SOME ASPECTS OF PURINE METABOLISM IN MUTANTS OF *OPHIOSTOMA MULTIANNULATUM* (HEDGC. & DAVIDS.) v. ARX

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I. GENERAL INTRODUCTION

During recent years a considerable interest has developed in both biological and chemical aspects of the nucleic acids and their components, the purines and pyrimidines.

Investigations into the origin and biosynthesis of the purine derivatives and their relation to the formation of the nucleic acids, subject of the present paper, make up only a small part of the work carried out in this field. Nevertheless a complete and detailed survey of the literature on this special limited subject would be far beyond the scope of this introduction. Therefore only those reports will be referred to, that are of direct importance for the underlying problem, especially since recently more detailed reviews have been published, *e.g.*, by BROWN (1948; 1950; 1953) and BUCHANAN (1951).

Thus far the experimental evidence concerning the biosynthesis and metabolism of purine compounds has been obtained mainly from two distinctly different lines of investigations.

By far the greater part of our present knowledge we owe to studies of both the *in vivo* and *in vitro* incorporation of isotopically labeled substances into purine compounds. These experiments have led to a concept of the principal pathways of purine biosynthesis that at the moment is almost generally accepted, as will be elucidated in the next section.

The second way of attacking the problem of purine biosynthesis is the study of the nature of the intermediary products with the aid of biochemically deficient mutants of microorganisms. It must be noted, however, that the data obtained in this way are for the present insufficient to draw a satisfactory picture of purine biosynthesis as a whole in these organisms. Nevertheless they have provided valuable additional evidence for the general concept mentioned above.

PURINE BIOSYNTHESIS AND METABOLISM STUDIED WITH ISOTOPICALLY LABELED COMPOUNDS

The scheme of figure 1 attempts to draw a picture of the present status of our knowledge about the intermediates and pathways in
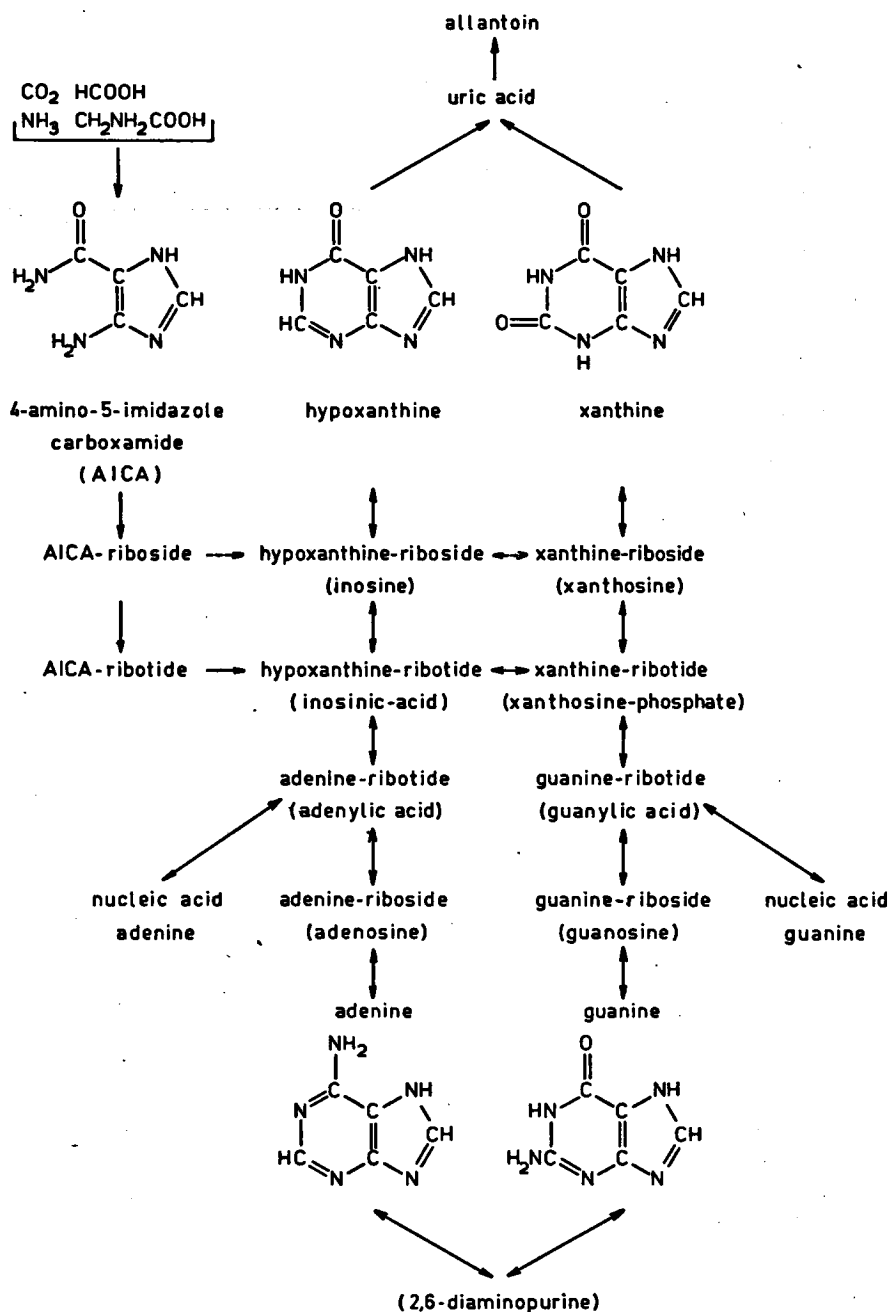
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allantoin

CO$_2$ HCOOH
NH$_3$ CH$_2$NH$_2$COOH

uric acid

4-amino-5-imidazole
carboxamide
(AICA)

hypoxanthine

xanthine

AICA-riboside ⟶ hypoxanthine-riboside ⟷ xanthine-riboside
(inosine)   (xanthosine)

AICA-ribotide ⟶ hypoxanthine-ribotide ⟷ xanthine-ribotide
(inosinic-acid)   (xanthosine-phosphate)

adenine-ribotide   guanine-ribotide
(adenylic acid)   (guanylic acid)

nucleic acid   adenine-riboside   guanine-riboside   nucleic acid
adenine   (adenosine)   (guanosine)   guanine

adenine   guanine

(2,6-diaminopurine)

Fig. 1. *General survey of reactions concerned with purine metabolism*

purine biosynthesis, as it is understood from experiments using the isotope tracer technique. These investigations consisted mainly of feeding experiments with pigeons and of *in vitro* incorporation studies with pigeon liver preparations. Useful additional evidence, however, has been obtained from feeding experiments with rats and from studies on sulfonamide inhibited *E. coli*.

It must be emphasized, however, that our understanding of the individual reactions is very rudimentary, as not one of these reactions has been obtained in a pure form. In several cases the arrows drawn are indicating reactions not yet known to occur, but which might occur on the basis of certain well-established analogous reactions.

Origin of the atoms of the purine ring

The report by BARNES and SCHOENHEIMER (1943) that in both the pigeon and the rat N^{15}-ammonium salts are incorporated into the cellular nucleic acids gave the impulse to a series of investigations which have resulted in an enormous extension of our knowledge about the origin of the atoms of the purine molecule. The leading principle of these experiments was that in animals — as in plants — the purines are synthesized from smaller compounds, normally occurring in the cells. Not until this had been established, the recent development became possible. It was still facilitated by the fact that purines could be obtained as excretion products.

SONNE and co-workers (1946; 1948), BUCHANAN and SONNE (1946) and BUCHANAN *et al*. (1948) examined the formation of excretory uric acid purine in pigeons, fed with isotopically labeled compounds, which were presumed to be purine precursors. After the isolation of uric acid from the excreta, it was subjected to various degradation procedures which permitted the localization of the labeled atoms in the purine ring. The experiments have led to the assumption that the purine skeleton originates from carbon dioxyde, formate, glycine and ammonia as is outlined in figure 2.
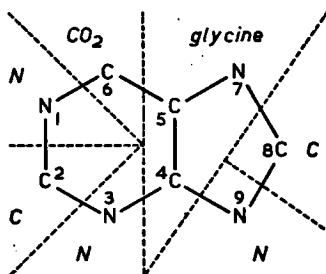

Fig. 2. *Origin of the atoms of the purine ring*
C: from 1-carbon source (formate).
N: from metabolic nitrogen pool (NH_3, NH_2 donors).

The possibility of glycine being the source of nitrogen 7 of uric acid in man had already been put forward by SHEMIN and RITTENBERG

(1947). KARLSSON and BARKER (1949) and ELWYN and SPRINSON (1950) demonstrated that in the pigeon intraperitoneal injection of labeled precursors resulted in an incorporation similar to that in the feeding experiments mentioned above.

Intermediary products in purine biosynthesis

EDSON, KREBS and MODEL (1936) found that in the pigeon hypoxanthine functions as an intermediary metabolite in uric acid synthesis. Pigeon liver preparations turned out to be able to synthesize hypoxanthine, ammonia simultaneously being incorporated, whereas the hypoxanthine formed could be converted into uric acid by a kidney preparation. GREENBERG (1948; 1949) and SCHULMAN et al. (1950) confirmed these results by demonstrating the incorporation of labeled uric acid precursors: bicarbonate, formate and glycine into hypoxanthine by pigeon liver homogenates.

In search for further intermediates in hypoxanthine synthesis GREENBERG (1949) observed that on incubation of pigeon liver homogenate with C^{14}-labeled formate only about 70 per cent of the total initial activity was accounted for as hypoxanthine. Paperchromatographic analysis of the reaction mixture revealed that it contained a second purine-like compound with considerable activity. This compound turned out to be inosinic acid (GREENBERG, 1950), whereas closer investigation (GREENBERG, 1951) showed the occurrence of a third substance containing activity, viz. inosine. Almost all activity initially present in the formate could be recovered from these three substances. Comparison of the rates of incorporation of C^{14} into inosinic acid, inosine, and hypoxanthine gave rise to the assumption that inosinic acid might be the first purine derivative, produced in this system, whereas inosine and hypoxanthine would be derived from it by reversible reactions.

These results could be fully confirmed by SCHULMAN and BUCHANAN (1952) who incubated pigeon liver homogenates with glycine-C^{14} in the presence of inosinic acid and inosine or hypoxanthine, and subsequently determined the activities of these three substances in the reaction mixture.

The immediate precursor of inosinic acid is supposed to be the pentose-phosphate derivative of 4-amino-5-imidazolecarboxamide. This aminoimidazolecarboxamide, henceforth referred to as AICA, was found to accumulate during sulfonamide bacteriostasis of *E. coli* and a variety of other bacteria (STETTEN and FOX, 1945). It was identified as such by SHIVE and co-workers (1947), who put forward the hypothesis that it might function as a purine precursor. This hypothesis was favoured by the fact that glycine exerts a stimulating effect on AICA accumulation in *E. coli*, as was reported by RAVEL et al. (1948) and by GOTS and LOVE (1954).

Experiments with pigeon liver preparations (SCHULMAN et al., 1950; SCHULMAN and BUCHANAN, 1952; BUCHANAN and SCHULMAN, 1953) gave additional support to the above-mentioned theory, since it was demonstrated, that C^{14}-labeled AICA was incorporated into purine

compounds. GREENBERG (1951), however, was unable to detect the presence of labeled AICA in such systems when the incubation was carried out in the presence of labeled formate or glycine. He concluded therefrom that AICA *per se* could not be an intermediate in purine synthesis. Because of the observed stimulating effect of ribose-phosphate on the formation of direct hypoxanthine precursors it was supposed that AICA riboside or ribotide might be involved in the biosynthesis of hypoxanthine. The discovery by BUCHANAN and SCHULMAN (1953) of an enzymatic exchange reaction between inosinic acid and formate strongly favoured the assumption that inosinic acid arises directly from AICA-pentose-phosphate by a reaction with formate, in which the citrovorum factor would function as "inosinic acid transformylase".

According to GREENBERG (1953) ATP is necessary for formate incorporation into inosinic acid. Because of the powerful stimulating effect of homocysteine upon this incorporation homocysteine derivatives are considered to represent active agents in transporting formyl groups, *e.g.*, by way of products as S-formylhomocysteine.

WILLIAMS and BUCHANAN (1953) found in yeast the same relationship between AICA, inosinic acid, inosine, and hypoxanthine as had been established in pigeon liver. The utilization of AICA for purine synthesis in the rat was previously reported by MILLER *et al.* (1950; 1952). MILLER (1954) showed that 2,4-dinitrophenol strongly affects this utilization together with the esterification of phosphate. This constitutes additional support for the concept that before ring closure can occur the ribotide of AICA must be formed.

Synthesis of other purine derivatives

Incorporation of one of the above-mentioned precursors, viz. formate, into polynucleotide purines was reported by MARSH (1949; 1951) who isolated radioactive nucleic acid adenine and guanine from the viscera of pigeons, fed with formate-C^{14}. This indicated a relation between the outlined biosynthesis of hypoxanthine and uric acid and the normal pathway of synthesis of other purine derivatives. As a matter of fact indications for such a relation have been found in quite a number of cases, in which labeled small purine precursors were found to be incorporated into polynucleotide purines, *e.g.*, by KOCH and his associates (1952) and BALIS *et al.* (1952) in *E. coli*, by ABRAMS and co-workers (1948), DiCARLO *et al.* (1950), and by ED-MONDS, DELLUVA and WILSON (1950) in yeast, by KALCKAR and RITTENBERG (1947), REICHARD (1949), MARSH (1949), HEINRICH and WILSON (1950), LEPAGE and HEIDELBERGER (1951), and LEPAGE (1952) in the rat, and by ABRAMS and GOLDINGER (1951; 1952) in the rabbit.

Although GREENBERG (1951) emphasized that there was no evidence of the *in vitro* formation of soluble adenine derivatives or polynucleotide purines in the above pigeon liver preparations, the formation of adenine nucleotides and hypoxanthine from added labeled adenine by pigeon liver homogenate as reported by GOLD-

WASSER (1952) strongly favours the idea that the biosynthesis of nucleotide and polynucleotide purines on one hand and of uric acid and its precursors on the other, proceeds at least partially by a common pathway.

Investigations by BROWN and his associates (1948a, b) demonstrated the incorporation of adenine into nucleic acids of the internal organs of the rat, whereas ABRAMS and GOLDINGER (1951) showed the incorporation of adenine into nucleic acids in rabbit bone marrow slices.

In the rat hypoxanthine and xanthine, however, failed to be converted into nucleic acid constituents (GETLER et al., 1949). Feeding of labeled hypoxanthine and xanthine resulted in a considerable labeling of excretory allantoin, a compound known from the work by BROWN et al. (1947) as an oxidation product of uric acid. This suggests that at least in the rat hypoxanthine and xanthine per se are not precursors of nucleic acid purines but rather have to be considered as intermediates in the breakdown process.

Incorporation of adenine into polynucleotides was reported for a variety of other organisms, of which may be mentioned yeast (KERR et al., 1951), E. coli (KOCH, PUTNAM and EVANS, 1952; BOLTON et al., 1952), Lactobacillus casei (BALIS and BROWN, 1951), L. leichmannii (WEYGAND et al., 1952), and Tetrahymena geleii (FLAVIN and GRAFF, 1951).

According to BROWN et al. (1948a, b) feeding of adenine-N^{15} results in incorporation of isotope into both nucleic acid adenine and guanine in the rat, thus indicating a possible pathway of synthesis of guanine via adenine. Similar observations were reported by KERR et al. (1951) for yeast.

ABRAMS and BENTLEY (1955a), using a soluble rabbit bone marrow extract, presented strong evidence that in this system exogenous adenine is transformed through a sequence of reactions by way of adenosine, inosine and xanthosine into guanosine. It is still possible, that in this sequence not the ribosides but the ribotides are involved, since the demonstration by ABRAMS and BENTLEY (1955b) of the formation of guanine from inosinic acid.

This does not imply, however, that the formation of guanine from adenine is a generally occurring process, for as was demonstrated by FLAVIN and GRAFF (1951) for Tetrahymena geleii and by WEYGAND et al. (1952) for L. leichmannii, sometimes adenine is utilized for the synthesis of nucleic acid adenine only. It stands as a remarkable fact that in these cases utilization of guanine for the synthesis of both nucleic acid guanine and nucleic acid adenine could be demonstrated.

The rat (ABRAMS, 1951) and yeast (KERR et al., 1951) are unable to bring about the conversion of guanine into adenine, although both utilize guanine to synthesize nucleic acid guanine. Between these two extremes there exist other situations, as in L. casei, where according to BALIS and BROWN (1951) and BALIS et al. (1952) maximum interconversion between adenine and guanine is represented.

It is presumed by some authors that 2,6-diaminopurine might be an intermediate in the adenine-guanine interconversion. It was shown

for the rat by BENDICH, FURST and BROWN (1950) and for *L. casei* by BALIS *et al.* (1952) that the behaviour of this compound is qualitatively similar to that of guanine. All these facts seem to favour the suggestion put forward by BROWN (1953) that the pathway leading first to adenine and thence to guanine may predominate in some species, whereas in others the reversed pathway is predominating.

BIOSYNTHESIS OF PURINES STUDIED WITH BIOCHEMICALLY DEFICIENT MUTANTS OF MICROORGANISMS

Biochemical mutations of microorganisms have presented valuable tools in the search for intermediary products in the biosynthesis of various biologically important compounds. According to TATUM (1946) the following presumptions are underlying investigations with such mutants: (1) a series of biochemical reactions in a given biosynthesis implies the cooperation of a corresponding series of enzymes and genes, and (2) the production of every enzyme is controlled by one specific gene. Thus every single gene controls a single biochemical reaction. Consequently the over-all synthesis may be blocked at different steps by mutation of different genes.

The growth of such mutant strains, therefore, in contrast to their parental strain, is dependent upon an exogenous supply of an additional substance.

All intermediary products which would have been formed normally in the reaction chain after the blocked reaction should have a growth-promoting effect equivalent to that of the ultimate product, whereas intermediates formed prior to the block should be inactive. As a further consequence one may expect the accumulation of one or more intermediary substances which are synthesized by enzymatic processes prior to the blocked one during growth of a mutant. Thus the ways are indicated by which these mutants permit the examination of the biosynthetic processes that lead to the formation of vital cellular constituents.

Although the above-mentioned presumptions (the so-called one gene-one enzyme hypothesis) have turned out to be too simple to give a sufficient explanation of all phenomena encountered at further examination, their consequences still hold good to a fairly large extent (GLOOR, 1955).

Biochemical mutants requiring purine derivatives for growth have been reported in various microorganisms, *e.g.*, in *Acetobacter* by GRAY and TATUM (1944), in *Ophiostoma* by FRIES (1945a, b; 1946; 1947), in *Neurospora* by PIERCE and LORING (1945) and by MITCHELL and HOULAHAN (1946), in *E. coli* by GOTS (1950) and by GOTS and LOVE (1954), and in *Aerobacter* by USHIBA and MAGASANIK (1952).

