investigated in studies of the kinetics of oxygen uptake and analyses of the interconversion of succinate, fumarate, malate and oxaloacetate.

2. Kinetic studies of oxygen uptake revealed that in intact liver mitochondria the rate-determining step of the oxidation of succinate is the rate of phosphorylation of ADP to ATP, whereas in mammary-gland mitochondria the oxidation of succinate is controlled by the rate of removal of oxaloacetate.

3. The overall rate of the removal of oxaloacetate added to liver mitochondria was not affected by a variety of conditions such as the simultaneous oxidation of succinate, or the addition of ADP or 2,4-dinitrophenol. During oxidative phosphorylation of ADP the addition of oxaloacetate inhibited the oxidation of succinate only to a limited extent, but, on disruption of the energy supply by the addition of 2,4-dinitrophenol, oxaloacetate completely inhibited the oxidation of succinate. Mammary-gland mitochondria metabolize oxaloacetate more slowly than do liver mitochondria, and in this system the oxidation of succinate is more readily inhibited by oxaloacetate.

4. The time-course of the inhibition of the oxidation of succinate by the addition of oxaloacetate and 2,4-dinitrophenol, as well as the stimulation of respiration on the addition of oxaloacetate to mitochondria in 'state 4', indicates that there is an energy requirement for the local removal of oxaloacetate in the vicinity of the succinate-oxidase sites.

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Studies in the Biosynthesis of Fungal Metabolites

5. THE UTILIZATION OF SULPHUR-CONTAINING AMINO ACIDS AS PENICILLIN SIDE-CHAIN PRECURSORS*

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During a preliminary survey of the influence of various amino acids on the biosynthesis of penicillins by *Penicillium chrysogenum*, it was observed that methionine stimulated the formation of a new antibiotic that was chromatographically distinct from the penicillins normally produced by this mould. In addition, the methionine analogues

ethionine, *S*-methyleysteine and *S*-ethylcysteine yielded a corresponding series of antibacterial metabolites that, on the basis of their relative R_f values, appeared to be homologically related.

Evidence is now presented that these amino acids are incorporated via a pathway involving oxidative decarboxylation, the resulting acids then serving as penicillin side-chain precursors in the normal manner.

To facilitate a radioautographic study, a convenient procedure, requiring minimum amounts of

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isotopically labelled precursors, was developed. This was based on the observation that washed mycelium from submerged cultures grown on a simple synthetic medium for 72 hr. at 25° continued to synthesize penicillins at a satisfactory rate while suspended overnight in 0.05 M-sodium phosphate buffer, pH 6.5. It was demonstrated radioautographically that this simple system effected synthesis of penicillins, since sodium [³⁵S]sulphate was readily incorporated both into the naturally occurring penicillins (presumed to be penicillin F, dihydropenicillin F and the K-type penicillins; cf. Karnovsky & Johnson, 1949) and, in the presence of phenylacetic acid, into benzylpenicillin. A similar system has been described by Halliday & Arnstein (1956) and successfully applied to a number of studies of penicillin biosynthesis (e.g. by Arnstein & Morris, 1960).

The volume of buffer required to resuspend the mycelium could be conveniently reduced to 1 ml., thereby allowing the economical use of high concentrations of radioactive precursors. Further, this system permitted the simultaneous investigation of a number of intermediates under uniform conditions by using mycelium from a single culture flask. In this manner the production of characteristic antibacterial substances by 0.1 % solutions of methionine, ethionine, *S*-methylcysteine and *S*-ethylcysteine was clearly demonstrated by bioautographic methods.

The methionine-stimulated product incorporated ¹⁴C from both L-[Me-¹⁴C]methionine and DL-[2-¹⁴C]methionine, thus indicating that most of the carbon skeleton of this precursor was utilized intact. The observed incorporation of DL-[³⁵S]methionine into this component was therefore anticipated; however, in this experiment the other penicillins were also radioactive, so that the methionine was apparently serving in addition as a source of the thiazolidine sulphur atom. In a parallel experiment, [ethyl-1-¹⁴C]ethionine was similarly incorporated into the antibacterial ethionine-stimulated component but not into the naturally occurring penicillins.

