*-diacetyl-L-[^{35}S]cysteine brucine salt were added successively three amounts of the corresponding non-isotopic compound (Table 2), essentially as described for the resolution of the [β-^{14}C]cystine. The butanol solution remaining after the third dilution with non-isotopic brucine salt was evaporated to dryness in a stream of air at 70° and finally *in vacuo*, giving a glass-like yellow gum (3·272 g.). This material was a mixture of the brucine salts of *N*, *S*-diacetyl-L-[^{35}S]cysteine (261 mg., 1·4 μc) and *N*, *S*-diacetyl-D-[^{35}S]cysteine (3·011 g., 692·5 μc). It was converted by methods already described into 'D-[^{35}S]cystine' (424·1 mg., 389 μc), which had $[\alpha]_D^{21} = +167{\cdot}8 \pm 0{\cdot}7°$ in N-HCl (*c*, 0·070) and thus contained 11% of the L enantiomorph. This value is in fair agreement with that of 8% calculated by the dilution technique. It can also be calculated that only approx. 0·09% of the total radioactivity of this material was associated with the L enantiomorph.

RESULTS

The present experiments (Table 3), as well as the results previously obtained with small-scale fermentations (Arnstein & Grant, 1954), show that L-cystine is used much more efficiently for penicillin biosynthesis than the D enantiomorph. As in the earlier work, degradation of the labelled penicillin (Table 4) showed that the β-carbon atom of cystine was incorporated exclusively into $C_{(5)}$ of penicillin. It was also found that the penicillamine moiety contained practically no isotopic nitrogen but that all the ^{15}N was incorporated into the side-chain

Table 3. *Conversion of* L- *and* D-*cystine, labelled with* ^{14}C, ^{15}N *and* ^{35}S, *into penicillin and mycelial cystine*

Solutions of L-[β-^{14}C, ^{15}N, ^{35}S]cystine (total 267·5 mg.; 4·22 μc ^{14}C, 3·11 atom % excess ^{15}N, 8·37 μc ^{35}S) or D-[β-^{14}C, ^{15}N, ^{35}S]cystine (total 267·5 mg.; 4·29 μc ^{14}C, 4·10 atom % excess ^{15}N, 9·56 μc ^{35}S) in N-HCl (about 3 ml.) were sterilized by Seitz-filtration and the filter was washed with N-HCl to give a final vol. of filtrate + washings of 5 ml. 1 ml. portions were added every 24 hr. to identical fermentations, the first addition being made at zero time, the last at 96 hr. after inoculation of the medium. At the end of the fermentation, i.e. 22 hr. after the final addition of cystine, the yields of sodium benzylpenicillin, calculated by bioassay, were 239 mg. and 120 mg., respectively. The amount of radioactivity incorporated into penicillin from the labelled L-cystine was 0·315 μc ^{14}C (7·5%) and 0·751 μc ^{35}S (9·0%), that from the D-cystine was 0·034 μc ^{14}C (0·79%) and 0·15 μc ^{35}S (1·6%). Penicillin was isolated from the broth as described in the text. The potencies of the penicillin *N*-ethylpiperidine salt in the L- and D-cystine experiments were respectively 1·037 and 1·019 times that of the corresponding derivative of International Standard penicillin (1670 i.u./mg.), the fiducial limits of the assay being ±1·4% ($P < 0{\cdot}05$). The standard error of all radioactivity assays was less than 5%. ^{15}N assays were accurate to ±0·003 atom % excess. The ^{15}N results in columns 3 and 4 were calculated from assays of material which had been diluted with carrier and are therefore less accurate, as indicated. The figures in parentheses in column 6 are the range of the dilution of ^{15}N incorporation into mycelial cystine (*A*/*C*). Other details are given in the Experimental section.

