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BIOSYNTHESIS OF NEBULARINE (PURINE 9-β-D-RIBOFURANOSIDE) INVOLVES ENZYMIC RELEASE OF HYDROXYLAMINE FROM ADENOSINE

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Key Word Index—*Lepista nebularis*; *Streptomyces yokosukanensis*; nebularine biosynthesis; purine 9-β-D-riboside and ribotide; adenosine; purine; enzymic hydroxylamine release; deamination.

Abstract—Biosynthesis of the antibiotic nebularine (purine-9-β-D-ribofuranoside) by *Lepista nebularis* and *Streptomyces yokosukanensis* has been studied and a novel enzymic activity is described which deaminates adenosine to release hydroxylamine. Use of ^{14}C-labelled nucleosides showed that adenosine was the more immediate precursor of nebularine. That formation of nebularine involves direct incorporation of adenosine and does not involve prior catabolism and re-use of catabolic fragments, was shown by locating 82% of the incorporated radioactivity from [8-^{14}C]adenosine in C-8 of nebularine. A crude nebularine-forming enzymic extract was fractionated by $(NH_4)_2SO_4$ precipitation and the activity recovered in the 100% satn supernatant; it was not sedimented by centrifuging for 90 min at 113 000 *g*. Further purification was achieved by chromatography of Sephacryl S-200 (173-fold) and on BrCN-activated Sepharose 4B (320-fold). Lability of the enzyme during concentration, by various techniques, obviated sequential use of these steps. Activity was not stimulated by pyridine nucleotides or flavins, and a range of metal ions were without effect. Various purine riboside analogues were not inhibitory, although some end-product inhibition was seen with nebularine. Gel-filtration and SDS–PAGE indicated a M_r of 9500–10 000 for the enzyme. That hydroxylamine is a product of the catalysed reaction was demonstrated chemically and by MS. Use of [^{15}N-amino]adenosine confirmed that the hydroxylamine originates from the 6-amino group of adenosine. The quantitative relationship between nebularine production and metabolism of adenosine to other compounds was studied. Of the total radioactivity from [8-^{14}C]adenosine recovered, 3% was in nebularine. The work describes the first reported natural occurrence, in a free state, of purine and its 5′-ribotide.

INTRODUCTION

Nebularine was first isolated as the active principle of a press-juice from *Lepista* (*Clitocybe*) *nebularis* Batsch exhibiting potent antibiotic activity against various mycobacteria, including *Mycobacterium phlei*, *M. avium* and *M. tuberculosis* and also against *Brucella abortus* [1, 2]. Elucidation of its structure by Löfgren and Lüning [3] as purine 9-β-D-ribofuranoside was the first demonstration of the occurrence of purine in a biological molecule, predicted by Fischer in 1907 [4]. Structurally, nebularine poses a unique biochemical problem. All known naturally occurring purines arise via an essentially similar pathway in plants, animals and microorganisms, and in each case this involves prior formation of the key biosynthetic product inosine 5′-monophosphate (IMP). The problem is that there is no known enzymic mechanism for complete removal of the 6-substituent and all other natural purine products possess a 6-substituent, almost always either hydroxyl or amino. Does, then, nebularine arise via a novel biosynthetic route resulting in formation of the purine ring system without involving IMP, or is there a previously undescribed enzyme catalysing complete removal of the C6-substituent? It is this question that the present investigation set out to answer in the context of a general study of nebularine biosynthesis.

Since its discovery in *L. nebularis*, nebularine has been shown to be produced by *Streptomyces yokosukanensis* [5] and by a novel *Microbispora* species [6]. The antibacterial activity of the compound is very selective [3] and whereas it strongly inhibits the growth of mycobacteria and *Brucella abortus*, it has no effect on *Sarcina lutea*, *Staphylococcus aureus*, *Escherichia coli*, *Aerobacter aerogenes* and *Bacillus subtilis*. Because of its pronounced cytotoxicity [3, 7–9], it has not current clinical application, although interestingly *L. nebularis* is considered edible [9].

RESULTS AND DISCUSSION

Examination of nebularine producers

Initially, the feasibility of culturing *L. nebularis* in the laboratory and using these cultures as the biosynthetic

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system for study was explored. Following standard mycological procedures, tissue excised from the fruiting bodies [9] was cultured axenically on a malt agar medium and optimum growth was obtained at 20° in the dark. Attempts to transfer this culture to chemically defined media, e.g. Czapek Dox agar, were, however, relatively unsuccessful in that poor growth resulted. Further, it was considered desirable to use liquid cultures to facilitate planned radioisotope incorporation experiments. Cultures were subsequently initiated in 25 or 50 ml liquid medium in 250-ml conical flasks using mycelial discs (5 mm diameter), taken aseptically from the malt agar cultures, as inocula. The media examined for this purpose were Czapek Dox, semisynthetic medium [10], and MN medium (Melin modified by Norkrans; Massart, P., personal communication). The composition of this latter medium is given in the Experimental.

Liquid cultures were incubated under a variety of conditions of light, temperature and orbital shaking rate. Samples were taken at regular intervals for assessment of growth and nebularine production. Optimal growth conditions were obtained using 25 ml of MN medium per 250 ml flask at 21° in the dark and with orbital shaking at 100 rpm. After five weeks, growth was 4–7 g (fresh weight) of mycelium per flask. Despite the growth, no significant amount of nebularine was, however, detectable in these cultures until seven weeks after inoculation, at which time it was detected in both medium and mycelium. Attempts to induce earlier nebularine production in the cultures by adding purine supplements, including IMP, were unsuccessful. Under the specified conditions, the production of nebularine by cultures of *L. nebularis* (Fig. 1) peaked at nine weeks and then declined sharply. Both the culture filtrate and the mycelium contained nebularine at that time.

