BIOSYNTHETIC INCORPORATION OF METABOLITE ANALOGUES

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I. INTRODUCTION

In 1940, Woods (155) found that *p*-aminobenzoic acid could reverse the inhibition by sulphanilamide of growth of haemolytic streptococci. He suggested that the enzyme involved in the utilization of *p*-aminobenzoic acid is subject to competitive inhibition by sulphanilamide; and that the inhibition is due to the structural similarity between sulphanilamide and *p*-aminobenzoic acid. Since then, the idea of competition at the active site of an enzyme between a metabolite analogue ("antimetabolite") and the normal substrate has been widely used as a basis for the design and synthesis of new growth-inhibitory compounds. Much new biochemical information has been obtained with such compounds, although few have so far achieved prominence in practical chemotherapy.

In the earlier work on antimetabolites, detailed growth inhibition studies were the main source of information on the mechanism of inhibition. For many years quantitative competitive relationships between growth of an organism and concentrations of antimetabolite and substrate in the medium were interpreted to

[1] I wish to thank Mr. Dennis Marshall for useful discussion on certain aspects of this review.

mean that an antimetabolite inhibited growth only by occupying the active site of the enzyme using the normal metabolite. With the development of chromatographic methods and the wider availability of labelled compounds, it has become possible to study biochemical transformations of analogues involving very small amounts of material. One of the most interesting developments from such work is the discovery that analogues of normal metabolites may be synthesised *in vivo* into more complex molecules.

An early example of such a synthesis concerned monofluoroacetic acid, a highly toxic compound, closely related in structure to acetic acid. The simplest explanation for its toxicity is that it interferes with the utilization of acetate. However, certain observations could not be explained on this view. Liébecq and Peters (80, 81) and Martius (93) put forward the idea that monofluoroacetate is metabolised some distance along the same path as acetate to form the true inhibitor. It was subsequently established that fluoroacetate is indeed condensed with oxaloacetate to form monofluorocitrate which was found to be a potent inhibitor of aconitase. To describe the phenomenon, Peters coined the term "Lethal Synthesis". This phrase neatly describes the main aspect of fluoroacetate toxicity. However, there are now a variety of metabolite analogues which are known to be incorporated *in vivo* into more complex molecules, and with many of these the relation of such incorporation to growth inhibition or toxicity is by no means so clear-cut. For these "Lethal Synthesis" would be an inaccurate description. We will consider here those inhibitory or toxic analogues of known normal metabolites for which there is evidence that incorporation occurs *in vivo* or in isolated enzyme systems to form analogues of more complex naturally occurring molecules.

Whether or not we include a particular example in such a definition is to some extent arbitrary. For instance, we will not discuss the organophosphorous insecticides which are transformed to active anticholinesterases through an *in vivo* change from =S to =O or by the formation of an N-oxide [although the latter change has been recently described as an example of "Lethal Synthesis" (115)]. These can be excluded for two reasons: (a) because there is no major increase in chemical complexity, and (b) because acetylcholine, the normal substrate of the inhibited enzyme, does not undergo a similar transformation. For a different reason we exclude metabolic changes in aromatic carcinogenic compounds and related substances which lead to an increase in chemical complexity (*e.g.*, the transformation of 2-naphthylamine to carcinogenic 2-amino-1-naphthol). With these we are, in general, ignorant of the normal substrates and products of the enzymes carrying out the transformations. Also excluded from consideration here is the large body of evidence concerning the detoxification of drugs which frequently involves the formation of more complex molecules (154b).

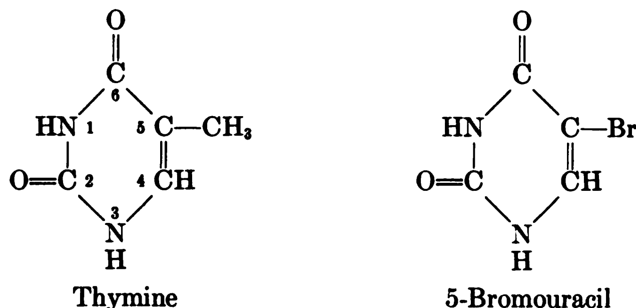
The examples described below include the incorporation of unnatural bases into deoxyribonucleic acids and ribonucleic acids; of amino acid analogues into proteins; and of a variety of structures into vitamins, coenzymes and related compounds.

II. ANALOGUES OF THE BASES IN NUCLEIC ACIDS

Large numbers of unnatural analogues of the purine and pyrimidine bases which occur in nucleic acids have been tested for effects on growth, particularly in relation to the problem of cancer therapy. There are now a number of well-authenticated examples of such analogues being incorporated into nucleic acids, replacing some of the corresponding normal base. These examples are of particular interest in view of the important functions now ascribed to nucleic acids for genetics and protein synthesis (136, 154). In the following sections we deal firstly with incorporation of analogues into deoxyribonucleic acids (DNA), secondly with incorporation into ribonucleic acids (RNA), and lastly with the biological effects of such incorporation. The conversion of analogues of the natural purines and pyrimidines to inhibitory nucleosides and nucleotides is considered in Section IV E.

A. Incorporation of analogues into DNA

1. 5-Halogenated uracils. The 5-halogenated uracils may be considered as analogues of thymine in which the methyl group is replaced by a halogen. Weygand *et al.* (151) grew *Streptococcus faecalis* on 5-bromouracil labelled with Br^{82}



Thymine 5-Bromouracil

They detected radioactivity in nucleic acids isolated from such cultures, but did not show whether the incorporation was into the DNA or the RNA. Dunn and Smith (27) and Zamenhof and Griboff (159) showed that substantial amounts of 5-bromouracil were incorporated into DNA of strains of *E. coli*, grown in the presence of the inhibitor and minimal amounts of thymine.

Under similar conditions 5-iodouracil was also incorporated into DNA of *E. coli* while both the bromo and iodo compounds appeared in the DNA of $T2_r$ phage grown on *E. coli* in the presence of the analogues (27). Under appropriate circumstances very large amounts of these analogues may be incorporated into the DNA.

Proof that the analogues were in fact incorporated into DNA was obtained firstly by isolation and characterisation of the 5′-deoxyribonucleotides of the analogues following enzymic hydrolysis of the DNA; and secondly by determi-

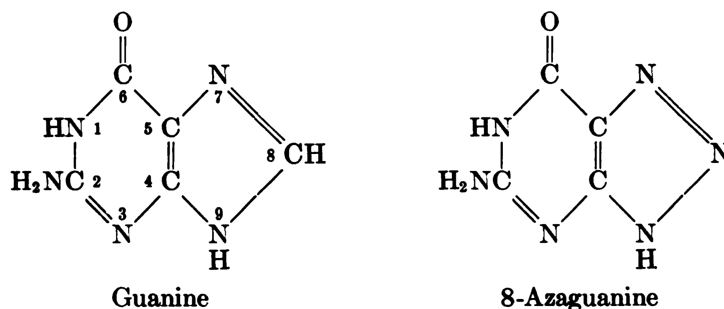
nation of the ratios of bases present which showed a deficiency of thymine corresponding closely to the amount of analogue present (27, 159).

In an experiment with *E. coli* 15T⁻ supplied with equal molar concentrations of the three analogues the chloro compound was incorporated to a greater extent than the other two analogues (26, 29). Moles of analogue per 100 moles of thymine plus analogue were: 5-chlorouracil 64; 5-bromouracil 31; 5-iodouracil 29. However, this relative effect is dependent on the conditions of the experiment.

The distribution of 5-bromouracil among the different molecules in a preparation of DNA has been studied by two different procedures—chromatographic fractionation and sedimentation in the ultracentrifuge. Using chromatography on Ecteola (a substituted cellulose derivative), Bendich *et al.* (6a) obtained a partial fractionation of DNA from *E. coli* grown in the presence of 5-bromouracil-2-C^{14}. The population of DNA molecules was heterogeneous with respect to incorporation of the analogue. DNA eluted from the column only at a high pH contained less 5-bromouracil than material eluted at lower pH values.

Using equilibrium sedimentation in a density gradient of cesium chloride Meselson *et al.* (102) showed that a mixture of normal DNA from T4 bacteriophage and DNA containing 5-bromouracil could be separated. The separation depends on the increase in effective density due to the presence of Br instead of CH_3. The normal DNA was homogeneous with respect to density and size. The DNA containing 5-bromouracil was heterogeneous with respect to effective density and the variation was found chromatographically to be related to the degree of substitution of thymine by 5-bromouracil.

2. 8-Azapurines. 8-Azaguanine has proved to be the most interesting of a series of 8-azapurine analogues synthesised by Roblin *et al.* (128)

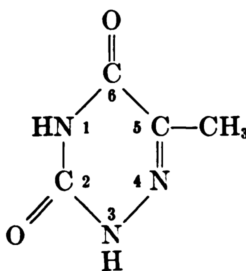


Guanine 8-Azaguanine

In a culture of *B. cereus* grown under conditions where 22% of the RNA guanine was replaced by 8-azaguanine the DNA appeared to contain small amounts of 8-azaguanine. After enzymic degradation of such DNA, a compound was isolated with the expected chromatographic and electrophoretic properties of 8-azaguanine deoxyribonucleoside (140). The amount was less than 1% of the deoxyguanosine.

However, no 8-azaguanine incorporation could be detected in the DNA of *E. coli* strains B/r and 55, nor in DNA of the $T2_r$ or $T2_r$ + phages grown on *E. coli* B/r in a medium containing 8-azaguanine under conditions where the analogue was incorporated into RNA.

3. 6-Azathymine. 6-Azathymine (4-azathymine on the numbering used here) and its deoxyriboside, azathymidine, inhibit growth of a variety of microorganisms.



Azathymine

Prusoff (121) showed that 6-azathymine-5-C^{14} is incorporated into the DNA of *Streptococcus faecalis* when the compound is supplied in the medium. Evidence was obtained for the presence of the analogue in polynucleotides from partially hydrolysed DNA. Up to 18% of the DNA thymine was replaced by the analogue.

B. Incorporation of analogues into RNA

1. 8-Azapurines. Several workers (7, 56, 103) reported that following administration of C^{14} labelled 8-azaguanine activity appeared in the RNA of various organisms. Mandel *et al.* (86, 87) showed that after alkaline hydrolysis of RNA from treated mice C^{14} could be detected in the nucleotide fraction and not in added carrier 8-azaguanine. With tobacco mosaic virus isolated from plants treated with 8-azaguanine it was possible to demonstrate the presence of the isomeric 8-azaguanosine 2′- and 3′-phosphates in alkaline hydrolysates of the virus RNA (94, 95). Further, because of the constant base composition of the virus RNA it could be shown from the ratio of bases present that the 8-azaguanine residues were in fact replacing about 3% of the guanine. The isomeric 8-azaguanylic acids have now been isolated from the RNA of a variety of organisms treated with 8-azaguanine (77, 140). The amount of guanine replaced is low in animal and plant systems, varying from less than 1% to about 3%. In several bacterial species the incorporation is also low, but in *Bacillus cereus* up to 40% of the RNA guanine may be replaced by 8-azaguanine.

This high level of incorporation into the RNA of *B. cereus* made it possible to study the distribution of the analogue relative to the normal base (98, 140). Shorter polynucleotides in the RNA preparations are richer in 8-azaguanine than longer ones. Partial fractionation of the products following digestion of the RNA with pancreatic ribonuclease suggests that 8-azaguanine occurs less frequently in the longer sequences of purines. Purine nucleoside 2′,3′ cyclic phosphates are not attacked by pancreatic ribonuclease so that any such compounds isolated from digests must have come from ends of chains (92). In digests of *B. cereus* RNA containing 8-azaguanine the guanosine 2′,3′ cyclic phosphate fraction is exceptionally rich in the 8-azaguanine analogue.

For example, in an RNA preparation that contained 22% 8-azaguanine over-

all, 69% of the material in the guanosine 2′,3′ cyclic phosphate fraction was the 8-azaguanosine compound. The reason for this is not yet understood. The 2′,3′ cyclic phosphates may have some special significance in RNA synthesis. Alternatively, an 8-azaguanine residue in this position may reduce the probability of further additions to the chain. What evidence there is favours the first hypothesis. Although total incorporation of analogue into *B. cereus* RNA increases greatly with time of growth in the presence of the analogue the relative amounts in the various fractions was about the same 5 min after addition of analogue as it was after 2 h. In RNA both from cells harvested after 5 min and from cells almost maximally inhibited by 8-azaguanine, the proportion of total cyclic phosphate ends (guanosine + 8-azaguanosine) to total (guanine + 8-azaguanine) was about the same (6 to 7%). Thus it is unlikely that an 8-azaguanine residue in this terminal position reduces the probability of further additions to the chain since this would lead to an increase in the proportion of chains with cyclic ends.

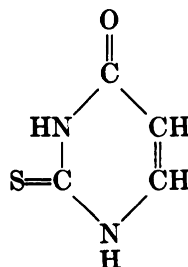
When the inhibition of growth of *B. cereus* by 8-azaguanine is annulled by addition of guanine to the medium the 8-azaguanine disappears from the RNA at a much greater rate than can be accounted for by dilution with new RNA containing no 8-azaguanine (140). This means that there is either a very rapid turnover of RNA or that the bases can exchange without rupture of the sugar-phosphate backbone. The second alternative is not supported by the observation of Mandel and Markham (88) that when the growth inhibition is annulled by the addition of guanylic acid, 8-azaguanine was ejected from the cells into the medium in the form of the nucleoside.

Recently a series of synthetic 8-azaguanine ribonucleosides have been made available to us by Dr. John Davoll (Parke Davis Co., London). These are the compounds with the ribose in the 9-position (the 'normal' compound) the 8-position and the 7-position of the 8-azapurine ring. All three nucleosides inhibited growth of *B. cereus* and *E. coli*, but not as strongly as the free base. Likewise they produced less incorporation of 8-azaguanine into the RNA. With TMV in tobacco plants, the "natural" 8-azaguanosine was slightly more effective than the free base in delaying virus multiplication. The 7- and 8-isomers were less effective. For all three nucleosides, both with the bacteria and with TMV, the 8-azaguanylic acid isolated from the RNA of inhibited cultures was of the "normal" configuration with the sugar in the 9-position. Thus the ribose must have been removed from the 7- and 8-positions before incorporation of the analogue.

