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.

We are indebted to Dr. P. D. Kulkarni, Miss I. Nalini, and Mr. D. N. Bilampelly for the bioassays reported here.

V. N. Deshpande and K. Ganapathi

Antibiotics Research Centre, Hindustan Antibiotics (P) Ltd., Pimpri (Poona District, India), May 17, 1957.

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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creased the initial rate of formazan production. In the cases of isocitric and malic dehydrogenases, triphosphopyridine nucleotide[10] was required in addition to a purine. It was not possible to demonstrate tetrazolium reduction with the following substrates, in the presence or absence of cofactor: glycine, serine, threonine, methionine, aspartic acid, spermine, histamine and 1,4-butanediamine.

The activating effects of inosine with various substrates

Substrate	Activity O.D. 520/10 min	
	No Inosine	Plus Inosine
Tyramine	0·000	0·920
Isoamylamine	0·000	0·860
Isocitrate	0·000	1·030
L-Malate	0·000	0·200
Benzylamine	0·175	0·830
Tryptamine	0·210	0·900
Choline	0·060	0·400
Succinate	0·150	0·790

In order to determine the specifity of the cofactor effect AMP, purine, hypoxanthine, adenine and uric acid were tested with several of the substrates. It was found that with succinate the first three compounds act in the same way as inosine (Table) and that adenine and uric acid are inactive, as they are with amines. It was also desirable to learn whether the cofactor which functions in tetrazolium reduction is also involved in the reduction of other dyes. Thus far it has not been possible to demonstrate this for methylene blue, 2,6-dichlorophenolindophenol or ferricyanide, although hypoxanthine is required in the specific case of sulphite/methylene blue[1].

Discussion.—Tetrazolium dyes are being used increasingly in enzymological and histochemical investigations, but in spite of this the kinetics and mechanism of their reduction are incompletely understood. BRODIE and GOTS[12] have adduced evidence that tetrazolium salts accept protons from flavoproteins, but the present data indicate that the transfer system is probably more complex than this. The existence of a cofactor for tetrazolium reduction has been suspected previously. SPRINZ and WALDSCHMIDT-LEITZ[13] detected requirement of a cofactor for succinic dehydrogenase when measured with tetrazolium. Diphosphopyridine nucleotide satisfied the requirement but, according to the authors, is probably not the natural factor. Others[14] have made similar findings. The present results demonstrate, first of all, that certain purines, nucleosides and nucleotides, respectively, participate in the enzymatic reduction of tetrazolium salts; and, secondly, that this effect is specific for tetrazolium salts among the electron acceptors tested, i.e. it does not apply to the same enzyme preparations catalyzing the reduction of ferricyanide, methylene blue, 2,6-dichlorophenol indophenol or oxygen. The nature and function of the cofactor is still somewhat speculative, but two possibilities can be envisaged: (1) The cofactor serves in proton transfer, serving at the pyocyanine or tetrazolium level. Because the molar ratio, monoformazan/purine, ranges between 0·5 and 1·5 it is evident that the cofactor does not behave like the usual catalytic agents. It is conceivable that immediately after transferring protons to the pyocyanine or tetrazolium the cofactor differs somewhat from the original compound, and it is unable to serve in the enzyme system, but that it is slowly reconverted to the original structure[1]. This would account for molar ratios greater than unity, in that a portion of the cofactor would move through the entire cycle quickly enough to be utilized in the dehydrogenation more than once. (2) An alternative hypothesis is that the cofactor accepts the dehydrogenated substrate residue, resulting in a 'bound', inactive form of the cofactor. However, it is difficult to reconcile such a function with the variety of substrates and of purified compounds which are effective as cofactors.

From a comparison of the chemical structure of active substances it would appear that the 6 and 8 positions on the purine are of special importance. Thus, adenine and certain other 6-substituted analogues are inactive, as is azaguanine. The activity of adenosine and AMP may be explicable by their prior deamination, enzymes for which are found in the rat liver, although adenase is absent.

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T. L. SOURKES and J. R. LAGNADO

Allan Memorial Institute of Psychiatry, McGill University, Montreal (Canada), September 2, 1957.

Zusammenfassung

Nach der vorliegenden Untersuchung können verschiedene Purine und einige ihnen nahestehende Verbindungen an der enzymatischen Reduktion der Tetrazole als Zwischenprodukte teilnehmen.

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[10] This compound, unlike DPN, cannot serve as cofactor in the test system containing amine as substrate.

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Oxygen Equilibrium of Human Myoglobins (Mb I and Mb II)[1]

In previous research, we demonstrated that at least three components (Mb I, Mb II, Mb III) are present in the human muscle and in preparations of crystallized myoglobin (Mb). These components differ from one another in their electrophoretic behaviour[2].

In this note, we give the results of experiments aimed at studying the oxygen equilibrium of Mb I and Mb II. It has not been possible to make experiments with Mb III, because of the small quantities in which it is present in Mb solutions.

We have found these studies interesting to investigate the possible physiological meaning of these pigments,

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[1] Aided by a grant from the Rockefeller Foundation.

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