The penicillin-like nature of these novel metabolites, separated chromatographically, was demonstrated enzymically by their ability to decolorize a specific penicillinase-starch-iodine spray (Thomas 1961b). This is consistent with isotopic data showing their derivation from sodium [³⁵S]sulphate. The absence of free amino groups was indicated both by their negative ninhydrin reactions and also their stability in the presence of phenylacetyl chloride under conditions that converted the co-existing 6-aminopenicillanic acid (I, R = H) to benzylpenicillin (I, R = C₆H₅·CH₂·CO).

To characterize these substances chemically, the product derived from *S*-ethylcysteine was investi-

gated since this precursor appeared to stimulate the formation of its characteristic penicillin more efficiently than the other three sulphur-containing amino acids. In addition, as with phenylacetic acid, it significantly inhibited the production of the naturally occurring penicillins, thereby facilitating the purification step. The major antibacterial component was readily freed from contaminating penicillins by recrystallization from aqueous *n*-butanol. The infrared-absorption spectrum was characteristic of an *N*-acyl derivative of 6-amino-penicillanic acid.

Elementary analysis of the dried sodium salt indicated that the molecular formula was

An *S*-ethyl determination under normal Zeisel conditions yielded an unexpectedly low value; however, corresponding analyses on *S*-ethylcysteine and *S*-methylcysteine were also very low, although ethionine and methionine behaved in the expected manner (Alicino, 1961). The usefulness of the Zeisel procedure for the functional-group analysis of thioalkyl compounds has been seriously criticized in a detailed study by Anderson & Zaidi (1962).

From the radioautographic results obtained with labelled methionine and ethionine it seemed reasonable to assume the presence of an *S*-ethyl group and, if the product was in fact an *N*-acyl derivative of 6-aminopenicillanic acid, then the formula of the required hypothetical side-chain precursor acid would be C₈H₉₋₁₀O₂S. From biosynthetic considerations a likely structure for this acid appeared to be *S*-ethylmercaptoacetic acid, C₄H₉O₂S, since this could arise simply from *S*-ethylcysteine by oxidative decarboxylation. The required penicillin would therefore be sodium 6-(ethylmercaptoacetamido)penicillanate

This product has been isolated by Behrens *et al.* (1948) from a *P. chrysogenum* fermentation carried out in the presence of ethylmercaptoacetic acid. Their crystalline penicillin was characterized by a specific inhibitory activity against *Staphylococcus aureus* of 1300 Oxford units/mg.; the penicillin derived from *S*-ethylcysteine showed an activity of 1080 Oxford units/mg. against our test strain of this organism (*S. aureus* 209P).

To test this structural possibility, an attempt was made to replace the precursors methionine, ethionine, *S*-methylcysteine and *S*-ethylcysteine by their corresponding oxidative decarboxylation products, namely β-methylmercaptopropionic acid, β-ethylmercaptopropionic acid, methylmercaptoacetic acid and ethylmercaptoacetic acid respectively. Washed suspensions of mycelium

supplied either with the amino acids or their corresponding mercapto acids produced chromatographically identical penicillins in each instance, thereby establishing the nature of their acyl substituents.

The designated structures of the penicillins derived from the above four sulphur-containing amino acids are shown in Table 1.

A similar pathway involving oxidative decarboxylation is operative in the formation of benzylpenicillin from phenylalanine. Although an obvious mechanism requires initial oxidation of the amino acid to the free acid corresponding to the penicillin side-chain precursor (or more probably its coenzyme A thio ester), a possible alternative pathway would involve direct condensation of the corresponding α -oxo acid with 6-aminopenicillanic acid before oxidative decarboxylation.

These results demonstrate that methionine can be metabolized simultaneously by two different pathways in *P. chrysogenum*. Thus the operation of a transmethylation and trans-sulphuration route is indicated from the above observation that [^{35}S]-methionine can label the thiazolidine sulphur of the naturally occurring penicillins, which is known to arise from cysteine (cf. Arnstein & Morris, 1960). This contrasts with the oxidative attack at the other end of the amino acid skeleton, leading to a specific penicillin with a side chain derived from β -methylmercaptopropionic acid.

Mazelis & Ingraham (1962) have described the enzymic degradation of methionine to β -methylmercaptopropionamide with horse-radish peroxidase. A related pathway leads to the biosynthesis of dimethyl- β -propiothetin in the alga *Ulva lactuca* (Greene, 1962). Ethyl β -methylmercaptopropionate, which was isolated from Hawaiian-pineapple pulp (*Ananas sativus*) by Haagen-Smit, Kirchner, Deasy & Prater (1945), is a further example of a product probably derived oxidatively from methi-

onine, although these authors suggested cysteine as a likely precursor.