From the RNA of a culture of *B. cereus* grown in the presence of 8-azaguanine, Mandel *et al.* (90) isolated sufficient of the 8-azaguanosine 2′- and 3′-phosphates for testing against a leukemia in mice. Although the test was necessarily on a small scale, and no analyses of the RNA from treated mice were made, there was no indication that the nucleotides were any more effective on a molar basis than the free base in prolonging the survival of mice bearing leukemia L-1210.

Various other 8-azapurine compounds when supplied to organisms become incorporated into RNA as 8-azaguanylic acid (97, 140) (Table 2). The question whether any of the 8-azapurines give rise to 8-azaadenylic acid in the RNA has not yet been adequately investigated.

2. 2-Thiouracil. Jeener and Roseels (66) reported that under conditions where the yield of tobacco mosaic virus was reduced by about 50% by S^{35} labelled 2-thiouracil the compound was incorporated into the virus RNA in an amount



2-Thiouracil

equivalent to about 20% of the virus uracil. We were able to confirm the incorporation of 2-thiouracil into the virus but in lesser amount (96). 2-Thiouridylic acid was identified in the hydrolysate by its electrophoretic and chromatographic properties. The distribution of S^{35} labelled 2-thiouracil in the virus RNA has been examined (89). The RNA was hydrolysed with pancreatic ribonuclease and the smaller products of the digestion were separated and characterised by chromatography and electrophoresis on paper. The dinucleotides adenylthiouridylic acid and guanylthiouridylic acid were detected. Of most interest was the high proportion of thiouridine and thiouridine-3′,5′-diphosphate. This might suggest that 2-thiouracil accumulates preferentially at the ends of polynucleotide chains. However, the question requires reinvestigating since it has been found more recently (126) that RNA preparations from tobacco mosaic virus may be quite heavily contaminated with substances yielding nucleosides and nucleoside diphosphates that are not an essential part of the virus RNA. The origin and significance of this contamination is not yet known.

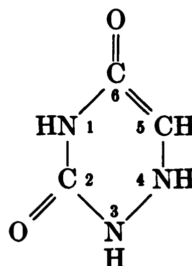
2-Thiouracil inhibits growth of *Bacillus megaterium*. Hamers (50) isolated a compound with the properties of 2-thiouridylic acid from the RNA of *B. megaterium* grown in the presence of S^{35} labelled 2-thiouracil. About 20% of the uracil in the RNA was replaced by 2-thiouracil.

3. 5-Halogenated uracils. From *E. coli* 15T⁻ grown in the presence of 5-chlorouracil, Dunn (26) isolated small amounts of a compound with the expected properties of 5-chlorouridylic acid. The 5-chlorouracil represented only about 2% of the uracil.

Recently 5-fluorouracil and some other 5-fluoropyrimidines have been synthesised (55). 5-Fluorouracil and 5-fluoroorotic acid showed marked inhibitory properties against a variety of microorganisms and transplanted tumours in rats and mice. 5-Fluorouracil C^{14} labelled in the 2-position was injected into mice bearing the Ehrlich ascites tumour. Mixed nucleic acids were isolated after 12 h from liver, spleen and tumour cells. Small amounts of 5-fluorouracil were identified in the mixture of free bases obtained after hydrolysis of the nucleic acids with $HClO_4$. 5-Fluorouracil was found only in the RNA fraction (54). However, to demonstrate that incorporation into polynucleotides had taken place it will

be necessary to isolate and characterise the appropriate mononucleotides containing 5-fluorouracil.

4. 6-Azauracil. Handschumacher (51) reported that "6-azauracil," a growth inhibitory analogue of uracil, was incorporated into the RNA of *Streptococcus faecalis* grown on a medium containing the analogue ("6-azauracil" = 4-azauracil on the ring numbering used here)



Azauracil

C. Biological effects of incorporation of analogues into nucleic acids

The incorporation of analogues of the purine and pyrimidine bases into nucleic acids is usually accompanied by an inhibition of growth. There are a number of observations which suggest that at least some of the growth inhibition may be due to the formation of non-functional nucleic acids. On the other hand in some systems there is definite evidence that nucleic acid which contains an unnatural analogue may still function. Furthermore, there is no reason to suppose that the analogues which are incorporated into nucleic acids inhibit growth through only a single mechanism. Observations relating to these problems are summarised below.

1. The infectivity of viruses. Data for the infectivity of three viruses containing analogues in their nucleic acids are summarised in Table 1. The normal preparations and those containing the analogue were compared on a basis of equal nucleic acid content. In preparations containing the analogues a substantial proportion of the particles were made non-viable. This is the most direct evidence that nucleic acid may be made non-functional by the presence of an analogue. However, this is no proof that the analogues interfere with actual reduplication of virus nucleic acids. For example, they might reduce infectivity of a virus preparation by interfering with the release of nucleic acid from the virus. It is now pos-

TABLE 1
Infectivity of viruses containing analogues of normal bases in their nucleic acids

Virus	Analogue	% Normal Base Replaced	Infectivity as a % of normal virus	Reference
TMV	8-Azaguanine	3	50	(95)
TYMV	8-Azaguanine	<1	40	(95a)
T2	5-Bromouracil	79	30	(27)
T2	5-Bromouracil	100	9	(83)

sible to prepare infectious tobacco mosaic virus RNA free of detectable protein (41). With this system it might be possible to obtain more conclusive evidence that 8-azaguanine incorporation affects nucleic acid function directly.

The effect of 2-thiouracil incorporation on tobacco mosaic virus is at present obscure. Commoner and Mercer (21) and Bawden and Kassanis (6) detected no difference in the infectivity of virus from normal and 2-thiouracil-treated leaves. Their methods, however, would not have detected small differences. Jeener (65) confirmed this result in more detailed experiments. When he compared normal virus and virus containing 2-thiouracil on an equal nucleic acid basis, there was no significant difference in the number of local lesions (infectious centres) produced in *Nicotiana glutinosa*. By contrast, when he inoculated leaves of *N. tabacum* with the same two preparations, the virus which contained 2-thiouracil multiplied at a substantially slower rate. Jeener interpreted this by suggesting that the nucleic acid in one virus particle might contain about eight viable units each independently capable of initiating infection. All eight units in a virus particle would have to be rendered sterile by 2-thiouracil incorporation, before the production of a local lesion would be prevented, but if a proportion of the eight were ineffective, rate of virus increase following infection by the particle would be reduced. While this hypothesis could explain Jeener's results, more definite evidence is needed concerning the existence of identical and independently acting sub-units of the virus nucleic acid. Physical evidence shows that the nucleic acid can be isolated as one large infective unit of MW about 2.1×10^6 (40). The radio-sensitive volume of the virus particle corresponds fairly closely to this [1.4–5.4×10^6 (42)]. These results suggest that the whole nucleic acid acts as a single infectious unit.

With the T2 bacterial virus, although a large proportion of particles may be made non-infectious by the incorporation of 5-halogenated uracils not all such particles are non-infectious. Litman and Pardee (83) using methods similar to those employed by Dunn and Smith (27) obtained a preparation of virus in which all the thymine was replaced by 5-bromouracil. In this preparation 9% of the particles were still viable. This experiment raises some difficult points. If *all* the thymine was replaced, what distinguished infective from non-infective particles? If, as is more likely, a small amount of thymine remained undetected in the preparation (probably not more than 1.0%) this would probably be distributed randomly among the particles. It might then be that the 9% containing most thymine were still infectious.

Most of the non-infective virus particles in preparations containing 5-bromouracil do not kill *B. coli* B (140a). On the other hand phage ghosts (the protein coats of virus particles from which the DNA has been released *in vitro*) are known to kill. It is thus possible that at least some of the non-infective particles produced by growth in a medium containing 5-bromouracil possess non-functional proteins. A further possibility is discussed in the next section.

This is a difficult problem to study since there appears to be no means of controlling the distribution of 5-bromouracil and the remaining thymine residues among the particles. However, useful information might be obtained by a sys-

tematic examination of the effects of various levels of analogue incorporation, particularly very high levels, on the proportion of viable particles.

2. Mutagenic effects of incorporation. Litman and Pardee (83) observed that among survivors in preparations of T_2 bacteriophage which contained 5-bromouracil there was a large proportion of plaque-type mutants. These were sometimes as much as 15% of the total infective progeny. The percentage of mutants was highest at the concentration of 5-bromouracil giving minimal infective yield. 5-Chloro- and 5-iodouracil, both of which are incorporated into phage DNA, also gave mutants; but 5-aminouracil (which is not incorporated) and uracil itself did not.

The appearance of these mutants could have been due either to a mutagenic action or to a selective enrichment of the mutants present in the original stock at a frequency of 10^{-3}. A statistical study of the mutant progeny occurring in a single-cell burst experiment demonstrated that the increase in plaque-type mutants occurred through mutation and not selection.

Litman and Pardee replated many of these mutants several times in the absence of 5-bromouracil, and almost all retained their mutant identity. The mutants must therefore be stable and do not need 5-bromouracil to reproduce. Even in the first mutant plaque formed there would have been many cycles of reproduction of the mutant under conditions where the amount of 5-bromouracil available would rapidly become negligibly small.

The chemical basis of this mutagenesis is quite obscure, but in view of the stability of the mutants, in the absence of 5-bromouracil, the significant structural changes in the DNA may well occur during the period of multiplication when 5-bromouracil is being replaced by thymine.

On one current hypothesis of DNA structure and function, reduplication of DNA involves a stage in which one intact polynucleotide chain acts as a template for the linear assembly of bases in some precursor form to make a second chain. The bases in the old and the new chain pair in a specific manner. It is suggested that appearance of a mutation may be caused by the replacement of one base by another in the chain.

A 5-bromouracil residue replacing thymine in a DNA chain might alter its immediate environment in such a way as to increase the probability that an incorrect base would pair with it during the reduplication process. A small number of such changes in a DNA molecule might give rise to a stable mutant, while larger numbers might make the DNA unable to function. On such a basis the replacement of any given set of thymine positions in a DNA molecule by 5-bromouracil would not in itself decide the fate of the molecule. We could get a mixture of normal particles, mutants, and sterile particles from a preparation of virus having all its thymine replaced by 5-bromouracil.

Zamenhof *et al.* (158, 160) examined the mutagenic effect of 5-bromouracil incorporation into the DNA of *E. coli*. They found the frequency of occurrence of a mutant to a form which was resistant to 5-bromouracil was unaffected by the presence of 5-bromouracil in the medium. They found no evidence for true mutagenesis for such characters as colony type and cell size. This question could

usefully be investigated using some clear-cut biochemical mutation as an indicator, as was used for a study of the mutagenic effect of thymine starvation on a strain *E. coli* (22). For example, Luzatti (84a) has recently found with *E. coli* 15T⁻ that incorporation of 5-iodouracil into the DNA is associated with an increase in the proportion of streptomycin-resistant mutants.

3. Effects on growth rate. This section summarises a variety of observations concerning the effects on growth of analogues which are incorporated into nucleic acids. Some of these indicate that the formation of non-functional nucleic acids may cause growth inhibition, while others show that analogues may actually support growth under certain conditions.

a. Correlation between incorporation and inhibition in the 8-azapurine series. In the action of 8-azapurines on TMV and two bacterial species we have no example so far of growth inhibition without incorporation into RNA or of incorporation without inhibition. Results of published (77, 95, 140) and some unpublished experiments are summarised in Table 2. With the methods used we could have detected incorporation of 8-azaguanine equivalent to about 0.1% to 0.5% of the guanine. The correlation observed is consistent with the idea that growth inhibition may be due to the production of non-functional RNA. Mandel (85) tested a series of compounds for their ability to annul the growth inhibition of *B. cereus* by 8-azaguanine. No compounds increased growth rate without decreasing incorporation of the analogue into RNA. Compounds with no effect on growth rate had no effect on incorporation. On the other hand he found that when restoration of the inhibited growth rate to normal was apparently complete, following addition of a natural purine compound, there was still a substantial proportion of 8-azaguanine in the RNA. Either the analogue was at this stage confined to a fraction of the RNA not essential for growth, or RNA containing the analogue was functioning normally.

b. Inhibition by the 5-halogenated uracils. With the incorporation of 5-haloge-

TABLE 2

Correlation between inhibition of growth and incorporation as 8-azaguanylic acid in the RNA of tobacco mosaic virus, E. coli and B. cereus

Compound	T.M.V.		*E. coli* B		*B. cereus* 569 H	
	Inhibition	Incorporation	Inhibition	Incorporation	Inhibition	Incorporation
8-Azaguanine	+	+	+	+	+	+
Dimethylamino-8-azaguanine	−	−	0	0	−	−
8-Azaadenine	+	+	+	+	−	−
8-Azahypoxanthine	−	−	+	+	−	−
8-Azaxanthine	−	−	−	−	+	+
8-Azaisoguanine	−	−	−	−	−	−
5(4)Amino-1,H-1,2,3-triazole-4(5)-carboxamide	+	+	+	+	−	−
8-Azaguanosine	+	+	+	+	+	+

+ = inhibition or incorporation; − = no inhibition or incorporation; 0 = not tested.

nated uracils into bacterial DNA there is, in general, a correlation between degree of growth inhibition and proportion of thymine residues replaced. With *E. coli* strain 15T⁻, where high incorporation occurs, growth usually ceases in about 4 hours, although with media containing higher levels of thymine growth may continue for longer than this (26). *E. coli* B continues to grow slowly even when the analogues are added at very low bacterial densities (26). Zamenhof *et al.* (161) found that in cultures of their thymine-requiring strain of *E. coli* which contained 5-bromouracil in the DNA a high proportion of cells were still viable. With the same strain, under conditions where there was no net increase in cell number or in DNA content per cell, 5-bromouracil could continue to be incorporated into the DNA (increasing from 8 to 48% of the thymine in 17 hours). Viability dropped from 88 to 61% over this period. It was suggested that this incorporation occurs through some form of exchange reaction. However, other work has indicated that the bases in DNA undergo very little exchange (13, 57, 139).