Because of the long delay in the production of nebularine by cultures of *L. nebularis*, attention was focused on another known nebularine producer *S. yokosukanensis*. Experiments to define optimal conditions for nebularine production by this streptomycete showed that complex media were better than chemically defined media. Of the media that were examined, a sucrose–tryptone medium, described in the Experimental, was the best. Cultures were grown in the dark at 28° in 250-ml portions of medium in 2-l conical flasks; orbital shaking at 120 rpm was used throughout. Under these conditions, the yield of nebularine was maximal at 96 hr in the medium and at 72 hr in the cells (Fig. 2). The total nebularine yield was highest at 96 hr with each 250 ml of culture yielding *ca* 25 mg (100 μmol). As a result of these studies, it was decided to use *S. yokosukanensis* for the biosynthetic investigation.

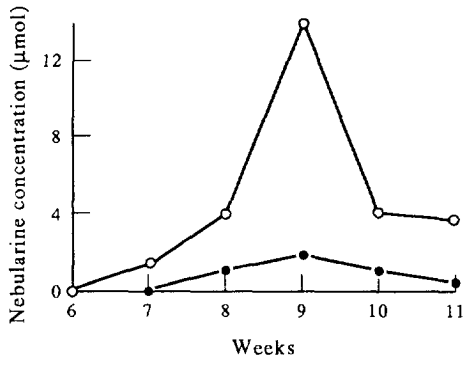

Fig. 1. Nebularine production by *Lepista nebularis* in axenic culture. Each datum is the total yield from six 25 ml cultures. ○—○ Mycelial extracts, ●—● culture filtrates.

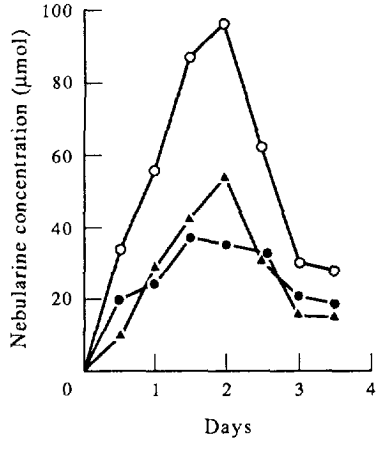

Fig. 2. Nebularine production by *Streptomyces yokosukanensis*. Data shown are the mean of two sets of experimental data with good reproducibility. ●—●, culture filtrate; ▲—▲, cells; ○—○, total in culture.

Development of an HPLC analysis for nebularine

The pilot studies described above involved extraction of nebularine and its identification by chromatographic, electrophoretic and spectrophotometric comparison with an authentic sample. Determination of nebularine required extraction, sequential paper chromatography in solvent systems (i) and (ii), followed by HV paper electrophoresis at pH 2.0, and finally UV-spectrophotometry (details in the Experimental). To facilitate the biosynthetic study, a more rapid, routine HPLC procedure was developed for determining the nebularine content of extracts. A reversed-phase column (Apex II ODS) was selected and isocratic elution with 0.2 M KH_2PO_4 containing 25% (v/v) methanol gave good resolution (Fig. 3). The overall per cent recovery of nebularine from the standard extraction procedure (Experimental) followed by HPLC, was determined by adding known amounts of nebularine to culture filtrates or to mycelia, as appropriate, immediately before the extraction process began. Recoveries of 92–96% were obtained both for culture filtrates and cells.

Examination of possible purine precursors of nebularine

Since IMP (6-hydroxypurine-9-β-D-riboside) is the end-product of the pathway of *de novo* purine biosynthesis

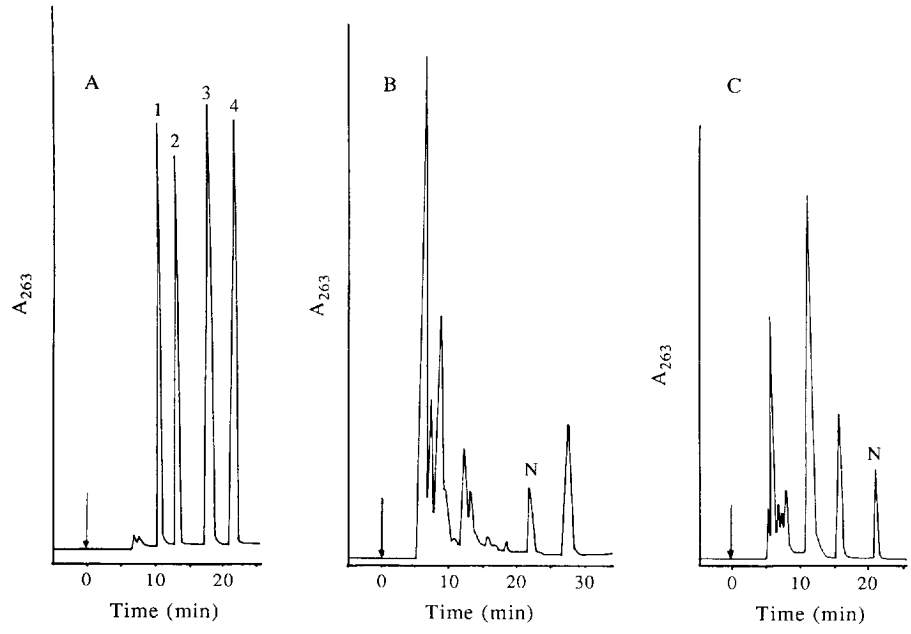

Fig. 3. Reversed-phase HPLC separation on Apex II ODS of nebularine from (A) a synthetic mixture of nucleosides: 1 adenosine, 2 xanthosine, 3 inosine, and 4 nebularine; (B) a cell extract from *S. yokosukanensis*; and (C) a culture filtrate from *S. yokosukanensis*.