The results of studies of different investigators on purine requiring mutants of *Neurospora*, *E. coli* and *Ophiostoma* permit the formulation of the schemes shown in figure 3.
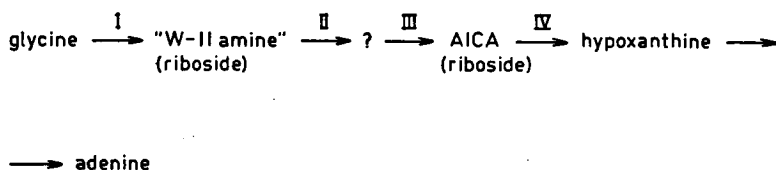
Adenine-deficient mutants

The most common type of purine-requiring mutants are the so-called adenine-less ones. Some mutants belonging to the first group show an absolute requirement for adenine derivatives, as was found, *e.g.*, in *Neurospora* (MITCHELL and HOULAHAN, 1946) and in *Ophiostoma* (FRIES, 1946).

Ophiostoma

$$? \xrightarrow{\text{I}} \text{AICA} \longrightarrow ? \xrightarrow{\text{II}} \text{hypoxanthine} \xrightarrow{\text{III}} \text{adenine} \xrightarrow{\text{IV}}$$
$$\text{(adenosine,}$$
$$\text{adenylic acid)}$$

$$\xrightarrow{\text{IV}} \text{2.6-diaminopurine} \xrightarrow{\text{V}} \text{guanine}$$
$$\text{(guanosine}$$
$$\text{guanylic acid)}$$

Fig. 3A. *Summary of information concerning the sequence of reactions in purine biosynthesis in Ophiostoma*

  I. Mutant utilizing adenine, hypoxanthine and AICA (FRIES *et al.* 1949).
 II. Mutant utilizing both adenine and hypoxanthine (FRIES, 1946).
III. Mutant requiring adenine (FRIES, 1946).
 IV. Mutant requiring guanine or 2,6-diaminopurine and accumulating adenine. "Sparing" effect of guanine upon adenine utilization by adenine-less mutants (FRIES 1949a, 1953).

Neurospora

$$? \xrightarrow{\text{I}} \text{purple pigment} \xrightarrow{\text{II}} ? \xrightarrow{\text{III}} \text{hypoxanthine} \xrightarrow{\text{IV}} \text{adenine}$$
$$\text{(inosine)} \qquad \text{(adenosine,}$$
$$\text{adenylic acid)}$$

$$\xrightarrow{\text{V}} \text{xanthine} \xrightarrow{\text{VI}} \text{guanine}$$
$$\text{(guanosine,}$$
$$\text{guanylic acid)}$$

Fig. 3B. *Summary of information concerning purine biosynthesis in Neurospora*

  I. Temperature-sensitive mutant requiring adenine or hypoxanthine when grown at temperatures above 26° C. (MITCHELL and HOULAHAN, 1946).
 II. Adenine or hypoxanthine-requiring mutant, accumulating purple pigment, considered as an adenine precursor. — Below 26° C. double-mutant I/II also produces this pigment, reaction II being blocked. Above 26° C. no pigment is formed by the double-mutant: biosynthesis is blocked at reactions I and II (MITCHELL and HOULAHAN, 1946).
III. Mutant utilizing adenine or hypoxanthine derivatives (MITCHELL and HOULAHAN, 1946).
 IV. Mutant with an absolute requirement for adenine compounds (MITCHELL and HOULAHAN, 1946).
  V. "Sparing" effect of xanthine upon adenine utilization by adenine-less mutants (FAIRLEY and LORING, 1949).
 VI. "Sparing" effect of guanine upon adenine utilization by adenine-requiring mutants (LORING and FAIRLEY, 1948).

E.coli

glycine $\xrightarrow{\text{1}}$ "W-II amine" $\xrightarrow{\text{II}}$? $\xrightarrow{\text{III}}$ AICA $\xrightarrow{\text{IV}}$ hypoxanthine $\longrightarrow$
(riboside)   (riboside)

$\longrightarrow$ adenine

Fig. 3C. *Summary of information concerning the pathway of purine biosynthesis in Escherichia coli*

  I. Stimulating effect of glycine upon purine precursor accumulation by an adenine-less mutant (GOTS and LOVE, 1954; LOVE and GOTS, 1955).
 II. Accumulation by an adenine-less mutant of a diazotizable amine, possibly a ribotide or riboside, which can be converted to AICA (LOVE and GOTS, 1955).
III. Mutant utilizing adenine, hypoxanthine and AICA (BEN ISHAI *et al.*, 1951; DAVIS, 1952).
 IV. Adenine or hypoxanthine-requiring mutant, accumulating AICA riboside (GOTS, 1950; 1953).

Mutants of the second group will grow equally well when instead of adenine hypoxanthine is added to the basal minimum nutrient solution. These mutants too have been reported in *Neurospora* and in *Ophiostoma* by the above-mentioned authors. This suggests that the final purine compounds are synthesized by way of hypoxanthine and adenine, in this order. The former mutants apparently are unable to convert hypoxanthine into adenine, whereas the latter are able to carry out this conversion and consequently have been blocked at an early step of the reaction chain.

A third group of adenine-deficient mutants is represented by *E. coli* strain B-96, as was reported by GOTS (1950). This strain is able to utilize adenine and hypoxanthine, but is different from the above-mentioned mutants since it accumulated during growth a non-acetylatable diazotizable amine, that was identified as AICA. It is therefore concluded that in this mutant as a result of the mutation the reaction involving ring closure does not proceed.

Recently an adenine-less strain (W-11) of *E. coli* was described (LOVE and GOTS, 1955) that accumulates a diazotizable amine of undetermined structure, when grown with either adenine or hypoxanthine. Evidence for the participation of glycine in the formation of the W-11 amine is furnished by the fact that the accumulation is stimulated by the former compound. It has been shown in a previous section that glycine stimulates AICA accumulation. The W-11 amine, however, was not identical with the latter compound, but it could be converted into AICA by strain B-96. Presumably the new amine is a precursor of AICA, though the latter could not replace purines in supporting growth of strain W-11. Possibly the *Neurospora* mutants 35203 and 70004, isolated by MITCHELL and HOULAHAN (1946), of which the former accumulated a purple pigment when grown with adenine or hypoxanthine and the latter was assumed to be blocked

in a reaction prior to that causing the accumulation of the pigment belong to the same group of mutants as strains B-96 and W-11 of *E. coli*. Too little is known, however, about the true nature of the reactions involved in these *Neurospora* mutants to be certain of this relation.

Adenine-less mutants whose growth is supported by AICA too were reported by FRIES *et al.* (1949) in *Ophiostoma* and by BEN ISHAI *et al.* (1951) in *E. coli*. These mutants represent a block still earlier in the sequence of reactions leading to the formation of the ultimate products.

With regard to the question as to at which level purine synthesis proceeds in the organism, adenine-less mutants have furnished additional evidence against the participation of the free bases as intermediates in the biosynthesis. FRIES (1946) reported that in adenine-less *Ophiostoma* adenosine and adenylic acid exerted a greater growth-promoting effect than adenine, when these substances were compared on a molar base. The formation of AICA riboside by *E. coli* mutants as shown by BEN ISHAI *et al.* (1951) and by GOTS (1953) together with the demonstration by LOVE and GOTS (1955) of the synthesis of the unknown AICA precursor as a riboside accumulated by strain W-11 of this organism, indicate that pentose incorporation is likely to take place at a very early stage of the biosynthesis.

It is worth while to mention that out of eight adenine-less *Neurospora* mutants studied, seven, that were able to grow with hypoxanthine, could utilize inosine too. The remaining one could utilize neither hypoxanthine nor inosine, but did grow with adenosine (MITCHELL and HOULAHAN, 1946). Thus it may be that the latter mutant — and similar mutants of other organisms — has lost the capacity to aminate inosine to form adenosine, rather than, as stated above, the ability to convert hypoxanthine into adenine. As to this it is possibly of some significance that according to MCELROY and MITCHELL (1946) *Neurospora* cells contain an adenosine deaminating enzyme, but no one that deaminates adenine.

Summarizing the results of the investigations cited above, we are led to the conclusion that, if the picture outlined in figure 1 is considered, our knowledge about purine biosynthesis obtained with biochemical mutants of different species fits rather well in this scheme, though it is certainly less complete. Far from complete is our understanding of the pathways of purine biosynthesis in the individual species of microorganisms, *e.g.*, in *Ophiostoma multiannulatum*, as becomes apparent from figure 3.

Guanine-deficient mutants

Mutants requiring guanine for growth have been reported in only a few cases. FRIES (1946, 1949a) isolated a strain of *Ophiostoma* that would only grow when guanine, guanosine, guanylic acid, or 2,6-diaminopurine was added to the basal minimum medium. Adenine could not be utilized, and even exerted an inhibitory effect upon growth of the mutant with guanine. According to FRIES (1953) this strain accumulates adenine when grown on a medium containing guanine

derivatives. It must, however, be noted that the true nature of the accumulated substance is not quite certain, since it was identified by way of its growth-promoting activity with respect to adenine-requiring strains.

The supposition made by FRIES (1949a, 1953) that from the above-mentioned adenine accumulation it follows that guanine is formed from adenine is favoured by another observation, which moreover indicates that the reaction is an irreversible one. It was found that growth with adenine of adenine-less strains, which are unable to utilize guanine for growth, is clearly stimulated by the latter compound. This was ascribed to the replacement of guanine that normally would have been formed from adenine, so that adenine is more efficiently utilized for the synthesis of cellular adenine compounds.

A similar "sparing" effect by exogenous guanine was demonstrated in an adenine-less *Neurospora* mutant by LORING and FAIRLEY (1948).

According to FAIRLEY and LORING (1949) xanthine too exerts a "sparing" effect upon adenine utilization by an adenine-less *Neurospora*.

MAGASANIK and BROOKE (1954) reported a guanine-requiring mutant of *Aerobacter aerogenes*, which accumulated a substance that was identified as xanthosine. This, together with the above-mentioned "sparing" effect of xanthine, seems to indicate that guanine synthesis proceeds by way of xanthine compounds. It was assumed by the authors that the accumulated xanthosine had originated from xanthosine-phosphate, which compound is rapidly dephosphorylated by phosphatase. Xanthosine-phosphate might be formed from inosine-phosphate, so that guanine synthesis would go on on the level of the ribotides. If this is true, it will be necessary to assume that xanthine *per se* — as adenine and hypoxanthine — is not an intermediary product in the biosynthesis, but lies on a secondary pathway, that involves ribosidation and phosphorylation of the free bases.

Whereas in *Ophiostoma* the formation of guanine from adenine is apparently an irreversible process as was shown above, in *Aerobacter* the reaction seems to be reversible. MAGASANIK and BROOKE (1954) described another mutant whose growth was supported not only by guanine, but also by xanthine, adenine and hypoxanthine. This means in the first place that purine synthesis has been blocked prior to the formation of hypoxanthine derivatives. But as a further consequence it means that apparently the requirements for hypoxanthine, adenine, xanthine or guanine are met by each of these substances, so that the formation of guanine from hypoxanthine can proceed also in the reversed direction.

FURTHER POSSIBILITIES OF INVESTIGATIONS WITH BIOCHEMICAL MUTANTS

The work described in the present paper was started in order to examine the further possibilities of investigations of purine metabolism with the aid of biochemical mutants of *Ophiostoma multiannulatum* (HEDGC. & DAVIDS.) V. ARX.

Obviously the discovery of new types of mutants will extend our

knowledge about the intermediary products involved in the biosynthesis of the purines. Therefore, the induction and isolation of purine-less mutants was undertaken in the hope that one or more new types might occur among the strains isolated.

Since the quantitative growth-responses of mutants to various substrates may give an insight into the true nature of the intermediates in a biosynthetic process, a more careful examination of the growth-promoting activities of various purine derivatives was undertaken. This seemed of importance as to the question whether the free bases or the ribosides or ribotides are involved in the biosynthesis.

Moreover it had to be expected that as a result of mutation *de novo* purine synthesis would not occur in purine-less strains. This seemed to offer the possibility to study the metabolism of supplemented purines and to investigate the utilization of the exogenous purine compounds for nucleotide synthesis. In order to realize this possibility the uptake of adenine derivatives from the culture medium by adenine-deficient mutants was determined together with the growth of the mutants and the purine and pyrimidine content of the mycelium formed.

II. INDUCTION AND ISOLATION OF ADENINE-DEFICIENT MUTANT STRAINS OF *OPHIOSTOMA*

The ascomycete *Ophiostoma multiannulatum* (HEDGC. & DAVIDS.) v. ARX was chosen for the present investigations because it is easy to handle and it grows rather rapidly on a synthetic medium. A strain of this fungus was obtained from the Centraal Bureau voor Schimmelcultures at Baarn (Netherlands). From this parent strain, which formed abundant masses of ascospores, monospore strains were isolated, which were used for the induction and isolation of biochemically deficient mutants.

INDUCTION OF MUTANTS

The induction of mutants was carried out on suspensions of wild type conidiospores, obtained in shaken cultures at 30° C. with a nutrient solution composed of the so-called "complete" medium (see below) plus 0.001 per cent inositol, as described by FRIES (1949b) and by WIKBERG and FRIES (1952). The conidia were filtered through a Schott G-1 glass filter (porosity $100—200\mu$) to remove mycelium possibly present, washed three times with distilled water by centrifugation and finally suspended in water to a concentration of $10^6—10^7$ cells per ml. 15 ml. of this suspension were placed in a Petri-dish of 10 cm. ø on a magnetic stirrer and exposed to ultraviolet irradiation from a Philips' TUV-25W tube lamp until about 99 per cent of the conidia had been killed. This required an irradiation time of $1\frac{1}{2}—2$ minutes under the conditions employed, *i.e.*, when the suspension was placed at a distance of 40 cm. under the UV source.

Isolation of Mutants

The isolation of mutants, induced by the UV irradiation was performed by way of a method quoted from FRIES (1947; 1948) and WIKBERG and FRIES (1952). The irradiated conidia were centrifuged and resuspended in 200 ml. of a minimum nutrient solution, containing glucose 20 g., ammoniumtartrate 5 g., KH_2PO_4 1 g., $MgSO_4$. $7H_2O$ 0.5 g., NaCl 0.1 g., $CaCl_2$ 0.1 g., $ZnSO_4.7H_2O$ 4.4 mg., $MnSO_4.4H_2O$ 4.0 mg., $FeCl_3.6H_2O$ 4.0 mg., thiamine 0.10 mg., pyridoxine 0.10 mg. and distilled water to make up a volume of 1 liter. This medium only permits germination and growth of the surviving wild type conidia, whereas the mutated conidia do not form hyphae.

The spores were kept for 24 hours at 20° C.; this temperature was chosen because it seems to favour hyphal growth and to suppress largely the conidial growth that is observed at 30° C. (FRIES, personal communication).

Subsequently the suspension was filtered through a Schott G-2 glass filter (porosity 40—100 μ). 36 hours later this filtration was repeated once more. By doing so an attempt was made to remove at least a part of the unmutated surviving conidia, which had germinated.

From the second filtrate a series of dilutions ranging from 1 : 10 to 1 : 10^4 was prepared. These were plated out in Petri-dishes with c. 15 ml. of a "complete" medium, consisting of the above nutrient salts and organic compounds of the minimum medium, 100 ml. of malt extract, 25 ml. of casein hydrolysate (equivalent to 250 mg. of casein), 25 mg. of yeast nucleic acid, 0.25 ml. of nucleic acid hydrolysate (equivalent to 50 mg. of yeast nucleic acid), 1 ml. of yeast autolysate, 2 per cent agar, and tap water to make up a volume of 1 liter. The dilutions were prepared so as to make certain that at least a number of dishes would contain an appropriate number of living conidia, i.e., not more than 70 and preferably not less than 20.

These conidia — mutated and unmutated ones — produced on the complete agar medium separate monoconidial mycelia, from which conidia were transferred as described by FRIES (1948) to Petri-dishes with minimum agar medium (minimum nutrient solution solidified with 1.5 per cent thoroughly washed agar).

Those conidia obtained from unmutated mycelia grew out rapidly on the minimum medium when incubated at 27° C., whereas inocula derived from mutant mycelia grew out very poorly or not at all. The latter inocula were cut out and transferred separately to slanted culture tubes containing complete agar medium, where development of mycelium could take place. To decide definitely whether an isolated strain was a biochemical mutant, it was transferred to a slanted tube with minimum agar medium. When after 10 days no mycelium had developed in this tube, the strain was decided to be a mutant.

Stock cultures of the mutants were kept on slanted culture tubes containing an enriched malt agar medium, composed of malt extract, casein hydrolysate, nucleic acid and nucleic acid hydrolysate and tap water, solidified with 2 per cent agar. Re-inoculation on fresh medium

took place once a month; every transfer was checked for reversal to wild type by simultaneous inoculation on minimum agar.

IDENTIFICATION OF MUTANTS

Since no attempt was made to carry out a selective isolation of purine-deficient strains, it will be evident that mutations were obtained involving the requirement of such vital cellular constituents as vitamins and amino acids too. The nature of the growth requirement was preliminarily established by transferring the mutants to a liquid minimum medium, supplemented with either yeast extract, or casein hydrolysate, or nucleic acid plus nucleic acid hydrolysate. Those mutants requiring a nucleotide constituent for growth responded to the supplemented nucleic acid and nucleic acid hydrolysate and — more or less — to yeast extract, that apparently contains also a certain amount of nucleic acid constituents.

The nature of the compounds required by the nucleotide-deficient strains isolated was established by investigating whether they would grow on a minimum nutrient solution supplemented with either adenine, guanine, uric acid, cytosine or uracil in mg. amounts. Simultaneously inoculated unsupplemented minimum solution served as a control. In all cases where growth occurred, eventually indicating the deficiency of the mutants, possible reversal to wild type was checked by appropriate controls.

MUTANTS OBTAINED

In the above-mentioned way eight mutant strains responding to adenine have been isolated and identified, viz. nos. 53, 59, 60, 64, 65, 66, 73, and 74. Through courtesy of Dr. Nils Fries from the Institute of Physiological Botany of the University of Uppsala (Sweden) three further adenine-requiring mutants were obtained, viz. his nos. 1671, 1202 and 870, that are referred to in this paper as nos. 57, 72 and 71.

The qualitative growth requirements of these adenine-less strains were studied more in detail by experiments in which besides of adenine, the growth-promoting activities of the following substrates were examined: adenylic acid, adenosine, inosinic acid, inosine, hypoxanthine and 4-amino-5-imidazolecarboxamide.

Petri-dishes, containing 15 ml. of minimum agar medium, in duplicate supplemented with 1 μM of the above substrates were inoculated with the adenine-deficient strains, together with two unsupplemented controls. After a week incubation at 27° C. the plates were observed for possible growth. In this, as well as in all growth experiments that will be described, transfer to unsupplemented medium was carried out to demonstrate that in those cases in which growth occurred, this was not due to reversal to wild type.

The results of one of these experiments are summarized in table I. Considering these data it appears that provisionally three distinctly different types of mutants can be discerned, viz. those growing only

TABLE I

Growth responses of adenine-less mutants of Ophiostoma to various purine derivatives

Mutants were grown on 15 ml. of minimum agar medium; supplements were added in 1 μM amounts.