c. 6-Azathymine. Prusoff (121) found that growth of *S. faecalis* was inhibited when azathymine was added to the medium either before inoculation or during the logarithmic phase of growth. Death of all the cells was observed only when the analogue was added during the logarithmic phase. By contrast the amount of analogue incorporated into the DNA was much greater when the analogue was added before inoculation (12 to 18% of thymine replaced, compared with 0.1 to 1.2% if analogue was added during logarithmic phase). A larger amount of analogue was found in the acid-soluble fraction of the irreversibly inhibited bacteria than in cultures where the analogue was added before inoculation. Some incorporation of azathymine into DNA of *S. faecalis* occurred even when the concentration of analogue in the medium was insufficient to inhibit growth. Prusoff suggests that loss of viability may be due to incorporation of the analogue into derivatives in the acid-soluble fraction, rather than into DNA (see p. 391).

d. Time lag in onset of decreased growth rate. With many inhibitors of bacterial growth, when the inhibitor is added to actively growing cells, inhibition begins almost immediately. With the 8-azapurines there may be a delay before growth inhibition is apparent (140). The length of the delay varies with concentration of inhibitor used and bacterial density at which inhibitor is added but is usually about 1 to 2 generations. Similar delays have been found in the inhibition of *E. coli* by 5-bromouracil and 5-iodouracil (20, 27, 29). The lag in development of growth inhibition could be interpreted as the time necessary for the accumulation of sufficient ineffective nucleic acid to affect the growth rate.

e. Examples where incorporated analogues support growth. Hitchings *et al.* (58–61) have shown that 5-bromouracil can partially replace thymine for the growth of *L. casei* in certain media. Further, with a number of apparent thymine analogues that inhibit growth, 5-bromouracil could reverse the inhibition in a similar way to thymine. For example, the inhibition of growth by 100 μg of 5-nitrouracil was nearly abolished by 10 μg of 5-bromouracil or by 2.5 μg of thymine. Prusoff (119) found with *Streptococcus faecalis* that when thymidine concentration in the medium was high 5-bromouracil inhibited growth. With

low levels of thymidine the same concentration of analogue increased growth. Prusoff made growth measurements at only one time so that these rather unexpected results may well be explained by differences in growth curves of the sort illustrated in Fig. 1 and discussed on p. 397.

In experimental leukemias in mice sublines which appear to be dependent on 8-azaguanine for optimal growth have been developed from lines originally sensitive to the analogue (78).

Among carnivorous protozoa, 8-azaguanine inhibited growth, and induced the production of abnormal giant forms in *Tokophrya infusionum* while in *Podophrya collini* the analogue had a growth-promoting effect and prevented appearance of giant forms (82). Both these effects were annulled by guanylic acid.

Incorporation of the analogues into nucleic acids was not investigated in any of the above experiments. However, it seems very likely that some incorporation would have been taking place, particularly in the experiments with 5-bromouracil.

4. Effects of incorporated analogues on the utilization of normal components for nucleic acid synthesis. If an analogue which is incorporated into nucleic acid replaces a proportion of the normal base without affecting the overall base composition it will obviously reduce the utilization of the normal base by the amount which it replaces. Apart from this, however, there appears to be little evidence that incorporated analogues interfere with the utilization of normal compounds.

Carlo and Mandel (17) found that 8-azaguanine had no significant effect on the rate of incorporation of C^{14} labelled guanine or 4-amino-5-imidazole carboxamide into nucleic acids of liver or Sarcoma S37 of mice. Under their experi-

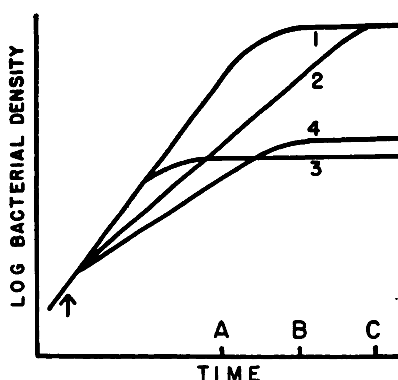


FIG. 1. Effects of 5-bromouracil on *E. coli* strain B, grown under conditions where thymine was required for growth (*i.e.*, on a medium containing sulphanilamide).

5-Bromouracil was added at time indicated by arrow. Curve 1: control with excess thymine. Curve 2: as for curve 1, plus 5-bromouracil. Curve 3: control with limiting amount of thymine. Curve 4: as for curve 3, plus 5-bromouracil. Possibe interpretations of the effect of 5-bromouracil if growth was measured only at time A, B or C.

A: from 1 and 2 inhibition; from 3 and 4 inhibition.

B: from 1 and 2 inhibition; from 3 and 4 stimulation.

C: from 1 and 2 no effect; from 3 and 4 stimulation.

mental conditions the analogue would have caused some inhibition of tumour growth and would itself have been incorporated into the tissues RNA. Although their results gave no indication of competitive inhibition of guanine incorporation by 8-azaguanine, it is probable that the amount of normal base incorporated would be reduced by about the amount replaced by the analogue. This amount may have been too small to detect in the mouse system. With *B. cereus*, where large amounts of 8-azaguanine are incorporated, Mandel and Markham (88) showed that guanylic acid incorporation into the RNA was decreased by approximately the amount of 8-azaguanylic acid incorporated. This result would be expected since the base composition of the RNA is little changed, with 8-azaguanine replacing some of the guanine (88a).

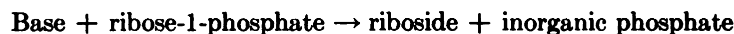
From experiments in which 8-azaguanine and P^{32} were added simultaneously to growing cultures of *B. cereus* Mandel and Markham (88a) concluded that the synthesis of guanylic acid was not impaired in inhibited cultures. Assuming that there is no buildup and breakdown of RNA without net increase in amount per cell, they suggest that in 8-azaguanine-inhibited cultures the amount of RNA is increased.

Creaser (23, 24) found that in washed suspensions of *Staph. aureus* 8-azaguanine was incorporated into the RNA. The presence of 8-azaguanine did not affect the amount of RNA formed as measured by the incorporation of radioactivity from C^{14} uracil into the nucleic acids.

Prusoff *et al.* (122) found that in tissue cultures of rabbit bone marrow and mouse Ehrlich ascites tumor, both thymidine and azathymidine inhibited the incorporation of the carbon of C^{14} formate into DNA thymine. The effect was much more marked with the natural nucleoside. They suggest that the effect of thymidine was by dilution and that of azathymidine by "inhibition." However, if the analogue was being incorporated into the DNA of these systems as it is into bacterial DNA (121), it could have been preventing formate incorporation in the same way as the natural compound.

Certain enzymes concerned in purine metabolism are strongly inhibited *in vitro* by 8-azaguanine. Feigelson and Davidson found that xanthine oxidase from cream (32) and adenosine deaminase from rabbit intestinal mucosa and adenocarcinoma 755 were strongly inhibited (33). It is not yet clear how such inhibitions could be related to the biological effects of 8-azaguanine.

As might be expected, studies with enzyme systems *in vitro* indicate that the analogues which are incorporated into nucleic acids can form nucleosides fairly readily. Thus Friedkin (36) showed with a preparation from horse liver that 8-azaguanine and 2-thiouracil will participate in a reaction of the type



Base + ribose-1-phosphate → riboside + inorganic phosphate

Friedkin and Roberts (38) measured the rates of formation of a number of pyrimidine deoxyribosides with a similar system. The amount of deoxyriboside formed from the following analogues relative to the amount of thymidine produced from thymine was: 5-chlorouracil 27%, 5-bromouracil 50%, 5-iodouracil 63%, 2-thiouracil 55%. Koch (75) obtained an extract from *E. coli* with trans-

ribosidase activity which could apparently exchange the hypoxanthine of inosine with several analogues including 5-bromouracil.

5. Enzyme formation. Creaser (23, 24) studied the effect of 8-azaguanine on enzyme formation in washed suspensions of *Staph. aureus*. The formation of the adaptive enzymes β-galactosidase and catalase in *Staph. aureus* Dunn strain harvested at various stages of growth is almost completely inhibited by 8-azaguanine. The formation of the constitutive enzymes glucozymase and catalase in *Staph. aureus* Duncan strain is much less sensitive to 8-azaguanine and completely insensitive at certain stages of growth. Inhibition of enzyme formation by 8-azaguanine could be annulled by the addition of guanine, xanthine, or hypoxanthine.

Rates of synthesis of RNA were not affected by 8-azaguanine but the analogue was incorporated into the RNA. None could be detected in the DNA. Creaser found that the incorporation of guanine into nucleic acid of *Staph. aureus* is not a reversible process, so presumably 8-azaguanine could be incorporated only during the synthesis of new RNA.

These results could well be explained by supposing that for adaptive enzyme formation a continuous production of new RNA is necessary, and that new RNA containing 8-azaguanine is ineffective in such a process. Creaser suggests that adaptive enzyme synthesis may be dependent on RNA with a short active life (and thus easily affected by 8-azaguanine incorporation at any stage), while constitutive enzyme production might be controlled by RNA with a long active life. This could explain why formation of glucozymase and catalase (in the Duncan strain) are not affected so markedly by 8-azaguanine and at some stages of cell growth are not affected at all.

An apparently similar inhibition of enzyme formation by 8-azaguanine and 2-thiouracil has been found with a strain of *Bacillus subtilis* (39). This strain under aerobic conditions in the presence of phosphate secretes small amounts of amylase into the medium. The addition of a suitable carbon source greatly increases this secretion of the enzyme. The enzyme secreted under these conditions appears to be newly formed in the cells after the addition of the carbon source. The secretion can be inhibited by the addition of 8-azaguanine or 2-thiouracil to the medium.

D. Discussion

The observations summarised above show that analogues which are incorporated into nucleic acids may have a variety of effects depending on the organism, the conditions of growth and the particular analogue. (i) They may lead to the formation of non-functional nucleic acids which are incapable of bringing about their own reduplication or the formation of new active proteins. (ii) Their presence may "unstabilise" deoxyribonucleic acid, leading to an increase in the frequency of mutation. (iii) They may effectively replace the natural base giving nucleic acid which functions normally. (iv) Besides being incorporated into nucleic acids, they may inhibit growth by interfering with enzyme reactions, either as the base or in the form of nucleosides or nucleotides (see p. 389).

At the present time we cannot make any firm predictions as to the likelihood of an analogue being incorporated into nucleic acids and we can say still less about how an analogue may affect nucleic acid function. Indeed, a study of the effects of a variety of analogues may give us some insight into the relative importance of different features of nucleic acid structure.

Whether an analogue is incorporated into nucleic acid and what effect it may have if incorporated will depend on two interrelated factors—the structure of the analogue and the metabolism of the organism involved.

1. Structural features of the analogue. There is one structural feature which may always be necessary for an analogue to be incorporated. This is the ability to form an N-glycoside link at the correct position in the ring (9 for purines, 3 for pyrimidines). For example, it is unlikely that the following guanine analogues would be incorporated into polynucleotides: 5-amino-7-hydroxy-1,2,4,6-tetraazaindene (which has a carbon at the position equivalent to 9 of purine); 5-amino-7-hydroxy(3,1,2)oxadiazolo(5,4-d)pyrimidine (which has 0 instead of N at the position equivalent to 9); and 7-methylguanine (which has no free hydrogen available at the 9-position).

All the unnatural bases so far known to be incorporated into nucleic acids are analogues of guanine, uracil, or thymine. These all have a keto substitution at position 6 of the pyrimidine ring. No analogues of adenine or cytosine have been shown to be incorporated. These two bases have an amino group in the 6-position.

Although too few analogues have been thoroughly examined to enable any definite statement to be made, there may be some special reason (*e.g.*, hydrogen bond formation in a double helical structure) why the amino group in this position is particularly sensitive to change. Structural alterations elsewhere in the molecule might bring about a change in the pK of the 6-amino group, or this substituent may be replaced by another. For example, the pK for the dissociation of the 6-amino group in 2,6-diaminopurine differs from that in the 6-position of adenine. In 6-mercaptopurine the amino group is replaced by —SH. Although both these compounds can be converted enzymatically to nucleotides (84, 127) they do not appear to be incorporated intact into nucleic acids.

Littlefield and Dunn (83a) have shown that 2-methyladenine, 6-methylaminopurine, and 6-dimethylaminopurine occur naturally in polyribonucleotides from various sources. This may nullify the suggestion made above. However, the amounts of these bases are rather small (0.1–0.6 moles/100 moles of uracil) and their significance in ribonucleic acid preparations remains to be established.

For those analogues that are incorporated, the relative importance of changes in size and shape and of changes in electronic configuration is difficult to assess. The change from an imidazole ring in guanine to triazole in 8-azaguanine brings about only a small change in size and shape, but the pK of both the amino and hydroxyl groups are lowered by about 2 pH units. With the 5-halogen analogues of thymine, under comparable conditions in *E. coli*, the proportion of thymine replaced decreased in the order 5-chloro-, 5-bromo-, and 5-iodouracil (29), although from the data of Pauling (110) the bromine atom appears to be closest

in size to the methyl group. However, as Dunn and Smith (29) point out, the relative effects of the three 5-halogenated uracils on growth and in replacement of thymine in DNA depend on growth conditions and strain of bacteria. Thus one cannot make generalisations regarding the effects of structure in this series. The fact that glucose-hydroxymethylcytosine is found in DNA from the T-even phages (135, 147, 157) suggests that size of the substituent in the 5-position of DNA pyrimidines may have a fairly wide degree of latitude.

With 5-chloro-, 5-bromo- and 5-iodouracils and also with 2-thiouracil and 6-azauracil the pK values of the —OH groups are about 2 units lower than for the corresponding normal compounds. This change seems to be a general feature of these analogues, and may well be of major importance in determining the structural and functional differences between a normal nucleic acid and that containing an analogue. However, Smith (140a) has recently shown that 5-ethyluracil can be incorporated into a thymineless strain of *E. coli* in small amounts. Here the pK of the 6 (OH) group, if changed at all, should be higher than that of thymine. A detailed physico-chemical comparison of incorporated analogues and the normal compounds, both as the bases and in nucleosides and nucleotides, might provide most useful information.