Table 1. Comparison of the incorporation of [8-^{14}C]inosine and [8-^{14}C]adenosine into nebularine by *Streptomyces yokosukanensis*

Preparation	Time substrate added (hr)	Radioactivity incorporated into nebularine (dpm) from: [8-^{14}C]Inosine	[8-^{14}C]Adenosine
Cultures	0	649	6265
Cultures	50	334	4866
Washed cells	96	275	6530
Cell homogenate	96	530	5306

Each datum was derived from two 250-ml cultures. To each 250-ml culture was added either 1 μCi of [8-^{14}C]inosine (sp. radioactivity 50 mCi mmol^{-1}) or 1 μCi of [8-^{14}C]adenosine (sp. radioactivity 50 mCi mmol^{-1}). Data are the mean of triplicate incubations with good reproducibility; for cultures they represent total nebularine from cells and culture filtrate at 96 hr. Washed cells were incubated for two days with precursors before extracting the nebularine. Homogenates were incubated for 2 hr.

and all known naturally occurring purines are derived from it, its riboside inosine was considered as a possible precursor of nebularine. Structurally, inosine can be considered to be 6-hydroxynebularine and conversion of the former would therefore require removal of the hydroxyl substituent. To test this possibility, [8-^{14}C]inosine was supplied aseptically to shaken cultures and cell preparations of *S. yokosukanensis*. Cultures were grown in 250 ml of medium in 2-l conical flasks as described in the Experimental. To one set of cultures, labelled inosine was added at zero time (inoculation) and to another at 50 hr; nebularine was extracted from both sets at 96 hr. To a third set of flasks, each containing four-day-old washed cells of *S. yokosukanensis* resuspended in 250 ml of fresh medium, a similar amount of radioactive inosine was added and the preparation incubated for a further two days before the nebularine was extracted. A crude cell homogenate, prepared from four-day-old cells of the streptomycete, was also incubated in a similar way and after two days, its nebularine content was extracted. After chromatographic and electrophoretic purification, the radioactivity of each of the nebularine samples was determined. The results (Table 1) show some small incorporation of radioactivity from [8-^{14}C]inosine into nebularine. However, replacing the inosine with [8-^{14}C]adenosine, under otherwise similar conditions, resulted in a 10-fold increase in the amount of radioactivity incorporated (Table 1). Since this was so with all three

systems examined, i.e. cultures, washed cells and homogenates, it was concluded that of the two naturally occurring ribosides inosine and adenosine, the latter was the more immediate precursor of nebularine. Although it was checked experimentally later in the investigation, guanosine was not at this stage considered to be a likely precursor because it is a disubstituted purine and would need to have both substituents removed.

Crude enzymic preparation

Structurally, adenosine would only require removal of its 6-amino group to be converted to nebularine; it was thought likely therefore that a single enzyme is involved in this process. To see if this enzymic activity could be extracted, cells of *S. yokosukanensis* were harvested from flask cultures by centrifuging and homogenized in potassium phosphate buffer (0.1 M; pH 7.0) using a prechilled mortar and pestle. The homogenate was centrifuged at 15000 *g* for 10 min at 4° and the supernatant examined for enzymic activity by incubating with [^{14}C]adenosine and determining the radioactivity of the synthesized nebularine. For comparison, similar incubations were simultaneously conducted with [8-^{14}C]inosine and [8-^{3}H]-guanosine. The results (Table 2) show substantial incorporation of radioactivity from [8-^{14}C]adenosine with much less incorporation from [8-^{14}C]inosine and [8-^{3}H]guanosine.

Because the crude enzymic preparation undoubtedly contained a variety of enzymic activities, it was necessary to eliminate the possibility that the observed incorporation of radioactivity from [8-^{14}C]adenosine into nebularine may have been indirect and attributable to prior catabolism of the adenosine followed by re-use of ^{14}C-labelled catabolic fragments. An experiment was therefore set up to locate the ^{14}C-label incorporated into the nebularine molecule from [8-^{14}C]adenosine. The nebularine produced was isolated, as described in the Experimental, and degraded by alkaline fission of the imidazole ring, resulting in specific release of C-8 as formate [11, 12]. Heating in the presence of mercuric acetate oxidized the latter to carbon dioxide; this was released by acidification [13, 14] and trapped in 2-phenylethylamine for determination of its radioactivity. To minimize loss of radioactive carbon dioxide, the entire degradation procedure was carried out in a sealed vessel (Experimental). Table 3 details the results from four separate experiments and indicates that 82% of the radioactivity incorporated from [8-^{14}C]adenosine into nebularine is located in C-8, i.e. adenosine is converted to nebularine without prior catabolism of the purine ring. This result means that the enzymic extract catalyses a novel reductive deamination

Table 2. Formation of nebularine by crude enzymic extract from *Streptomyces yokosukanensis*

Substrate	Radioactivity incorporated into nebularine: Total radioactivity (dpm)	Specific radioactivity (dpm μmol^{-1})
[8-^{14}C]Inosine	694	560
[8-^{14}C]Adenosine	6400	2927
[8-^{3}H]Guanosine	542	440

In each case, 1 μCi of substrate was used per 250 ml culture; the specific radioactivity of the inosine and adenosine was 50 mCi mmol^{-1}, and that of the guanosine was 10 mCi mmol^{-1}. Incubation was for 2 hr. The results are the mean of triplicate determinations with good reproducibility.

Table 3. Specific degradation of the enzymically formed ^{14}C-nebularine to release C-8 as carbon dioxide

Replicate	Total radioactivity in nebularine (dpm)	Radioactivity in C-8 released as CO_2 (dpm)	Per cent of total radioactivity located in C-8
1	3146	2854	91
2	2746	1985	72
3	3575	2895	81
4	3241	2755	85

Replicates are four separate experiments.

in which the 6-amino group of adenosine is replaced by a hydrogen atom.

Partial purification and properties of the enzyme

The crude enzymic extract, prepared as described in the Experimental, was subjected to fractional precipitation with ammonium sulphate. Protein precipitates were redissolved in potassium phosphate buffer (0.1 M; pH 7.0) dialysed overnight against a large excess of the same buffer, and assayed for activity in deaminating [8-^{14}C]adenosine to [8-^{14}C]nebularine. Unexpectedly, it was found that although various protein fractions were obtained, activity was entirely confined to the 100% saturation supernatant. Nevertheless, this proved to be a useful step giving a 94-fold purification with a 78% recovery (Table 4).

The dialysed 100% saturated supernatant was loaded on to a column (1.6 × 31.5 cm) of Sephacryl S-200; elution was with potassium phosphate buffer (30 mM; pH 7.0) at a flow rate of 15 ml hr^{-1} and fractions (1.5 ml) were collected automatically. The elution profile (Fig. 4) showed a single peak of enzymic activity and fractions 29–35, which comprised the peak, were pooled and designated the Sephacryl S-200 fraction. From the original crude extract, this represented a 173-fold purification (Table 4).