+ : good growth; +— : weak growth; — : no growth

Supplement	Mutant strain no.										
	57	60	65	66	53	59	64	71	74	72	73
Adenylic acid	+	+	+	+	+	+	+	+	+	+	+
Adenosine	+	+	+	+	+	+	+	+	+	+	+
Adenine	+	+	+	+	+	+	+	+	+	+	+
Inosinic acid	+—	+—	+—	+—	+—	+—	+—	+—	+—	—	—
Inosine	+	+	+	+	+	+	+	+	+	—	—
Hypoxanthine	+	+	+	+	+	+	+	+	+	—	—
AICA	+[1]	—	—	—	—	—	—	—	—	—	—
None	—	—	—	—	—	—	—	—	—	—	—

[1] 20 ml. of liquid minimum medium supplemented with 1 μM of AICA and inoculated with a heavy inoculum.

when adenine derivatives are present in the medium, those whose growth is supported by hypoxanthine derivatives too, and those who are able to utilize, in addition to the former substrates, AICA. With respect to the latter type it was reported by FRIES, BERGSTRÖM and ROTTENBERG (1949) that mutant no. 57 could be grown on a medium supplemented with AICA, provided a heavy inoculum was used. In fact this strain did not grow on the plate with AICA, but it showed good growth on liquid minimum medium to which this substance was added.

MATERIALS

The organic and inorganic chemicals used throughout this study as constituents of nutrient media were common commercial preparations.

Liquid malt-extract was used for the "complete" and malt-agar media.

Yeast autolysate was prepared in the following way: 1000 g. of baker's yeast were suspended in 1000 ml. of distilled water and kept for c. 18 hours at 55° C. Subsequently the resulting autolysate was centrifuged and the supernatant was used for the nutrient media.

Casein hydrolysate was obtained by boiling 10 g. of commercial casein for 18 hours with 100 ml. of 25 per cent sulfuric acid. After neutralization with Ba(OH)$_2$ and removal of the BaSO$_4$ precipitated, the final volume was adjusted to 1 liter.

Yeast nucleic acid was obtained from Hoffman-La Roche S.A., Basle (Switzerland). Nucleic acid hydrolysate was prepared by autoclaving 1 g. of yeast nucleic acid with 5 ml. of N NaOH for 30 minutes at 15 pounds steam pressure.

Agar for the solid minimum medium was washed for at least ten days with distilled water that was refreshed once a day. Subsequently it was soaked for two days with ethyl alcohol and finally the alcohol was washed out carefully with distilled water.

Unless stated otherwise the nutrient media were sterilized as such by autoclaving for 30 minutes at 15 pounds steam pressure.

Adenylic acid (adenosine-3-phosphate), adenosine, adenine, hypoxanthine, cytidine, uridine and uracil were obtained from Hoffmann-La Roche, inosinic acid (inosine-5-phosphate) and inosine from the Bios Laboratories, New York (U.S.A.) and cytosine from Light & Co Ltd., Colnbrook (England).

4-Amino-5-imidazole carboxamide was prepared as the hydrochloride according to the method described by SHAW and WOOLLEY (1949) *). The over-all yield of the synthesis was 29 per cent. The compound melted at 254—256° C. (decomp.). It had a maximum UV absorption at 267 mμ (with a characteristic shoulder between 230 and 245 mμ) and showed a positive test for non-acetylatable diazotizable amine (BRATTON and MARSHALL, 1939); the absorption maximum of the azo dye lay at 540 mμ.

III. GROWTH CHARACTERISTICS OF DIFFERENT ADENINE-DEFICIENT *OPHIOSTOMA* MUTANTS

GROWTH OF ADENINE-LESS MUTANTS WITH VARIOUS PURINE DERIVATIVES

As was pointed out in the introductory chapter examination of the various growth requirements of mutant strains blocked at different preps of a reaction chain may give an insight into the sequence of the conversions involved.

The qualitative test of the growth requirements, described in the stevious chapter (see table I), demonstrated that the compounds supporting growth of adenine-less mutants may be divided into three groups, viz. the adenine derivatives, the hypoxanthine derivatives and AICA, thus indicating the possible sequence of conversions in adenine biosynthesis from AICA *via* hypoxanthine derivatives.

In order to effectuate a further analysis of this reaction sequence quantitative data about the growth-promoting activities of each of the above-mentioned substances had to be obtained. For this purpose representatives of the different types of mutants whose growth is supported by either one or more of the above-mentioned groups of substrates were grown in 100 ml. Erlenmeyer flasks containing liquid minimum medium supplemented with known amounts of the desired compounds in a final volume of 20 ml. In each experiment some flasks containing 20 ml. of unsupplemented nutrient solution were inoculated to serve as controls. After the inoculation the flasks were incubated at 27° C. At regular time intervals growth was measured as mg. of dry weight of the mycelium formed.

The mycelium was filtered off through a dry filter paper disc of 2 cm. ø and of known weight. The filter with the mycelium was dried in an oven for 16 hours at 105° C., allowed to cool in a desiccator and weighed. The dry weight was always determined on duplicate cultures, the values listed being the means. The determinations had a standard deviation of c. 5 per cent $\left(\sigma = \sqrt{\dfrac{\Sigma(\bar{x} - x_1)^2}{n-1}} \right)$, as was calculated from a series of 20 determinations in duplicate.

Growth curves were obtained by plotting the dry weight of the mycelium against the duration of growth. A typical set of such curves is presented in fig. 4, showing the growth of mutant no. 65 with 2.0, 0.6 and 0.2 μM of adenosine respectively. In the figure this growth

is compared with growth of the wild type parental strain no. 13 on the unsupplemented solution.

Apparently even 2 μM of adenosine were not sufficient to restore the growth of the mutant to the rate shown by the parental strain. After the lag phase of about 2 days initially the growth rates were
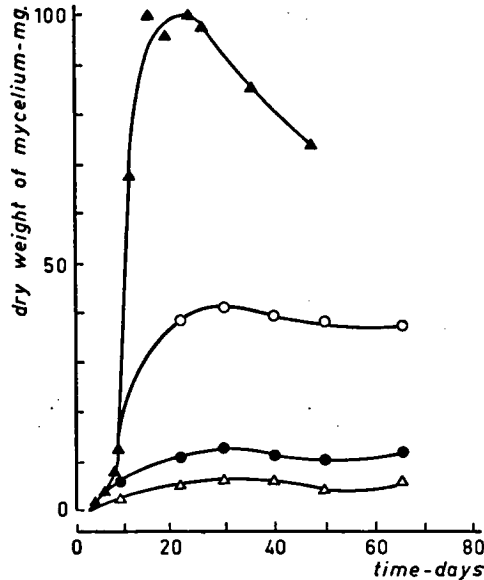

Fig. 4. *Growth curves of Ophiostoma multiannulatum*
Solid triangles: wild type strain 13, grown on 20 ml. unsupplemented minimum nutrient solution at 27° C.
Open circles, solid circles and open triangles: mutant strain 65 grown on 20 ml. minimum nutrient solution supplemented with 2.0, 0.6 and 0.2 μM of adenosine respectively.

equal, but soon the curve for the mutant fell off, whereas growth of the wild type strain proceeded almost linearly to reach the maximum after c. 20 days. In this experiment the mutant attained the maximum dry weight after about 30 days. This time, however, varies from one experiment to another and is depending on the mutant strain used.

A generally occurring tendency is that at lower substrate concentrations the maximum is attained somewhat later than at higher. After the maximum dry weight has been reached, a decrease in weight is found, but often this decrease is followed by another increase in weight, probably caused by synthesis of new mycelium at the expense of substances originating from the old mycelium by partial autolysis. A similar phenomenon has been described by FRIES (1949a).

To compare the growth-promoting effects of various substrates at different concentrations the maximum dry weights attained were plotted against the concentration of the supplement. This was done with mutants 57, 65, 53 and 73, grown with 2.0, 0.6 and 0.2 μM respectively of the following substrates: adenosine-3-phosphoric acid,

adenosine, adenine, inosine and hypoxanthine. The dose-response curves thus obtained are shown in figure 5. They turned out to be straight lines, indicating a close relationship between the concentration of the growth substance and the maximum dry weights of the mycelium formed. This simple relation made it relatively easy to compare the growth rates obtained with different substrates.
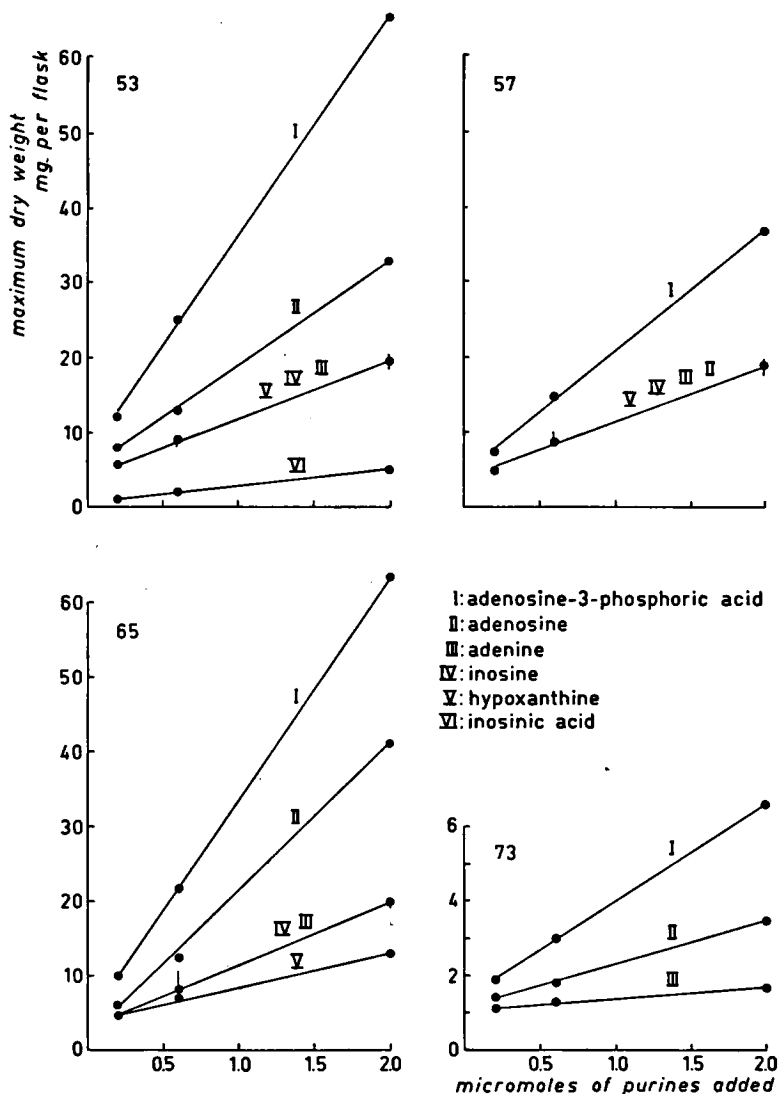

Fig. 5. *Dose-response curves of adenine-less Ophiostoma mutants grown with different purine derivatives*

Mutants were grown at 27° C. on 20 ml. minimum nutrient solution containing known amounts of the various purine derivatives.

Inosinic acid turned out to have only a weak growth-promoting activity, as could be demonstrated by preliminary experiments and as is shown in figure 5 for strain no. 53. Obviously mutant no. 73 did not grow at all with inosinic acid since it has become evident from the data of table I, that this strain is able to utilize adenine derivatives only.

The most striking feature of this figure is that apparently adenylic acid on a molar base was 2—3 times as effective as was adenine in supporting growth of adenine-less mutants. Somewhat less pronounced adenosine too was more effective than adenine except for the case of mutant no. 57, in which these substrates had equal activities. It appears that the activities of adenine, inosine and hypoxanthine were almost equal, though mutant 65 grew less well with inosine. Finally it is seen that strain no. 73 utilized its adenine derivatives very inefficiently. A similar behaviour of an adenine-less strain was reported by FRIES (1945). It is difficult to explain this phenomenon; one might suppose it to be due to the accumulation of some growth-inhibiting adenine analogue.

In accordance with earlier observations normal inoculation did not result in any appreciable growth of mutant 57 on the minimum medium supplemented with AICA. When, however, a very small amount of hypoxanthine or adenine (0.01 μM per flask) was given in addition to AICA, good growth was obtained at a concentration of 2 μM of the latter substance per flask, as is shown in figure 6. The maximum dry weight was even higher than that with an equal amount of adenosine; it was attained later because of the exceptionally long lag phase. At the 0.6 and 0.2 μM levels, however, AICA was quite ineffective in supporting growth. With 0.01 μM of hypoxanthine as only supplement the dry weight did not exceed 2.5 mg.

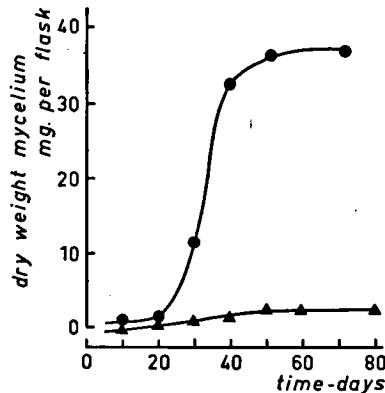Since mutant no. 57 apparently has been blocked at a step prior to

Fig. 6. *Growth of adenine-less Ophiostoma strain 57 with 4-amino-5-imidazole carboxamide (AICA)*

The mutant was grown at 27° C. on 20 ml. minimum nutrient solution supplemented with 2 μM AICA plus 0.01 μM hypoxanthine (*circles*) and as control with 0.01 μM hypoxanthine only (*triangles*).

ring closure, in which process presumably formate participates as the source of carbon 2 of the purine ring (cf. figs. 1 and 2), the possibility exists that the utilization of AICA by the mutant is limited by the endogenous supply of formate. Thus, addition of exogenous formate to the medium containing AICA and a trace of hypoxanthine or adenine might result in a stimulation of growth.

Experiments, however, in which mutant 57 was grown on a minimum nutrient solution containing 2 μM of AICA and 0.01 μM of hypoxanthine per flask with addition of 4 μM of sodium formate did not support the above supposition. The maximum dry weights did not exceed that of controls to which no formate had been added. Apparently in this case the supply of endogenous formate is sufficient to cover the requirements.

THE EFFECT OF OTHER NUCLEOTIDE CONSTITUENTS

In the section of chapter I dealing with guanine biosynthesis in mutants mention has been made of the "sparing" effect of guanine upon growth of adenine-less strains with adenine. Examination of such an effect provides another method to investigate the biosynthetic relations between different compounds. So it could be concluded from the above-mentioned "sparing" effect that adenine or adenine derivatives are precursors in the biosynthesis of guanine. In the following experiment the "sparing" effect of guanine was demonstrated with mutant no. 53.

In the same experiment it was investigated whether possibly pyrimidines exerted a "sparing" effect upon growth of the adenine-less mutant too. For, though it is generally assumed that the biosyntheses of purines and pyrimidines proceed by separate pathways, yet a few reports seem to indicate the existence of a relation between these processes. So HAMMARSTEN et al. (1950) were able to demonstrate that in rats administration of cytidine-N[15] resulted in labeling of desoxyribonucleic acid (DNA) adenine. PIERCE and LORING (1947) demonstrated an inhibitory effect of adenosine and adenosine-3-phosphate upon growth of pyrimidine-less Neurospora mutants. They assumed that to ensure normal growth in the mycelium an equilibrium between purines and pyrimidines had to exist, which was disturbed by adding the adenine derivatives. But in view of the fact that adenine has an inhibitory effect upon growth of guanine-deficient mutants, though it is likely to be a guanine precursor, the possibility of a connection between purine and pyrimidine biosynthesis has to be considered.

The possibility of an effect of guanine, cytosine, cytidine, uracil and uridine was examined by growing mutant no. 53 on 20 ml. of minimum nutrient solution containing 1 μM of adenine and to which the respective nucleotide constituents had been added in 1 μM amounts. The maximum dry weights obtained were compared; growth on the adenine containing solution without supplement served as control. The results of this experiment are listed in table II.

It is seen that in agreement with the findings of FRIES (1949a)

guanine had a distinct "sparing" effect. The free pyrimidine bases apparently had no effect at all, but the ribosides behaved differently,

TABLE II

The effect of other nucleotide constituents upon growth of adenine-less Ophiostoma with adenine

Maximum dry weights of mutant no. 53 grown on 20 ml. of minimum nutrient solution supplemented with adenine plus either guanine, cytosine, cytidine, uracil or uridine, as derived from growth curves.

Supplement	Maximum dry weight	
	mg.	% of control
1 μM adenine (control)	11.2	100
1 μM guanine	0.0	—
1 μM adenine + 1μM guanine	14.4	129
1 μM cytosine . ., ...	0.0	—
1 μM cytidine	0.0	—
1 μM uracil	0.0	—
1 μM uridine	0.0	—
1 μM adenine + 1 μM cytosine	11.4	102
„ + 1 μM cytidine	9.7	85
„ + 1 μM uracil	11.3	101
„ + 1 μM uridine	14.4	129 .

cytidine having an inhibitory, uridine a stimulating effect upon growth in presence of adenine.

With regards to the latter phenomenon it is worth while to mention an experiment in which the effect of adenine upon the growth of a pyrimidine-deficient *Ophiostoma* mutant was investigated. Mutant no. 52 (utilizing cytosine, cytidine and uridine, but not uracil) was grown with 0.6 μM of uridine and 0.6 μM of adenine and the maximum dry weight attained was compared with that with 0.6 μM of uridine only. From the results, shown in table III, it became evident that adenine had a distinct stimulating effect, growth with adenine being 124 per cent of the growth of the control.

TABLE III

Effect of adenine upon growth of pyrimidine-less Ophiostoma with uridine

Maximum dry weights of mutant no. 52 (utilizing cytosine, cytidine and uridine, but not uracil) grown on 20 ml. of minimum nutrient solution containing uridine plus adenine, as derived from growth curves.

Supplement	Maximum dry weight	
	mg.	% of control
0.6 μM uridine (control)	42.1	100
0.6 μM adenine	0.0	—
0.6 μM uridine + 0.6 μM adenine	52.0	124

IV. EVIDENCE FOR THE ACCUMULATION OF PURINE PRECURSORS BY ADENINE-DEFICIENT MUTANTS

As was pointed out in the introductory chapter, in biochemical mutants of microorganisms the normal sequence of reactions leading to the biosynthesis of a vital substance has been blocked. The effect of the block can be overcome by the addition to the minimum nutrient medium of one of the products formed either by the blocked reaction or by one of the reactions which come next to it. Thus, together with growth, the biosynthesis of intermediates formed normally prior to the blocked reaction may be restored too. Since, however, no further conversion can take place, accumulation of the intermediates—or of derivatives formed by secondary reactions—may occur.

A way to discover such an accumulation is to examine whether the culture filtrate, after the growth factor originally present has been removed, contains a growth factor or factors for mutants which have been blocked one or more steps before in the same biosynthetic reaction chain. When growth of these mutants is made possible by the filtrate it can be concluded that a product intermediary between the two blocks—or a derivative thereof—is accumulated. When no growth of the mutants occurs, this may indicate that no accumulation takes place, but the possibility remains that the accumulated substance is converted to a metabolically inactive derivative.

The above-mentioned method was chosen to investigate the possibility of accumulation of purine precursors by the adenine-less mutants mentioned in the foregoing chapters.

Demonstration of growth factors in culture filtrates

It was investigated whether growth-promoting substances were present in filtrates from cultures of mutants 57, 65, 66, 53, 59, 64, 71 and 72.

The filtrates were obtained as follows. A mutant was grown at 27° C. in ten 200 ml. Erlenmeyer flasks containing 50 ml. minimum nutrient solution supplemented with 7.5 μM of adenine. After an incubation time of 6–7 weeks, in which maximum growth was attained, the mycelium formed was removed by filtration. Prior to the removal the mycelium was checked for possible reversal to wild type. If this turned out to have occurred the corresponding filtrate was discarded. The remaining culture fluids were combined and sterilized by filtration through a Schott G–5 glass filter.