With the more refined analytical methods now available, previously undetected bases are being found in nucleic acids (20a, 24a, 28, 83a). Each of these opens up new possibilities for the design of analogues. A thymineless strain of *E. coli* contains small amounts of 6-methylaminopurine in its DNA (28). Under certain conditions (*e.g.*, with low thymine or in the presence of 5-aminouracil) the amount of this base can be substantially increased. Under the same conditions the amount of thymine in the DNA is reduced, but 6-methylaminopurine does not appear to be replacing thymine residues in the DNA (29a).

These results emphasise that it is impossible to make any satisfactory generalisations at this stage about what types of change in the structure of a base may lead to an analogue which is incorporated into nucleic acids.

2. Effects of host metabolism. An analogue that is potentially capable of entering the structure of a nucleic acid will only do so if the metabolic processes of the organism under study can deal with the compound in an appropriate way. The analogue must not be altered or degraded to inactive material. It must be able to be incorporated into whatever type of "precursor" molecules are involved in nucleic acid synthesis under the particular conditions of growth. For example, *B. coli* will not normally use externally supplied thymine or thymidine for synthesis of DNA, and 5-bromouracil when added to the medium is not incorporated into DNA. The precusor of DNA thymine is presumably made by a route not involving thymine or thymidine. However, when *B. coli* is grown on a medium containing sulphanilamide (supplemented with certain amino acids and purines) the cells become dependent on externally supplied thymine. Under these conditions 5-bromouracil is incorporated into DNA (29).

The rate of synthesis of nucleic acid may affect incorporation of an analogue. As noted above, when grown under conditions where it does not require thymine *B. coli* does not incorporate 5-bromouracil into its DNA. However, the analogue

is incorporated in substantial amounts into the DNA of T_2 phage when grown on such bacteria. This is probably due to the fact that the rate of DNA synthesis infected cells is about four times normal, so that the system becomes short of DNA thymine and uses 5-bromouracil (29).

At the present time a substantially larger number of analogues are known to be enzymically converted to the nucleoside than are known to be incorporated into nucleic acids. Nevertheless, it may well be that with some analogues lack of *in vivo* conversion to the nucleoside prevents incorporation into nucleic acids; or the rate of conversion may limit the amount of incorporation. Incorporation of 5-bromouracil may have been limited by the rate of conversion to the deoxyriboside in the thymine-requiring strain of *E. coli* studied by Zamenhof and Griboff (160). These bacteria were more strongly inhibited by 5-bromouracil deoxyriboside than by 5-bromouracil; and thymidine was much more effective than thymine in annulling the inhibition.

It has been evident for some time that different organisms may vary widely in the way they metabolise an analogue. As new compounds are developed they should be tested in a wide variety of systems. In a particular organism an analogue may not be incorporated for reasons that have little to do with nucleic acid structure. Analogues which are not incorporated when supplied as the base might be when supplied as a nucleoside or nucleotide. There are well over 10 possible types of nucleotide for a given base. With certain compounds (*e.g.*, 8-azaguanine), analogue precursors are known which might also be supplied as a nucleoside or nucleotide. Thus the number of possible forms in which an analogue could be supplied is quite large.

The possibilities of achieving selective toxicity with incorporated analogues of nucleic acid bases may be somewhat more favourable than with amino acid analogues that enter protein structure. The four major bases in each kind of nucleic acid are almost universally distributed, but recently several additional bases have been isolated. These may occur in rather small amounts. If a base occurred in a virus or tumour nucleic acid and not in the host cell, it might be possible to design an analogue which would be specifically incorporated into the tumour or virus nucleic acid. The only example so far known of a base peculiar to an agent producing disease is the occurrence of 5-(hydroxymethyl)-cytosine in the DNA of the T-even phages. However, not many nucleic acids have yet been exhaustively investigated for the presence of bases occurring in small amounts.

RNA is found in all cells (except certain animal spermatozoa). In many cells phosphorus and bases are incorporated at appreciable rates into RNA even when the total amount of RNA is not increasing (for references see 139). Thus, in this respect the situation is similar to that with proteins. An analogue that is incorporated into virus or tumour RNA would most probably be incorporated into host RNA as well. However, with viruses which contain RNA the template may be irreversibly inactivated by incorporation of an analogue, whereas inactivated cellular RNA might be renewable from another template (*e.g.*, DNA).

In contrast to RNA, the DNA in non-dividing cells appears to be rather stable.

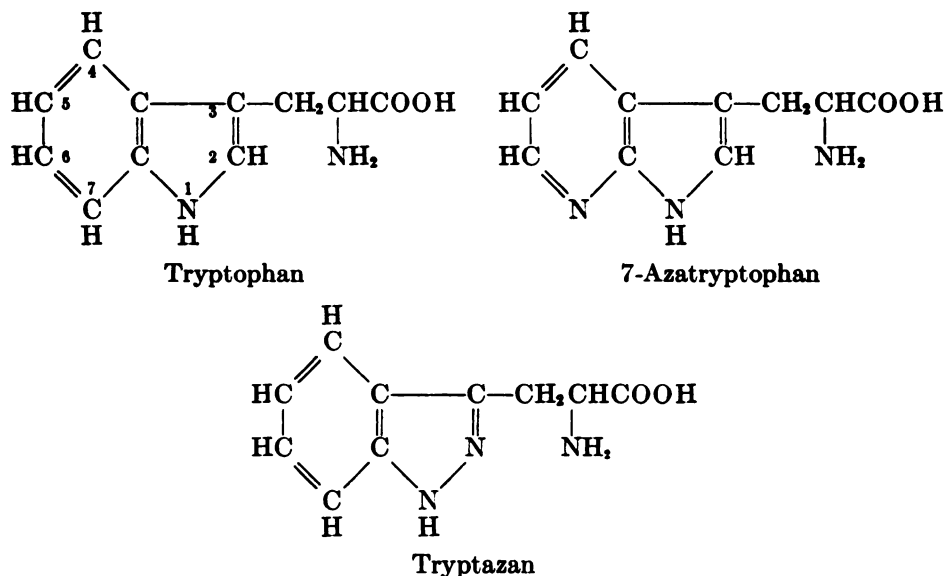
Other factors being equal, a tissue in which rapid cell division is occurring should incorporate considerably more of an analogue into its DNA than one where only a small proportion of the cells are undergoing division. Thus since DNA in tumours and viruses replicates much more rapidly than host cell DNA it might be more inactivated by an analogue. However, it has been shown that 5-bromouracil in substantial amounts enters the DNA of cultures of *E. coli* under conditions where very few cells appeared to be dividing (161). This result is not in accord with the general evidence for the stability of DNA in non-dividing cells, and remains to be explained.

III. AMINO ACID ANALOGUES IN PROTEINS

The possibility of inhibiting growth by interfering with protein synthesis is an obvious one, and many analogues of the naturally occurring amino acids have been synthesised and tested. Some are active growth inhibitors. In earlier work it was assumed that such analogues acted either by interfering with the synthesis of the normal compound, or by preventing its incorporation into protein. More recently it has been found that several analogues are themselves incorporated into proteins, and there is some evidence that such proteins may be biologically ineffective.

A. Analogues of tryptophan

Several analogues of tryptophan are active inhibitors of growth in various systems. At least two of these appear to be incorporated into proteins.



For a tryptophan-requiring mutant of *E. coli* (#19–2) 7-azatryptophan allowed an increase in turbidity, protein and RNA content in the absence of tryptophan (109). Tryptazan also allowed such increases but 5-methyltryptophan did not. Growth with the first two analogues stopped after RNA, protein,

turbidity or viable cell count had approximately doubled. 7-Azatryptophan was isolated from acid-hydrolysed bacterial residues by chromatography and identified by its position, its fluorescence in ultraviolet light and its reaction with ninhydrin. There was approximately 0.5% azatryptophan in the bacterial protein preparations, about the amount expected if azatryptophan was replacing tryptophan in the newly formed protein. Bacteria exposed to azatryptophan for only 4 min showed none of the compound in their proteins.

Brawerman and Yčas (12) obtained similar results with tryptazan in another tryptophan-deficient mutant of *E. coli* (✻567). When an actively growing culture of this organism was transferred to a medium without tryptophan, synthesis of both protein and nucleic acid ceased. Synthesis of both these materials was rapidly restored by the addition of tryptazan. Growth in the presence of the analogue led at most to a doubling in the amount of protein. These workers took advantage of a strong absorption peak at about 255 mμ not given by tryptophan to show the presence of the analogue in bacterial protein preparations. Chromatography of alkaline hydrolysates of the proteins showed the presence of substantial amounts of the analogue—equal to about half the amount of tryptophan (judged by eye from the published elution diagrams of Brawerman and Yčas). No direct evidence is given in either paper that tryptazan or 7-azatryptophan was present in peptide linkage, and the possibility remains that at least some of the compound was adsorbed onto the proteins. However, although they give no quantitative figures there is clear evidence from the elution diagrams of Brawerman and Yčas that in the sample where tryptazan is present the amount of tryptophan (relative to tyrosine) is substantially reduced. This is good evidence that the analogue was in fact replacing tryptophan in the protein.

Sharon and Lipmann (131) studied the reactivity of a series of tryptophan analogues with a tryptophan-activating enzyme from pancreas using two tests—amino acid hydroxamate formation and an amino acid-dependent pyrophosphate-ATP exchange. The following tryptophan analogues were active in both these tests: 7-azatryptophan, tryptazan, 6-fluorotryptophan, and 5-fluorotryptophan. Another group of analogues were not activated, and these inhibited the activation of tryptophan. These included β-methyltryptophan, 5-hydroxytryptophan, 5-methyltryptophan, 6-methyltryptophan.

Sharon and Lipmann suggest that those analogues which are activated by the tryptophan-activating enzyme are incorporated into proteins and inhibit growth through such incorporation, while those that are not activated are not incorporated, and inhibit growth through inhibiting the activation of tryptophan. If this is so, then 5-fluoro- and 6-fluorotryptophan should be found to be incorporated into proteins.

Pardee *et al.* (109) examined the biological activity of proteins produced in the presence of several analogues. A number of inducible and constitutive enzymes were not produced (as measured by activity) in the absence of tryptophan or in the presence of 7-azatryptophan, tryptazan, or 5-methyltryptophan. However, the first two analogues allowed the development of increased activity of serine deaminase and ureidosuccinic acid "synthetase". T2 bacteriophage produced in *E. coli* B in the presence of 7-azatryptophan contained at least 0.4% of the

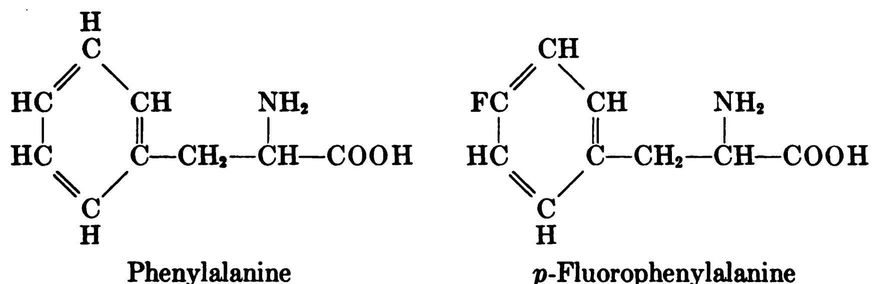
analogue in its protein. A large proportion of the phage particles were non-viable. This is perhaps the most direct evidence so far produced to show that incorporation of an amino acid analogue may lead to the production of defective protein.

Halvorson *et al.* (49) found that tryptazan inhibited growth, enzyme synthesis and the utilization of the free amino acid pool in yeast.

The incorporation into proteins of the various amino acids in the pool was equally inhibited by tryptazan, and there was no accumulation of peptides. These results might well be explained by the formation of ineffective proteins containing the analogue.

B. Analogues of phenylalanine

1. p-Fluorophenylalanine. *p*-Fluorophenylalanine is inhibitory for *Lactobacillus arabinosus*. However, when the concentration of phenylalanine in the medium is sub-optimal, low concentrations of the analogue stimulate growth (3). Baker



Phenylalanine

p-Fluorophenylalanine

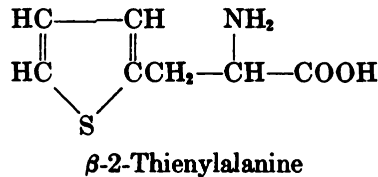
et al. (4) give evidence that under such growth conditions *p*-fluorophenylalanine may be incorporated into the bacterial proteins.

Munier and Cohen (106) studied the effect of *p*-fluorophenylalanine in *E. coli*. The addition of 10^{-3} M DL *p*-fluorophenylalanine to cultures in the logarithmic phase of growth resulted in a change to a linear growth rate. At the same time synthesis of protein became linear. However, the quantity of valine incorporated into protein, per unit mass of cells formed, was the same in inhibited and control cultures.

From hydrolysates of protein fractions from inhibited cultures they isolated *p*-fluorophenylalanine by paper chromatography. This was unlikely to be an adsorbed contaminant since protein fractions from bacteria grown in the presence of *p*-fluorophenylalanine and chloramphenicol did not contain the analogue. Proteins formed in the presence of the analogue contained 23 % less phenylalanine and 47 % less tyrosine than proteins from normal cultures. Thus it appears that *p*-fluorophenylalanine may partially replace both tyrosine and phenylalanine in the proteins of inhibited bacteria.

Synthesis of the enzyme β-galactosidase induced by thiomethylgalactoside was not inhibited by *p*-fluorophenylalanine. Less enzyme was formed per unit time, but the amount formed per unit of new bacterial mass was the same as in control cultures. In contrast, adaption of *E. coli* K_{12} to using lactose, maltose, or xylose was inhibited.

2. β-2-Thienylalanine. β-2-Thienylalanine inhibits growth of *E. coli* in a similar way to *p*-fluorophenylalanine (106)



β-2-Thienylalanine

Munier and Cohen state that the analogue is incorporated into the bacterial proteins. In the presence of this analogue, in contrast to *p*-fluorophenylalanine, the formation of β-galactosidase activity is suppressed.