During a separate study of the metabolites of a species of *Cephalosporium* grown in the presence of methionine, the formation of α -hydroxy- γ -methylmercaptobutyric acid was observed. This was identified on the basis of its elementary analysis, which corresponded to a molecular formula of $\text{C}_5\text{H}_9\text{NaO}_3\text{S}$, and also from spectral data by comparison with methionine and β -methylmercaptopropionic acid. The infrared-absorption spectrum showed a peak at 3330 cm^{-1} characteristic of a hydroxyl substituent. The nuclear-magnetic-resonance spectrum (measured in D_2O solution, with tetramethylsilane as an external standard) showed peaks at τ values of: 7.82, singlet ($\text{S}-\text{CH}_3$); 5.78, multiplet ($\alpha\text{-CH}$); 7.38, multiplet ($\gamma\text{-CH}_2$) and approx. 7.92, multiplet ($\beta\text{-CH}_2$). These data are consistent with the designated structure:

The same acid has been described as a metabolite of methionine produced by the fungus *Oidium lactis* (Akobe, 1936). On the addition of the *Cephalosporium* product at 0.1% concentration to a buffer suspension of *P. chrysogenum* mycelium in the usual system, it was readily converted into an antibacterial component chromatographically indistinguishable from the penicillin derived from methionine, probably via a common α -oxo acid intermediate.

Although the utilization of both methylmercaptacetic acid and ethylmercaptacetic acid as penicillin side-chain precursors is known from the studies of Behrens *et al.* (1948), the formation of the corresponding penicillin homologues from the β -alkylmercaptopropionic acids has not been previously described. The yields of these latter penicillins were lower than those obtained from the corresponding acetic acids.

Table 1. *Penicillins derived from sulphur-containing amino acids*

Precursor	Penicillin side chain (R)
Methionine	β -Methylmercaptopropionyl ($\text{CH}_3 \cdot \text{S} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CO}$)
Ethionine	β -Ethylmercaptopropionyl ($\text{C}_2\text{H}_5 \cdot \text{S} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CO}$)
S-Methylcysteine	Methylmercaptoacetyl ($\text{CH}_3 \cdot \text{S} \cdot \text{CH}_2 \cdot \text{CO}$)
S-Ethylcysteine	Ethylmercaptoacetyl ($\text{C}_2\text{H}_5 \cdot \text{S} \cdot \text{CH}_2 \cdot \text{CO}$)

EXPERIMENTAL

Cultural conditions. A mutant strain of *P. chrysogenum*, grown on moist corn, was used to inoculate a sterile natural medium (50 ml. in each 250 ml. flask) of the following composition: corn-steep liquor, 40 ml.; lactose, 20 g.; glucose, 5.0 g.; NaNO_3 , 3.0 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.5 g.; KH_2PO_4 , 0.5 g.; CaCO_3 , 5.0 g.; water, 1 l.

After incubation for 24 hr. at 25° on a rotary shaker (280 rev./min., 2 in. diameter stroke), 2.5 ml. samples of the resulting broth were added to 500 ml. conical flasks each containing 100 ml. of the sterile synthetic medium: lactose, 30 g.; glucose, 10 g.; ammonium lactate, 6.0 g.; potassium acetate, 3.5 g.; $\text{NH}_4\text{H}_2\text{PO}_4$, 3.0 g.; Na_2SO_4 , 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g.; CaCl_2 , 0.04 g.; FeCl_2 , 0.03 g.; ZnCl_2 , 0.01 g.; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.01 g.; cupric acetate monohydrate, 0.004 g.; water, 1 l. This medium is based on that of Halliday & Arnstein (1956).

Incubation was continued for 3 days and this culture used as an inoculum (2.5%, v/v) for fresh flasks of the above synthetic medium. This decreased the concentrations of constituents of the original natural medium carried over from the previous inoculum. The resulting culture was used as a source of mycelium for studies on washed cells.

Penicillin synthesis by mycelial suspensions. The broth from a 3-day fermentation (at pH 6.2) was filtered under vacuum, the mycelium was washed with water and filtration was continued until all excess of moisture had been removed; the typical wet weight from a single flask was 10 g. Samples (0.7 g.) of freshly filtered mycelium were suspended in 2 ml. portions of 0.05M-sodium phosphate buffer, pH 6.5, contained in test tubes (1 in. $\times$ 4 in.). One-half of these amounts (i.e. 0.35 g. of mycelium in 1 ml. of buffer) was sometimes used. Specific precursors (amino acids and alkylmercapto acids as described in the text) were dissolved (0.1%) in the buffer solution before the addition of mycelium.