The presence of a growth factor in the filtrate was established in the following way: 20 ml. portions of the filtrate were brought together with 10 ml. of sterile minimum medium of twice the usual concentration in 100 ml. Erlenmeyer flasks. Subsequently the flasks were inoculated with all above-mentioned strains and incubated for 43 days, after which the dry weights of the mycelia formed were determined as usual.

The flasks inoculated with the strain from which the filtrate had

originally been obtained served as control. Since no appreciable growth occurred the conclusion seemed correct that adenine or growth factors derived from adenine were absent, or at the most present in such small amounts that they could not enable growth. Table IV summarizes the results of this experiment.

TABLE IV

Accumulation of purine precursors by adenine-less Ophiostoma mutants

Filtrates were obtained from 6–7 weeks old cultures of different mutant strains grown on 50 ml. of minimum nutrient solution supplemented with 7.5 μM of adenine. — 20 ml. portions, sterilized by filtration, were combined with 10 ml. of twice concentrated sterile minimum solution and reinoculated with each of the mutant strains used. — Dry weights were determined after 43 days incubation at 27° C.

Nutrient solution: concentrated minimum solution plus filtrate of mutant no.:	Mg. dry weight of mutant no.:							
	57	65	66	53	59	64	71	72
57	*0.9*	0.8	1.2	0.9	0.5	1.0	1.6	0.7
65	**39.5**	*0.5*	0.4	0.7	0.4	0.7	1.3	0.5
66	**11.5**	0.7	*0.5*	1.1	1.3	1.1	1.7	0.6
53	0.7	0.3	0.3	*0.8*	0.4	0.9	0.5	0.2
59	0.6	0.6	0.9	0.6	*0.5*	0.7	1.3	0.4
64	1.4	0.7	0.5	0.9	0.9	*1.0*	0.9	0.4
71	1.2	0.9	1.2	0.8	0.5	1.2	*2.1*	0.5
72	1.6	0.7	0.7	0.7	1.1	0.5	0.9	*0.3*

From these data it becomes evident that the filtrates obtained from cultures of strains 65 and 66 contain a substance or substances that enable growth of strain 57. With respect to the other filtrates there is no evidence of any appreciable accumulation of growth-promoting substances since the dry weights obtained may be considered negligible.

No complete quantitative data could be obtained with mutant 60, since this strain shows a very frequent reversal to the wild type when grown on liquid media. Indications, however, were found that this strain too accumulated a substance which functioned as a growth factor for mutant 57.

The strains 73 and 74 have not been investigated, because they had not yet been isolated when the above-mentioned experiment was undertaken.

IDENTIFICATION OF THE ACCUMULATED COMPOUND

Since mutant 57 is the only one able to utilize AICA it seemed worth while to examine whether the substance accumulated by the adenine-less strains 65 and 66 was identical with AICA, its riboside or its ribotide.

This was achieved in the following way. First the culture filtrates were examined for the presence of a non-acetylatable diazotizable

amine by way of the BRATTON and MARSHALL (1939) test. As a control
the filtrates of the other adenine-less strains were tested too.

The filtrates were obtained by growing the mutants for three
weeks in 200 ml. Erlenmeyer flasks containing 100 ml. of minimum
nutrient solution supplemented with 5 mg. of adenosine to ensure
good growth. Glucose had been autoclaved separately and was added
to the medium before inoculation. This was done because upon
sterilization of glucose containing nutrient media brownish coloured
products were formed, which might interfere with the colorimetric
BRATTON and MARSHALL (BM) test.

This test was performed as follows. The filtrate was freed from cellular material
by filtration through a Schott G–4 glass filter and the volume was adjusted to
100 ml. To 20 ml. of filtrate 0.4 ml. of a solution of 2 per cent acetic anhydride were
added. The reaction mixture was allowed to stand for 30 minutes at room tempera-
ture to complete the acetylation of acetylatable amines possibly present. Then
5 ml. 0.8 N sulfuric acid and 0.5 ml. of a 0.1 per cent aqueous sodium nitrite solution
were added to bring about the diazotization of the non-acetylatable amines possibly
present. This required less than five minutes as could be shown with pure AICA.
Excess nitrite was removed by the addition of 0.5 ml. of a 0.5 per cent solution of
ammonium sulfamate. Three minutes. later 0.5 ml. of 0.1 per cent N-naphthyl-
ethylenediamine dihydrochloride in water was added. The presence of a non-
acetylatable diazotizable amine was concluded from the development of a red
colour, due to the formation of an azo dye.

The original nutrient solution turned out to give a negative BM
test. The results of the BM test with the culture filtrates of the adenine-
deficient mutants are shown in table V.

TABLE V

Presence of non-acetylatable diazotizable amine in culture filtrates of adenine-less mutants
Filtrates were obtained from 3 weeks old cultures grown on 100 ml. of minimum
nutrient solution supplemented with 5 mg. of adenosine. +: positive Bratton–
Marshall (BM) test; —: negative BM test.

Mutant no.	BM test in filtrate	Absorption maximum of azo dye
		mμ
57	—	—
60	+	540
65	+	540
66	+	540
53	—	—
59	—	—
64	—	—
71	—	—
74	—	—
72	—	—
73	—	—

It followed that the filtrates of mutants 65 and 66—and also of 60,
which fortunately had not reverted to wild type during growth—
contained a non-acetylatable diazotizable amine. The azo dyes

showed a maximum absorption at 540 mμ. As was mentioned in the last section of chapter II, the azo dye of AICA too had a maximum absorption at 540 mμ. According to Love and Gots (1955) the azo dye of AICA riboside has the same absorption maximum. It is therefore concluded that mutants 60, 65 and 66 are able to accumulate AICA or a closely related compound.

The second step in the identification procedure consisted of an attempt to isolate the amine in a more or less pure state by paper-chromatography.

For this purpose the filtrates had to be concentrated. Concentration by evaporation *in vacuo* at 25° C. or by lyophilization resulted in a complete loss of the diazotizable compound.

When the concentrate was chromatographed on paper with *n*-butanol saturated with water as solvent (*cf.* chapter V) one strongly UV absorbing band at R_F 0.44 was observed on the chromatograms.

After elution of this band with water the eluate showed an absorption maximum at 300 mμ. It gave no reaction for diazotizable amine nor the pentose reaction with orcinol (Albaum and Umbreit, 1947).

It was assumed that the product found had originated by decomposition of the diazotizable amine, probably by the high concentration of acid in the concentrate (the original filtrate had a *p*H of about 4.5). It also might be that it was rapidly oxidized. That's why certain precautions had to be taken during the concentration.

When the concentration was carried out *in vacuo* under nitrogen and care was taken that the solution remained neutral or slightly alkaline, the resulting concentrate turned out to contain a considerable amount of diazotizable amine.

To a small portion of the concentrate some AICA was added. This portion and the remainder were deposited on 6 sheets of Whatman no. 1 filter paper and chromatographed with 80 per cent *n*-propanol in water (*v*/*v*). Pure AICA was chromatographed separately.

The chromatograms of the concentrated filtrate showed two distinct UV absorbing bands at R_F 0.45 to 0.55 and R_F 0.58 to 0.68 respectively. Also when AICA had been added only these two bands could be discerned. Since the latter compound had an R_F of 0.57 it apparently moved along with the substance in the faster moving band.

After elution with water the eluates of both absorbing bands showed a positive Bratton–Marshall reaction.

The eluate of the slower substance showed a positive orcinol test for pentose, the faster compound gave no reaction with the orcinol reagent.

Both eluates had an absorption maximum at 267.5 mμ, which is also the maximum for AICA and AICA riboside (Love and Gots, 1955).

In view of these results it was concluded that the faster substance was identical with AICA, whereas the more slowly moving compound was AICA riboside or ribotide.

The latter statement had to be verified, as it was not certain whether the sugar present in the eluate was actually associated with the diazotizable amine. For this purpose the eluate was concentrated

in vacuo and rechromatographed with 60 per cent *n*-propanol in water (*v*/*v*) in a NH_3 atmosphere. The chromatogram showed one UV absorbing band at R_F 0.66.

The eluate still gave the Bratton-Marshall reaction and also the orcinol reaction. The UV absorption had its maximum at 267.5 mμ.

For these reasons it was concluded that the substance isolated was AICA riboside or ribotide. The fact that the R_F value with 60 per cent propanol (0.66) was similar to that found by GREENBERG (1952) for AICA riboside from a culture of sulfonamide inhibited *E. coli* (R_F 0.65), is in favour of the assumption that the compound accumulated by the present *Ophiostoma* mutant was also AICA riboside.

The last step in identifying the accumulated growth substances consisted in the proof that the isolated compounds could actually support growth of strain 57. For this purpose a few drops of the eluates obtained after the first time chromatographing were added to flasks containing 20 ml. unsupplemented minimum medium. The flasks were inoculated, partly with strain 57, partly with strain 65 as a control, and incubated at 27° C.

After 10 days good growth was observed in the flasks containing the presumed AICA riboside and inoculated with strain 57. A somewhat less growth occurred in the flasks containing the substance which was presumed to be identical with AICA.

In the flasks inoculated with strain 65 no growth occurred, indicating that growth substances other than AICA and AICA riboside were absent.

SUBSTANCES INFLUENCING THE ACCUMULATION

In chapter I mention was made of the stimulating effect of glycine and threonine upon AICA accumulation by *E. coli*. This effect was interpreted in terms of glycine being a precursor of AICA.

It seemed desirable to investigate whether the observed accumulation of AICA compounds by mutant 65 was also stimulated by glycine and related compounds. Moreover, as experiments with isotopically labeled formate had demonstrated that this compound may be a source of the ureide carbon of AICA, the possibility of a stimulating effect of this substance was investigated too.

For this purpose strain 65 was grown on an adenosine containing minimum nutrient solution, supplemented with glycine, serine, threonine, sodium formate and glycine plus sodium formate respectively. As was indicated before glucose was autoclaved separately and added before inoculation.

After three weeks incubation at 27° C. the amounts of AICA compounds in the culture filtrates were determined by way of the Bratton–Marshall test. The extinctions of the colored solutions were read at 530 mμ against suitable blanks. The values found were expressed in μg. AICA.

The results of these experiments are summarized in table VI.

Glycine, as well as serine and threonine exerted a distinct stimulating effect upon the accumulation of AICA compounds. This is quite in

TABLE VI

The effect of glycine, serine, threonine and formate upon the accumulation of AICA compounds
Strain 65 was grown at 27° C. in flasks containing 20 μM of adenosine in 100 ml.
minimum nutrient solution, supplemented with glycine, serine, threonine, sodium
formate or glycine plus sodium formate. — After 3 weeks the amounts of AICA
compounds were determined by way of the Bratton–Marshall test. Extinctions
were read at 530 mμ. The values found were expressed as μg. AICA.

Supplement	Dry weight mycelium	AICA formed per 100 mg. dry weight
	mg.	μg.
None	381	8.0
10 mg. glycine	412	11.4
20 mg. dl-serine	310	13.5
20 mg. dl-threonine	400	11.4
10 mg. sodium formate	366	7.9
10 mg. glycine + 10 mg. sodium formate	403	9.3

consistence with the view that glycine functions as a purine precursor.
The rather high amount of AICA formed with serine suggests that
this compound is perhaps a more immediate precursor than glycine.

No effect of formate could be observed. Apparently the endogenous
supply of formate was sufficient to cover the requirement.

V. METHODS USED IN THE STUDY OF PURINE
INCORPORATION BY ADENINE-LESS
OPHIOSTOMA MUTANTS

The study of the incorporation of purines into mycelial nucleotides
and polynucleotides by adenine-less mutants involved (*a*) the deter-
mination of the purine uptake from the nutrient solution during
growth, (*b*) the quantitative analysis of the purines and pyrimidines
present in the mycelium formed, and (*c*) the determination of purines
and pyrimidines excreted into the medium by the mycelium.

The methods used for the determination of purine uptake (*a*) and
purine and pyrimidine excretion (*c*) were almost identical and will,
therefore, be discussed together in the section dealing with the estima-
tion of purines and pyrimidines in culture fluids.

QUANTITATIVE SEPARATION AND DETERMINATION OF MYCELIAL PURINES AND PYRIMIDINES

The purines and pyrimidines present in the mycelium as free bases,
nucleosides or nucleotides can be separated from the polynucleotides
by extraction with acid. Afterwards they can be determined separately.

The quantitative separation and determination, however, of the
constituents of the acid insoluble nucleic acids require a preceding
hydrolysis. For the present study hydrolysis with 12 N perchloric acid,
as recommended by MARSHAK and VOGEL (1951), was chosen. This

method resulted in the liberation of the free purine and pyrimidine bases.

To obtain uniformity in the subsequent separation and determination procedures the acid soluble fraction too was subjected to the perchloric acid treatment, to release the free bases.

Preparation of hydrolysates

The mycelium to be analyzed was obtained by filtration from cultures grown on a liquid nutrient medium. It was washed thoroughly with distilled water, alcohol and ether, dried in an oven at 105° C., powdered and stored in a desiccator over calcium oxyde.

From the mycelium a fraction containing the free bases, nucleosides and nucleotides was obtained as follows. 30 mg. of dried powdered mycelium were brought together with 1 ml. of ice-cold 5 per cent trichloroacetic acid (TCA), stirred until the powder had been thoroughly wetted and kept for 2 hours in the refrigerator at 0° C. Subsequently the mass was centrifuged in the cold and the residue washed twice with 2 ml. portions of ice-cold 5 per cent TCA. The combined supernatants were evaporated on a boiling water-bath until near dry; 0.16 ml. of 12 N $HClO_4$ were added and the hydrolysis was carried out for 1 hour at 100° C. The hydrolysate was made up to 1 ml. with distilled water and centrifuged. The clear supernatant was used for the analysis.

The residue after the TCA treatment contained the acid insoluble polynucleotides. MARSHAK and VOGEL (1951) developed a procedure for the quantitative micro-analysis of polynucleotide purines and pyrimidines without prior isolation of the nucleic acids. For this purpose the TCA residue was hydrolyzed directly with perchloric acid and the resulting hydrolysate was used for further analysis. This method was adapted for *Ophiostoma* mycelium in the following way.

The TCA residue was placed in an open test tube of 10 cm. length and a diameter of 1.5 cm. 0.16 ml. of 12 N $HClO_4$ were added and the tube was kept for 1 hour at 100° C. in a boiling water-bath with occasional stirring. After the addition of *c.* 1 ml. of distilled water the hydrolysate was centrifuged and the residue was washed three times with small amounts of distilled water. The acid supernatant and the aqueous washings were combined and concentrated to a final volume of 1 ml. After centrifugation the supernatant was used for the analysis.

When only the total amounts of free and bound purines and pyrimidines were determined the hydrolysate was prepared by hydrolyzing 30 mg. of dry powdered mycelium without prior TCA extraction.

Paperchromatographic separation of mixtures of purine and pyrimidine bases

The separation of mixtures of purine and pyrimidine bases was performed by paperchromatography.

Known volumina of the solutions to be analyzed were deposited as spots on a 60 × 60 cm. sheet of Whatman no. 1 filter paper. At 6.5

cm. from the upper end of the sheet the "starting line" of the chromatogram was pencilled, indicating the points at which the solutions were dispensed by means of a capillary micropipette, allowing the measurement of 5.5 μl. $\pm$ 0.1 μl. (standard deviation).

Usually for quantitative work three spots of the solution to be analyzed were deposited at 3 cm. distances in order to obtain sufficient amounts for the spectrophotometric determination of the separated components.

After the deposition of the acid solutions these were neutralized on the paper with gaseous ammonia for at least 20 minutes. Excess ammonia was removed by keeping the sheets in a continuous stream of air during 30 minutes.

Care was taken that the amounts of the individual bases per spot were not less than 2 μg. and not more than 6 μg., since when the amount is too small detection on the paper becomes difficult, whereas too high amounts are badly separated. As far as mixtures of the pure bases were concerned this was only a matter of using suitable concentrations and appropriate volumina of the solutions per spot. As for hydrolysates of mycelium preliminary separations had to be carried out in order to establish the appropriate volumina of the hydrolysate to be deposited per spot on the paper. These volumina varied from 5.5 up to 82.5 μl.

The separation was performed by descending chromatography at 22° C. The solvent used was n-butanol saturated with distilled water; the bottom of the chromatography chamber was provided with equal amounts of n-butanol and water. 16 hours proved to be sufficient for a complete separation. In this time the solvent front travelled c. 30 cm. Then the position of the front was marked and the chromatogram was dried upside down at room temperature in a continuous stream of air. Finally the positions of the separated components were localized by observing the chromatogram in ultraviolet light, obtained from a Philips' TUV–15W tube, supplied with a Schott UG–5 filter; the purines and pyrimidines appeared as dark spots on a faintly fluorescing background.

Figure 7 shows schematically a chromatogram of a perchloric acid hydrolysate of wild type *Ophiostoma* mycelium, of a mixture of adenine, guanine, cytosine, uracil and thymine in 2 N HClO$_4$, and of solutions of the individual purine and pyrimidine bases in HClO$_4$, used as markers.

Thus the qualitative composition of the mycelium with regards to purines and pyrimidines could be established.

It appeared that the quantity of mycelium powder used (30 mg.) contained only detectable amounts of adenine, guanine, cytosine and uracil. This indicated that besides the free bases, nucleosides or nucleotides only a measurable quantity of ribonucleic acid (RNA) was present in the hydrolysate. Apparently the concentration of desoxyribonucleic acid (DNA) was too low to allow even a qualitative determination, since no thymine could ever be detected on chromatograms of hydrolyzed mycelium.

From the scale on the left side of figure 7 the R_F values can be read.

These values differ considerably from those given by VISCHER and CHARGAFF (1948a) for the separation of purines and pyrimidines with *n*-butanol saturated with water. The differences not only concern the absolute values, but even the relative positions of the separated components. Compared with the present data VISCHER and CHARGAFF found the positions of adenine and uracil reversed. It seems difficult to explain this phenomenon. On the other hand HOTCHKISS (1948) found
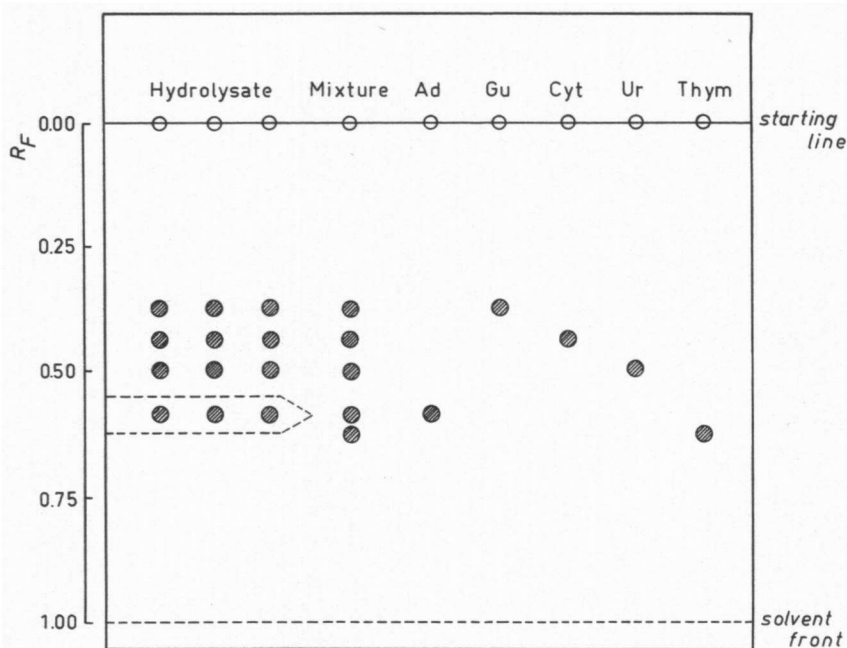

Fig. 7. *Outlines of a chromatogram of pure purine and pyrimidine bases compared with a perchloric acid mycelium hydrolysate*
Solutions of adenine (*Ad*), guanine (*Gu*), cytosine (*Cyt*), uracil (*Ur*) and thymine (*Thym*) in 2 N HClO$_4$ and a mixture of these solutions were chromatographed on paper together with a perchloric acid hydrolysate of *Ophiostoma* mycelium. — *Solvent used*: *n*-butanol saturated with water.

a sequence of R_F values similar to that presented here. It is true that guanine did not move at all in the chromatograms described by HOTCHKISS, but this was undoubtedly due to the fact that the separation was carried out in a NH$_3$ atmosphere.