As BrCN-activated Sepharose 4B has proved effective in separating purine riboside deaminases [15–17], use of this hydrophobic affinity matrix for separation of the nebularine-forming enzyme was examined. A 10-ml sample of the partially purified enzyme [100% $(NH_4)_2SO_4$ supernatant] was percolated through a column (2 × 10 cm) of BrCN-activated Sepharose 4B followed by 1 bed-volume of potassium phosphate buffer (0.1 M; pH 7.0). Elution was then begun with a similar phosphate buffer containing NaCl (0.25 M). The first 15 ml of the eluate, which contained the total activity, was collected and dialysed against 0.1 M phosphate buffer (pH 7.0) to remove NaCl. The resulting fraction (Table 5; affinity chromatography fraction) represented a 320-fold purification over the original crude extract with 79% recovery. Unfortunately, lability during concentration of the activity in the Sephacryl S-200 peak fractions (Fig. 4) meant that the affinity step could not be used to extend the purification protocol. Various standard techniques for concentrating proteins were tried, including ultrafiltration, freeze-drying and dehydration with Aquacide (polyethylene glycol M_r 8000), but all caused substantial loss of activity. Ultracentrifugation (113 000 *g* for 90 min) was tried in case the activity was particulate, but no sediment was obtained. The activity was also heat-labile.

Since it appeared that enzyme is catalysing a reductive deamination, the effects upon its activity of a variety of redox compounds were examined at a final concentration of 5 mM. NAD^+, NADH, $NADP^+$, NADPH, FAD and ascorbic acid were tested, singly and in various combinations, but no stimulation was observed. Because of its involvement in deaminations, pyridoxal 5-monophosphate was added at a similar concentration, but it was also without effect. Separate examination of a range of metal ions (Al^{3+}, Ba^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}) at a final concentration of 1 mM similarly had no detectable effect on the activity of the enzyme.

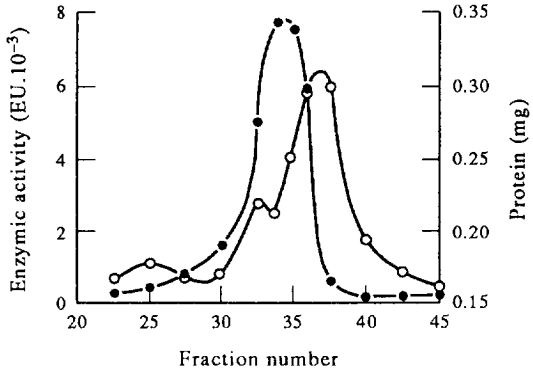

Fig. 4. Elution profile of the nebularine-forming enzyme from *S. yokosukanensis* on a column of Sephacryl S-200 eluted with a K-phosphate buffer (30 mM; pH 7.0). ●—●, Enzymic activity; ○—○, protein content.

Table 4. Partial purification of the crude enzymic activity from *Streptomyces yokosukanensis*

Step	Total protein (mg)	Total activity (EU)	Specific activity (EU mg^{-1}) protein)	Recovery (%)	Fold purification
Crude extract	178.7	5876	33	100	1
100% saturated $(NH_4)_2SO_4$ supernatant	1.48	4565	3084	78	94
Sephacryl S-200 (pooled fractions 29–35)	0.39	2214	5677	39	173

EU = radioactivity (dpm) incorporated hr^{-1} from [8-^{14}C]adenosine (1 μCi) into [8-^{14}C]nebularine. The sp. radioactivity of the adenosine was 50 mCi mmol^{-1}. The data shown are the means of triplicate determinations with good reproducibility.

Table 5. Partial purification of the crude enzymic activity using affinity chromatography on BrCN-activated Sepharose 4B

Step	Total protein (mg)	Total activity (EU)	Specific activity (EU mg^{-1}) protein)	Recovery (%)	Fold purification
Crude extract	193	5600	29	100	1
100% saturated $(NH_4)_2SO_4$ supernatant	1.72	4631	2693	83	93
BrCN-activated Sepharose 4B, affinity step	0.48	4455	9281	79	320

EU = radioactivity (dpm) incorporated hr^{-1} from [8-^{14}C]adenosine (1 μCi) into [8-^{14}C]nebularine. The sp. radioactivity of the adenosine was 50 mCi $mmol^{-1}$.

The effect of some possible substrate–analogue inhibitors was examined; the group included 2'-dideoxyadenosine, 7-deazaadenosine (tubercidin) and kinetin (6-furfurylaminopurine 9-β-D-riboside). Similarly, the purine ribosides inosine and guanosine were tested, as was also the end-product nebularine. None of them had any observable effect, with the exception of nebularine which exhibited end-product inhibition and caused a reproducible 30% decrease in the control value.

Examination of the activity of the nebularine-forming enzyme in citrate–phosphate and Tris–HCl buffers at various pH values, showed a typical activity curve with an optimum at pH 7. Activity was unaffected by addition of either EDTA (1 mM) or mercaptoethanol (0.5 mM). Estimation of its M_r was made by gel-filtration chromatography [18] using a column of Sephacryl S-200 (1.6 × 31.5 cm) and the reference proteins, insulin chain A, M_r 2900; insulin, M_r 5700; cytochrome c, M_r 12 400; carbonic anhydrase, M_r 29 000; and bovine serum albumin, M_r 67 000. A good linear relationship was obtained and the M_r of nebularine was shown to be between 8900 and 10 700. Using SDS–PAGE [19] and the reference proteins bovine serum albumin (M_r 67 000), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 29 000), soybean trypsin (M_r 21 500), lysozyme (M_r 14 400), cytochrome c (M_r 12 400) and insulin (M_r 5700), gave a value of 9600 ± 300 for nebularine. Thus, essentially similar estimates were obtained by the two methods, indicating a M_r of 9500–10 000 for nebularine. A M_r of this size would represent a very small protein molecule containing *ca* 70 amino acid residues. For comparison, lysozyme has a M_r of 14 400, and milk lipase and horse muscle acyl phosphatase have M_r of 7000 [20] and 8450 [21], respectively.