	Specific radioactivity (μc/m-mole) or atom % excess ^{15}N			Dilution of isotope	
Isotope	Cystine* added to fermentation (*A*)	Penicillin isolated (*B*)	Cysteic acid derived from mycelial cystine (*C*)	Penicillin (*A*/*B*)	Mycelial cystine (*A*/*C*)
	L-Cystine experiment				
^{14}C	1·91	0·47	0·35†	4·1	5·5
^{15}N	3·11 ± 0·003	0·83‡ ± 0·01	0·52 ± 0·09	3·75 ± 0·05	6·0 (5·1–7·25)
^{35}S	3·79	1·12	0·77	3·3	4·9
	D-Cystine experiment				
^{14}C	1·94	0·10	0·08†	19·4	24·2
^{15}N	4·09 ± 0·003	0·16‡ ± 0·01	0·10 ± 0·085	25·6 ± 1·6	40·9 (22·1–273)
^{35}S	4·33	0·44	0·285	9·9	15·2

* The specific radioactivity of this substance is given as μc/m-mole cysteine. Where necessary, the specific radioactivity and ^{15}N content of the cystine used are corrected for the presence of the optical enantiomorph, which was practically free of isotopic tracer (see Experimental section).

† Calculated by difference from the radioactivity of cysteic acid and that of the cysteic acid sulphur after conversion into benzidine sulphate.

‡ Calculated for one N atom being derived from the precursor (see degradation, Table 4).

Table 4. *Degradation of labelled penicillin isolated from fermentations containing* L- *or* D-[β-^{14}C, ^{15}N, ^{35}S]*cystine*

Details of the fermentations, assay and degradation are given in Table 3 and in the Experimental section. In order to compare the ^{15}N content of different compounds, the ^{15}N results are given on the basis of all the isotope being located in one N atom, i.e. the atom % ^{15}N excess in the compounds was multiplied by the number of nitrogen atoms. For numbering of the penicillin molecule see Arnstein & Grant (1954).

Compound	Atom number in thiazolidine and β-lactam rings of penicillin	Specific radioactivity (μc/m-mole) or atom % excess ^{15}N: L-Cystine expt. ^{14}C, ^{35}S	L-Cystine expt. ^{15}N	D-Cystine expt. ^{14}C, ^{35}S	D-Cystine expt. ^{15}N
Benzylpenicillin *N*-ethylpiperidine salt	All	1·59	0·83±0·01	0·54	0·16±0·01
$BaCO_3$ from acid-labile CO_2	7	Inactive	—	Inactive	—
Isopropylidenepenicillamine HCl	(1+2+3)	1·13	0·04	0·44	None
Benzidine sulphate	1	1·11	—	0·43	—
Benzylpenilloaldehyde 2:4-dinitrophenylhydrazone	(5+6)	0·47	0·80±0·015	0·12	0·165±0·015
2-Naphthalenesulphonylglycine	(5+6)	0·44	0·80±0·003	0·09	Not determined
$BaCO_3$ from glycine carboxyl carbon atom	5	0·47	—	0·10	—
Formaldehyde dimedon from glycine α-carbon atom	6	Inactive	—	Inactive	—

Table 5. *Conversion of* L-[β-^{14}C, ^{35}S]*cystine into penicillin and into mycelial cystine and serine*

The experimental conditions were similar to those described in Table 3, but less labelled cystine was used. L-[β-^{14}C, ^{35}S]-Cystine (34·6 mg., containing 1·7 mg. almost completely non-radioactive D-cystine, present in the ^{14}C-labelled cystine as explained in the Experimental section; 0·546 μc ^{14}C, 0·507 μc ^{35}S) was divided into six equal portions which were added every 24 hr. starting at zero time. After 139 hr. the fermentation medium contained 345000 i.u. corresponding to 202 mg. sodium benzylpenicillin (0·0822 μc ^{14}C, 0·0817 μc ^{35}S). Penicillin was isolated in the usual way. Cystine was isolated from the mycelium as cysteic acid and the radioactivity in the β-carbon atom of the mycelial serine was determined as described in the Experimental section. The standard error of all radioactivity determinations was less than 5%.

Isotope	Specific radioactivity (μc/m-mole): Cystine added to fermentation* (*A*)	Penicillin isolated (*B*)	Cysteic acid derived from mycelial cystine (*C*)	Formaldehyde dimedon from β-carbon atom of mycelial serine (*D*)	Dilution of isotope: Penicillin (*A*/*B*)	Mycelial cystine (*A*/*C*)	β-Carbon atom of mycelial serine (*A*/*D*)
^{14}C	2·01	0·145†	0·087‡	0·0035	14·3	23·0	572
^{35}S	1·87	0·144	0·093	—	13·0	20·1	—

* Calculated as μc/m-mole cysteine.

† Calculated by difference from the molar radioactivity of penicillin *N*-ethylpiperidine salt and that of benzidine sulphate (Table 6).