In order to enable the estimation of the quantities of the various compounds present on the papergram, strips containing the three spots of each separated substance were cut out from the paper as is indicated by the broken lines in figure 7. The strips were eluted with a descending flow of N HCl (for guanine) or 0.1 N HCl (for the other compounds). Finally the volumina of the eluates were adjusted to 3 ml.

Quantitative determination of separated purines and pyrimidines

The quantitative determination of adenine, guanine, cytosine and uracil, the bases that could be detected in the mycelium after hydrolysis, is in general most easily effected by way of their UV absorption. The extinction values, preferably read at the absorption maxima, are directly proportional to the concentrations. This, however, only holds when solutions of pure compounds are used.

Figure 8 shows the absorption spectra of 0.5 mg. per cent solutions of adenine, cytosine, and uracil in 0.1 N HCl and of guanine in N HCl. The optical densities of the solutions were read against HCl blanks in 1 cm. cuvettes in a Beckman model DU quartz spectrophotometer. The logarithms of the extinction values (*log E*) were plotted against the wavelength.

The molar extinction values for the pure bases, calculated from the data mentioned above are listed in table VII. For purpose of comparison some data cited from literature have been included. It appears that the values found agree very well with the latter.

Errors may arise when the quantitative determination of the components of purine and pyrimidine mixtures, separated by chromatography, is concerned. Absorbing materials in the filter paper, interfering with the spectrophotometric determination, may be a source of these errors. Furthermore the determination becomes unreliable when the separated components are not recovered quantitatively from the paper-chromatograms.

TABLE VII

Molar extinction coefficients of purines and pyrimidines

ε : extinction coefficient computed from absorption measurements at the wavelength λ.—λ max.: wavelength of absorption maximum. — Column A: values calculated from figure 8. Column B: values calculated from data quoted from VISCHER and CHARGAFF (1948a). Column C: values quoted from MARKHAM and SMITH (1954).

Substance	Solvent	λ max.	λ	$\varepsilon \times 10^{-3}$		
		$m\mu$	$m\mu$	A	B	C
Adenine . . .	0.1 N HCl	262.5	260	12.65	12.56	13.0
Guanine . .	1.0 N HCl	249	249	11.37	11.42	—
Cytosine . . .	0.1 N HCl	275	275	10.49	—	10.5
Uracil	0.1 N HCl	259	259	8.21	8.19 [1])	—

[1])   Value for uracil in water. Comparison is allowed because of the identity of the absorption spectra of uracil in water and in HCl (*cf.* HOTCHKISS, 1948). The uracil used in the present study had a $\varepsilon \times 10^{-3}$ at 259 $m\mu$ in water of 8.24.

With regards to the determination of the quantitative composition of mixtures of the pure substances the above-mentioned errors appeared negligible, as will be demonstrated by the following experiments.

A mixture of adenine, guanine, cytosine and uracil in 2 N HClO$_4$ was chromatographed. The concentration of the mixture and the volumina deposited on the paper were chosen so that the eluates should contain 0.5 mg. per cent of the separated compounds.
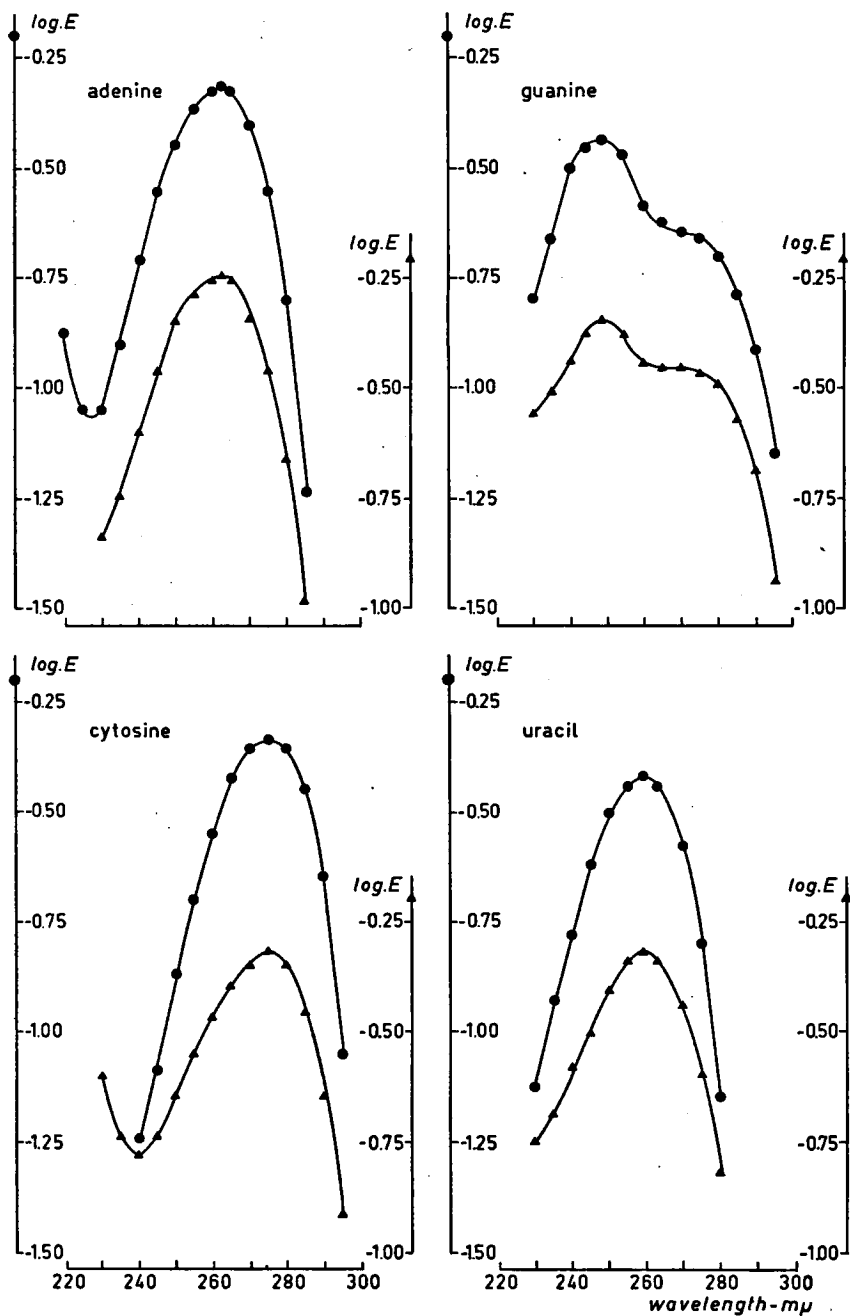
Fig. 8. *Absorption spectra of purines and pyrimidines*
Circles: spectra of 0.5 mg. per cent solutions of pure adenine, guanine, cytosine
and uracil.
Triangles: spectra of the bases obtained from a papergram of a perchloric acid
mycelium hydrolysate.
Solvents: 0.1 N HCl for adenine, cytosine and uracil; 1 N HCl for guanine.

Adenine, cytosine and uracil were eluted with 0.1 N HCl, guanine with 1 N HCl. The absorption spectra of the eluates were determined by reading the optical densities against HCl eluates of the corresponding areas of a $HClO_4$ blank, chromatographed simultaneously. *Log E* was plotted against the wavelength.

The curves obtained turned out to cover entirely those given in figure 8 for solutions containing 0.5 mg. per cent of the various bases. This means that absorbing materials eluted from the paper are compensated for by the performance of the blank determination, and that no loss of material occurs during the separation and elution procedures. Consequently the quantitative determination of the separated bases is achieved most accurately by way of the extinction values at their absorption maxima.

The latter statement, however, does not hold for eluates of chromatographed mycelium hydrolysates. In figure 8 are also plotted the absorption spectra of the eluates containing adenine, guanine, cytosine and uracil obtained after paperchromatography of 247.5 μl. (3 × 82.5 μl.) of a perchloric acid hydrolysate of 30 mg. of dry wild type *Ophiostoma* mycelium. Provided the purines and pyrimidines were sufficiently separated from other UV absorbing materials present in the hydrolysate, their absorption curves should have parallelled those of the pure compounds, since in the graphs *log E* was plotted. The values for the maxima, depending on the concentrations, might have constituted the only differences between the curves.

As is seen from the figure, actually this holds only fairly well for the eluate containing adenine. This eluate, therefore, appears least contaminated with other absorbing substances. Obviously this is due to the fact that adenine is the compound which moves most rapidly on the chromatograms.

The absorption curves of the eluates containing guanine, cytosine and uracil showed considerable deviations, when compared with the curves for the pure substances, indicating that they did contain appreciable amounts of materials with an unspecific absorption.

To make allowance for the unspecific absorption which is a source of errors in the quantitative determination, various correction methods have been proposed. From these the one elaborated by VACHER (1949) was chosen for this study. Recently this method has been discussed in detail by STIENSTRA (1954). It requires extinction measurements at three different wavelengths, viz. at the absorption maximum and at appropriate equal distances on each side of the maximum. The difference Δ between the maximum extinction value and the mean of the two other extinction values is directly proportional to the concentration of the solution under investigation, provided the course of the unspecific absorption is a linear one in the region in which the readings are taken. For this reason the wavelength intervals were chosen so that within the limits of error the absorption curves in this region parallelled those for the pure substances.

For the evaluation of unknown concentrations from absorption readings use was made of the value $\Delta \frac{1 cm.}{1 \mu g.}$, i.e., the Δ value for solutions containing 1 μg. per ml., calculated from readings in a 1 cm. cuvette. The $\Delta \frac{1 cm.}{1 \mu g.}$ values were determined with solutions containing 10 μg. per ml. of adenine, guanine, cytosine or uracil in HCl. They were obtained from extinction measurements against HCl blanks. The spectrophotometer readings and calculations are summarized in table VIII.

The wavelength intervals chosen are rather small, which gives rise

to a certain degree of inaccuracy in the quantitative determinations.

TABLE VIII

Calculation of $\Delta \frac{1 \text{ cm.}}{1 \mu g.}$ values for solutions of adenine, guanine, cytosine and uracil in HCl The solutions contained 10 μg. per ml. of the pure bases. Extinctions were read in 1 cm. cuvettes against HCl blanks. $\Delta \frac{1 \text{ cm.}}{1 \mu g.}$: see text.

Substance	Solvent	Wavelength	Extinction per cm.	$\Delta \frac{1 \text{ cm.}}{1 \mu g.}$
		mμ		
Adenine. . . .	0.1 N HCl	255	0.878	0.0104
		262.5	0.943	
		270	0.800	
Adenine. . . .	2.0 N HCl	255	0.912	0.0103
		262.5	0.969	
		270	0.820	
Guanine . . .	1.0 N HCl	244	0.699	0.0042
		249	0.734	
		254	0.685	
Cytosine . . .	0.1 N HCl	270	0.879	0.0063
		275	0.937	
		280	0.869	
Uracil	0.1 N HCl	255	0.713	0.0030
		259	0.741	
		263	0.708	

Taking larger intervals would enlarge the accuracy, but this is made impossible when working with hydrolysates by the large deviations in the absorption spectra.

ESTIMATION OF PURINES AND PYRIMIDINES IN CULTURE FLUIDS

In order to investigate the adenine consumption by adenine-less mutants during growth the adenine content of the culture medium had to be determined.

As the possibility existed that purine and pyrimidine derivatives were excreted by the mycelium the culture filtrates had to be examined for the presence of these compounds too.

Determination of adenine in nutrient solutions

The quantitative spectrophotometric determination of comparatively small amounts of adenine in autoclaved nutrient solutions proved to be quite unsatisfactory, even when use was made of the above-mentioned correction method of VACHER. This was due to the very high unspecific absorption shown by the medium itself. Consequently UV absorption measurements could only be performed on highly diluted solutions, which brought about a high inaccuracy in the adenine determinations.

For this reason an at least partial purification was necessary. Paperchromatographic procedures were likely to give bad results because of the high salt content of the solutions. Therefore, purification was attempted by way of ion exchange chromatography. It turned out that in this way the interfering substances could be removed

and spectroscopically pure adenine could be obtained from nutrient media and culture filtrates.

The method used has been described by COHN (1949). The solution to be analyzed was made 2 N for HCl and placed on a 5 cm. × 0.8 cm². column of the strongly acid cation exchange resin *Imac C–12* [1]) (H+, 70–140 mesh).

Before operating the resin was washed thoroughly with distilled water. After the column had been poured 5 per cent HCl was passed through until the *pH* of the effluent remained constant. 50 ml. turned out to be sufficient for this purpose. Next the column was washed with *c.* 300 ml. of distilled water till the effluent was neutral to litmus and free from Cl⁻. Then 30 ml. of 5 per cent ammonia were passed through. When the effluent was strongly alkaline the resin was washed with water again to neutral reaction. Eventually 50 ml. of 5 per cent HCl were used to bring the resin into the H+ form. Excess HCl was removed by washing with distilled water.

The adenine containing solution passed through the column at a rate of *c.* 3 ml. per minute. Afterwards the resin was washed with distilled water until the extinction per cm. at 262.5 mμ ($E_{262.5}$) of the effluent was less than 0.005.

The adenine adsorbed by the resin was eluted with 2 N HCl. The eluate was collected in 50 or 100 ml. fractions of which the $E_{262.5}$ was measured against 2 N HCl. The presence of adenine was established by determination of the UV absorption spectrum of the various fractions.

The spectrum of the adenine eluted from the column proved to be identical with the absorption shown by pure adenine. It was concluded therefrom that no other UV absorbing material was present, the adenine obtained being spectroscopically pure.
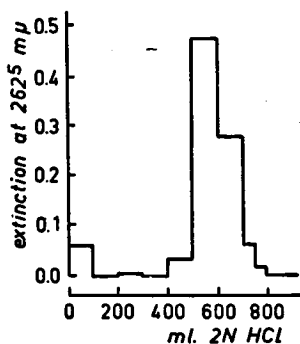

Fig. 9.   *Elution diagram of adenine recovered from autoclaved nutrient solution by ion exchange chromatography*

20 ml. minimum nutrient solution were supplemented with 897 μg. of adenine and autoclaved. The solution was made 2 N for HCl and passed through a 5 cm. × 0.8 cm². column of Imac C–12 (H+, 70–140 mesh). The column was washed with water and adenine eluted with 2 N HCl at a rate of 3 ml. per minute. The effluent was collected in 50 or 100 ml. fractions. — The adenine content of the various fractions was calculated from the extinction values at 262.5 mμ in 1 cm. cuvettes. — Recovery: 100 per cent.

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[1])   Handelsmij. N.V. "Activit", Amsterdam.

Figure 9 shows the elution diagram of adenine which had been added to minimum nutrient solution before autoclaving.

It appears that adenine came off the resin in the eluate volume between 400 and 750 ml. This was quite reproducible in other experiments. The recovery was 100 per cent; in other experiments the recoveries ranged from 96 to 102 per cent.

As in these experiments the total volume of the adenine containing fractions amounted to 350 ml., the determination of amounts of adenine smaller than 500 μg. became very inaccurate. Therefore the method had to be modified so as to be able to determine amounts down to 10 μg. of adenine in 20 ml. of autoclaved nutrient solution or culture filtrate.

The adenine containing solution was passed over the column, which afterwards was washed with 100 ml. of distilled water. Then it was washed with 2 N HCl followed by distilled water. From the diagram of figure 9 it is evident that this could be done without fear of a loss of adenine, provided no more than 300 ml. of HCl were used. For routine determinations 200 ml. turned out to be sufficient. By these washings a more or less considerable amount of UV absorbing material other than adenine was removed. Finally adenine was washed off the resin with 30 ml. of 5 per cent ammonia.

The eluate was freed from ammonia and concentrated on a boiling water-bath. The concentrate was made 2 N for HCl, adjusted to a volume of 5 ml. and read in the spectrophotometer against 2 N HCl.

Determination of purines and pyrimidines excreted by the mycelium

The presence of excreted purines and pyrimidines in the culture medium of growing mutants was established in the following way.

The culture filtrate was made 2 N for HCl and kept for 1 hour in a boiling water-bath to bring about hydrolysis of the purine and—at least partially—of the pyrimidine derivatives present. Then it was placed on the cation exchange column. After the passage of the solution the column was washed with distilled water till the effluent was neutral to litmus and Cl^--free. Next 30 ml. 5 per cent ammonia were passed through.

The ammoniacal effluent was concentrated on a boiling water-bath and subsequently subjected to a paperchromatographic analysis as was described before.

Never any other purine derivative than adenine was found in culture filtrates which were treated in the above way. It was present only in filtrates of young cultures, grown on an adenine containing medium, and decreased rapidly. Thus no excretion of purines seemed to have occurred.

The same holds for the pyrimidine derivatives: no indications were found for the excretion of pyrimidines by the mycelium. It is felt, however, that in the latter case the evidence is far from conclusive, since the above-mentioned method enabled only partially the determination of pyrimidines.

RELIABILITY OF THE METHODS USED

The reliability of the methods so far described in this chapter was checked by the following experiments.

Paperchromatographic separation and spectrophotometric determination of purines and pyrimidines in mycelium hydrolysates

First of all the methods described were tested by the performance of an experiment involving the separation and subsequent quantitative determination of the components of a mixture of pure adenine, guanine, cytosine, and uracil in 2 N $HClO_4$. This solvent was chosen because the mycelium hydrolysates used were also approximately 2 N for $HClO_4$. The results are shown in table IX.

For the determination of mycelial purines and pyrimidines the mycelium was hydrolyzed with 12 N perchloric acid. It seemed appropriate to investigate whether this treatment caused losses or interconversions. The latter possibility should not be ruled out, as VISCHER and CHARGAFF (1948b) demonstrated that a considerable conversion of cytosine to uracil occurred on treatment with 10 per cent HCl or with a mixture of equal amounts of 10 N HCOOH and 1 N HCl.

The last column of table IX shows the recoveries obtained after paperchromatographic separation of a purine/pyrimidine mixture which had been heated with 12 N $HClO_4$ for 1 hour at 100° C.