The enzyme-catalysed reaction and release of hydroxylamine

Enzymic deaminations are usually either hydrolytic or oxidative, and result in release of the amino group as NH_3 with its replacement by a hydroxyl or oxo group. At this time, the reaction in which adenosine is deaminated to nebularine is unique in that the amino group of the substrate is replaced by a H-atom. Consideration of possible mechanisms led us to the idea that hydroxylamine rather than NH_3 may be released. To test this, a sensitive method of detecting hydroxylamine described by Hirschel and Verhoeff [22], and modified by Feigl [23] was further adapted for the present purpose. The test is based on the oximation by hydroxylamine of 2,3-butanedione monoxime to yield 2,3-butanedione dioxime which precipitates as a bright red complex with Ni^{2+} ions. With our adaptation (Experimental), as little as 1 μg (30 nmol) of hydroxylamine could be detected. Applying this to a reaction mixture in which a dialysed enzyme preparation had been incubated for 4 hr with adenosine (5 mm) in a potassium phosphate buffer (0.1 M; pH 7.0) gave a pronounced positive reaction. Confirmation of the release of hydroxylamine was sought by mass spectrometry, but as hydroxylamine is relatively unstable in aqueous solution, it was first trapped in the reaction mixture as a nitrile by refluxing with 4-chlorobenzaldehyde. The nitrile was extracted into ether and then transferred to hexane for mass spectrometry. A reference sample of 4-chlorobenzonitrile was prepared for comparison. The electron-impact mass spectra (EI-MS) of the two samples (Fig. 5a, b) both show prominent ions at m/z 137 $[M]^+$ (base peak), m/z 102 $[M - Cl]^+$, m/z 75 $[M - (CN + HCl)]^+$, m/z 110 and m/z 50, confirming presence of 4-chlorobenzonitrile in the refluxed reaction mixture and hence, of hydroxylamine in the untreated enzymic reaction mixture at the end of the incubation period. To test further the hypothesis that this hydroxylamine originates from the 6-amino group of adenosine during its enzymic deamination to nebularine, [^{15}N-amino]adenosine was synthesized and the foregoing experiment repeated with hydroxylamine again being trapped as 4-chlorobenzonitrile. The EI-MS (Fig. 5c) of the refluxed ^{15}N-reaction mixture showed a prominent ion at m/z 138, another at m/z 103, corresponding to $[(^{15}N)M]^+$ and $[(^{15}N)M - Cl]^+$, respectively, and as with the ^{14}N-sample, a peak at m/z 110. Chemical ionization-MS of the same sample corroborated these

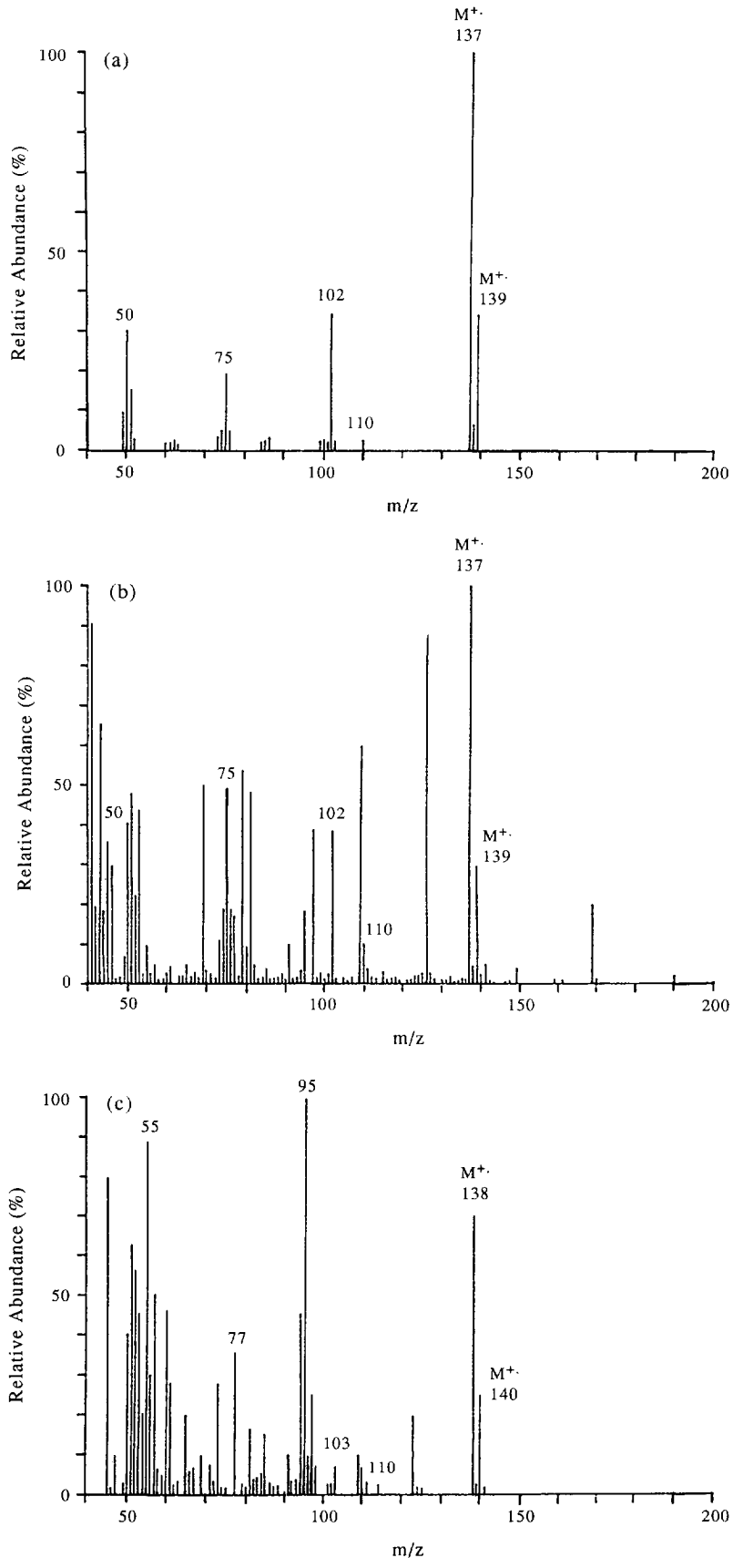

Fig. 5. Mass spectra of (a) an authentic sample of 4-chlorobenzonitrile, (b) the enzymic reaction mixture, with adenosine as substrate, refluxed with 4-chlorobenzaldehyde, and (c) the enzymic reaction mixture, with [6-^{15}N]-adenosine as substrate, refluxed with 4-chlorobenzaldehyde. (EI: 70 eV; source temp. 210°; pressure 10^{-6} mBar.)