‡ Calculated by difference from the radioactivity of cysteic acid and that of the cysteic acid sulphur after conversion into benzidine sulphate.

Table 6. *Degradation of labelled penicillin isolated from a fermentation containing* L-[β-^{14}C, ^{35}S]*cystine*

Details of the fermentation and degradation are given in Table 5 and in the Experimental section. The numbering of the penicillin atoms is as in Table 4. For the degradation 29·0 mg. of the radioactive penicillin *N*-ethylpiperidine salt was diluted with 170·0 mg. non-radioactive carrier. The figures in parentheses in column 3 are the calculated radioactivities corresponding to the undiluted penicillin.

Compound	Atom number in thiazolidine and β-lactam rings of penicillin	Specific radioactivity (μc/m-mole)
Penicillin *N*-ethylpiperidine salt	All	0·0416 (0·286)
Isopropylidenepenicillamine HCl	(1+2+3)	0·0220 (0·151)
Benzidine sulphate	1	0·0206 (0·141)
Benzylpenilloaldehyde 2:4-dinitrophenylhydrazone	(5+6)	0·0199 (0·137)

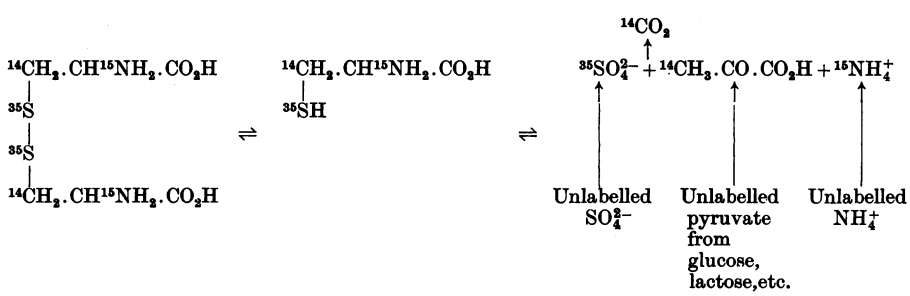
amide group. As expected, the penicillin also contained radioactive sulphur. In the experiment with L-cystine, labelled with isotopic carbon, nitrogen and sulphur (Table 3), the calculated ratios of isotopes ($^{35}S:^{15}N:^{14}C$) in the precursor and in penicillin were similar, being 1·99:1·63:1 and 2·49:1·77:1, respectively. The $^{15}N:^{14}C$ ratios show excellent agreement, but the sulphur of the added cystine appeared to be converted into penicillin with a significantly lower dilution than the other isotopes, resulting in an unexpectedly high $^{35}S:^{14}C$ ratio. In this experiment, the cystine isolated as cysteic acid from the mycelium at the end of the fermentation was found to contain ^{35}S, ^{15}N and ^{14}C in the ratio 2·43:1·64:1, which is essentially the same as that in the penicillin. However, the incorporation of all three isotopes into the mycelial cystine was significantly less than into penicillin and a similar result was obtained in the experiments with similarly labelled D-cystine and also with L-[β-^{14}C, ^{35}S]cystine added to a fermentation in much smaller amount (Table 5). In the latter experiment (Tables 5 and 6) the $^{35}S:^{14}C$ isotope ratios in the precursor, in cystine from the mycelium and in the penicillin showed a somewhat better agreement (0·93, 1·07 and 0·99, respectively), although the sulphur isotope was still to some extent preferentially utilized. The radioactivity of the β-carbon atom of the serine present in the mycelium was also determined and found to be only about 4% of the radioactivity due to ^{14}C in the cystine from the same source and 2·4% of that of the $C_{(5)}$ of the isolated penicillin.

DISCUSSION

The efficient utilization of L-cystine, labelled with ^{14}C in the β-position, for penicillin biosynthesis, which was shown in the preceding paper (Arnstein & Grant, 1954), suggested that this amino acid may be an immediate precursor of penicillin. Since penicillin formation in this case would involve only reactions in which the cystine molecule remains intact apart from a reduction to cysteine, L-[β-^{14}C, ^{15}N, ^{35}S]cystine should give rise to penicillin in which the isotopic carbon, nitrogen and sulphur are located at carbon-atom 5, the nitrogen of the side-chain amide group and the sulphur atom, respectively. Furthermore, the three isotopes should be incorporated into these positions to an equal extent.

