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MICROBIAL RIBOFLAVIN BIOSYNTHESIS AND THE ISOLATION OF A NEW GUANINE DERIVATIVE

by

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A dissertation submitted in partial fulfillment
of the requirements for the Degree of
Doctor of Philosophy in The
University of Michigan
1959

Doctoral Committee:

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Associate Professor Saul Roseman
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"Will you tell me, please, which way I ought to go from here?" asked Alice.
"That depends a good deal on where you want to get to." said the cat.

(Alice's Adventures in Wonderland)

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ABBREVIATIONS

DPN	Diphosphopyridine nucleotide
DPNH	Reduced diphosphopyridine nucleotide
TPN	Triphosphopyridine nucleotide
ATP	Adenosine triphosphate
GMP	Guanosine-5'-phosphate
FMN	Flavin monomucleotide
FAD	Flavin dinucleotide
Compound G	6,7-dimethyl-8--ribityllumazine
Compound V	6-Methyl-7-hydroxyribolumazine
PGA	Phosphoglyceric acid
PRPP	5-Phosphoribosylpyrophosphate
AMP	Adenosine-5'-phosphate

INTRODUCTION

While the conversion of vitamins to cofactors and the role of these cofactors in specific enzyme reactions is well documented, our knowledge of the pathways of the biosynthesis of many of the vitamins is limited. One of the reasons for this dearth of study is that the vitamins generally are formed in minute quantities, and the levels of the enzyme concerned reflect the minor nature of these pathways. This does not mean that these routes of synthesis are unimportant. On the contrary, because of the role of cofactors in enzymatic reactions, these pathways assume considerable significance.

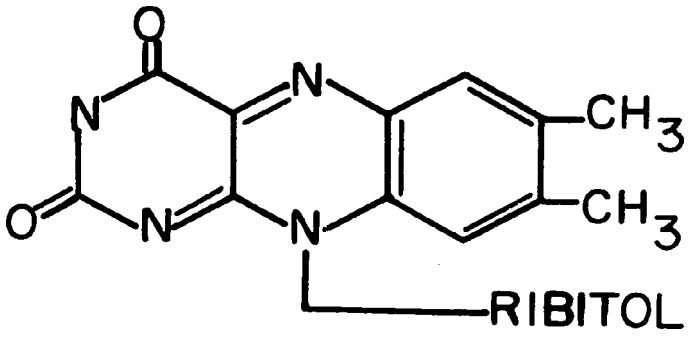
Because of the unique structures of most vitamins it might be expected that their biosynthesis would involve novel biochemical mechanisms not found in the metabolism of the major foodstuffs. Certainly the classification of a compound as a vitamin depends on the inability of a particular organism to carry out one or more of the unique reactions leading to the synthesis of the compound.

In occasional organisms, the pathway of synthesis of certain vitamins has been shown to be much faster than the normal requirements of the organism, and the vitamin accumulates. Thus a number of microorganisms show an exaggerated pathway of riboflavin biosynthesis (1-4). Prominent among

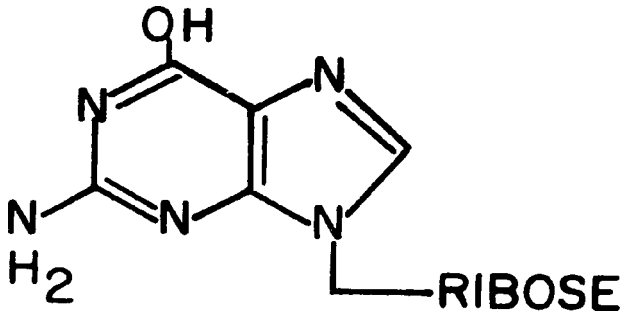
these are the closely related molds, Eremothecium ashbyii and Ashbya gossypii. In this instance since these organisms have been used for the commercial production of riboflavin, the nutritional and environmental requirements for optimum synthesis of the vitamin have been extensively studied (5-10). For these reasons, E. ashbyii was the organism chosen in the present study.

The first suggestion for the route of biosynthesis of riboflavin came in 1951 from Woolley (11) who, arguing on structural grounds, suggested that 1,2-diamino-4,5-dimethylbenzene (Figure 1) might be a common intermediate in the biosynthesis of riboflavin and vitamin B_{12}. To investigate this possibility he synthesized 1,2-diamino-4,5-dichlorobenzene and tested this analog for its inhibitory effect on the growth of 26 species of microorganisms drawn from widely divergent classes. The dichloro compound inhibited the growth of those organisms which had no nutritional requirement for riboflavin or vitamin B_{12} but was without effect on those which required both vitamins. Diaminodimethylbenzene or 1,2-diaminobenzene competitively overcame the inhibition. On the other hand, riboflavin and B_{12} had little effect. Furthermore, 1,2-dimethyl-4,5-diaminobenzene met the requirements for riboflavin in some organisms though not in a very impressive way.