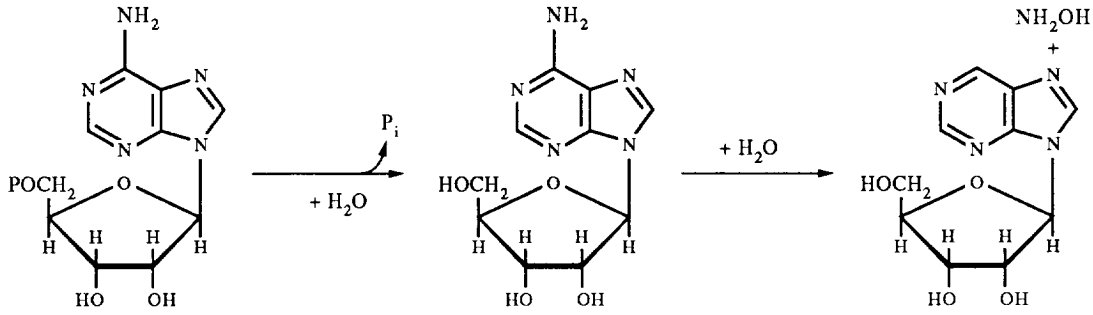

Fig. 6. The biosynthetic route by which nebularine arises in *Streptomyces yokosukanensis*.

findings with a molecular ion at m/z 156 $[M+NH_4]^+$, and peaks at m/z 120 and m/z 110. It is thus concluded that the hydroxylamine released does originate from the 6-amino group of adenosine.

Production of hydroxylamine by biological systems was first suggested by Blom [24] who postulated that it was an intermediate in nitrogen fixation by microorganisms. Early experimental support for *in vivo* hydroxylamine production came almost entirely from the work of Virtanen and his associates [25–27]. Later it was reported to be an intermediate in heterotrophic [28, 29] and autotrophic [30] ammonia oxidation. Recently, evidence has been presented that hydroxylamine is released during glutamine oxidation by the cyanobacterium *Phormidium uncitanum* [31]. There have also been several reports of the further metabolism of hydroxylamine, both oxidatively and reductively, by microorganisms [32–42], and the enzymes involved, hydroxylamine oxidase (EC 1.7.3.4) and hydroxylamine reductase (EC 1.7.9.9.1), have been shown to be flavoproteins [42].

Consideration of the properties of the enzyme, especially its low M_r (9500–10000) and what is, in effect, its hydroxylation of the amino group of adenosine, suggests that it may be of cytochrome P_{450}-type. Whatever the nature of the enzyme, however, the reaction catalysed is a novel one in purine metabolism. The route of nebularine biosynthesis in *S. yokosukanensis*, indicated by the present findings, is summarized in Fig. 6.

Nebularine synthesis and adenosine metabolism by S. yokosukanensis

Having established adenosine as the immediate precursor of nebularine, the question arose as to how quantitatively important nebularine synthesis is in relation to adenosine metabolism by *S. yokosukanensis*. To investigate this, [8-^{14}C]adenosine was supplied to cultures of the organism and its metabolic fate traced. Acid-soluble metabolites were identified by co-chromatography in several solvent systems, HV paper electrophoresis and UV-spectrophotometry. In this way, ten ^{14}C-labelled purine bases, ribosides and ribotides were identified; these were adenine, guanine, purine, adenosine, inosine, guanosine, nebularine, AMP, GMP and nebularine 5-phosphate (Table 6). Of the radioactivity originally supplied as adenosine (222×10^4 dpm) only 10% (225×10^3 dpm) was recovered. This means that 90% of the exogenously supplied adenosine was metabolized to compounds not detected or determined. Some part of this would be attributable to nucleic acid synthesis and the bulk of the remainder to catabolism to carbon dioxide. Of the total radioactivity recovered (Table 6), over 55% was in unmetabolized [8-^{14}C] adenosine, 20% in inosine and 3% in nebularine.

Table 6. Distribution of radioactivity in purine metabolites recovered after incubating cultures of *Streptomyces yokosukanensis* with [8-^{14}C]adenosine

Compound isolated	Radioactivity recovered	
	dpm	per cent of total
AMP	13754	6.1
GMP	2016	0.9
Nebularine 5-phosphate	1598	0.7
Adenosine	125472	55.6
Guanosine	7213	3.2
Inosine	45866	20.4
Nebularine	6374	2.8
Adenine	20553	9.1
Guanine	1434	0.6
Purine	•1102	0.5

Each culture was supplied with 1 μCi [8-^{14}C]adenosine; specific radioactivity 50 mCi mmol^{-1}. Standard incubation conditions were used (Experimental). Results are a typical set with good reproducibility.

The finding of purine amongst the catabolites is not surprising in view of the relatively common occurrence of the enzymic hydrolysis of nucleosides to their corresponding free bases, but it represents the first identification of free purine as the product of a biological system. Similarly, the formation of nebularine 5′-monophosphate

Table 7. Nebularine and nebularine 5′-phosphate contents of mycelium of *Lepista nebularis* and cells of *Streptomyces yokosukanensis*

Organisms	Nebularine (μmol 100 g^{-1} fresh wt)	Nebularine 5-phosphate (μmol 100 g^{-1} fresh wt)
Lepista nebularis	0.49	0.12
Streptomyces yokosukanensis	2.46	0.69

The culture of *L. nebularis* was 10 weeks old and that of *S. yokosukanensis* was 72 hr old. Data are the mean of triplicate HPLC determinations (s.d. ± 5%).

is not unexpected, but the present report is the first to describe this compound as a natural metabolite. The corresponding triphosphate has, however, been found in ascites cells treated with nebularine [43]. Since it is unavailable commercially, identification of nebularine 5′-phosphate necessitated synthesizing a reference sample of the compound. This was done by enzymic phosphorylation of nebularine [44–46] using a phosphotransferase preparation obtained from wheat shoots, and with 4-nitrophenyl phosphate as the phosphate donor. 4-Nitrophenol was removed from the incubated reaction mixture by solvent extraction and the nebularine 5′-phosphate was then separated chromatographically on a column of Dowex-1 × 4 anion exchange resin. Details are given in the Experimental.