In the present work, degradation of the penicillin obtained in such an experiment showed that the isotopes were indeed located in the expected positions. However, only the carbon and nitrogen isotopes were incorporated with similar dilutions. In all experiments, a small but significant excess of radioactive sulphur relative to the other isotopes was found in the penicillin. The unexpectedly high utilization of cystine sulphur may be due to the endogenous synthesis of cystine containing radioactive sulphur but relatively little isotopic carbon or nitrogen.

One mechanism by which cystine labelled originally with isotopic sulphur, carbon and nitrogen might be converted into cystine containing relatively more isotopic sulphur than carbon and nitrogen is illustrated below.



In the experiment with labelled D-cystine (Table 3), the $^{35}S:^{15}N:^{14}C$ ratios in the added cystine, in the penicillin and in the mycelial cystine were found to be 2·24:2·11:1, 4·4:1·6:1 and 3·56:1·25:1, respectively. It appears that in this case the isotopic sulphur is converted into penicillin and into the L-cystine of the mycelium with about twice the efficiency of the utilization of ^{14}C. The change in the $^{15}N:^{14}C$ ratio also indicates that relative to the carbon some of the nitrogen is lost during the conversion of the D-cystine into penicillin or into the mycelial cystine.

It is known (cf. Arnstein & Grant, 1954) that cystine is oxidized to carbon dioxide during the fermentation and it is likely that in this reaction the sulphur of cystine is converted into sulphate, possibly by a pathway similar to that known to occur in animal tissue (Medes & Floyd, 1942; cf. Kearney & Singer, 1953). Sulphate is used efficiently for penicillin biosynthesis (Lester Smith & Hockenhull, 1952) and is, of course, also a precursor of cystine, since it can serve as the sole sulphur source in fermentation media. By analogy with the metabolism of cystine in animal tissues, pyruvate has been assumed to be

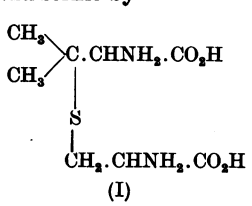
the primary sulphur-free metabolic product, but such an assumption is not essential to the present discussion. The above sequence of reactions leading to the degradation and resynthesis of cystine would be expected to result in a greater dilution of the carbon and nitrogen isotopes than of the sulphur isotope, because much more unlabelled carbon (as glucose, lactose, etc.) and nitrogen (as ammonia) than sulphur (as sulphate) are present in the fermentation medium. Including the relatively small amount of cystine added, the fermentation solution contains approximately 16 g. C, 2 g. N and 0·2 g. S/l.

In support of the above hypothesis that the unexpectedly high incorporation of ^{35}S into penicillin is due to re-incorporation of radioactive sulphate during cystine synthesis, it was found that the cystine present in the mycelium at the end of the fermentation also contained a definite excess of radioactive sulphur, compared with the carbon and nitrogen isotopes. In fact, the $^{35}S:^{15}N:^{14}C$ ratios in the mycelial cystine and in the relevant atoms of penicillin were practically the same. Moreover, a decrease in the quantity of labelled cystine added from 1·0 to 0·14 m-mole decreased the extent of preferential utilization of the sulphur isotope. It is likely that in this case less of the labelled cystine was oxidized to sulphate. It is also of interest that, at the lower level, cystine was used for penicillin synthesis with about twice the efficiency, 15% of the added ^{14}C being converted into penicillin compared with 7·5% at the higher level.

In the preceding paper (Arnstein & Grant, 1954) it was assumed that the lower specific radioactivity of the penicillin compared with the cystine added to the fermentation was due to the dilution of the labelled amino acid by unlabelled cystine synthesized endogenously by the mould. This interpretation is supported by the present results, which show that the isotope contents of the cystine incorporated into the mycelial protein and of the penicillin are of the same order of magnitude. On the assumption that the added labelled cystine mixes freely with the endogenously produced cystine, it may be calculated that about 2–5 m-moles of cystine are synthesized during the course of a 1 l. fermentation. One explanation of the differences in the absolute values of the isotope contents of the mycelial cystine and of the penicillin, which range from 25 to 50%, may be that protein synthesis occurs mainly during a stage of the fermentation in which the amount of endogenous cystine synthesized is somewhat larger than that produced during the period of penicillin biosynthesis. Most of the cystine incorporated into the mycelial protein would thus have a slightly lower radioactivity than that used for penicillin formation. This interpretation is in agreement with the observation that mycelium formation by *Penicillium chrysogenum* WIS 48–701 on a corn-steep medium is essentially complete before the beginning of penicillin production (cf. Anderson *et al.* 1953).