TABLE IX

Quantitative separation and determination of mixtures of pure adenine, guanine, cytosine and uracil

A standard solution of the four bases in 12 N $HClO_4$ was prepared. Part of this solution was heated for 1 hour at 100° C. (solution A). The remainder was kept at room temperature (solution B). — For the analysis 0.16 ml. of solutions A and B respectively were made up to 1 ml. with distilled water. Aliquots were chromatographed with *n*-butanol saturated with water. Adenine, cytosine and uracil were eluted from the papergram with 0.1 N HCl, guanine with 1 N HCl. Concentrations were determined from the optical densities of the eluates read at the absorption maxima against eluates of the corresponding areas of a $HClO_4$ blank, chromatographed simultaneously. — Recoveries were calculated as mg. of the individual bases per ml. of standard solution.

Substance	Amount per ml. standard solution	Amount recovered			
		Without heating[1])		After heating[2])	
	mg.	*mg.*	%	*mg.*	%
Adenine. . . .	6.14	6.19	101 ± 0.8	6.14	100
Guanine. . . .	5.81	5.94	102 ± 2.0	5.56	96
Cytosine. . . .	8.25	7.94	96 ± 1.6	7.94	96
Uracil	8.50	8.31	98 ± 0.7	8.50	100

[1]) Average values of 19 separations with calculated standard deviations.
[2]) Average values of 2 separations.

It appears that in the course of the separation no appreciable amounts of the bases were lost. Moreover, the perchloric acid treatment apparently did not cause any loss or interconversion.

The next experiment showed that in the mycelium hydrolysates no substances were present which interfered with the quantitative determination of purines and pyrimidines, provided the latter was carried out according to the correction method of VACHER.

30 mg. of dry wild type mycelium were hydrolyzed with perchloric acid as was described before. The amounts of adenine, guanine, cytosine and uracil present in the hydrolysate were determined by way of quantitative paperchromatographic analysis.

To another 30 mg. of the same mycelium sample known amounts of adenine, guanine, cytosine and uracil were added. Likewise this mixture was subjected to the perchloric acid treatment, whereupon the quantities of the four bases in the hydrolysate were determined.

The results of the analysis are listed in table X. From the differences between the amounts of purines and pyrimidines found in both hydrolysates the recoveries could be computed.

TABLE X

Recovery of added purines and pyrimidines from a hydrolysate of wild type Ophiostoma mycelium
To 30 mg. of dry mycelium powder 203 μg. of adenine, 131 μg. of guanine, 128 μg. of cytosine and 147 μg. of uracil were added. The mass was hydrolyzed for 1 hour at 100° C. with 0.16 ml. of 12 N HClO₄, made up to 1 ml. and chromatographed on paper with *n*-butanol saturated with water. — Likewise 30 mg. of mycelium without additions were hydrolyzed and chromatographed. —The concentrations of the eluates of the separated purines and pyrimidines were computed according to VACHER from UV absorption readings. Recoveries were calculated from the differences between the total amounts found in both hydrolysates.

	Adenine	Guanine	Cytosine	Uracil
	μg.	μg.	μg.	μg.
Mycelium + additions	322	254	200	233
Mycelium	114	124	73	91
Additions recovered . . .	208 *(102%)*	130 *(99%)*	127 *(99%)*	142 *(97%)*

These data show that the bases added to the mycelium prior to hydrolysis were almost completely recovered.

In all experiments of this kind the mycelium was hydrolyzed for 1 hour. Preceding experiments had shown that this was sufficient to bring about a total hydrolysis of the purine and pyrimidine compounds without the occurrence of losses. Table XI shows the purine and pyrimidine composition of hydrolysates obtained after 0.5, 1, 2 and 4 hours hydrolysis of 30 mg. amounts of dry wild type Ophiostoma mycelium.

Apparently a hydrolysis of less than 1 hour was insufficient for a complete liberation of the free bases, in particular the pyrimidines. A prolonged hydrolysis up to 4 hours did not result in any significant increase in the amounts of free purines and pyrimidines found.

The quantitative determination of the composition of the acid soluble and insoluble mycelium fractions was carried out on TCA extracts and TCA residues respectively. One could imagine that the TCA treatment might cause a loss of purines and pyrimidines. In

TABLE XI

Effect of duration of hydrolysis upon the purine and pyrimidine composition of mycelium hydrolysates

30 mg. of dry wild type Ophiostoma mycelium were hydrolyzed with 0.16 ml. of 12 N $HClO_4$. The hydrolysate was adjusted to 1 ml. and aliquots were analyzed by paperchromatography with *n*-butanol/water followed by spectrophotometry according to VACHER.

Duration of hydrolysis	Purine and pyrimidine composition hydrolysate			
	Adenine	Guanine	Cytosine	Uracil
hrs.	μg.	μg.	μg.	μg.
0.5	130	149	0	74
1	158	161	50	130
2	151	159	57	128
4	157	160	52	133

order to examine this possibility the amounts of purines and pyrimidines in the TCA extract and in the TCA residue of 30 mg. mycelium were determined and their sum compared with the amounts found in untreated mycelium.

Table XII shows the results of two of these experiments.

TABLE XII

Determination of TCA soluble and TCA insoluble purines and pyrimidines in wild type Ophiostoma mycelium

30 mg. of dry mycelium were extracted for 2 hours with 1 ml. of ice-cold 5 per cent TCA. The residue was washed twice with 2 ml. of ice-cold 5 per cent TCA. — Extract plus washings were concentrated and hydrolyzed with $HClO_4$. The TCA residue was hydrolyzed in the same way. At the same time 30 mg. of untreated mycelium were hydrolyzed. — The hydrolysates were subjected to a quantitative paperchromatographic and spectrophotometric analysis.

	μg. purines and pyrimidines in 30 mg. mycelium			
	Adenine	Guanine	Cytosine	Uracil
1 *Untreated mycelium* *15 days old*	108	120	60	81
TCA treated mycelium				
TCA extract	51	60	42	48
TCA residue	57	57	21	36
Sum.	108	117	63	84
2 *Untreated mycelium* *28 days old*	57	62	37	46
TCA treated mycelium				
TCA extract	20	28	20	27
TCA residue . . .	34	30	15	18
Sum.	54	58	35	45

These data indicate that the sum of the separately determined acid soluble and insoluble fractions fairly equalled the amount of purines

and pyrimidines found on analysis of untreated mycelium and that no serious losses of material occurred.

Quantitative determination of adenine in nutrient solutions

Using the ion exchange procedure the quantitative determination of adenine or adenylic acid in nutrient solutions and in culture filtrates gave quite satisfactory recoveries as is demonstrated by the data in table XIII.

Known amounts of adenine or adenylic acid were added to 20 ml. of minimum nutrient solution and autoclaved. Afterwards the solutions were made 2 N for HCl. Adenylic acid being present they were heated for 1 hour at 100° C. to liberate free adenine. Subsequently the solutions were subjected to the quantitative analysis.

In stead of minimum nutrient solution in some experiments the culture filtrate of an adenine-less mutant was used. The mutant had been grown for 16 days on an adenine containing liquid medium. During growth a large amount of UV absorbing compounds other than adenine were excreted into the medium. Consequently the UV absorption of the nutrient solution increased considerably. It seemed desirable to investigate whether the absorbing substances interfered with the determination of adenine.

A preceding analysis showed that in the 16 days growth period the adenine originally present had disappeared from the medium. The recovery of added adenine from the culture filtrate is also shown in table XIII.

TABLE XIII
Quantitative determination of adenine and adenylic acid in nutrient solutions

Solution A: 20 ml. of minimum nutrient solution. — Solution B: 20 ml. of culture filtrate obtained from a 16 days old culture of the adenine-less strain 53 grown on minimum medium supplemented with adenine. The filtrate contained no adenine. — Known amounts of adenine or adenylic acid were added to solutions A and B. Subsequently these were autoclaved and made 2 N for HCl. The adenylic acid containing solutions were heated for 1 hour at 100° C. — The solutions were passed over a 5 cm. × 0.8 cm². column of *Imac C-12* (H+, 70–140 mesh) at a rate of 3 ml./min. The column was washed with 100 ml. water, 200 ml. 2N HCl and 350 ml. water successively. Adenine was washed off with 30 ml. 5 per cent ammonia and determined in the concentrated eluate (NH$_3$–free, 2N for HCl) by UV spectrophotometry using the correction method of VACHER.

Solution	Substance added	Amount added	Amount recovered	
		µg.	µg.	%
A	Adenine	945	950	100.5
,,	,,	270	270	100.0
,,	,,	59	61	103.4
,,	,,	10	10	100.0
,,	Adenylic acid	730	725	99.3
B	Adenine	270	270	100.0
,,	,,	135	140	103.7
,,	,,	14	13	92.9

It is seen that good recoveries were obtained of amounts down to 10 µg. per 20 ml. of solution. Although the use of the correction method

appeared necessary, the amount of absorbing material interfering with the spectrophotometry could be diminished to such an extent that the quantitative determination met with no difficulties.

VI. RESULTS OF PURINE INCORPORATION STUDIES

INTRODUCTION

The experiments which will be presented in this chapter were carried out in order to examine the metabolism of the purines added as growth factors to the nutrient medium of adenine-deficient *Ophiostoma* mutants.

The studies of the incorporation of exogenous substrates into cellular components are often hampered by the ocurrence of a considerable *de novo* synthesis of these compounds. So it is difficult to distinguish between the metabolism of the added substances and that of similar compounds synthesized by the organism itself. This difficulty, however, has been overcome to a large extent by the development of the isotope tracer technique.

A second source of troubles encountered is that the addition of a metabolite may result in a considerable decrease in the normal synthesis of this compound (*cf.* BALIS *et al.*, 1952). Also, the rate of conversion of an added compound is not necessarily equal to its normal rate of conversion.

Obviously these complications are avoided when heterotrophic organisms are used. Therefore, biochemical mutants seem to offer excellent tools for incorporation studies. Since a reaction has been blocked there will be no *de novo* synthesis at all of any product formed normally by way of this reaction. The organism has been made "artificially heterotrophic" for these compounds.

In spite of the opportunity which is offered by these mutants for the study of the metabolism of the substances for which they are deficient, little or no attempts at all have been made into this direction. This may at least in part be due to the fact that it is doubtful whether in every biochemical mutant the *de novo* synthesis of the growth substance is completely absent. As has been shown by LEIFER *et al.* (1950) and by YANOFSKY and BONNER (1952) sometimes a reaction may be blocked as long as no growth occurs whereas it proceeds under conditions which do permit growth. Thus, even in investigations with biochemical mutants the picture may be complicated by the occurrence of a *de novo* synthesis.

Such a phenomenon was described by ABRAMS (1952) for an adenine-less mutant of *Saccharomyces cerevisiae*. Experiments in which the mutant was grown with unlabeled adenine in the presence of glycine-C^{14} led the author to assume that during growth a considerable purine synthesis took place. In contrast with the wild type, which shows a strongly reduced synthesis in the presence of exogenous purines, the *de novo* synthesis by the mutant appeared to be proportional to the amount of adenine added. Since the mutant did not contain

appreciable quantities of acid soluble nucleotides it was presumed that the mutation had resulted in a merely quantitative reduction of purine biosynthesis, causing an insufficient supply of these metabolically indispensable nucleotides. So growth was made impossible and purine synthesis reduced to zero. The addition of adenine would lead to the formation of nucleotides and thus enable growth and purine synthesis to start.

It seems inappropriate here to enter into details, but it should be noted that this view was not completely supported by the experimental data. The picture is complicated by the fact that apparently the mutant used consisted of two types, one being colourless and one forming a red pigment during growth. Moreover, the growth requirements of the mutant were not fully clear.

Nevertheless it will be evident that when the incorporation of adenine by adenine-deficient *Ophiostoma* mutants is studied, the possibility of a *de novo* purine synthesis has to be considered in order to avoid a misinterpretation of the results obtained.

EXPERIMENTAL

The present study has been restricted to the investigation of purine metabolism in the adenine-less mutant 53. This strain belongs to the group of mutants which could be grown not only with adenine derivatives but also with hypoxanthine and inosine as was shown in chapters II and III. It would have been obvious to investigate in the first instance a mutant with an absolute requirement for adenine derivatives. Because of the fact, however, that the mutants of the latter kind available showed a very poor growth, any mutant of the former type was chosen.

The uptake and incorporation of adenine and adenylic acid were examined. The latter compound was chosen because of its high growth-promoting activity as compared with adenine. It should be possible to establish whether this was a matter of a more efficient utilization of adenylic acid.

The experiments were planned as follows. 100 ml. Erlenmeyer flasks containing 20 ml. of sterile minimum nutrient solution supplemented with known amounts of adenine or adenylic acid were inoculated with a few drops of a suspension of strain 53 conidiospores in distilled water. The number of flasks inoculated varied from one experiment to another, ranging from 50 to 160. All flasks were incubated together at 27° C.

At regular intervals after inoculation the dry weight of the mycelium formed was determined. At the same time the amount of adenine or adenylic acid which had disappeared from the nutrient solution was measured and a quantitative determination of all mycelial compounds which presumably had been formed from the added purine was performed.

The latter analysis consisted of the determination of the total amounts of adenine and guanine obtained after $HClO_4$ hydrolysis

of the TCA soluble and TCA insoluble mycelium fractions. As there are indications for a connection between purine and pyrimidine metabolism, as was shown already in chapter III, the amounts of cytosine and uracil in these fractions were determined too.

For purine and pyrimidine determinations mycelium samples had to be collected by filtration from a number of flasks. This number varied depending on the age of the cultures, i.e., on the amount of mycelium formed per flask.

In the first instance samples were taken before the uptake of the added purine was complete. Thus the rate of this uptake could be estimated.

Moreover samples were taken and analyzed after complete depletion of the growth factor. In this way it could be established whether the purines taken up were accumulated in the mycelium, further assimilation taking place gradually in the course of growth. Besides it was possible to decide whether as a result of the addition of exogenous purines a *de novo* purine synthesis occurred. If under these conditions purines were synthesized by the mutant the total amounts of purines per culture should increase. When no synthesis occurred this amount would at the most remain at a constant level.

Eventually some samples were collected after growth had reached its maximum. At this stage dissimilation processes are predominating. As these seemed of less importance for the present study they will not be discussed in detail.

One experiment was performed in which the amounts of purines and pyrimidines in wild type mycelium were determined during growth. This was done to enable a comparison between the formation of nucleotides from minor constituents, as occurs in the wild type, and nucleotide synthesis from added exogenous purines, as is achieved by the mutant. For this purpose the parental wild type strain 13 was grown in a number of Erlenmeyer flasks containing unsupplemented minimum nutrient solution. The determinations of the dry weight of the mycelium formed and of its purine and pyrimidine composition were carried out as was described above.

RESULTS

Purine metabolism in wild type Ophiostoma

Table XIV gives the results of analyses of wild type mycelium of strain no. 13, grown on unsupplemented minimum nutrient solution. At different intervals during growth the dry weight of the mycelium formed was determined and sufficient amounts of mycelium were harvested to enable a quantitative analysis of the purine and pyrimidine composition of the TCA insoluble and TCA soluble fractions.

Figure 10 shows the amounts of TCA soluble and TCA insoluble purines and pyrimidines in the mycelium per culture at various stages of the development. The broken lines indicate the changes in the amounts of these compounds when growth ceased as a result of a

D. STEGWEE

TABLE XIV

Purine and pyrimidine contents of mycelium of wild type strain no. 13 of Ophiostoma multiannulatum

The mould was grown at 27° C. in 100 ml. Erlenmeyer flasks containing 20 ml. of unsupplemented minimum nutrient solution. — At various stages of growth the dry weight of the mycelium formed was determined. The purine and pyrimidine content of the mycelium was determined after total hydrolysis of the TCA insoluble and TCA soluble fractions.

Days after inoculation	Mg. dry weight of mycelium	μMoles of purines and pyrimidines per mg. of dry mycelium											
		TCA insoluble fraction				TCA soluble fraction				Total			
		Adenine	Guanine	Cytosine	Uracil	Adenine	Guanine	Cytosine	Uracil	Adenine	Guanine	Cytosine	Uracil
4	1.6	.015	.013	.006	.014	.024	.020	.011	.023	.039	.033	.017	.037
6	3.9	.015	.012	.007	.012	.027	.022	.010	.028	.042	.034	.017	.040
8	8.6	.015	.013	.006	.014	.024	.022	.011	.025	.039	.035	.017	.039
11	67.6	.015	.013	.006	.012	.024	.022	.011	.027	.039	.035	.017	.039
15	101.2	.014	.012	.006	.011	.013	.011	.010	.014	.027	.023	.016	.025
18	96.2	.007	.005	.003	.006	.013	.012	.010	.015	.020	.017	.013	.021
23	100.0	—	—	—	—	—	—	—	—	—	—	—	—
26	97.6	—	—	—	—	—	—	—	—	—	—	—	—
48	73.6	—	—	—	—	—	—	—	—	—	—	—	—

lack of nutrients or growth substances. In this phase autolysis led to a breakdown of purines and pyrimidines.

From the data summarized in table XIV and in figure 10 the following facts become apparent.

Initially the total amounts of mycelial purines and pyrimidines per culture increased rapidly. This was followed by a decrease, when growth had ceased and autolysis became predominating.

Until after the 11th day the increase of mycelial purine and pyrimidine derivatives exactly parallelled growth. This can be seen from the figures given in table XIV, showing that the total content of the individual purines and pyrimidines in the mycelium, expressed as μM per mg. of dry weight, remained constant up to and including the 11th day. During this period the molar proportions of the bases relative to adenine were: adenine 1.00, guanine 0.85, cytosine 0.43, uracil 0.97.

After the 11th day this ratio changed considerably with respect to cytosine. This was due to the fact that the total amount of this substance still increased, whereas the total amounts of adenine, guanine and uracil underwent no appreciable change from the 11th to the 15th day.

During this period the dry weight of the mycelium was still increasing. The amounts of nucleic acid purines and pyrimidines increased too. Apparently the adenine, guanine, and uracil in this fraction were derived from the TCA soluble fraction. The mean molar proportions of the different bases in the nucleic acid as a fraction of the total

nucleic acid purines and pyrimidines were: adenine 0.32, guanine 0.28, cytosine 0.13, uracil 0.27.
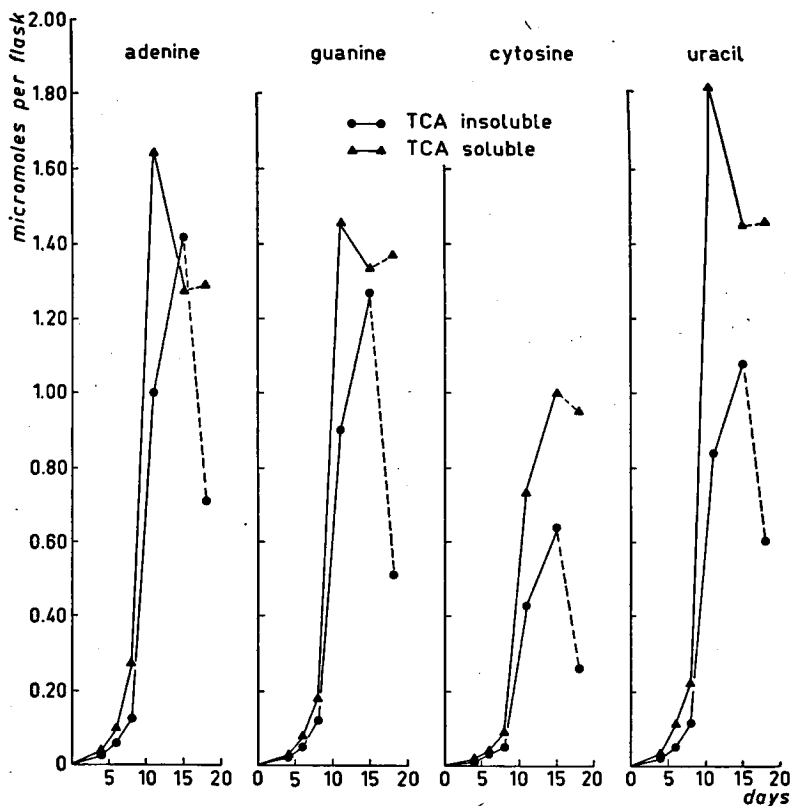

Fig. 10. *Purines and pyrimidines in wild type Ophiostoma mycelium*

Wild type strain 13 was grown at 27° C. in flasks containing 20 ml. unsupplemented minimum nutrient solution. At regular intervals the amounts of purines and pyrimidines in hydrolysates of the TCA soluble and TCA insoluble mycelium fractions were determined.