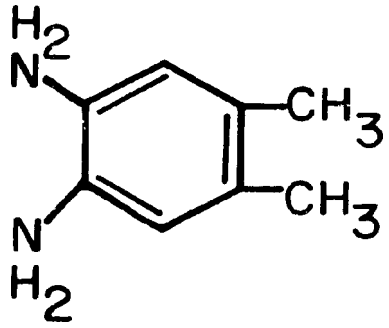
Later Woolley and Pringle (12) reported the synthesis and study of the inhibitory activity of 35 compounds related to 1,2-diamino-4,5-dimethylbenzene. Some of these



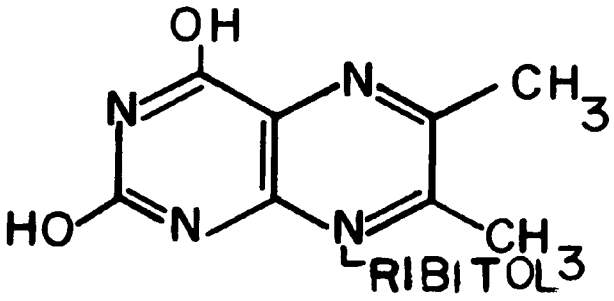
RIBOFLAVIN



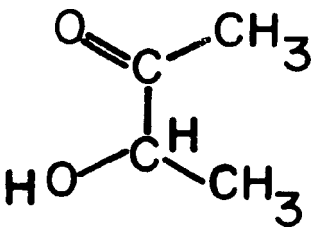
GUANOSINE



1,2 DIMETHYL-3,4-DIAMINO BENZENE



COMPOUND G



ACETOIN

FIG. 1 The Structure of Certain Compounds of Possible Interest in Riboflavin Synthesis.

compounds did not show inhibition of growth yet decreased the synthesis of vitamin B_{12} in B. megatherium. This idea has been confirmed by Foster and Pittillo (13) who found that 1,2-diamino-4,5-dichlorobenzene preferentially inhibited riboflavin production over growth in B. subtilis.

More recently Plaut has taken another line of approach, namely a study of the ultimate precursors of riboflavin (14,15). In growing cultures of A. gossypii, the riboflavin pyrimidino ring is labeled by the same precursors which bring about the labeling of the pyrimidino portion of purines. In contrast radioactive acetate entered into the synthesis of the aromatic ring of riboflavin.

The pattern of labeling in the ribitol moiety was such that it could have been formed from a pool of pentose which was synthesized equally by the phosphogluconic acid pathway and by the transketolase-transaldolase reactions (16). However, the route of synthesis of pentose in this organism has never been studied.

Convincing evidence for the direct contribution of purines to the synthesis of riboflavin came from the work of McNutt using cultures of E. ashbyii (17,18). With uniformly labeled C^{14} -adenine in the culture medium, 94% of the activity in the riboflavin appeared in the pyrimidino ring, distributed equally between the four carbon atoms. On the other hand starting with 8-C^{14} adenine only a trace of the activity entered riboflavin.

While the studies of Plaut and McNutt implicated purine derivatives as intermediates in riboflavin biosynthesis, they did not rule out the possibility that purines entered as an alternate route somewhere along the true pathway. Evidence that a purine derivative was indeed on the direct pathway was provided by Goodwin et al. (19). They found that a small pool of adenine added to the culture decreased the incorporation of 2-C^{14}-serine into riboflavin. This would not be expected if the serine label did not pass obligatorily through a purine intermediate.

