Biosynthesis of Benzylpenicillin by Mycelial Suspensions of *Penicillium chrysogenum*

The biosynthesis of benzylpenicillin by the mycelia of *Penicillium chrysogenum* involves the utilization of the immediate precursors L-cysteine, the non-nitrogenous moiety of valine, phenylacetic acid (PA), and an amino group donating compound (amino acid ?)[1]. The exact mechanism of synthesis and the enzyme systems involved require to be elucidated and work is in progress in this laboratory towards this end. In view of the observations of HALLIDAY and ARNSTEIN[2] and of DEMAIN[3] published recently, we report our results on the biosynthesis of benzylpenicillin by the resting cells of *P. chrysogenum*.

We utilized the mycelia grown (in the fermentors used for manufacture) in corn steep-peanut meal-lactose medium with sodium phenylacetate added every 6–8 h. The mycelial suspension was withdrawn from the fermentor at the specified hours, a measured volume taken, the mycelia separated from the broth by squeezing with cloth and washed twice or thrice by resuspending in sterilised distilled water and squeezing with cloth (this consistently removed all the penicillin adhering to the mycelium). The washed mycelium was resuspended in 0·01 *M* phosphate buffer (pH 7·0) to make up to the original volume of the broth, 100 ml of the mycelial suspension distributed into each of the 500 ml conical flasks and the compounds to be studied added to the individual flasks. These were shaken in a rotary shaker giving 250 r.p.m. in a room maintained at 24°C. Samples (5 ml) were taken at the required intervals and the penicillin content of the filtered solution determined by bioassay according to the method of HUMPHRY and LIGHTBOWN[4].

Mycelia from different batches were used for the different sets of experiments and so to check the variations there was included in every trial a control flask with PA (0·05%) and lactose (1%). The washed mycelium suspended in buffer or lactose alone produced only negligible amounts of penicillin for a few hours. Tables I, II, and III give some of the results obtained indicating the relative effectivenesss of the various carbohydrates added. Sucrose gives the best results of the sugars, and phenylacetamide seems to be better than PA.

Table I

39 h mycelium (batch 1270), washed, and resuspended in phosphate buffer (P. amide: Phenylacetamide)

Constituents added	Penicillin titre in units per ml after			
	4 h	8 h	12 h	16 h
PA (0·05%)	47	96	120	124
PA (0·05%) + Lactose (1%)	55	155	210	300
P.amide (0·05%)	67	136	–	–
P.amide (0·05%) + Lactose (1%)	78	240	300	353

The following results, not reported in the tables to save space, were also obtained: arabinose, xylose, sodium and ammonium acetates (the sodium salt giving better results than the ammonium salt), sodium lactate and pyruvate (the two free acids showing inhibition),

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Table II

36 h mycelium (batch 1240), washed, and resuspended in phosphate buffer

Constituents added	Penicillin titre in units per ml after					
	4 h	8 h	12 h	16 h	20 h	24 h
PA (0·05%) + Lactose (1%) . . .	10	35	65	156	227	348
PA (0·05%) + Sucrose (1%) . . .	34	100	188	242	321	370
PA (0·05%) + Glucose (1%) . . .	5	23	43	122	184	247
PA (0·05%) + Mannose (1%) . .	8	26	35	145	188	232
PA (0·05%) + Fructose (1%) . .	6	34	62	145	214	273

Table III

34 h mycelium (batch 1239), washed, and resuspended in phosphate buffer.

Constituents added	Penicillin titre in units per ml after					
	4 h	8 h	12 h	16 h	20 h	24 h
PA (0·05%) + Lactose (1%). . .	16	45	106	216	280	296
PA (0·05%) + Fructose (1%) . .	20	65	117	232	233	221
PA (0·05%) + Starch (1%) . . .	14	50	90	77	0	0
PA (0·05%) + Dextrin (1%) . . .	17	28	120	184	225	315

arachis oil and oleic acid were found to increase the rate of synthesis of penicillin, though not as effectively as lactose. Sodium benzylpenicilloate, prepared from benzylpenicillin, was inefficient as the penicillin precursor. The seed mycelium (prepared in corn steep liquor-sucrose medium by inoculating with the spores and aerating for 48 h and used to inoculate the fermentors) did not produce any penicillin as such or with PA, lactose, L-cystine and L-valine. The penicillin-producing capacity of the mycelium in the fermentor varies with the age of the mycelium, rising to a peak at 36–45 h and then declining. The observation of HALLIDAY and ARNSTEIN[2] that crushing the mycelium destroys the penicillin producing capacity is confirmed.