The tubes containing the buffered mycelial suspensions were incubated on the rotary shaker at 25° for 16 hr., after which the mycelium was separated by filtration under vacuum and the filtrate examined chromatographically.

Radioactive-precursor experiments. The following labelled precursors (obtained commercially) were incorporated into penicillins by addition to the buffered suspensions of *P. chrysogenum* mycelium, sometimes in the presence of other unlabelled precursors (e.g. Na₂³⁵SO₄ plus 0.1% phenylacetic acid): L-[Me-¹⁴C]methionine (2 mc/m-mole), DL-[2-¹⁴C]-methionine (1 mc/m-mole), DL-[³⁵S]methionine (10 mc/m-mole), DL-[ethyl-¹⁴C]ethionine (1 mc/m-mole) and Na₂³⁵SO₄ (2 mc/m-mole). The labelled amino acids and Na₂³⁵SO₄ were added to give final concentrations of 5 μ C/ml. and 50 μ C/ml. respectively.

After incubation, 50 μ l. portions of the filtrates were chromatographed and the resulting chromatograms placed in contact with Kodak Medical X-ray film (Blue Brand) which was, after a suitable exposure period (3–6 days), developed in the usual manner.

Chromatography and detection of penicillins. Whatman no. 1 paper was buffered with a solution of 10% (w/v) citric acid monohydrate adjusted with aqueous saturated NaOH to pH 5.7 and dried at room temperature. The sheets were spotted and equilibrated for 30 min. at about 23° in a chromatographic tank containing a saturated atmosphere of diethyl ether and water before the addition of solvent (water-saturated diethyl ether).

After a development time of approximately 2 hr., the sheets were dried and examined for penicillin-like components (a) by placing them in contact with agar inoculated with *Sarcina lutea* (for 4 min.) and observing the zones of inhibition of growth after incubation at 37° overnight, or (b) by radioautography as above, or (c) by their enzymic capacity to decolorize a specific colorimetric spray containing penicillinase (Thomas, 1961b). The penicillins derived from methionine, ethionine, *S*-methylcysteine and *S*-ethylcysteine were characterized by their mobilities relative to that of benzylpenicillin, namely 0.54, 0.94, 0.39 and 0.88 respectively.

Production of sodium ethylmercaptomethylpenicillin [sodium 6-(ethylmercaptoacetamido)penicillanate]. Ten 2 l. conical flasks, each containing 500 ml. of the above synthetic medium, were inoculated (2.5%, v/v) with a 3-day culture grown on the same medium as described

above and incubated at 25° on a rotary shaker. At 72 hr., *S*-ethylcysteine was added in sterile water to give a final concentration of 0.1%, and after a further 24 hr. the combined cultures were filtered. The resulting pale-yellow filtrate (4.5 l.) was adjusted from an initial pH of 6.5 to pH 2.5 with conc. HCl before extraction with *n*-butyl acetate (one-third vol.). The extract was shaken with M-sodium phosphate buffer, pH 7.0 (one-third vol.), and the aqueous phase re-extracted with *n*-butyl acetate (one-third vol.) at pH 2.5. This fraction was finally extracted with water (one-third vol.) and adjusted to pH 6.5 with dilute NaOH. The aqueous layer was concentrated at room temperature *in vacuo* to 10 ml., *n*-butanol (35 ml.) was added, and the mixture was further concentrated: a pale-yellow crystalline deposit (85 mg.) separated.

Chromatographic analysis showed that this product consisted predominantly of the penicillin derived from *S*-ethylcysteine. This was purified by repeated recrystallization from aqueous *n*-butanol until chromatographically homogeneous, yielding colourless needles (33 mg.). (Found: C, 42.1; H, 5.7; N, 8.2; Na, 5.8; S, 17.2; *S*-Et (Zeisel), 3.5. Calc. for sodium ethylmercaptomethylpenicillin, C₁₂H₁₇N₂NaO₄S₂: C, 42.4; H, 5.1; N, 8.2; Na, 6.8; S, 18.8; *S*-Et, 19.5%.)

SUMMARY

1. Incubation of buffered mycelial suspensions of *Penicillium chrysogenum* with methionine, ethionine, *S*-methylcysteine and *S*-ethylcysteine led to the formation of a characteristic series of penicillin derivatives.