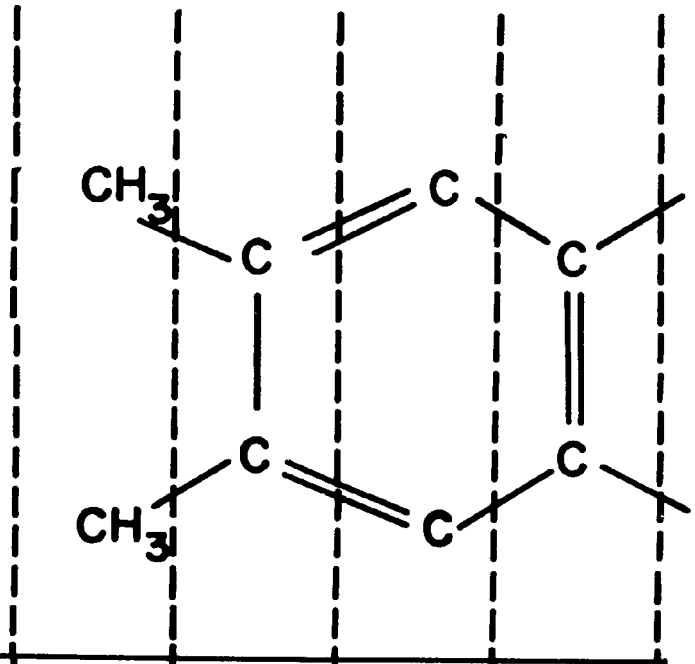
The first indication of an intermediate compound between purines and riboflavin came in 1956 when Masuda (20) reported the isolation of 6,7-dimethyl-8-ribityllumazine (compound G) in cultures of E. ashbyii (see Figure 1). Maley and Plaut have found compound G in A. gossypii and have confirmed its structure. In addition these workers demonstrated the incorporation of C^{14}-formate into compound G (21). This compound is of special interest in view of its structural similarity to riboflavin. In fact riboflavin can be formed by the chemical condensation of dimethylribityllumazine with acetoin (20). Recently Kawada et al. (22) reported the conversion of compound G to riboflavin in dialyzed extracts of E. ashbyii. The source of the 4 extra carbons is still unknown. The same conversion also is reported to occur in extracts of E. coli (23) and A. gossypii (24).

On the basis of chemical experiments, and on the basis of finding large amounts of acetoin in cultures of E. ashbyii, Masuda suggested that the aromatic ring of riboflavin is derived biologically from acetoin or diacetyl-like fragments (20). In confirmation of this idea, Goodwin et al. found that labeled acetoin was incorporated into the aromatic ring of riboflavin more effectively than labeled acetate 2-C^{14} (25).

While at first it might be reasonable to accept acetoin and compound G as precursors of riboflavin, certain observations are difficult to reconcile with this idea.

Thus an extensive study of the metabolism of compound G in 5 riboflavin-producing organisms and with extracts made from them in various ways (26) showed that while C^{14}-labeled compound G was oxidized with whole cell suspensions of some of the organisms, in no case was any radioactivity detected in riboflavin.

The studies of Plaut are not in accord with acetoin being an intermediate. Acetate-1-C^{14} and acetate-2-C^{14} were incorporated into the xylene ring of riboflavin according to Figure 2, as the only known mechanisms of synthesis of acetoin are from acetaldehyde or pyruvate; if acetoin were an obligatory intermediate in the synthesis of the aromatic ring of riboflavin, then one would expect that carboxyl-labeled acetate would not label the ring because the conversion of acetate to acetoin through pyruvate or acetaldehyde, and via the Krebs cycle, would involve the loss of



$CH_3\overset{+}{C}OOH$	8	77	8	73
$\overset{+}{C}H_3COOH$	71	36	63	33

FIG. 2

The Incorporation of C^{14} Acetate into the Xylene Ring of Riboflavin after Plaut (15).
The figures refer to counts per minute per micromole X 10^{-3}.

the carboxyl group of the acetate as carbon dioxide. (See Figure 3).

Acetate-2-C^{14} carried through this same pathway should lead to equal labeling of all 4 carbons of acetoin and if acetoin were a precursor of riboflavin, in turn, the ring should be labeled equally; the data of Plaut can be explained only by assuming that acetoin is not an intermediate or that a direct reduction of acetate to acetaldehyde takes place in this organism. At present the evidence for the occurrence of such a reduction in yeast or higher organisms is weak (27).

In disagreement with Plaut's results Klungsøyr (28) showed that in standing cultures acetate-1-C^{14} did not enter the aromatic ring of riboflavin.

On structural grounds and on the basis of the labeling data of Plaut (16) one assumes that the ribitol in riboflavin is derived from ribose in a nucleotide or nucleoside. However, in one study with E. ashbyii, uniformly labeled guanosine appeared to lose its pentose during conversion of the purine moiety to riboflavin (29). However, this result does not exclude the possibility that ribitol in riboflavin is derived from ribose in a nucleotide. The experiment did not exclude the possibility that the isotope in the pentose portion of the guanosine was diluted by endogenous carbohydrate. The involvement of a nucleotide or nucleotide-like compound rather than the free base in riboflavin biosynthesis is suggested by some experiments of Kawada et al.