Natural occurrence of nebularine 5′-phosphate

Using the prepared sample of nebularine 5′-phosphate as a reference, the possible natural occurrence of this compound in the mycelia, cell and culture filtrates of *L. nebularis* and *S. yokosukanensis* was investigated. None could be detected in culture filtrates of either organism, but it was found in mycelia and cells of both (Table 7). The amount of nebularine 5′-phosphate in mycelia of *L. nebularis* was *ca* one-third of the nebularine content, and in cells of *S. yokosukanensis* the content of nebularine 5′-phosphate was *ca* one-quarter of that of nebularine (Table 7). Whereas previous studies with ascites cells [43] had shown that nebularine could be phosphorylated to the 5′-triphosphate, the author noted that nebularine 5′-triphosphate is not incorporated into the RNA of *E. coli* and that the cytotoxicity of nebularine is therefore unlikely to be related to the functioning of a species of RNA. It is possible, however, that the nebularine phosphates inhibit key enzymes of nucleotide metabolism in sensitive organisms.

EXPERIMENTAL

Materials. Nebularine and other purine derivatives were obtained from Sigma (London) Chemical Co; 2′,3′-dideoxyadenosine was purchased from Calbiochem Novabiochem (U.K.) Ltd. Malt extract agar, malt extract broth, potato dextrose, tryptic soy and Czapek Dox media were obtained from Oxoid Ltd. Radiolabelled compounds were from Amersham International PLC; $^{15}NH_3$ was purchased from Aldrich Chemical Co. Ltd. Apex II ODS reversed phase packing for HPLC was obtained from Jones Chromatography, Hengoed, Mid-Glam, U.K.

Lepista nebularis (Batsch: Fr.) Kummer was collected locally by Drs C. R. Hipkin and J. M. Milton; axenic cultures were prepd by Dr J. M. Milton [9]. *Streptomyces yokosukanensis* was obtained from the American Type Culture Collection, Maryland, U.S.A.

Culture media and conditions. Lepista nebularis was cultured on (i) 2% malt agar (oxoid CM59), (ii) semisynthetic medium [10] containing glucose, malt extract or yeast extract, $MgSO_4$ and KH_2PO_4, (iii) Czapek Dox medium, and (iv) NM medium [Massart, P., personal communication], consisting of (g l^{-1}) glucose (10), malt extract (2.8), $(NH_4)_2HPO_4$ (0.25), $MgSO_4 \cdot 7H_2O$ (0.15), KH_2PO_4 (0.5), NaCl (0.025), $CaCl_2$ (0.05), and (mg l^{-1}) thiamine (0.1), biotin (0.005), $MnCl_2 \cdot 4H_2O$ (0.01), $ZnCl_2$ (0.007), H_3BO_3 (0.01), $CuCl_2 \cdot 2H_2O$ (0.002), $FeSO_4 \cdot 7H_2O$ (0.005), $NaMoO_4 \cdot 2H_2O$ (0.001), plus $FeCl_3$ (12). Agar plates and liquid cultures were incubated at 21° in the dark, the latter with orbital shaking (50 rpm). Liquid cultures were grown in 250-ml flasks, each containing 25 ml medium.

For *S. yokosukanensis*, growth was at 28° on agar containing (g l^{-1}) glucose (4), yeast extract (4), malt extract (10) and $CaCO_3$ (2) [Dyson, P. J., personal communication]. Liquid cultures of the streptomycete were grown at 28° with orbital shaking (120 rpm) on a medium containing (g l^{-1}), DIFCO tryptic soy broth (30), sucrose (100), yeast extract (10), $MgCl_2$ (10) and $CaCl_2$ (1.47) [47]. For experiments with washed cells, cells were aseptically resuspended in sterile minimal medium [48] and incubated for 2 days at 28° on an orbital shaker (120 rpm). For liquid culture, 2-l baffled conical flasks [48] were used, each containing 250 ml of medium.

Cultures of *S. yokosukanensis* were inoculated with a spore suspension (2.5 ml per 250 ml culture) prepd from reconstituted freeze-dried cultures grown for 2–4 days on the agar medium described above. The spore suspension was obtained using 10 ml of sterile H_2O dish^{-1}. After being aseptically filtered through non-absorbent cotton wool, the suspension was centrifuged (1000 *g* for 10 min at 4°), and the pellet resuspended in a similar vol. of sterile aq. glycerol (20% v/v). Suspensions were stored for short periods at −20°; for longer periods storage was at −70°.