2. Radioautographic studies demonstrated the partial incorporation of methionine and ethionine.

3. *S*-Ethylcysteine was the most effective precursor and gave rise to a penicillin that was readily purified and shown to be the *N*-ethylmercaptoacetyl derivative of 6-aminopenicillanic acid. This penicillin is readily synthesized in *P. chrysogenum* from ethylmercaptoacetic acid.

4. The utilization of these amino acids involves the oxidative loss of the carboxyl group, the resulting acids serving as penicillin side-chain precursors in the usual manner, although the sequence of steps involved is not known.

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The Effect of the Oral Administration of Leucine on the Metabolism of Tryptophan

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The occurrence of pellagra in an endemic form in a population subsisting mainly on the millet jowar (*Sorghum vulgare*) has been briefly reported by Gopalan & Srikantia (1960). Both jowar and maize have one common feature with regard to their amino acid composition, namely a high content of leucine. The possible role of amino acid imbalance resulting from a relative excess of leucine in the pathogenesis of pellagra was therefore suggested. In the present investigation the effects of the oral administration of leucine on the metabolism of tryptophan and nicotinic acid have been studied.

METHODS

Experimental procedure. Six patients suffering from pellagra (pellagrins) and six apparently normal male adults, between the ages of 25 and 45 years, formed the subjects for the study. They were kept in hospital and put on a standard diet, based on rice, wheat flour, dhal and vegetables, providing them with approximately 2300 kcal., 50 g. of protein and 50 g. of fat/day. The protein was derived entirely from vegetable sources, and constituted about 8% of the total calories. Preliminary studies had indicated that the urinary excretion of some nicotinic acid metabolites usually became stabilized on this diet by the third or fourth day. Therefore 24 hr. collections of urine were made on the fifth, sixth and seventh days of the standard diet. The subjects were then given 10 g. of pure L-leucine daily, in a single dose, taken with one of the main meals of the day, for five days. Then 24 hr. urine collections were again made on the second, third, fourth and fifth days of the supplementation with leucine. The supplement of leucine was then withdrawn, but the collection of urine samples was continued for 4 more days. The samples of urine were collected over 15 ml. of acetic acid and analysed without delay.

In four other normal volunteer adults the effects of simultaneous ingestion of (a) tryptophan and leucine, and of (b) nicotinic acid and leucine, on the urinary excretion of

tryptophan metabolites were studied. Subjects were maintained on the standard diet described above, and 24 hr. urine samples were collected on the fifth, sixth and seventh days. On the morning of the eighth day, at 9 a.m., 5 g. of DL-tryptophan was given orally, and the urine was collected till 9 p.m. At 9 p.m. another load at 5 g. of DL-tryptophan was given, and urine was collected for the next 12 hr. Samples (24 hr.) were collected from the morning of the ninth day for the next 3 days. The subjects were then given 5 g. of leucine twice a day for 5 days; on the fifth day they received 5 g. of tryptophan in addition to the leucine. Urine collections were made in the manner described above. For the load test with nicotinamide the experimental procedure was identical except that 100 mg. of nicotinamide/dose was used instead of tryptophan.

Analytical methods. Tryptophan was determined by the method of Albanese & Frankston (1945). Nicotinic acid was determined by the cyanogen bromide method of Swaminathan (1939), after the removal of interfering substances by the method described by Nandi & Banerjee (1949). Quinolinic acid was measured as the increase in nicotinic acid, after autoclaving at 15 lb./sq.in. for 60 min. and calculation by the method of Sarett (1951). *N*-Methylnicotinamide was measured by the fluorimetric method of Carpenter & Kodicek (1950). The 6-pyridone of *N*-methylnicotinamide was determined by the method of Rosen, Perlzweig & Handler (1948) and Rosen, Perlzweig & Leder (1949). Indican was determined by the colorimetric method after development of the colour with Obermeyer's reagent in the presence of thymol and then extracted with chloroform. Indolylacetic acid, both free and total, was measured by the method described by Weissbach, King, Sjoerdsma & Udenfriend (1959). 5-Hydroxyindolylacetic acid was determined by the modified method of Macfarlane and co-workers, as described by Dalglish (1958).

RESULTS

The amounts of some metabolites of tryptophan and nicotinic acid excreted in 24 hr. in the urine of normal subjects and of pellagrins are shown in