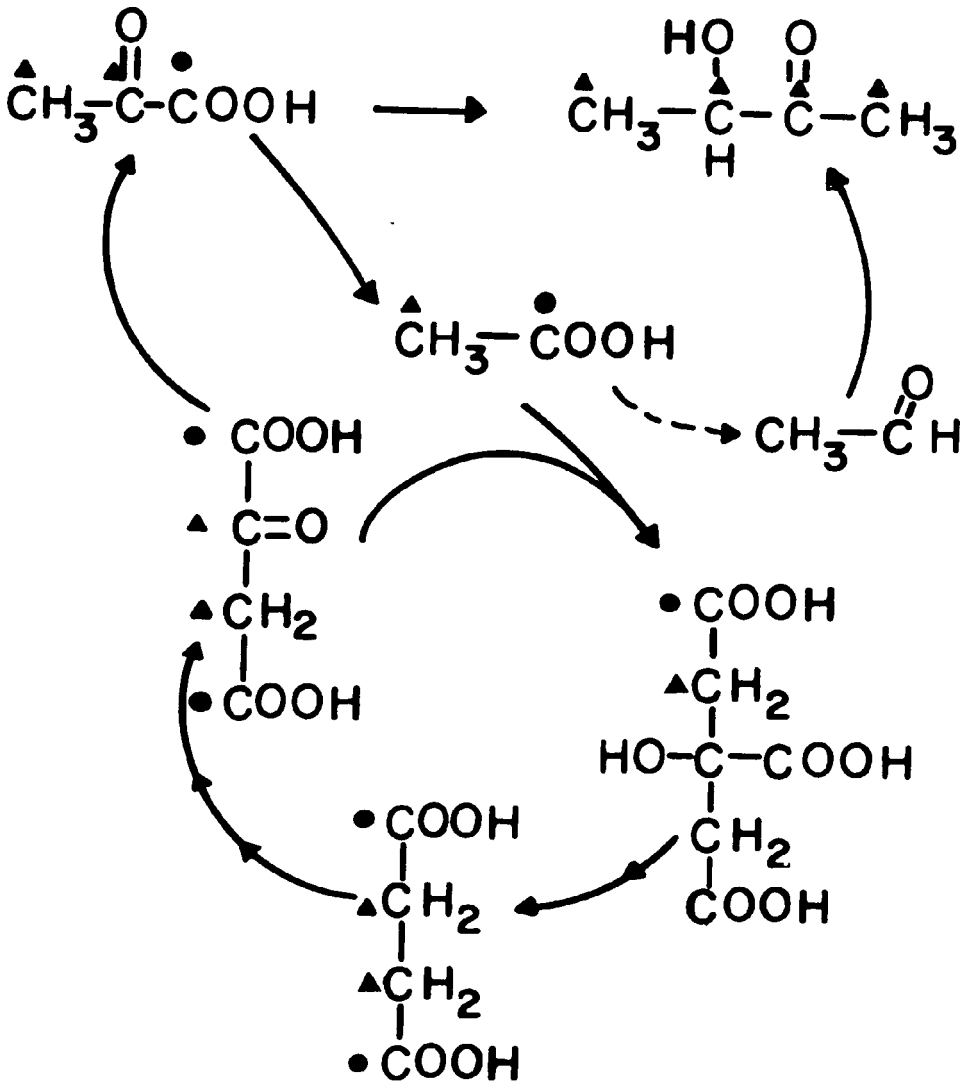


FIG. 3

The Theoretical Incorporation of Acetate into Acetoin.

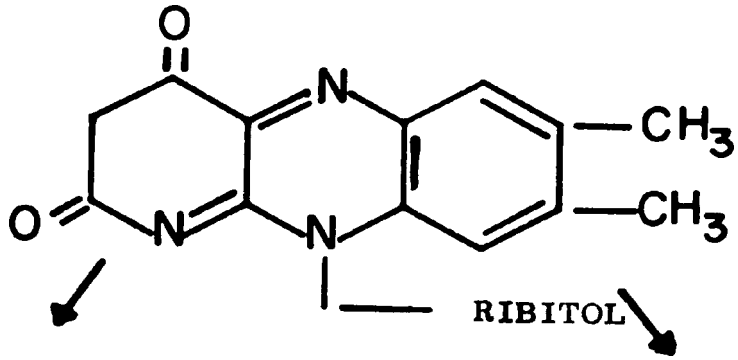
Note that barring the direct reduction of acetate to acetaldehyde (dotted arrow), the carboxyl of acetate is not incorporated into acetoin - while the methyl group is incorporated equally into the 4 carbons of acetoin.

with model substrates (30). While diamino-uracil inhibited the synthesis of riboflavin in E. ashbyii, N-4-methyldiamino-uracil was converted to 6,7,8-trimethyllumazine.