From the 15th day, when the maximum dry weight was reached, breakdown processes predominated, resulting in a rapid fall of the components of the acid insoluble fraction. The acid soluble fraction, however, did not decrease further.

Purine metabolism in adenine-less Ophiostoma grown with adenine

Mutant 53 was grown in flasks containing 20 ml. of minimum nutrient solution supplemented with 7μM of adenine. During growth were determined: (a) the dry weight of the mycelium formed, (b) the amount of adenine in the nutrient solution, and (c) the amounts of purines and pyrimidines in the TCA insoluble and TCA soluble mycelium fractions.

The growth curve of the mutant is shown in figure 11. The amounts of adenine in the nutrient solution at different stages of the development of the mycelium were plotted too. It appears that this amount decreased very rapidly to zero within the first 8 days after inoculation. Nevertheless growth continued for some 3 weeks more, the dry weight increasing from about 14 to about 50 mg.

This was a rather surprising result as it is generally assumed that growth stops as soon as the growth factor has been used up.

The following experiment demonstrated that the adenine originally present in the medium had not been converted to an unknown growth-promoting derivative, which had been excreted again into the medium, but had escaped from the analysis.

After 12 days growth of mutant 53 on the medium containing 7 μM of adenine, the mycelium was removed from the flasks. The filtrates were sterilized by filtration and inoculated again with conidia of strain 53. The flasks were incubated at 27° C.

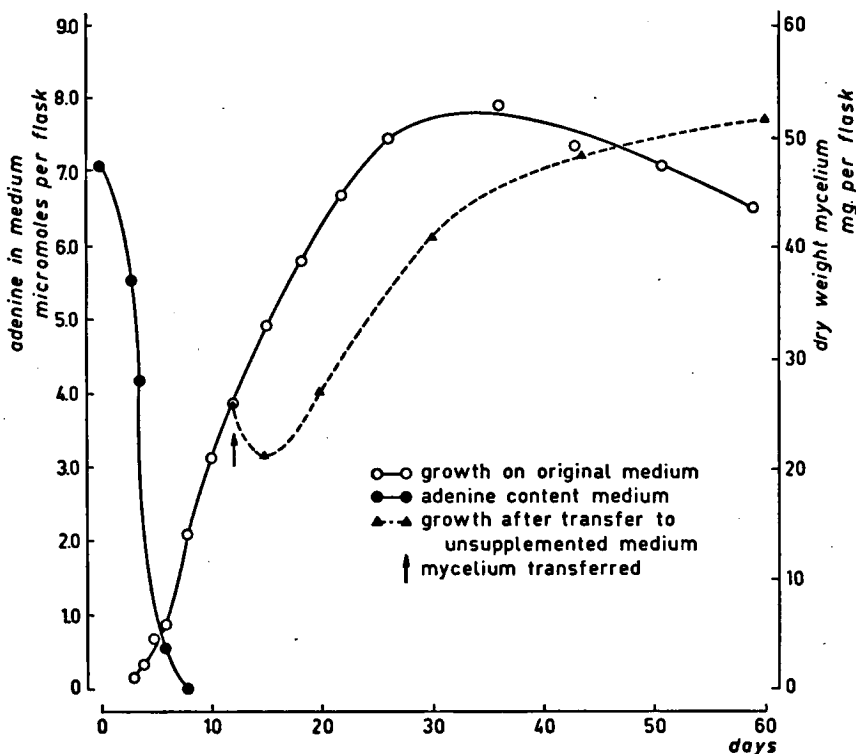No growth was observed over a period of 38 days. This indicated

Fig. 11.   *Growth of adenine-less Ophiostoma with adenine*
Mutant strain 53 was grown at 27°C. in flasks containing 20 ml. minimum nutrient solution supplemented with 7 μM of adenine. At the 12th day after inoculation part of the cultures were transferred to fresh unsupplemented minimum nutrient medium and incubated again at 27° C.

that the filtrates did not contain any growth-promoting substance. So it must be assumed that the growth observed after the added adenine had been consumed was due to a storage of adenine containing compounds in the mycelium before it was used for the synthesis of derivatives needed for growth.

The latter could be verified as follows. The mycelia obtained from the 12 days old cultures were washed thoroughly with sterile distilled water and transferred aseptically to Erlenmeyer flasks containing 20 ml. of unsupplemented minimum medium. The flasks were incubated at 27° C. and at regular intervals the dry weight of the mycelium was determined.

The results can be seen in figure 11 too, the course of the dry weights after the transfer being indicated by the broken line. At first a slight drawback occurred, probably caused by damage inflicted upon the mycelium by the transfer. Later on the growth curve became exactly similar to the curve obtained with the cultures on the original solution. The maximum dry weights obtained in both cases were essentially equal.

These results seemed to support the above assumption put forward to explain the continued growth of the adenine-less mutant without an exogenous supply of adenine. As will, however, be shown later on in this chapter indications were found that this hypothesis was inadequate too.

The purine and pyrimidine contents of strain 53 mycelium grown

TABLE XV

Purine and pyrimidine contents of mycelium of mutant strain no. 53 of Ophiostoma multiannulatum grown with adenine

The mutant was grown at 27° C. in 100 ml. Erlenmeyer flasks containing 20 ml. of minimum nutrient solution supplemented with 7.0 μM of adenine. — At various stages of growth the dry weight of the mycelium, the amount of adenine in the medium and the amounts of purines and pyrimidines in the mycelium after total hydrolysis of the TCA insoluble and TCA soluble fractions were determined.

Days after inoculation	μM of adenine in medium	Mg. dry weight of mycelium	μMoles of purines and pyrimidines per mg. of dry mycelium											
			TCA insoluble fraction				TCA soluble fraction				Total			
			Adenine	Guanine	Cytosine	Uracil	Adenine	Guanine	Cytosine	Uracil	Adenine	Guanine	Cytosine	Uracil
0	7.0	—	—	—	—	—	—	—	—	—	—	—	—	—
3	5.6	0.5	—	—	—	—	—	—	—	—	—	—	—	—
4	4.1	1.9	.032	.026	.015	.026	.021	.018	.008	.024	.053	.044	.023	.050
6	0.6	6.0	—	—	—	—	—	—	—	—	.030	.031	.020	.033
8	0.0	14.2	.009	.009	.004	.006	.016	.013	.013	.019	.025	.022	.017	.025
10	0.0	21.0	—	—	—	—	—	—	—	—	.017	.015	—	.018
12	—	25.6	.005	.005	.003	.004	.009	.006	.011	.013	.014	.011	.014	.017
18	—	39.0	.004	.004	.003	.003	.006	.005	.010	.007	.010	.009	.013	.010
26	—	48.0	.003	.003	.002	.002	.004	.004	.010	.006	.007	.007	.012	.008
36	—	52.2	.000	.000	.000	.000	.003	.005	.007	.006	.003	.005	.007	.006

on the nutrient solution with 7 μM of adenine and collected at different intervals in the course of the experiment are listed in table XV.

The total amounts of adenine, guanine, cytosine and uracil present per culture in the TCA insoluble and TCA soluble mycelium fractions have been plotted against time in figure 12. Again the broken lines refer to the phase in which autolysis led to a breakdown of purines and pyrimidines. From the 8th day the values plotted represented the total amounts of purines and pyrimidines per culture, as from that day the medium did not contain any purines or pyrimidines (see chapter V). Thus, it may be calculated that of the adenine, originally added, 10 per cent is accounted for as mycelial purines, *i.e.*, adenine and guanine.
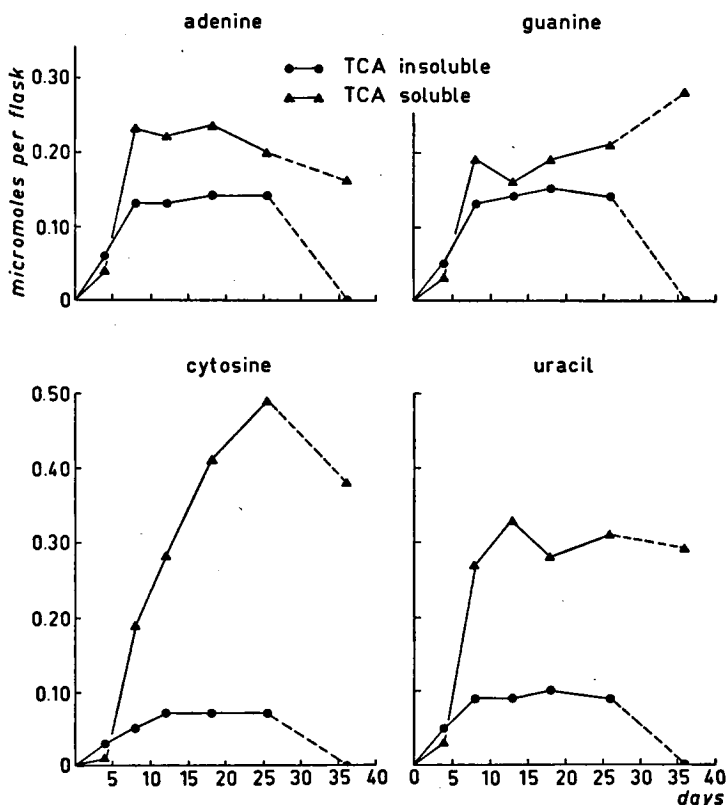
Fig. 12.   *Purines and pyrimidines in mycelium of adenine-less Ophiostoma*
Mutant strain 53 was grown at 27° C. in flasks containing 20 ml. minimum nutrient solution supplemented with 7 μM of adenine. At regular intervals the amounts of purines and pyrimidines in hydrolysates of the TCA soluble and insoluble mycelium fractions were determined.

In various respects these data are considerably different from those obtained with the wild type. In the mutant too a sharp initial increase

in mycelial adenine, guanine, uracil and cytosine was observed. However, the total amounts of the former three substances remained at a constant level, as soon as the added adenine had been completely taken up from the medium, *i.e.*, from the 8th day. Only the amount of cytosine still increased after this day.

The important bearing of these findings is, that evidently in this mutant no *de novo* purine synthesis occurred, when it was grown with adenine. Moreover, the synthesis of uracil seemed to be associated in some way with purine metabolism. This will be discussed later on.

Unlike the situation found in the wild type, the initial increase in adenine, guanine and uracil did not parallel growth. This can be seen from the figures in table XV, showing that the amounts of these substances per mg. of mycelium decreased gradually. The molar proportions relative to adenine, however, remained approximately constant at adenine 1.00, guanine 0.88, uracil 1.05. As a result of the more rapid increase of cytosine, the cytosine/adenine ratio increased from 0.44 at the 4th day to 1.91 at the 26th day of growth.

It should be noted that in the early stages of development the purine and pyrimidine content of the mutant was very high as compared with the wild type. Obviously this was due to the fact that in the mutant the TCA insoluble fraction contained initially about twice as much purines and pyrimidines as in the wild type. The molar proportions of the bases as a fraction of the total bases found were almost identical, being adenine 0.32, guanine 0.27, cytosine 0.15, uracil 0.26.

From the time when no added adenine was left in the medium the amounts of TCA insoluble nucleic acid purines and pyrimidines remained remarkably constant. The same holds for adenine, guanine and uracil in the TCA soluble fractions.

When mutant no. 53 was grown with 2 μM of adenine the results obtained on analysis of the medium and the mycelium were quite comparable to those mentioned above for growth with 7 μM of adenine.

The only difference was that the efficiency of adenine utilization appeared to be somewhat higher, 15 per cent of the added adenine being accounted for as mycelial purines. 6 per cent of the adenine added, *i.e.*, 40 per cent of the adenine incorporated, could be recovered as nucleic acid adenine and guanine.

Purine metabolism in adenine-less Ophiostoma grown with adenylic acid

In the next experiment mutant no. 53 was grown on 20 ml. of minimum nutrient solution supplemented with 2 μM of adenosine-3-phosphoric acid. In the course of development the dry weight of the mycelium formed was determined. Adenylic acid in the medium was determined after hydrolysis to adenine. Finally the amounts of purines and pyrimidines in both TCA insoluble and TCA soluble fractions of the mycelium were determined as has been indicated before.

The results of this experiment are shown in table XVI.

The results with adenylic acid were partly similar to those obtained with adenine.

TABLE XVI

Amounts of purines and pyrimidines in mycelium of mutant no. 53 of Ophiostoma multiannulatum grown with adenylic acid

The mutant was grown at 27° C. in 100 ml. Erlenmeyer flasks containing 20 ml. of minimum nutrient solution supplemented with 2 μM of adenosine-3-phosphoric acid. — At various stages of growth the dry weight of the mycelium, the amount of adenylic acid in the medium (after hydrolysis to adenine) and the amounts of purines and pyrimidines in the mycelium after total hydrolysis of the TCA insoluble and TCA soluble fractions were determined.

Days after inoculation	μM adenylic acid in medium	Mg. dry weight of mycelium	μMoles of mycelial purines and pyrimidines per flask											
			TCA insoluble fraction				TCA soluble fraction				Total			
			Adenine	Guanine	Cytosine	Uracil	Adenine	Guanine	Cytosine	Uracil	Adenine	Guanine	Cytosine	Uracil
0	2.0	—	—	—	—	—	—	—	—	—	—	—	—	—
10	0.5	2.9	.035	.035	.014	.035	.106	.051	.009	.051	.141	.086	.023	.086
12	0.2	3.6	—	—	—	—	—	—	—	—	—	—	—	—
15	—	10.4	.070	.063	.030	.060	.100	.032	.028	.040	.170	.095	.058	.100
17	0.0	13.0	.097	.085	.047	.084	.068	.017	.057	.020	.165	.102	.104	.104
22	—	20.4	.136	.112	.055	.110	.031	.000	.090	.000	.167	.112	.145	.110
36	—	56.0	—	—	—	—	—	—	—	—	—	—	—	—

Adenylic acid disappeared rapidly from the nutrient solution. After the disappearance growth continued, as was also observed when adenine was used as growth factor.

The total amounts of adenine, guanine, and uracil in the mycelium remained approximately constant after adenylic acid had been used up, *i.e.*, after the 15th day of growth. Cytosine showed a continued increase during this period. About 15 per cent of the adenylic acid added could be accounted for as mycelial adenine and guanine.

However, a striking difference was constituted by the behaviour of the individual mycelium fractions. The amounts of purines and pyrimidines in the TCA insoluble fraction of the mycelium continued to increase after the 15th day, evidently at the expense of the TCA soluble fraction. Except for cytosine, the components of the latter fraction showed a sharp decrease from the 17th day.

Efficiency of purine utilization

It is evident from the data presented in table XV and XVI and in figure 12, that the greater part of adenine or adenylic acid present in the nutrient solution was metabolized without giving rise to mycelial nucleotide constituents, especially in the initial phase of growth. So it may be calculated from the figures of table XV that after the first four days of growth only 6 per cent of the adenine consumed was accounted for as mycelial purines. From the fourth till the sixth day the percentage was again about 6 per cent, whereas from the sixth till the eigth day 56 per cent of the adenine taken up was accounted for as mycelial adenine and guanine.

It therefore seemed obvious that spores and young mycelium were able to perform a considerable enzymatic destruction of purine derivatives, whereas older mycelium utilized these substances more efficiently for the synthesis of nucleotide constituents.

This hypothesis was checked by studying the destruction of adenine and adenylic acid by conidiospores. Experiments like this, with whole cells, are only of importance for the present problem if use is made of "resting cells", which will not assimilate the added purine derivatives. Consequently care had to be taken that no growth could occur. This was performed by using a medium from which the essential nutrients, glucose and magnesium, had been omitted. It was assumed that when in this medium in the presence of conidospores a decrease in the amounts of added purines occurred, this was only due to a destruction by the spores.

Suspensions of conidiospores of the wild type strain 13 and of mutant strain 53 were prepared as was outlined in chapter III, by growing the strains at 30° C. in shaken cultures on a medium containing inositol. After the spores had been thoroughly washed they were suspended in sterile distilled water. Finally the number of spores per ml. was determined.

Aliquots of the suspensions were brought together with liquid minimum medium from which glucose had been omitted and in which $MgSO_4$ had been replaced by an equivalent amount of Na_2SO_4. Next adenine or adenosine-3-phosphoric acid was added.

At various intervals after incubation the amounts of adenine or adenylic acid present in the solutions were determined, the latter

TABLE XVII

Destruction of adenine and adenylic acid by "resting" conidia of Ophiostoma multiannulatum
A suspension of conidiospores of wild type strain no. 13, containing 1.6×10^7 cells per ml. and a suspension of conidiospores of adenine-less strain no. 53, containing 2.1×10^8 cells per ml. were used. — 1 ml. portions of these suspensions were transferred to flasks containing 20 ml. of minimum nutrient solution without glucose and with an equivalent amount of Na_2SO_4 in stead of $MgSO_4$. This medium did not permit growth. It was supplemented with either 7.0 μM of adenine or 2.7 μM of adenosine-3-phosphoric acid per flask.—The flasks were incubated at 27° C. Adenine and adenylic acid were determined spectrophotometrically, the latter compound after hydrolysis to adenine. The controls contained no spores.

Time hours	Adenine					Adenylic acid				
	μM per flask			per cent destroyed		μM per flask			per cent destroyed	
	Control	Wild type conidia	Mutant conidia	Wild type conidia	Mutant conidia	Control	Wild type conidia	Mutant conidia	Wild type conidia	Mutant conidia
0	7.0	7.0	7.0	—	—	2.7	2.7	2.7	—	—
12	—	6.4	5.9	8.6	15.7	—	2.5	2.4	7.4	11.1
24	—	6.0	5.2	14.3	25.7	—	2.1	1.8	22.2	33.3
38	—	5.9	4.3	15.7	38.7	—	1.8	1.5	33.3	44.4
60	7.0	5.9	4.1	15.7	41.4	2.7	1.7	0.7	37.0	74.1

substance as adenine after hydrolysis with 0.5 N hydrochloric acid at 100° C. for 30 minutes.

The results of these experiments are listed in table XVII.

It is evident that even under conditions which did not permit growth a considerable destruction of adenine and especially of adenylic acid was brought about by conidiopores, wild type as well as mutant ones. Although the amount of mutant conidia was 13 times that of the wild type conidia, the purine destruction by the mutant spores was only 2 times that by the wild type conidia. Apparently the latter possess by far the most active enzyme systems for purine destruction.

Microscopic examination revealed that during the experiment germination of the spores had taken place, though no further development of mycelium was observed over a period of 6 days. Especially in the solutions containing adenylic acid a rather abundant formation of small hyphae occurred. This was probably due to the presence of ribose (-phosphate) originating from adenylic acid. In the solutions containing adenine the spores had formed much less hyphae.