C. Ethionine

Ethionine CH_3CH_2—S—$CH_2CH_2CH(NH_2)COOH$, is an analogue of methionine in which the methyl group is replaced by an ethyl group.

Levine and Tarver (79) fed rats with ethionine C^{14} labelled in the methylene group of the ethyl residue. The labelled analogue was found in the tissue proteins and various indirect lines of evidence suggested that the ethionine was actually incorporated into protein. There is also an indication that ethionine may be incorporated into proteins of the Ehrlich ascites carcinoma (125). More conclusive chemical evidence for incorporation was obtained in experiments with the protozoon *Tetrahymena pyriformis* (47, 145). When this organism was grown in the presence of labelled ethionine the label was taken up by the proteins. Most of the label was present in ethionine (equivalent to about 0.08% of the protein) although there was a small amount in aspartic and glutamic acids. From partial acid hydrolysates of the labelled proteins Gross and Tarver isolated peptides containing ethionine.

Berg (8) isolated an enzyme from yeast which catalyses an L-methionine dependent exchange of inorganic pyrophosphate with adenosine triphosphate. None of the other naturally occurring amino acids was active in the system but DL-ethionine catalysed a slow exchange. This suggests that the analogue can substitute for methionine in amino acid activating reactions. However, Berg considered that the effect may have been due to contamination of the analogue preparation with traces of methionine.

Lee and Williams (78a) reported that ethionine inhibited the increase in tryptophan peroxidase activity found in the liver after administration of L-tryptophan to rats. They suggested that the ethionine interfered with the utilization of methionine in the formation of new enzyme. However, it may well be that ethionine was synthesised in the liver into new protein to produce inactive enzyme.

D. Norleucine

Norleucine (α-amino-n-caproic acid) is an isomer of leucine which has not been shown unequivocally to occur normally in proteins. Following injection

of C^{14} labelled norleucine into lactating cows, Black and Kleiber (10) isolated norleucine from casein preparations. The norleucine did not appear to be present as a contaminant, but no direct evidence was given for the existence of the amino acid in peptide linkage. If norleucine was a normal component of the casein it was present in concentrations of less than 1.6 mg/100 g casein. About 4.7 mg of norleucine per 100 g casein was found following injection with the labelled compound.

E. Selenium poisoning

In many areas of the Western United States, soils contain relatively large amounts of available selenium. This is taken up by plants. There is some evidence [reviewed by Painter, up to 1940 (108)] that selenium analogues of sulphur compounds are formed in the plants. In grains most of the selenium occurs in the protein fraction. Farm animals feeding on seleniferous plants may develop chronic selenium poisoning. It seems likely that the toxicity of seleniferous plants for grazing animals is largely due to the presence of organic selenium compounds. Selenium appears in the body proteins of animals fed seleniferous diets. The selenium appearing in dog serum proteins is non-dialyzable over a range of pH values (100).

p-Bromobenzene administered to animals is detoxified by conjugation with cysteine and methionine, being excreted in the urine as *p*-bromophenylmercapturic acid. Moxon *et al.* (105) found that all the selenium in the blood of steers was in the protein precipitate. The amount present could be greatly reduced by the administration of *p*-bromobenzene to the animals. This is indirect evidence that selenium analogues of methionine and cysteine may have been present in the blood proteins.

McConnell and Wabnitz (101) studied the forms in which selenium was found in the liver proteins of the dog following injection of $Se^{75}Cl_4$. Small amounts of Se appeared in the liver proteins. Following acid hydrolysis of the proteins, radioactivity appeared in compounds having the following properties expected of amino acids: retention on and elution from Dowex 50 resin; mobilities on paper chromatography; precipitation by amino acid precipitants such as cuprous chloride; and liberation from the copper salt by the thiocyanate-pyridine method. On two-dimensional paper chromatography most of the radioactivity had the R_f values expected for selenomethionine and selenocystine.

Inhibition of yeast growth by selenium (supplied as selenic acid) is annulled by methionine. The action of methionine is specific. Only the L-isomer is active. A casein hydrolysate freed of methionine was inactive (34). Selenomethionine has been identified chromatographically in enzymatic hydrolysates of proteins from *E. coli* grown in the presence of radioactive selenite (154a). Cohen and Cowie (19a) found that selenomethionine would support the growth of a methionine-requiring strain of *E. coli* in a methionine-free medium. Mudd and Cantoni (105a) studied the functioning of selenomethionine in transmethylation reactions. When the analogue was incubated with methionine-activating enzyme

prepared from rabbit liver or yeast, it was utilized as well as or better than methionine. They also showed that Se-adenosyl selenomethionine could serve as a methyl donor.

Thus the evidence suggests that selenium may be metabolized into amino acids where sulphur is normally present and that these analogues may be incorporated into proteins. The toxicity of selenium may well be largely due to such syntheses, although some analogues containing selenium appear to function like the natural compounds.

F. Discussion

There seems to be little doubt that at least some of the analogues noted above are incorporated into peptide linkage in proteins (*e.g.*, ethionine into *Tetrahymena* proteins). There is also good evidence, for some, that proteins from organisms grown in the presence of an analogue may be ineffective (*e.g.*, T2 bacteriophage grown in the presence of 7-azatryptophan). So far there appears to be no example where a well characterized protein has been isolated, shown to contain an analogue in peptide linkage and shown to be biologically ineffective.

For a number of proteins it is now known that substantial parts of the structure can be removed or changed without altering biological activity (for references see 2). It may well be that an incorporated amino acid analogue would only affect function of the protein when replacing the normal amino acid in some critical position or positions. Amino acid analogues which can be incorporated in well characterized proteins may have a part to play in future detailed studies of the active sites of proteins.

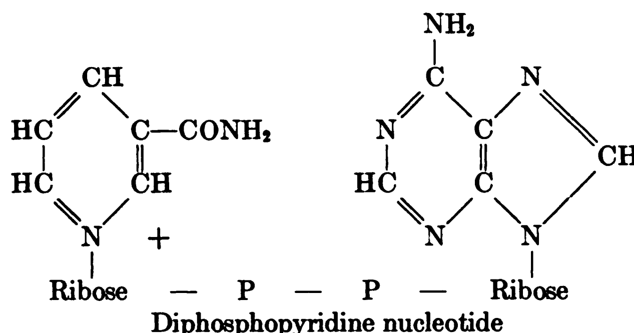
Proteins from normal cells, cancer cells and viruses contain very much the same selection of about twenty amino acids. At the present time there appears to be little reason to hope for the development of an amino acid analogue that would be incorporated specifically into say a virus protein and not the proteins of the host cell. A greater rate of protein synthesis with viruses or tumor cells might lead to differential incorporation, but this kind of difference is not likely to be an adequate basis for useful selective action. Multiplication of T2 bacterial virus is strongly inhibited by 10 μg/ml of 7-azatryptophan, infective centres (infected bacteria yielding virus) being reduced to about 2% of the input value in two hours. Uninfected bacteria could survive and multiply under these conditions (109).

Burton (16a) has shown that the synthesis of T_2 phage DNA is dependent on the formation of new proteins in the first five minutes after infection. Most of the effect of these amino acid analogues in preventing virus synthesis may be on this critical early stage.

IV. ANALOGUES OF VITAMINS, COENZYMES AND RELATED COMPOUNDS

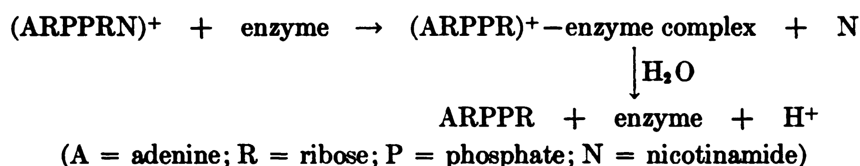
A. Diphosphopyridine nucleotide analogues

Diphosphopyridine nucleotide (DPN, coenzyme I, or cozymase) is a dinucleotide containing adenylic acid and nicotinamide ribonucleotide. The molecule functions as a hydrogen carrier in a wide variety of biochemical reactions. It occurs ubiquitously in plants and animals.

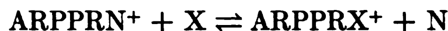


Diphosphopyridine nucleotide

1. Reactions involving diphosphopyridine nucleotidases. Enzymes known as DPNases, which have been found in various animal tissues, catalyze the cleavage of the DPN molecule to give free nicotinamide and adenine-ribose-P-P-ribose. Zatman *et al.* (162) found that a DPNase preparation from beef spleen was inhibited by nicotinamide in a non-competitive but reversible way with respect to DPN. To account for this they suggested a two step mechanism for the reaction as follows:



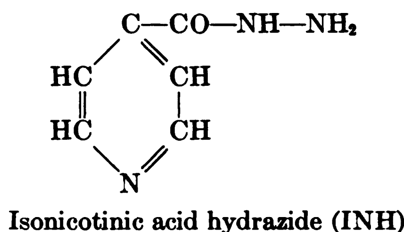
The proposed (ARPPR)⁺-enzyme complex is a covalent linked compound in which the energy of the nicotinamide-ribose bond is conserved. On this hypothesis the inhibition of enzyme by nicotinamide would depend on a competition between the nicotinamide and water for the ARPPR⁺-enzyme complex. The hypothesis predicts that in the presence of inhibiting concentrations of nicotinamide exchange should occur between free nicotinamide and that bound in the DPN molecule. Using C^{14} labeled nicotinamide, Zatman *et al.* (162) showed that such exchange occurred. They suggested that reactions of the following type might also be possible:



$$ARPPRN^{+} + X \rightleftharpoons ARPPRX^{+} + N$$

where X is a molecule structurally similar to nicotinamide and the product is an analogue of DPN.

The first analogue of nicotinamide shown to take part in such a group exchange reaction was isonicotinic acid hydrazide (INH) (163), a compound with antituberculous activity.

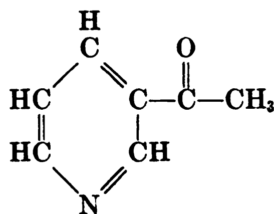


Isonicotinic acid hydrazide (INH)

DPNases from various animals were roughly equally inhibited by nicotinamide, but there were striking species differences in the inhibition by INH (164). For example, crude homogenates of beef spleen or brain were inhibited more than 50% by 7.5×10^{-4} M INH. The corresponding tissues from rat and mouse were little affected by 2.5×10^{-2} M INH. INH is about 10 times more effective than nicotinamide in inhibiting beef spleen DPNase. The INH inhibition is reversible but is influenced by DPN concentration.

Zatman *et al.* (165) describe the isolation and properties of the analogue of DPN in which INH replaces nicotinamide. This analogue (ARPPR·INH) appeared in 75% yield when INH was incubated with pig brain DPNase and DPN. DPN could be regenerated from ARPPR·INH by incubation with pig brain DPNase and nicotinamide. With "sensitive" DPNases the rate of ARPPR·INH formation from DPN and INH is only about 1% of that with "insensitive" sources (43, 165). ARPPR·INH is at least twice as effective as free INH in inhibiting the INH-sensitive DPNases. ARPPR·INH neither was reduced by DPN-requiring enzyme systems (*e.g.*, crystalline yeast alcohol dehydrogenase, rabbit muscle lactic dehydrogenase) nor did it affect the reduction of DPN by such systems.

Kaplan and Ciotti (68) described the preparation of the 3-acetylpyridine analogue of DNP (APDPN) from a reaction mixture containing pig brain DPNase, DPN and 3-acetylpyridine. APDPN was isolated from neoplastic tissues in mice, following injection of the animals with 3-acetylpyridine (71).



3-Acetylpyridine

APDPN has many properties similar to those of DPN. It can be reduced enzymatically or with hydrosulphite, and it forms complexes with reagents such as cyanide, bisulphite and dihydroxyacteone.

These workers also prepared the DPN analogues containing pyridine-3-aldehyde, pyridine, β-picoline and 3-methylpyridyl carbinol. The first of these could be reduced enzymatically. The others could not be reduced either enzymatically or chemically. The activities of DPN, APDPN and the pyridine-3-aldehyde analogue (Py3AL DPN) have been compared in a number of dehydrogenase systems (69). The comparative activities of the three structures varied with the enzyme system tested. For example, with yeast alcohol dehydrogenase APDPN reacts at about 10% the rate of DPN and Py3AL DPN reacts at a slower rate still. With liver glutamic dehydrogenase both the analogues react faster than DPN. Thus the grouping $-C\begin{matrix}\nearrow O \\ \searrow NH_2\end{matrix}$ in the 3-position of the pyridine nucleus is clearly not essential for DPN activity.

In the oxidation of triose phosphate by muscle triosephosphate dehydrogenase Py3AL DPN, INH·DPN and marsalid DPN were effective inhibitors (marsalid-isopropyl derivative of INH). Py3AL DPN appears to have a much greater affinity for the enzyme than DPN. A number of other DPN analogues were inert (70).

6-Aminonicotinamide is a nicotinamide analogue that is toxic for mice. Nicotinamide and nicotinic acid protect mice against the lethal effect. From a reaction mixture containing pig brain DPNase DPN and 6-aminonicotinamide, Johnson and McColl (67) isolated the 6-aminonicotinamide analogue of DPN in good yield. This analogue was also detected in liver and kidney of mice treated with 6-aminonicotinamide, and in rat tumor tissue. This analogue of DPN was found to be inactive in the yeast alcohol dehydrogenase system.

The role of the analogues of DPN containing INH or marsalid in growth inhibitions by these compounds is not clearly established. Other factors may well be involved. For example, it has been suggested that chelate metal complex formation is concerned in the antituberculous activity (129). However, Albert (1) found no correlation between affinity for metals and biological effects for a series of compounds structurally related to INH. It may be that there is some as yet undiscovered factor of major importance in the activity of INH.

2. 5-Fluoronicotinic acid. Hughes (63) examined the effect of a series of halogen-substituted nicotinic acids in three bacterial species. 5-Fluoronicotinic acid was the most effective inhibitor of both growth and DPN synthesis.