The poor utilization of D-cystine for mycelial protein and penicillin biosynthesis confirms the earlier work with micro-fermentations, in which D-cystine was found to be a less efficient penicillin precursor than the L enantiomorph. It is probable that D-cystine is converted into L-cystine before it is used for penicillin formation, since the relative incorporation of both enantiomorphs into penicillin and into the cystine of the mycelial protein were very similar. The inversion of D amino acids is well known, especially in animals, which can use the D enantiomorphs of certain essential amino acids for growth. In experiments with ^{15}N-labelled leucine and γ-phenyl-α-aminobutyric acid it has been demonstrated that rats are able to convert the D into the corresponding L amino acids. In this reaction most of the labelled amino nitrogen was lost, presumably because the corresponding keto acids were formed as intermediates (Ratner, Schoenheimer & Rittenberg, 1940; du Vigneaud *et al.* 1939). It is therefore of interest that in the fermentation the conversion of D-cystine into L-cystine takes place largely without loss of the labelled amino group. It is possible that in this case an imino acid is an intermediate or, alternatively, the ammonia formed by deamination may be only in slow equilibrium with the unlabelled ammonia of the fermentation medium. This would result in reamination of the intermediate keto acid with ammonia of substantially the same isotope content as that of the D amino acid.

While the present experiments with labelled cystine indicate that this amino acid is an important penicillin precursor they provide relatively little information about the individual reactions involved in the conversion of cystine into penicillin. The first step consists presumably of a reduction to cysteine, which may then condense with valine or a close derivative of this amino acid. Alternatively, cysteine (or cystine) may first react with phenylacetic acid to give phenylacetylcysteine as an intermediate. Condensation of cysteine and valine may lead either to a dipeptide or to ββ-dimethyllanthionine (I). Since (I) could possibly also arise from penicillamine and serine by



(I)

analogy with the condensation of serine and homocysteine to cystathionine, it was important to

compare cystine and serine as possible intermediates in penicillin biosynthesis. Earlier experiments (Arnstein & Grant, 1954) had shown that labelled serine was converted into penicillin, but with a somewhat higher dilution than cystine. More conclusive evidence that serine is not an intermediate in the synthesis of penicillin from cystine is provided by the observation that the serine present in the mycelial protein of a fermentation to which ^{14}C-labelled cystine had been added was only very weakly radioactive compared with the cystine from the same source or with the isolated penicillin. It is therefore likely that serine is used for penicillin biosynthesis only after conversion into cystine by a substantially irreversible reaction.

The complete elucidation of the mechanism by which cysteine is converted into penicillin must await further experiments. It is of interest, however, that the participation of penicillamine appears to be excluded by the present work, which suggests that the penicillamine moiety of penicillin is derived from valine. Penicillamine should therefore no longer be regarded as a naturally occurring amino acid but solely as an artifact produced by the chemical degradation of penicillin.

SUMMARY

1. The preparation of L- and D-cystine, labelled with ^{14}C in the β-position, ^{15}N or ^{35}S, is described. By appropriate dilution with unlabelled carrier, each optically active form was obtained practically free from labelled enantiomorph.

2. The conversion of L- and D-[β-^{14}C, ^{15}N, ^{35}S]- and L-[β-^{14}C, ^{35}S]-cystine into penicillin and into the cystine of the mycelial protein has been studied.

3. Penicillin and cysteic acid from the mycelial cystine were chemically degraded in order to ascertain the distribution of the isotopes.

4. The labelled L-cystine was a more efficient precursor of penicillin and of mycelial cystine than the D enantiomorph.

5. The ratios of the ^{14}C, ^{15}N and ^{35}S isotopes in the mycelial cystine and in the labelled atoms of penicillin were identical and similar to that of the L-cystine which had been added to the fermentation.

6. It is concluded that cystine is a direct precursor of penicillin, probably after reduction to cysteine. Its role in penicillin biosynthesis is discussed.

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