In view of the fact that the destruction of adenylic acid surpassed that of adenine, it seems likely that the destruction of purines by conidiospores is closely associated with the germination process.

VII. DISCUSSION

A. The pathway of purine biosynthesis

Nucleic acids are found in all living cells, both animal and vegetable. The well-known work of Caspersson and his collaborators (*cf.* Caspersson, 1947; 1950) has provided strong evidence for a relationship between the amount of nucleic acids present per cell and the rate of protein synthesis. Recently Gale (1955) was able to demonstrate that nucleic acids are directly responsible for amino acid incorporation into proteins, presumably by functioning as templates for the polypeptide chains to be formed. Thus, there is good reason to expect that nucleic acid synthesis and growth are closely related processes.

Research into the biosynthesis of the nucleic acids and of their constituent parts, the purines and pyrimidines, has received much attention. Especially during the last decade, when new techniques became available, a rapid progress has been made in this field. The results of this research made it possible to design a general pattern for the biosynthesis of purine derivatives, as was shown in chapter I. It should be noted that this general concept was obtained by compilation of rather fragmentary evidence gathered from experiments with very different objects. In all cases the knowledge about purine metabolism in any particular organism is far from complete. This applies in a large measure to *Ophiostoma*, as is seen in figure 3A.

The present study was intended to provide more detailed information about the biosynthesis and metabolism of purine compounds in this mould. For this purpose a comparative biochemical investigation into the utilization of various purines for nucleic acid synthesis by adenine-less mutants was undertaken. The following principles were underlying this study.

(i)   As was mentioned in detail in the introductory chapter, comparison of the growth requirements of different adenine-less strains may elucidate the normal pathway of purine biosynthesis by a proper arrangement of the compounds involved.

(ii)   More detailed evidence concerning the nature of the actual intermediates in purine synthesis is obtained by studying the accumulation by some mutants of precursors which can be utilized by others.

(iii)   Further indications can be obtained by examining the efficiency of utilization of the various purine growth factors. It may be assumed that a substance which is more closely related to the ultimate products of the biosynthetic reaction chain than other compounds, is more efficiently utilized for growth.

(iv)   Eventually additional evidence regarding the pathway of purine synthesis is furnished by investigation of the "sparing" effect which can be exerted by certain compounds upon the utilization of precursors.

It must be pointed out that an unequivocal interpretation of the results obtained with the adenine-less mutants is only possible, if one is certain that no de novo synthesis occurred in the presence of the growth factors. In this respect the investigations of LEIFER et al. (1950) and YANOFSKY and BONNER (1952), already mentioned in the introduction to chapter VI, may be referred to as an instance that this situation is not a priori realized in mutants.

The possibility of de novo purine synthesis during growth was investigated with strain 53 grown with either adenine or adenylic acid. From the results summarized in figure 12 and table XVI it can be seen that after the exogenous purine source had been depleted, no further increase in the amount of mycelial purines was observed. This proves that no purine synthesis occurred but from the added substances. As the growth of the other mutant strains used was quite comparable with that of strain 53, it may be assumed that the above statement has general validity.

PURINE SYNTHESIS IN ADENINE-LESS OPHIOSTOMA

With respect to their growth requirements the mutants used can be divided into three categories (cf. table I). The mutants belonging to the first category (strains 72 and 73) can only be grown in the presence of adenine, adenosine or adenylic acid. Growth of the mutants of the second group (strains 53, 59, 64, 71 and 74) is supported by the adenine compounds and also by hypoxanthine, inosine and inosinic acid. Finally the third category (strain 57) requires for growth either the adenine or hypoxanthine compounds or AICA.

Thus three groups of substances promoting growth of adenine-less mutants can be discerned, viz. the adenine derivatives, the group of hypoxanthine derivatives and the AICA compounds, of which only AICA was studied.

Growth factors adenine, adenosine and adenylic acid

Within the group of adenine derivatives marked differences in growth-promoting activities were observed (figure 5). By far the highest maximum dry weights were obtained with adenylic acid, then followed adenosine and finally adenine.

According to the principle (*iii*) outlined before this means that in the mutants purine synthesis proceeds by way of adenine, adenosine and adenylic acid, in this order.

It must be emphasized that the above principle may only be applied if equal amounts of the various substances are available for the synthetic processes in the cells. However, it is conceivable that the growth substances are destroyed before they can be incorporated into cell constituents. If this destruction proceeds with unequal velocity for the various compounds different amounts become available for nucleotide synthesis. The possibility also exists that the cell membranes possess a different permeability for the various growth substances, which likewise results in an unequal supply of material to the systems which bring about nucleic acid synthesis.

Investigation of this problem revealed that as a matter of fact a considerable destruction of the added purines occurred, especially in the initial phase of growth, as is apparent from the data of table XV and figure 12. The experiment summarized in table XVII demonstrated that germinating spores are largely able to bring about the destruction of both adenine and adenylic acid. But, what is far more important, it also showed that the destruction rates are equal for both compounds.

Actually the relative amounts of purines recovered from the mycelium were equal, whether the mutant was grown with adenine or with adenylic acid. It has been mentioned that when mutant 53 was grown with 2 μM of adenine 15 per cent of the added substance was accounted for as mycelial adenine and guanine. When in stead of adenine an equimolar amount of adenylic acid was used, likewise 15 per cent was recovered as mycelial purines as is apparent from table XVI.

Thus it must be concluded that adenylic acid is a better source of nucleic acid purines than adenine. This is confirmed by the data of tables XV and XVI. 89 per cent of the amount of adenylic acid taken up was used for nucleic acid synthesis, whereas for adenine this value was only 40 per cent.

This efficient utilization of adenylic acid constitutes strong evidence that in the mutants this compound is synthesized from adenine by way of adenosine and that it is an immediate precursor of nucleic acid purines.

Growth factors hypoxanthine, inosine and inosinic acid

Within the group of hypoxanthine derivatives the most remarkable feature was the very weak growth-promoting activity shown by inosinic acid as compared with hypoxanthine and inosine. The activities of the latter compounds were equal and of the same order of magnitude as that shown by adenine.

When again the efficiency principle is applied, the conclusion is that inosinic acid is converted to hypoxanthine or inosine. At first sight this may seem rather peculiar, but it must be noted that the inosinic acid used was inosine-5-phosphate, contrary to the corresponding adenine derivative, which was adenosine-3-phosphate. One could imagine that, this adenosine-3-phosphate being an intermediate in purine biosynthesis, its immediate precursor might be inosine-3-phosphate. So it is not inconceivable that in the mutant inosine-5-phosphate had to be converted to inosine-3-phosphate by way of hypoxanthine and/or inosine before being converted to nucleic acid constituents. Unfortunately inosine-3-phosphate was not available to check this hypothesis. It should have had an activity comparable with adenylic acid, or at least higher than hypoxanthine and inosine.

Growth factor AICA

As was stated above, of the third group of substances promoting growth of adenine-less mutants only the effect of AICA was studied. Yet some information could be obtained with respect to the possible participation of AICA derivatives in purine biosynthesis.

The investigations into the accumulation of purine precursors by adenine-less mutants (chapter IV) revealed that among the mutants of the above-mentioned second category some strains were present belonging to a type hitherto unknown in *Ophiostoma*. These mutants (strains 65 and 66, and possibly also 60) differed from the other strains by their ability to accumulate a purine precursor during growth.

This precursor supported growth of only strain 57, which is able to utilize AICA. Consequently the accumulated compound should be classed with the group of AICA derivatives. Additional evidence that the product was an AICA compound was furnished by the fact that the culture filtrates containing the precursor showed a positive Bratton–Marshall reaction for non-acetylatable diazotizable amine. The absorption maximum of the azo dye formed in this reaction was at 540 mμ, which value is also found for AICA.

 On further paperchromatographic examination of the culture fluid of one of the present mutants, viz. strain 65, it could be shown that two diazotizable amines were present. Both had a maximum UV absorption at 267.5 mμ, the maximum for AICA. One of them turned out to be identical with the latter compound on account of its chromatographic behaviour. The other compound proved to contain a pentose and was therefore and also because of its behaviour on the paperchromatograms considered to be identical with AICA riboside.

As was also shown in chapter IV, under certain conditions, *e.g.*, when acids are present, the accumulated substances are rapidly decomposed. The product of this decomposition gave no longer the Bratton–Marshall reaction or the orcinol reaction for pentose and had a maximum UV absorption at 300 mμ. This strongly reminds of the findings of Love and Gots (1955) who isolated from a culture of an adenine-less *E. coli* together with a diazotizable amine a non-diazotizable substance with an absorption maximum at 300 mμ.

Both isolated compounds, AICA and AICA riboside were able to support growth of strain 57. This constitutes evidence that the riboside too is involved in purine biosynthesis in this mutant. As no quantitative information is available about the relative growth-promoting activities of the free amine and the riboside, for the present no decision can be made about the order in which these substances are participating in this biosynthesis.

THE MAIN LINE OF PURINE BIOSYNTHESIS IN WILD TYPE OPHIOSTOMA

In normal wild type *Ophiostoma* the essential purine compounds (nucleic acids and nucleotides) are synthesized from minor consti-tuents. Evidence for the pathway along which this synthesis proceeds can be obtained from the results with adenine-less mutants described in the preceding section, as is obvious from the following considerations.

The mutants investigated utilized adenylic acid most efficiently of all compounds tested. Consequently this substance was the most immediate precursor of nucleotide purines. Apparently the mutants had in common the ability to convert adenylic acid to the ultimate purine compounds required for growth. So this conversion may be considered as a part of the normal pathway of purine biosynthesis.

Now the question arises what is the normal pathway of synthesis of adenylic acid. The growth requirements of the three groups of mutants, which could be discerned, indicate that the formation of adenine compounds proceeds by way of AICA and hypoxanthine or derivatives of these compounds. As both adenine and adenosine could be converted to adenylic acid, three ways are conceivable by which the normal biosynthesis of this substance can proceed, viz. at the level of the free bases, by way of the ribosides or by way of the ribotides.

Both AICA and its riboside were active growth factors for strain 57. As AICA riboside was not available in a sufficient amount no quanti-tative data could be obtained concerning the activity of this sub-stance. However, the high growth-promoting activity shown by the free amine, leads to some important conclusions.

When mutant 57 was grown with 2 μM of AICA plus 0.01 μM of hypoxanthine the maximum dry weight obtained was 36 mg.; with 0.01 μM of hypoxanthine only the maximum dry weight amounted to 2.5 mg. (figure 6). Consequently the maximum mycelium pro-duction which might be attributed to AICA was 33.5 mg. This value far exceeds the mycelium production with equimolar amounts of either adenine, adenosine, hypoxanthine or inosine and is only slightly less than the value for the maximum dry weight with 2 μM of adenylic acid.

From these findings it must be concluded that the synthesis of adenylic acid from AICA cannot possibly proceed by way of hypoxanthine and adenine, nor by way of inosine and adenosine, unless it is assumed that the adenine and hypoxanthine compounds are broken down by the fungus to a far greater extent than AICA.

Far more probable, however, is the hypothesis that purine biosynthesis proceeds at the ribotide level and that AICA ribotide is the normal intermediate. Actually the products found to accumulate during growth of strain 65 were AICA riboside and free AICA. So it must be assumed that these are degradation products of AICA ribotide. A similar assumption was made by GREENBERG (1952) with respect to the accumulation of AICA riboside by sulfonamide inhibited *E. coli*. Here too experimental data pointed to the ribotide being the actual product of accumulation.

As a further consequence of purine synthesis proceeding at the level of the ribotides it has to be assumed that a hypoxanthine ribotide is involved too. In this connection the most likely intermediate is inosine-3-phosphate.

According to GREENBERG (1953) ATP is necessary for the conversion of AICA ribotide to inosinic acid. The observations that the utilization of AICA by mutant 57 requires the presence of a catalytic amount of hypoxanthine or adenine are consistent with this view. Apparently growth has to be initiated and some ATP formed before AICA can be converted.

Little is known about the precursors of AICA (ribotide) in *Ophiostoma*. The observed stimulating effects of glycine, serine and threonine upon the accumulation of AICA compounds by strain 65 (table VI) provide strong evidence that glycine (and indirectly serine and threonine) is a precursor of these AICA derivatives. This is in agreement with the results obtained with isotopically labeled glycine in the study of uric acid synthesis in the pigeon, mentioned in chapter I.

In view of the "sparing" effect of guanine upon adenine utilization by mutant 53, shown in table II, one has to assume that in *Ophiostoma* guanine compounds can be synthesized from adenine and its derivatives. A higher mycelium production was observed when guanine had been added to the adenine containing nutrient solution. Apparently guanine in a measure is able to replace adenine as a precursor of nucleotide purines. As guanine *per se* did not support growth of adenine-less mutants, it should be concluded that it cannot function as a source of nucleotide adenine in *Ophiostoma*. It seemed, therefore, reasonable to assume that the above "sparing" effect is due to a—partial—replacement of adenine in the synthesis of guanine compounds, resulting in a more efficient utilization of the former substance.

As a matter of fact the "sparing" effect of guanine, found earlier by FRIES (1949a), together with the observed "adenine" accumulation by a guanine-less mutant (FRIES, 1953) constitute the main evidence for the formation of guanine by way of adenine. Investigations with

other organisms, mentioned in chapter I, demonstrated that in the normal biosynthetic process both compounds probably are derived from a common precursor, viz. inosinic acid. It seems quite possible that in *Ophiostoma* the reactions proceed along similar lines, both adenine and guanine compounds being derived from inosine-3-phosphate. The above-mentioned experimental results are in no way incompatible with such an assumption.

Summarizing, the following provisional scheme for the pathway of purine biosynthesis in *Ophiostoma multiannulatum* is proposed.

One of the first purine precursors is glycine. This compound, presumably by combining with formate, carbon dioxide, ammonia and ribose-3-phosphate, gives rise to AICA ribotide. The latter is converted to inosine-3-phosphate, in which reaction ATP is involved. Inosine-3-phosphate is aminated to adenosine-3-phosphate, which compound is participating in nucleotide synthesis. Guanylic acid, which likewise participates in this process, is formed either from adenosine-3-phosphate or from inosine-3-phosphate by way of an irreversible reaction.

B.   PURINE METABOLISM

RELATIONS BETWEEN PURINE AND PYRIMIDINE METABOLISM

From the foregoing it is evident that the substances supporting growth of adenine-less mutants are converted to both cellular adenine and guanine compounds.

The data summarized in figure 12 and table XVI indicate that the synthesis of cytosine compounds proceeds independently of purine synthesis, as the amount of cytosine in the mycelium still increased when no more mycelial purine compounds were formed.

The formation of uracil compounds stopped when purine synthesis had ceased. This suggests that uridine or uridylic acid synthesis is closely related to the formation of purine containing mycelium constituents. Such a relation also followed from the experiments summarized in tables II and III. Uridine exerted a "sparing" effect upon adenine utilization by the adenine-less strain 53, while adenine had a similar effect upon uridine utilization by the pyrimidine-less strain 52.

In view of the latter results one would be inclined to assume that adenine and uracil compounds are interconvertible, or that both are derived from a common precursor. However, for the present any further comment regarding the problem of the relation between purine and pyrimidine biosynthesis is a matter of pure speculation.

NUCLEIC ACID SYNTHESIS

The experiments mentioned in chapter VI and already discussed in section A of this chapter demonstrated that added adenine and adenylic acid were rapidly incorporated into nucleic acids by growing

mutants. In the first days of growth nucleic acid synthesis proceeded faster than in the wild type, resulting in a higher content of nucleic acids per mg. of dry mycelium. This is in agreement with the principle, outlined before, that more immediate precursors are utilized more efficiently.

The average purine and pyrimidine composition of the mutant nucleic acids was equal to the composition of the wild type polynucleotides. The molar proportions of the bases as a fraction of the total bases found were approximately: adenine 0.32, guanine 0.28, cytosine 0.14 and uracil 0.26. This indicates that in the mutants no irregularities occurred in nucleic acid synthesis. The absence of detectable quantities of thymine demonstrated that ribonucleic acids constituted the bulk of the nucleic acids present.

It must be noted that only the values found with comparatively young mycelium, harvested before maximum growth is reached, can be compared. As can be seen from table XII, experiment 2, in 4 weeks old wild type mycelium the molar proportions of the bases found in the acid insoluble fraction was: adenine 0.34, guanine 0.26, cytosine 0.18 and uracil 0.22. This indicates that in older cultures the original proportionality is disturbed by the occurrence of autolysis.

The greater part of the quantity of mycelium ultimately obtained was synthesized during the period in which no more nucleic acid synthesis occurred. The mutant grown with 7 μM of adenine produced in this period 38.0 mg. of mycelium, i.e., 73 per cent of the ultimate amount; with 2 μM of adenylic acid 36.6 mg. or 65 per cent of the total amount of mycelium was synthesized during the above-mentioned period. This resulted in a lowering of the nucleic acid content of the mutant mycelium till below the level found in the wild type.

It is generally accepted that protein synthesis depends upon nucleic acid metabolism. According to CASPERSSON (1947, 1950) a simple kinetic relationship would exist between nucleic acid content and the rate of protein synthesis.

From the data mentioned in chapter VI it may be calculated that mutant 53 grown with 7 μM of adenine produced 186 mg. of mycelium (dry weight) per μM of nucleic acid purine. Grown with 2 μM of adenine this value was 182 mg. Cultivated under similar conditions with 2 μM of adenylic acid the mutant formed 201 mg. mycelium per μM of nucleic acid purine eventually present.

If there is really such as a relation between nucleic acids and proteins the above data only permit the statement that apparently a given quantity of nucleic acid is responsible for the synthesis of a definite amount of protein. The ultimate decision with regards to this problem has to be put off for the future.

SUMMARY

(1)   UV-induced adenine-less mutants of the ascomycete *Ophiostoma multiannulatum* (Hedgc. et Davids.) v. Arx have been used to elucidate the normal pathway of purine biosynthesis and metabolism in this mould.

(2)   Investigation of the qualitative growth requirements of different mutant strains provided evidence that the normal pathway of nucleotide synthesis proceeds by way of 4-amino-5-imidazole carboxamide (AICA), hypoxanthine and adenine, or their ribosides or ribotides.

(3)   This view was supported by the discovery of a mutant type hitherto unknown in *Ophiostoma*, accumulating AICA riboside and AICA during growth. These mutants are unable to perform the conversion of AICA derivatives to hypoxanthine compounds. Glycine, serine and threonine stimulated the accumulation and consequently are considered as AICA precursors.   '

(4)   A comparative quantitative investigation into the efficiency of utilization of the various purine growth substances strongly suggested that the synthesis of nucleotide and nucleic acid adenine and guanine proceeds by way of AICA ribotide, inosine-3-phosphate and adenosine-3-phosphate.

(5)   Adenosine-3-phosphate was considered as the immediate precursor of nucleic acid purines. A quantitative analysis of growth and of purine metabolism revealed that 89 per cent of the total amount of adenosine-3-phosphate taken up could be accounted for as nucleic acid purines. For adenine this value was only 40 per cent.

(6)   In the mutants a close relationship exists between the amount of nucleic acids per culture and the production of mycelium, approximately 190 mg. dry matter being formed per μM of nucleic acid purines. This indicates that in the presence of a given amount of nucleic acid a definite quantity of protein is synthesized.

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