The washed suspensions of *Lactobacillus arabinosus* studied by Hughes could use added nicotinic acid, nicotinamide, nicotinamide riboside or nicotinamide ribonucleotide for synthesis of DPN. 5-Fluoronicotinic acid was equally effective in inhibiting DPN synthesis from any of these added precursors. Uptake of added DPN was little affected by the analogue.

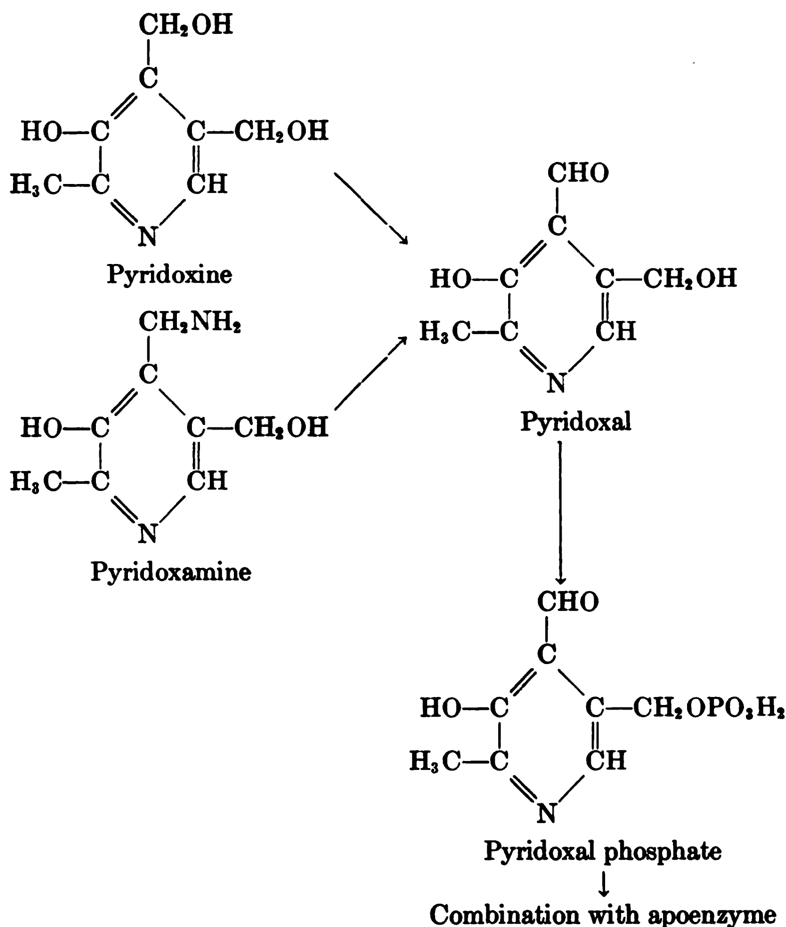
When the inhibitor and the nicotinic acid derivative were added simultaneously the inhibition was of a competitive type. Inhibition was greatest with the lowest concentrations of nicotinic acid derivative and least with the highest concentration of the normal compound. However, the reversal of inhibition was never complete. When the cells were incubated with 5-fluoronicotinic acid before the addition of the normal compound, the magnitude of the inhibition was not affected by the concentration of normal compound, but depended mainly on the time of preincubation with inhibitor. The effectiveness of the inhibition was increased some 100- to 1000-fold by preincubation of the cells with the inhibitor. If cells were washed free of excess inhibitor after preincubation DPN synthesis was found to be still inhibited. For the uptake and retention of 5-fluoronicotinic acid a glycolytic reaction had to be going on at the same time.

Hughes considered that these results could be best explained if the inhibitor is metabolized some way along the same path as nicotinic acid. He isolated a material from *Staph. aureus* which was tentatively identified as 5-fluoronicotinamide but the quantities of material available were inadequate for full identification. 5-Fluoronicotinic acid is taken up and bound by yeast cells and Hughes suggested that this would be a good starting source for the necessary further chemical work.

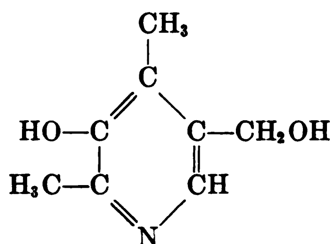
It would be of interest to determine the activities of 5-fluoronicotinic acid and 5-fluoronicotinamide in the enzyme system from human erythrocytes which can synthesize nicotinamide ribonucleotide directly from the free base and 5-phosphoribosyl-1-pyrophosphate (118).

B. Analogues of vitamin B_6

The coenzyme form of the vitamin B_6 group is pyridoxal phosphate, but pyridoxine, pyridoxal and pyridoxamine are effective in the diet and have all been shown to be converted to pyridoxal phosphate. Pyridoxal phosphate is the coenzyme for a number of enzymes, including amino acid decarboxylases and transaminases. Reactions leading to the formation of holoenzyme may be summarized as follows:



4-Desoxypyridoxine is an analogue of pyridoxine which can cause signs of vitamin deficiency when fed to animals.



4-Desoxypyridoxine

Umbreit and Waddell (146) studied the mode of action of this compound on the activity of the enzyme tyrosine decarboxylase from *Streptococcus faecalis*. Desoxypyridoxine might act by (i) inhibiting conversion of pyridoxine and pyridoxamine to pyridoxal, (ii) by inhibiting the formation of pyridoxal phosphate from pyridoxal, (iii) by interfering with the combination of the pyridoxal phosphate with apoenzyme, (iv) by being itself converted to desoxypyridoxal phosphate and competing with the normal compound for apoenzyme, or displacing it from holoenzyme, (v) by interfering with the decarboxylation of tyrosine by holoenzyme, (vi) by a combination of any or all of the above effects.

Umbreit and Waddell did not examine the first possibility noted above. However, they found that desoxypyridoxine had no effect on the combination of pyridoxal phosphate with apoenzyme even when it was allowed prior access to the apoenzyme. Likewise there was no effect of desoxypyridoxine on the formation of pyridoxal phosphate when the supply of ATP was the limiting factor. However, when ATP was in excess and pyridoxal in limiting amount desoxypyridoxine caused some inhibition of enzyme action.

These results suggested that desoxypyridoxine itself might be converted to the phosphate when the pyridoxal level was low, and that the desoxypyridoxal phosphate so formed might be the enzyme inhibitor. This possibility was tested using a synthetic preparation of desoxypyridoxine phosphate. This material had no significant effect on the decarboxylation of tyrosine once the normal coenzyme was attached. However, if desoxypyridoxine phosphate was added before or at the same time as pyridoxal phosphate substantial enzyme inhibition occurred.

These experiments suggest that desoxypyridoxine inhibits tyrosine decarboxylase by being converted to the phosphate which then competes with normal coenzyme for apoenzyme. The normal compound has a greater affinity for the enzyme than the analogue. This proposed mechanism of action helps to explain the results of growth studies. Administration of desoxypyridoxine produces pathological and biochemical symptoms characteristic of vitamin B_6 deficiency, but this occurs only when the B_6 supply is suboptimal (116). With adequate amounts of B_6 even high ratios of desoxypyridoxine had little effect. Presumably it is only when B_6 is deficient that there is opportunity for the phosphorylation of desoxypyridoxine and combination of the phosphate with apoenzymes.

Various apoenzymes using pyridoxal phosphate vary in their affinity for 4-desoxypyridoxine phosphate. For example, under conditions where apotyrosine decarboxylase from *E. coli* is about 35% inhibited and apotransaminase from

pig heart 100% inhibited, apocysteine desulphhydrase (presumably from mouse liver) was not affected (25).

The conversion of tryptophan to indole by tryptophanase requires pyridoxal phosphate as coenzyme. Gooder and Happold (44) studied the effects of a number of compounds structurally related to tryptophan. Their results were consistent with the idea that coenzyme and apoenzyme are linked through the phosphate group of the coenzyme. The substrate would be linked through its amino group to the aldehyde group of the coenzyme, and through its ring nitrogen atom and terminal carboxyl group to the apoenzyme. This mechanism could explain directly the inhibition by 4-dexoxypyridoxine phosphate. The analogue can combine with apoenzyme through the phosphate group but cannot make its contribution to the three point combination with the substrate, since it has an unreactive methyl group in the 4-position.

Hurwitz (64) studied a number of structures related to pyridoxal. Kinase preparations from *Streptococcus faecalis* and yeast which phosphorylated pyridoxal in the presence of ATP also phosphorylated other compounds besides desoxypyridoxine which were unsubstituted in the 6-position and which contained a hydroxymethyl group in position 5 of the pyridine ring. These included two other unnatural analogues, 4,5-dihydroxymethyl-2-methylpyridine and 3-amino-4,5-dihydroxymethyl-2-methylpyridine. The phosphorylated analogues were inhibitory for tyrosine decarboxylase.

Olivard and Snell (107) studied the effect of ω-methylpyridoxal, ω-methylpyridoxamine, and ω-methylpyridoxal phosphate on growth and some enzymic activities of *Streptococcus faecalis*. The relative activation of different apoenzymes by pyridoxal phosphate and ω-methylpyridoxal phosphate varied widely. For example, activation was about the same for both compounds with phenylalanine-glutamic acid transaminase. For cysteine desulphhydrase activation by the analogue was about $\frac{1}{300}$ that for the normal coenzyme.

C. Analogues of vitamin B_{12}

Several natural compounds with vitamin B_{12} activity have been isolated. These differ in the nucleotide they contain. The bases isolated include adenine, 2-methyladenine, and 5,6-dimethylbenzimidazole. The range of bases known that can apparently provide a structure suitable for B_{12} activity has recently been substantially widened. Some of these bases are inhibitors of growth in certain systems.

A strain of *E. coli* (#113-3) which requires vitamin B_{12} can synthesize the vitamin when provided with factor B (the non-nucleotide portion of B_{12}) and an appropriate nucleotide or base (*e.g.*, adenine, 2-methyladenine, or 5,6-dimethylbenzimidazole). Ford *et al.* (35) found that this strain of *E. coli* could synthesize compounds with B_{12} activity for *Ochromonas malhamensis* when supplied with factor B and a range of bases structurally related to adenine or benzimidazole (*e.g.*, 5,6-dichlorobenzimidazole, 5-nitrobenziminazole, 4-chloro-1,2-benztriazole, 2,8-dichloroadenine, 2-methylthioadenine, 2,6-diaminopurine). The new factors produced, which presumably contained these bases in the molecule, varied in their activity for *Ochromonas* compared with the compound containing

5,6-dimethylbenzimidazole; for example, the compound formed with benzimidazole had 36% of this activity; those formed with 2-methylthioadenine and 2,6-diaminopurine were inert.

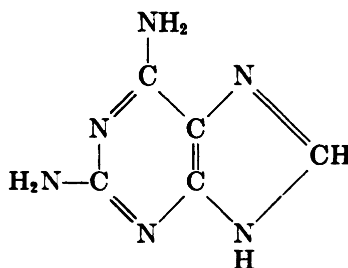
D. A possible analogue of vitamin B_1

2,4-Diamino-5-phenylthiazole (Daptazole or Amiphenazole) has a range of apparently unrelated pharmacological actions. Daptazole shows a structural resemblance to the thiazole part of thiamine. Schulman (134) studied the effects of Daptazole on rats fed on a diet free of vitamin B_1. Low doses of Daptazole (10 mg/kg) given together with the pyrimidine part of thiamine (2-methyl-4-amino-5-brom-methyl-pyrimidine dihydrobromide) (2.5 mg/kg) prevented rats from developing thiamine deficiency symptoms. Larger doses (5 times the above amounts) led to a rapid development of thiamine deficiency symptoms, which could be reversed by the administration of thiamine. The same rapid onset of the avitaminosis was not produced by either drug alone. On the basis of these observations Schulman suggests that Daptazole may be synthesized into more complex compounds which normally contain thiamine. A chemical investigation of this possibility should prove interesting.

E. Nucleosides and nucleotides of growth-inhibitory bases

A wide range of naturally occurring nucleotides have been isolated. These may have important functions in a variety of processes, although definite functions have not yet been established for some of the more recently discovered compounds. A number of analogues of the naturally occurring purines and pyrimidines are now known to be converted to nucleosides and nucleotides either by isolated enzyme systems or *in vivo*. For some analogues there is evidence that toxicity or growth inhibition may be due, at least in part, to these transformations.

1. 2,6-Diaminopurine. For a wide variety of systems 2,6-diaminopurine is a powerful growth inhibitor. This compound does not appear to be incorporated as such into nucleic acids, but it may in some systems act as a precursor of nucleic acid, adenine and guanine (5).



2,6-Diaminopurine

Kornberg and Pricer (76) found an enzyme from yeast which catalyzed the direct phosphorylation of adenosine and of 2,6-diaminopurine riboside (2-amino adenosine) at the 5′-position. Fifteen other nucleosides were not active. When suitably coupled with phosphopyruvate, pyruvate kinase, and myokinase the

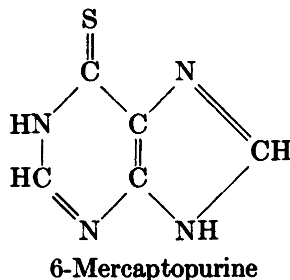
enzyme converts adenosine and 2-amino adenosine to ATP and 2-amino adenosine triphosphate, respectively. Wheeler and Skipper (152) presented evidence suggesting that 2,6-diaminopurine is incorporated into the ATP fraction of mouse tissues.

In a comparative study of purine utilization and growth in strains of *Lactobacillus casei* which were sensitive and resistant to 2,6-diaminopurine, Elion *et al.* (31) obtained results which could be most simply explained by supposing that 2,6-diaminopurine is converted to an inhibitory analogue of some compound normally containing adenine. Direct evidence for such conversion has been obtained in *E. coli* strain B. In this organism 2,6-diaminopurine is methylated to 2-methylamino-6-aminopurine and both these bases are converted to the nucleoside 5′-phosphate and to polyphosphates (127). Addition of adenine to the medium annulled the growth inhibition and prevented incorporation of these bases into the nucleotide fraction. In a mutant of *E. coli* B which is resistant to 2,6-diaminopurine, neither this compound nor 2-methylamino-6-aminopurine was converted to nucleotides. These observations support the idea that growth inhibition by 2,6-diaminopurine may be due to the formation of analogues of cofactors which normally contain adenine.