Isolation of nebularine. To minimize artefact formation resulting from the action of non-specific phosphatases on ribotides, $HClO_4$ was used as extractant [49] and subsequently neutralized at 4° with KOH to precipitate $KClO_4$. Subsequently, it was found that with nebularine quantitatively similar recovery was achieved more quickly using aq. EtOH (25% v/v) and this was adopted for routine use. Mycelia were extracted 3 ×; on each occasion the homogenate was stirred continuously for 2 hr and the pooled extracts were clarified by centrifuging at 15 000 *g* for 10 min. Nebularine was adsorbed from the pooled extract and from culture filtrates, on to 'Norit OL' charcoal using a batch procedure [50] allowing 1 g of charcoal 100 ml^{-1} of extract or culture filtrate. The suspension was stirred continuously for 20 hr at 4° and the charcoal collected by centrifuging (5000 *g*; 30 min). After washing this 2 × with H_2O, nebularine was eluted in aq. acetone (70% v/v; pH 2) by stirring for 20 hr at 4°. The eluate was neutralized with NaOH, centrifuged to remove remaining traces of charcoal and Dowex-50W × 8 (300) cation exchange resin added (5 g 100 ml^{-1}). After stirring for 6 hr at 4°, the resin was collected by filtration, washed with H_2O, and nebularine eluted with 2.5 M HCl by again stirring for 6 hr at 4°. After filtration, the eluate was evapd *in vacuo* at 40°. The nebularine was further purified by sequential paper chromatography in (i) 1-BuOH–17 M HOAc–H_2O (60:15:25), (ii) 2-PrOH–aq. NH_3 (sp. gr. 0.88)–H_2O (70:10:20), (iii) 2-PrOH–HCl (37%)–H_2O (130:33:37) and (iv) EtOH–aq. NH_3 (sp. gr. 0.8)–H_2O (80:10:20). HV electrophoresis was also used with a formic acid–HOAc buffer (1.5 M, pH 2) and a current of 50 mA (35 V cm^{-1}) for 1.5 hr.

Determination of nebularine and its 5′-phosphate. Nebularine from the foregoing isolation procedure was determined spectrophotometrically (λ_{max} 262 nm: ε_{max} 5.9×10^3; pH1) [7]. In the absence of published data, the same values were used for nebularine 5′-phosphate. For determination of nebularine in crude extracts or enzymic incubations, an HPLC procedure was developed (Fig. 3). An Apex II ODS reversed phase column was used with isocratic elution (0.2 M KH_2PO_4; 25% aq. MeOH; 1:1 by vol.) at a flow rate of 0.5 ml min^{-1}. The eluate was monitored at 263 nm. Both for the full isolation procedure and for HPLC analysis, recovery was assessed by adding known amounts of nebularine to the material being extracted. For the former an average of 82% was found and for the latter 94%.

Preparation of nebularine 5′-phosphate. To a soln (pH 4) of nebularine (40 mM) and 4-nitrophenyl phosphate (0.6 M) was added an equal vol. of a crude phosphotransferase prepn from wheat [46]. The mixt. was incubated at 37° for 24 hr and the reaction terminated by heating to 100°. After cooling, the prepn was clarified by centrifuging and the supernatant washed several times with diethyl ether to remove 4-nitrophenol. After evapn to dryness *in vacuo*, the residue was redissolved in H_2O, adjusted to pH 8.0 with aq. NH_3 and filtered through a Millipore MF-filter (0.45 μm pore size). It was then loaded on to a column (1.8 × 20 cm) of Dowex-1 × 4 (400) formate and the column washed with H_2O (200 ml). The nucleotide was eluted in 0.25 M formic acid at a flow rate of 1 ml min^{-1} and 10-ml frs collected automatically at 4°; A_{263} was monitored. Frs 40–50, which contained the nebularine 5′-phosphate, were pooled and evapd *in vacuo* at 40°. The identity of the compound was confirmed by chromatography, HV electrophoresis and UV-spectrophotometry.

Determination of protein. The method of Lowry *et al.* [51] as modified by Bailey [52] was used throughout the work described.

Degradation of [8-^{14}C]*nebularine.* [8-^{14}C]Nebularine was degraded chemically to release, specifically, C-8 as CO_2. The process, based on reactions described in refs [11–14], was effected in 50-ml conical flasks, each modified by having a small well (10 mm diameter × 15 mm depth) fused centrally into the base. 2-Phenylethylamine (0.5 ml) was placed in the centre well and the nebularine sample was placed in the outer region. After hermetically sealing the flask with a Subaseal, 10 ml of 40 mM NaOH was introduced into the outer region using a hypodermic syringe. After 96 hr at room temp., 10 ml of 0.1 M acetic acid was added in the same way to the outer compartment and the flask heated at 95° for 15 min. The evolved CO_2 was trapped by the amine in the centre well and after cooling, aliquots of this were taken for scintillation counting.

Scintillation counting. Radioactivity was determined by scintillation counting samples (0.5 ml) in 5-ml portions of Optiphase Ria Luma scintillation fluid (LKB).

Detection of hydroxylamine. The method used was an adaptation of that of Feigl [23]. 2,3-Butanedione monoxime (4 g) was dissolved in 25 ml H_2O and to it was added 300 ml of a soln of $NiSO_4 \cdot 7H_2O$ (5.5 g) in aq. NH_3 (2.5%). After shaking, the mixt. was filtered and NaOH (1 M) added dropwise until the colour changed from violet to yellow–green. The soln was made up to 500 ml with H_2O and used to impregnate filter paper discs (Whatman 3 MM). The oven-dried (40°) papers were used to test for hydroxylamine. A small drop of the sample from a capillary tube, placed on the paper disc, gave a characteristic red positive test if hydroxylamine was present. The sensitivity limit was 1 μg.

Preparation of 4-chlorobenzonitrile. The method of van Es [53] was used on a smaller scale, starting with 10 mmol of 4-chlorobenzaldehyde, 10 mmol of hydroxylamine, 1.25 g sodium formate and 15 ml of formic acid (98–100%). The nitrile was recrystallized from H_2O, giving fine needles. A similar procedure was used with enzymic reaction mixts being examined for hydroxylamine, except that these mixts replaced the authentic hydroxylamine sample. The product was dissolved in hexane for MS.

Mass spectrometry. Samples were examined in a VG 12-253 quadrupole mass spectrometer. Both electron impact and chemical ionization were used; conditions are specified with the data.

Preparation of [6-$^{14}NH_2$]*adenosine.* Direct amination of 6-chloropurine riboside was used. A soln of 100 mg of 6-chloropurine in 5 ml MeOH was prepd and satd at −12° with 1 l of $^{15}NH_3$. This was heated in a sealed, Teflon-lined stainless steel bomb at 100° for 10 hr. After cooling, the mixt. was evapd to dryness *in vacuo* at 40°

and the residue was crystallized from 2 ml of H_2O. Sequential chromatography and electrophoresis, similar to that described for nebularine above, were used to purify the compound and, together with UV-spectrophotometry, to confirm its identity.

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