The use of the washed mycelial suspensions as adopted in these experiments excludes the metabolites found in the fermented broth from participating in the biosynthesis, and satisfies the two conditions now taken to be essential for penicillin synthesis, viz. the mycelium should not be actively multiplying and the pH of the medium should be about 7·0[5]. The following picture of penicillin production by the mycelium emerges from the present studies. The mycelium prepared as seed to inoculate the big fermentors probably lacks the enzyme systems essential for penicillin synthesis. The mycelium in the production fermentors gradually builds up endogenously the enzyme systems essential for biosynthesis as well as for providing energy for the synthetic steps and also the substrates except PA. For continued penicillin production at a good rate, the mycelium requires PA from the exogenous source and also energy which seems to be provided by some saccharides, arachis oil, oleic acid, and acetate acting as substrates.

Fuller details of the work and discussion of the results will be published elsewhere.

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Résumé

Les auteurs ont étudié les effets des divers hydrates de carbone (glucose, fructose, mannose, galactose, xylose, arabinose, lactose, amidon et dextrine), du pyruvate, du lactate, de l'acétate, de l'huile d'arachide, etc. sur la biosynthèse de la pénicilline par les cellules lavées de *P. chrysogenum*.

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On the Role of Purines in the Enzymatic Reduction of Tetrazolium Salts

In the past, three prime functions of purines and their derivatives in the cellular economy have been elucidated, namely in the formation of nucleoproteins, in energy-conservation mechanism and in some transfer reactions of carbohydrates and phospholipides. That still other functions exist for this important group of compounds is attested to by the role of hypoxanthine in the oxidation of sulphite[1], cysteine[2], and fatty acids[3], and of adenosine-5′-monophosphate (AMP) in L-amino acid decarboxylation[4]. It has also been recently found[5] that a wide variety of such compounds function in tetrazolium salt reduction. Thus, purine, hypoxanthine and related compounds[6] can replace the heat-stable factor, found in various animal tissues and in yeast, which is necessary for the enzymatic reduction of tetrazolium salts[7]. The purpose of the present communication is to report the findings that this activation by purines and purine derivatives, although first detected by us in the case of an amine dehydrogenating system, is observed with quite diverse substrates when a tetrazolium salt is used as the terminal electron acceptor.

Methods.—Washed particles of homogenized rat liver or, alternatively, solubilized enzyme preparations[8], were used as sources of enzyme. Reaction mixtures contained, in a final volume of 2·0 ml, 40 μmoles of phosphate buffer, pH 7·4; 20 μmoles of substrate; 4·8 μmoles of triphenyltetrazolium chloride or neotetrazolium chloride (expressed in monotetrazolium units); 0·4 μmole of pyocyanine; 0·1–0·3 μmole of cofactor (i.e. purine, etc.); and 28–35 mg (dry wt.) of dialyzed liver extract. Pyocyanine was added to aid the transfer of hydrogen from substrate to the tetrazolium[9], but it was not an obligatory component. The solutions were incubated in a waterbath at 37° for 15–40 min at which time the cofactor was added. This preincubation of substrate and enzyme assured maximal rate of tetrazolium reduction. The appearance of formazan with amine substrates usually depended upon the addition of the cofactor, but occasionally a small amount was formed before supplementation. The reaction was followed by the increase in absorbance of the reaction mixtures at 520 mμ, read directly in the Coleman Junior Spectrophotometer. Under these conditions formazan production has been observed to proceed briskly for a short period, reaching a plateau in 5–15 min. This was the case when hypoxanthine, xanthine, purine, guanine, inosine and diphosphopyridine nucleotide (DPN) were used. At the plateau the molar ratio of 'formazan produced: cofactor added' varies between 0·5 and 1·5. Adenine, 6-methylpurine, azaguanine, uric acid and alloxan were inactive in this test system[6].

Results.—Many substrates were tested as hydrogen donors with a tetrazolium salt as the ultimate acceptor (Table). Among the amines, tyramine and isoamylamine had an absolute requirement for this cofactor; with tryptamine and benzylamine the rate of reduction of the dye was markedly accelerated by the cofactor. Choline and succinate were also dehydrogenated in the unsupplemented reaction mixtures but addition of cofactor in-

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