2. Purine riboside. 9-β-D-Ribofuranosylpurine (Nebularin) occurs naturally in the mushroom *Agaricus nebularis* and is inhibitory for *Mycobacteria*. It is extremely toxic for mice (14). Gordon *et al.* (45, 46) injected rats with 9-β-D-ribofuranosylpurine labeled with C^{14} in the 8-position. From the livers of such animals they were able to isolate by chromatography on Dowex-1 resin three radioactive peaks in the positions expected for the nucleoside 5′-mono-, di- and triphosphates. The identity of the 9-β-D-ribofuranosylpurine-5′-phosphate was established. At least some of the high toxicity of purine riboside may be due to the formation of analogues of the adenosine-5′-phosphates. This idea is supported by the observation that the toxicity of purine riboside in sarcoma 180 tissue culture is most effectively annulled by AMP and ATP (9). The blocking of toxicity of the riboside required much higher concentrations of normal metabolites than that produced by free purine.

In rats purine is relatively non-toxic and only 0.7% of the administered dose was subsequently found in the soluble nucleotide fraction. In contrast, with purine nucleoside, which is highly toxic, the greater part of the dose appeared in the soluble nucleotide fraction (45a).

3. 6-Mercaptopurine. 6-Mercaptopurine has achieved prominence in recent years on account of its ability to inhibit certain types of neoplastic growth.



6-Mercaptopurine

It has been suggested (30) that the analogue may be incorporated into nucleic acids in the mouse, but so far there appears to be no decisive evidence that such incorporation occurs.

Friedkin (37) prepared 6-mercaptopurine deoxyriboside by the action of horse liver purine nucleoside phosphorylase on a mixture of the free base and dicyclohexylammonium deoxyribose-1-phosphate.

Recently an inosinic acid pyrophosphorylase has been prepared from beef liver which catalyses a reaction between 5-phosphoribosylpyrophosphate and 6-mercaptopurine to form 6-mercaptopurine ribotide (84). 6-Mercaptopurine riboside had about the same activity as the free base against adenocarcinoma 755 in mice (137). Lines of *Streptococcus faecalis* and of adenocarcinoma 755 resistant to 6-mercaptopurine were also resistant to the riboside. Such resistant lines of *S. faecalis* did not metabolise hypoxanthine, guanine, or 6-mercaptopurine. Susceptible cells formed 6-mercaptopurine ribotide from the base (12a).

4. 8-Azaguanine. Smith and Matthews (140) identified 8-azaguanosine and 8-azaxanthosine in the soluble fraction from cells of *Bacillus cereus* grown in the presence of 8-azaguanine. Mandel and Markham (88, 88a) have identified 8-azaguanosine-5′-phosphate in similar extracts. Brockman *et al.* (12a), working with resistant and sensitive lines of leukemia L1210, found that the nucleoside and one or more nucleotides of 8-azaguanine were formed in susceptible cells. Resistant cells did not form significant amounts of these compounds. Similarly with strains of *Streptococcus faecalis*, a nucleotide containing 8-azaguanine was produced by a sensitive line but not by an 8-azaguanine-resistant culture. This inability to form 8-azaguanine ribotide was associated with an inability to form ribonucleotides from natural purines such as xanthine and guanine.

5. Nucleotides of other purine analogues. Way and Parkes (148) have synthesised the 5′-nucleotides of a variety of purine analogues using enzyme preparations from liver and yeast which catalyse a reaction between 5-phosphoribosylpyrophosphate and the free bases. These included 8-azaadenine, 8-azaxanthine and the guanine, adenine, and hypoxanthine analogues of the pyrazolo(3,4,d)-pyrimidine series (as well as 8-azaguanine, 6-mercaptopurine and 2,6-diaminopurine).

6. 6-Azathymine. Prusoff (120) gave evidence for the formation of 6-azathymidine from added 6-azathymine in resting cells of *S. faecalis*. The effect of this analogue on viability of the organism was greatest if the analogue was added during the logarithmic phase of growth, and under these conditions there was the most incorporation of the analogue into the acid-soluble fraction (121). The nucleoside is more inhibitory than the base for several bacterial species (123).

7. 6-Azauracil. 6-Azauracil inhibits growth of several experimental tumours (48) and a variety of microorganisms (52). Its action is annulled by uracil.

The riboside of 6-azauracil was much more effective than the free base in inhibiting Sarcoma 180 in tissue culture (130). A conjugated compound of 6-azauracil, probably the riboside, is excreted in the urine of mice receiving 6-azauracil (149).

Handschumacher (51) reported that 6-azauracil ribofuranoside is a major metabolite of 6-azauracil in *S. faecalis*. Another compound was tentatively

identified as 6-azauracil riboside-5-phosphate. The riboside is also produced from the base in *E. coli* (138). Bacterial populations develop resistance to 6-azauracil. The development of such resistance is associated with an inability of the cells to convert the analogue to the riboside (51a). Cells resistant to the base were still inhibited by the riboside.

8. Discussion. It is very likely that at least some of the nucleoside and nucleotide analogues synthesised *in vivo* from the bases will be found to be responsible for the observed toxicity or inhibition of growth. So far, however, there is no conclusive evidence for this with any of the bases. It should be possible to choose suitable *in vitro* systems for carrying out tests when the nucleotide analogues become available in sufficient quantity. For example, it would be of some interest to test guanine analogues in the system described by Keller and Zamecnik (72). This is an enzyme system from rat liver which incorporates amino acids into protein. It has guanosine di- or tri-phosphate as an essential requirement. GTP is apparently involved in the transfer of an activated amino acid to peptide linkage by an as yet unknown mechanism (62).

Several examples are now known where the development of resistance to a purine or pyrimidine analogue, in a line of initially sensitive cells, is associated with a loss of the ability to form a nucleoside or nucleotide containing the analogue. These observations are of substantial interest in the field of experimental cancer chemotherapy and should encourage a more intensive investigation of synthetic nucleoside and nucleotide analogues.

V. ANALOGUES OF ORGANIC ACIDS AND CARBOHYDRATES

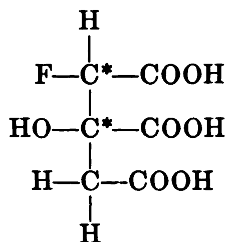
A. *Monofluoroacetic acid*

The mechanism of the toxicity of fluoroacetate and related compounds has recently been reviewed in detail by Peters (113). This section gives a brief summary of the main points.

Monofluoroacetate, the active principle of the poisonous plant, *Dichapetalum cymosum*, is highly toxic for animals, although the LD_{50} varies widely with species. The attack is mainly on either the heart or the central nervous system. In 1949 Lièbecq and Peters (80, 81) put forward the hypothesis that fluoroacetate is metabolised like acetate and enters the tricarboxylic acid cycle, resulting in "jamming" of the cycle accompanied by the accumulation of citrate. A similar hypothesis was proposed independently by Martius (93).

The first *in vivo* evidence in favour of this idea came from the work of Buffa and Peters (15) and Potter and Busch (117). Buffa and Peters found that when rats were injected with sodium fluoroacetate relatively large amounts of citrate accumulated in heart, kidney and brain tissue. Buffa *et al.* (16) incubated fluoroacetate with homogenates of guinea pig and ox kidney cortex (with added fumarate and ATP). From such mixtures they isolated a mixed tricarboxylic acid fraction containing small amounts of combined fluorine but no fluoroacetate. Per unit of fluorine these fractions were more active than fluoroacetate in preventing the disappearance of citrate. Peters *et al.* (114) further purified the inhibitor from fluoroacetate in kidney tissue. They obtained in crystalline form a monofluorotricarboxylic acid which they considered to be monfluorocitric acid.

Fluoroacetate has not been found to have activity against any isolated enzymes *in vitro*. However, fluorocitric acid is a powerful inhibitor of the activities of the enzyme aconitase, which catalyses conversions between the three tricarboxylic acids citrate, *cis*-aconitate and *iso*citrate. The activity of fluorocitric acid as an inhibitor of the enzyme varies with both the source of the acid, and with the state of the enzyme. The introduction of a fluorine atom in citric acid gives two optically active centres with four possible isomers.



Fluorocitric acid

Synthetic fluorocitric acid will be a mixture of the four isomers, which have not yet been separated. The enzymatically formed acid should be a single isomer or a mixture of two. Fluorocitric acid produced enzymically from fluoroacetate inhibits purified aconitase in a competitive manner (112). The effect of enzymatic fluorocitrate on particle preparations from kidney is much greater than on the free enzymes.

The synthetic acid is about half as active as the enzymatic acid when tested against kidney particle preparations. This would be consistent with the idea that only one optically active centre was concerned in the inhibition. However, with soluble aconitase the synthetic acid is much more inhibitory than the enzymatic. The different effect with soluble aconitase and the "particles" is not yet properly understood.

Speyer and Dickman (141) put forward a theory which could explain the different activities of natural and synthetic monofluoroacetic acid against aconitase. They suggest a common intermediate for citric, *cis*-aconitic and *iso*citric acids. They visualise this as an aconitase-Fe^{++}-carbonium ion-cysteine complex. They ascribe differences in activity of their four postulated chelate isomers to the enhanced acidity of the carboxyl group adjacent to the fluorine-substituted methylene group in two of the isomers, and to the influence of fluorine substitution on the formation of the carbonium ion in the other two. They suggest that the latter effect will give rise to more potent inhibition. Further testing of this theory would be greatly facilitated by the separation of the four isomeric fluorocitric acids.

To account for the formation of fluorocitrate the simplest idea would be that fluoroacetate is activated by the same set of enzymes as acetate, and that citric and fluorocitric acid are formed upon the same condensing enzyme. This view is supported by the fact that relatively small amounts of acetate interfere with the formation of fluorocitrate from fluoroacetate by kidney particle preparations (111).

Brady (11) and Marcus and Elliot (91) synthesised fluoroacetyl-coenzyme A and showed that it gave fluorocitrate in the presence of oxaloacetate and condensing enzyme. Thus it seems almost certain tha fluoroacetyl-coenzyme A is on the path of synthesis to fluorocitric acid. However, there are a number of observations discussed by Peters (111, 113) which show that this simple hypothesis is insufficient, and that at some stage in the synthesis of fluorocitric acid there may be activation centres which are independent of the acetate-activating and fatty acid-activating centres.

The relation of citrate accumulation to the observed physiological effects in fluoroacetate poisoning is by no means clear. The effects in nervous tissue do not appear to be due to the sequestration of divalent metal ions by citrate; neither is there any conclusive evidence that the convulsions are initiated by depletion of the energy supply.

If the active fluoroacetyl fragment is built into the other types of molecule besides fluorocitrate, a variety of effects might be possible. In *Dichapetalum toxicarium* a fluoro long chain acid is present and this is presumably built up from a fluoro two carbon compound (113). The fluoroacetyl fragment might be built into a fluorosterol, fluoroacetylcholine, or fluoroaminoacids. These possibilities have not yet been thoroughly investigated.

B. 2-Deoxyglucose

2-Deoxyglucose, a compound closely related to glucose, inhibits glucose fermentation in yeast and in various normal and tumour tissues. Wick *et al.* (153) showed that the extrahepatic tissues of the rabbit oxidised only trace amounts of C^{14} labelled 2-deoxyglucose. The compound did not affect oxidation of injected acetate, suggesting that the inhibition occurs in the glycolytic process. In *in vitro* tests using rat kidney phosphoglucoisomerase 2-deoxyglucose-6-PO_4 competitively inhibited formation of ketose from glucose-6-PO_4. They conclude that the primary block in the breakdown of glucose in extrahepatic tissues may be due to the competitive inhibition by 2-deoxyglucose-6-PO_4 of the conversion of glucose-6-PO_4 to ketose. This hypothesis would be strengthened by the isolation of the phosphorylated analogue from the tissues.

Purified glycogen from the carcasses of animals injected with C^{14} 2-deoxyglucose contained about 5% of the radioactivity (153). The form in which this C^{14} occurs requires further investigation but it may be that this glucose analogue is synthesised into glycogen.

VI. CHOLINE ANALOGUES

Choline is a relatively simple molecule with several important functions. (a) It serves as a source of methyl groups in transmethylation reactions. (b) It is part of the structure of the phospholipid lecithin. Phospholipids may have a number of important functions in the absorption, storage, transport and metabolism of fatty acids. Choline is known to be a lipotropic factor, *i.e.*, its administration restricts the accumulation of fat in the liver. (c) Choline, $(CH_3)_3 \equiv N^+—CH_2CH_2OH$, is part of acetylcholine, a molecule vital in nerve function.

The triethyl analogue of choline inhibits the growth of rats, and the inhibition can be alleviated by choline or less effectively by methionine (144). The triethyl analogue has lipotropic activity (19) but possesses no labile methyl groups (104). The analogue can be used intact in the synthesis of new phospholipid molecules when fed to rats (99). Keston and Wortis (73) present evidence suggesting that the triethyl analogue interferes with acetylcholine formation in the mouse. This effect was abolished by choline.

The methyl groups of choline may be derived from methionine. Ethionine inhibits growth of rats, and either choline or methionine alleviates the inhibition (142).

Stekol and Weiss (143) fed rats with ethionine labelled with C^{14} in the methylene carbon of the ethyl group. From the tissues they isolated radioactive choline. This radioactivity was confined to the trimethylamine part of the molecule. They found that cysteine was formed from the sulphur of S^{35} labelled ethionine. This suggests the formation of homocysteine from the ethionine. If this occurs the ethionine must be deethylated. Thus the evidence strongly indicates that the ethyl analogue of choline was synthesised *in vivo* following the administration of ethionine. However, further chemical evidence will be needed to prove the presence of the ethyl group in the choline molecule and to determine whether the mono-, di-, or triethyl analogue or a mixture of these is formed.

If we assume that the triethyl analogue of choline is formed from ethionine it provides an interesting example of a transformation where the analogue synthesised can replace the normal compound in one function (lipotropic activity) but not in others (transmethylation and acetylcholine formation). The growth inhibition by ethionine may be even more complex. The analogue may be incorporated into proteins (p. 380) and it is quite possible that ethyl analogues of other important compounds which normally contain methyl groups may be formed.

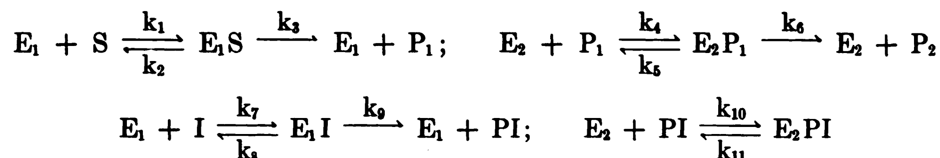
Arsenocholine, in which the nitrogen of choline is replaced by arsenic, possesses no labile methyl groups, but shows lipotropic activity. When rats were fed arsenocholine chloride, arsenic appeared in the lecithin. The arsenic was probably present as the analogue of choline (150). Rats fed on a diet containing 1% of the analogue showed no toxic symptoms after one week. The tripropyl analogue of choline is much more toxic than the triethyl compound (18). These analogues could well be reinvestigated using the more sensitive analytical methods now available.

VII. INCORPORATION OF ANALOGUES IN RELATION TO COMPETITIVE INHIBITION

A theory has been developed by which the results of growth studies with antimetabolites can be used to indicate the mode of action of the inhibitor (53, 132, 133, 156). To apply this theory, growth is measured in the presence of a range of concentrations of analogue and normal compound. In a simple case, if the ratio of concentration of normal substrate to concentration of analogue for a given degree of inhibition is constant, it is inferred that the analogue inhibits

growth by competing with normal compound for sites on the enzyme using the normal metabolite.

From the variety of examples now known where the added inhibitor is metabolised along the same path as the normal compound, it would appear that growth studies alone may be inadequate to define the site at which a compound inhibits. Consider the simplest situation in which the added inhibitor is metabolised one stage along the same path as the normal compound to form the actual competitive inhibitor:



$$E_1 + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_1S \xrightarrow{k_3} E_1 + P_1; \qquad E_2 + P_1 \underset{k_5}{\overset{k_4}{\rightleftharpoons}} E_2P_1 \xrightarrow{k_6} E_2 + P_2$$

$$E_1 + I \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E_1I \xrightarrow{k_9} E_1 + PI; \qquad E_2 + PI \underset{k_{11}}{\overset{k_{10}}{\rightleftharpoons}} E_2PI$$

Where E_1 = enzyme metabolising normal compound; E_2 = enzyme metabolising the product of E_1; S = normal compound; and P_1 and P_2 are successive products of S; I = added inhibitor; PI = real inhibitor (an analogue of P_1).

If we assume that PI acts competitively (*i.e.*, $k_{11} \neq 0$) then this situation cannot be distinguished from the simple one where I is a competitive inhibitor of S.

Quesnel (124) has put forward a theory of competitive antagonism which he suggests can distinguish between the situation where the metabolite analogue inhibits in the form supplied and where the analogue is metabolised along the same path as the normal compound to form the real inhibitor. This treatment is inadequate in several respects.

To apply his theory, Quesnel has to assume that the inhibition by the real inhibitor (produced by the metabolism of the added compound) acts non-competitively. The growth rate will then be dependent only on the proportion of the second enzyme occupied by inhibitor (E_2PI in the notation outlined above).

If all the second enzyme E_2 is not occupied by P_1 and PI, then P_1 and therefore S should influence growth rate. If E_2 is fully occupied by P_1 and PI then it can be shown that Quesnel's equations reduce to the more limited case treated by Shive and Macow (133). Further, it is not possible to determine whether this non-competitive inhibition is occurring at the first or at a later enzyme step. The essential distinction in fact made by Quesnel in attempting to apply his theory is between competitive and non-competitive inhibition rather than, as he considers, between inhibition by the added compound and by its metabolic product.

On Quesnel's treatment, an added analogue that is metabolised to the true inhibitor is stated to give a plot of S/I against 1/I with a negative slope. Inhibitors that act directly in the form supplied give a plot with a positive slope. In the process of obtaining the numerical values for his figures from published experimental results, Quesnel uses a plot of I against S. The question as to whether the slope of his curves is positive or negative depends on whether the plot of I against S intercepts the inhibitor or substrate axis. The intercept may

occur close to the origin, and whether the inhibitor or substrate axis is actually intercepted may well be decided by experimental error (*e.g.*, in the data from Hitchings *et al.* (60) for inhibition of *L. casei* by 5-bromouracil. This point was treated by Quesnel as a negligible correction factor.

Thus the theory of competitive antagonism developed so far does not distinguish between analogues which inhibit in the form supplied and those that are metabolised along the same path as the normal compound to form inhibitory or non-functional products.

A lag of 1 to 2 generation times in the onset of inhibition of bacterial growth is a common feature of many analogues that are incorporated into proteins (7-azatryptophan and tryptazan) and into nucleic acids (8-azaguanine into RNA; 5-halogenated uracils into DNA). There may also be a delay in the relief of an inhibition by added normal metabolite. Indeed, it was such a lag following the addition of guanylic acid that first led Kidder and Dewey (74) to suggest that 8-azaguanine might be inhibitory to the growth of *Tetrahymena* through being metabolised in the same way as guanine. Delay in the onset of inhibition may turn out to be a useful feature in some systems for indicating that the inhibitor is being incorporated into larger molecules. However, the absence of such a delay would not rule out incorporation into more complex molecules. For example, there is no delay in the onset of bacterial inhibitions produced by *p*-fluorophenylalanine or by 5-chlorouracil. Such analogues may inhibit growth by some other mechanism as well as by incorporation into large molecules.

The possible complexities of growth inhibition by incorporated analogues are well illustrated by data on inhibition of *E. coli* by 5-bromouracil (26) (Fig. 1). In such a situation measurement either of initial rates or of amount of growth at any particular time after addition of inhibitor could be quite misleading. In fact it is rather unlikely that any theoretical treatment of data on growth inhibition could distinguish the variety of effects possible with an antimetabolite that is incorporated *in vivo* into more complex molecules.

VIII. SUMMARY

In the few years that chromatographic and isotope techniques have been used in the study of antimetabolite action they have demonstrated the inadequacy of earlier interpretations of the way in which such compounds inhibit growth. It is now clear that a wide variety of metabolite analogues do not simply inhibit biosynthetic enzymes directly but instead are converted into more complex compounds. This finding suggests new approaches to the problem of selective growth inhibition. In addition, there are new possibilities for studying the relationship between chemical structure and biological function. Perhaps outstanding here is the mutagenic effect of the incorporation of 5-halogenated uracils into DNA. Further studies of this phenomenon may lead to a more detailed knowledge of the mutation process.

The following sections give a summary of present knowledge on the structural changes and biochemical effects of incorporated analogues.

1. Structural changes in incorporated analogues. Table 3 presents a summary of

TABLE 3

Structural changes in incorporated analogues of normal metabolites

Normal metabolite	Analogue	Structural change	Analogue known or assumed to be formed	System
Guanine	8-Azaguanine	— N = for — CH =	Unnatural RNA	Bacteria, viruses, plants, mammalian tissues
Uracil	6-Azauracil	— N = for — CH =	Unnatural RNA, nucleosides and nucleotides	Bacteria, mice
Thymine	6-Azathymine	— N = for — CH =	Unnatural DNA, nucleosides and nucleotides	Bacteria
Tryptophan	7-Azatryptophan	— N = for — CH =	Unnatural proteins	Bacteria, bacterial viruses
	Tryptazan			
Thymine	5-Chlorouracil	— Cl for — CH_3		
	5-Bromouracil	— Br for — CH_3	Unnatural DNA	Bacteria, bacterial viruses
	5-Iodouracil	— I for — CH_3		
Uracil	5-Chlorouracil	— Cl for — H	Unnatural RNA	Bacteria
Uracil	5-Fluorouracil	— F for — H	Unnatural RNA	Mouse tissues
Acetate	Fluoroacetate	— F for — H	Fluorocitrate	Animal tissues
Nicotinic acid	5-Fluoronicotinic acid	— F for — H	DPN analogue	Bacteria
Phenylalanine	*p*-Fluorophenylalanine	— F for H	Unnatural proteins	Bacteria
5,6-Dimethyl-benziminazole	5,6-Dichlorobenziminazole	two — Cl for two — CH_3	Analogue of vitamin B_{12}	Bacteria
Adenine	2,8-Dichloroadenine	two — Cl for two — H	Analogue of vitamin B_{12}	Bacteria
Uracil	2-Thiouracil	= S for = 0	Unnatural RNA	Viruses, bacteria
Adenine	6-Mercaptopurine	= S for — NH_2	6-Mercaptopurine ribotide	Beef liver enzymes
Pyridoxal	4-Deoxypyridoxine	— CH_3 for — CHO	4-Deoxypyridoxine phosphate	Bacteria
Glucose	2-Deoxyglucose	— H for — OH	2-Deoxyglucose-6-phosphate	Rat tissues
Adenine	Purine	— H for — NH_2	Purine riboside phosphates	Mice
Adenine	2,6-Diaminopurine	— NH_2 for — H	2,6-Diaminopurine riboside phosphates	Mice, bacteria

Methionine	Ethionine	CH_3CH_2 for CH_3	Unnatural proteins; triethylcholine	Animal tissues, protozoa
Choline	Triethylcholine	$(CH_3CH_2)_3$ for $(CH_3)_3$	Analogue of lecithin	Rats
Choline	Arsenocholine	As for N	Analogue of lecithin	Rats
Cysteine	Selenocysteine	$=$ Se for $=$ S	Unnatural proteins	Animals and plants
Methionine	Selenomethionine	$=$ Se for $=$ S	Unnatural proteins	Animals and plants
Nicotinamide	Isonicotinic acid hydrazide	$-CONHNH_2$ in *p*-position for $-CONH_2$ in *m* position	Analogue of DPN	Enzymes from pig brain
Nicotinamide	3-Acetyl pyridine	$-COCH_3$ for $-CONH_2$	Analogue of DPN	Enzymes from pig brain
Nicotinamide	Pyridine-3-aldehyde	$-CHO$ for $-CONH_2$	Analogue of DPN	Enzymes from pig brain
Nitocinamide	6-Aminonicotinamide	$-NH_2$ for $-H$	Analogue of DPN	Enzymes from pig brain, *in vivo* in mice

the structural changes present in some metabolite analogues which are known or assumed to be incorporated by biological systems into more complex molecules. Such analogues have resulted most frequently from replacement of a hydrogen or a methyl group by a halogen atom, or from substitution of a nitrogen for a carbon in a heterocyclic ring.

In 1952 Woolley (156) listed the analogues which had been found to antagonise normal metabolites. The changes noted in Table 3 form a fairly representative selection from those given by Woolley.

2. Biochemical effects of incorporated analogues. Perhaps the most useful feature of this review is to focus attention on the diversity of effects that a structural analogue of a normal metabolite may have. We conclude with a summary of the possible biochemical effects of incorporated analogues. This summary includes some examples where the suggested biosynthesis is not fully proven, and some where the transformation has so far been demonstrated only with enzyme systems *in vitro*.

a. Competitive inhibition of an enzyme reaction, the analogue acting in the form supplied to the organism (*e.g.* inhibition of xanthine oxidase by 8-azaguanine).

b. Synthesis through one or more enzymic steps along the same path as the normal compound, or a similar path, to form a competitive inhibitor of an enzyme metabolising a product of the normal compound (*e.g.*, the conversion of fluoroacetate to fluorocitrate).

c. Synthesis into an analogue of a coenzyme or vitamin molecule. The "holoenzyme" formed by combination of the analogue with apoenzyme may function as well as or better than the normal holoenzyme (*e.g.*, the acetylpyridine analogue of DPN with liver glutamic dehydrogenase), or may function less efficiently (*e.g.*, the acetylpyridine analogue of DPN with yeast alcohol dehydrogenase), or may be ineffective (*e.g.*, desoxypyridoxal phosphate with tyrosine decarboxylase).

d. Synthesis into an analogue of a molecule with several diverse activities, the analogue being able to replace the normal compound in some activities but not in others (*e.g.*, the synthesis of the ethyl groups of ethionine into triethylcholine).

e. Incorporation into proteins which may either function normally (*e.g.*, serine deaminase in *E. coli* containing 7-azatryptophan) or be ineffective (*e.g.*, protein of T2 bacteriophage containing 7-azatryptophan).

f. Incorporation into nucleic acids. Such nucleic acids may: (i) be incapable of initiating either their own reduplication or the production of functional proteins (*e.g.*, TMV nucleic acid containing 8-azaguanine; inhibition of adaptive enzyme formation in *Staph. aureus* by 8-azaguanine), (ii) be "unstabilized" leading to an increase in the production of mutants (*e.g.*, DNA of T_2 virus containing 5-bromouracil), (iii) function apparently normally (*e.g.*, DNA of T2 virus containing 5-bromouracil; DNA of *L. casei* and *E. coli* containing 5-bromouracil, at least under some conditions).

g. Incorporation into storage or structural molecules where they would pre-

sumably be inert until they re-enter general metabolism (*e.g.*, 2-deoxyglucose-6-phosphate into glycogen).

The study of the incorporation of metabolite analogues is a rapidly developing field. At the 1950 meeting of the New York Academy of Sciences on Antimetabolites (102a) the subject was barely mentioned. Taking the four two-year periods since then, the number of papers referred to in this review is as follows: *1950–51*, 13; *1952–53*, 23; *1954–55*, 39; *1956–57*, 82. From these figures it may not be unreasonable to expect well over 100 papers relevant to the subject in the next two years.

A claim that a metabolite analogue causes inhibition by being incorporated *in vivo* into a larger molecule is received with skepticism unless suitable chemical and biochemical evidence is forthcoming. On the other hand, there are numerous papers dealing with antimetabolites in which it is suggested, frequently on the basis of growth data only, that the analogue acts in the form supplied to the organism. There may be a change of emphasis in the near future so that such a claim will need to be supported by direct evidence about the fate of the analogue *in vivo*.

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