Various other pteridines which might be of importance in riboflavin biosynthesis have been reported. Masuda (31) and Forest and McNutt (32) report the isolation of a violet fluorescent pteridine identified as 6-methyl-7-hydroxyribo-lumazine from cultures of E. ashbyii. It is unlikely that this pteridine is a riboflavin precursor on structural grounds. It seems to be derived from the condensation of pyruvate with a diaminopyrimidine derivative.

Two pteridines, the crithidia factor (33) and a crystal-line unidentified pteridine from grasshopper eggs (34), have been reported to spare the requirements of riboflavin in some organisms. The significance of these pteridines in the biosynthetic pathway of riboflavin is still obscure.

The most significant developments in riboflavin bio-synthesis are summarized in Figure 4.



The Pyrimidine Ring

1948	Purines found to stimulate riboflavin synthesis
1954	Plaut finds glycine CO_2 and formate label ring
1955	McNutt finds purine rings incorporated as unit
1956	Masuda finds compound G
1958	Compound G is incorporated into riboflavin

The Aromatic Ring

1951	Woolley suggests 1,2-dimethyl 3,4-diamino benzene
1954	Plaut studies incorporation of acetate
1956	Masuda suggests diacetyl as intermediate
1958	Goodwin finds C^{14} diacetyl incorporated

The Ribitol

1955	Labeling from glucose similar to a pentose
1958	McNutt finds guanosine ribose not incorporated

FIG. 4 The most significant developments in the biosynthesis of riboflavin.

SCOPE OF INVESTIGATION

In this investigation we have tried to find ways of studying the detailed pathway of the biosynthesis of riboflavin. Although the overall picture of the conversion of purines into riboflavin has been well documented, the intermediate steps in the conversion are still very obscure. With the exception of compound G, no other intermediate has been isolated in this obviously complex series of reactions.

We approached this problem by attempting to bring about the accumulation of intermediates in the biosynthesis of riboflavin. A knowledge of such intermediates might have some practical applications in the designing of antibiotics, as a large majority of microorganisms synthesize riboflavin, whereas mammals require the vitamin preformed.

The incorporation of purines into riboflavin can be postulated to require: (1) the opening of the imidazole ring, (2) the loss of the C-8, (3) the reduction of the ribose of a nucleotide or its replacement by a ribitol grouping, and (4) the introduction of the xylene ring either by building it on the open ring compound from "acetate" or diacetyl units or by adding it as a preformed unit. The order of these hypothetical reactions is unknown, the replacement of ribose by ribitol presumably might take place at any point in the sequence.

Our preliminary experiments with E. ashbyii suggested that the conversion of purines to riboflavin occurred at the oxidation level of guanine or xanthine rather than that of adenine or hypoxanthine.

To detect intermediates which may accumulate normally, we studied the acid-soluble fraction of whole cells grown in the presence of radioactive guanine. When no such intermediates were detected we attempted to bring about their accumulation by the use of certain inhibitors of riboflavin synthesis, chosen because of their apparent preferential inhibition on riboflavin synthesis over the growth of the organisms. This investigation led to negative results but may provide insight into these multiple enzyme systems.

When the above experiments failed to yield the desired results, we studied a cell free system to determine whether it was capable of carrying out the conversion of guanine into riboflavin. If such an overall reaction could be demonstrated the system could be fractionated by conventional methods. However, only very minor overall incorporation was obtained. Instead guanine was converted to a new guanine derivative (compound X). This compound has been isolated, and studied. It remains to be determined whether this guanine derivative is an intermediate in riboflavin synthesis.

MATERIALS AND METHODS

I. Biological Material:

Two strains of E. ashbyii were used. (1) A strain obtained from Professor Baddiley's laboratory in Newcastle, England was used for the inhibitor studies. This strain originally had been obtained from a commercial house engaged in riboflavin synthesis. (2) A strain provided by Northern Regional Research Laboratories (#Y1363) was used for most of the extract experiments. This strain had the advantage of making much more riboflavin. Dr. D. H. Hayes has studied the growth and the riboflavin production of various strains and his data indicate that the maximum rate of riboflavin synthesis per dry weight of mycelium occurs around 36 hours using a 5% inoculum under the conditions of growth we were using. Figure 5 is taken from the work of Dr. D. H. Hayes.

Growth Conditions - For inhibitor studies it was felt advisable to use a synthetic medium for these reasons:

1. The effect of the inhibitors was suspected to be more pronounced under these conditions.

2. Less dilution of the added C^{14}-guanine was expected to occur in synthetic media.

3. The stimulation of riboflavin synthesis by purines was apparent only with synthetic media.

The synthetic media of Goodwin et al. (35) were used. For extract experiments it was felt that cells which are

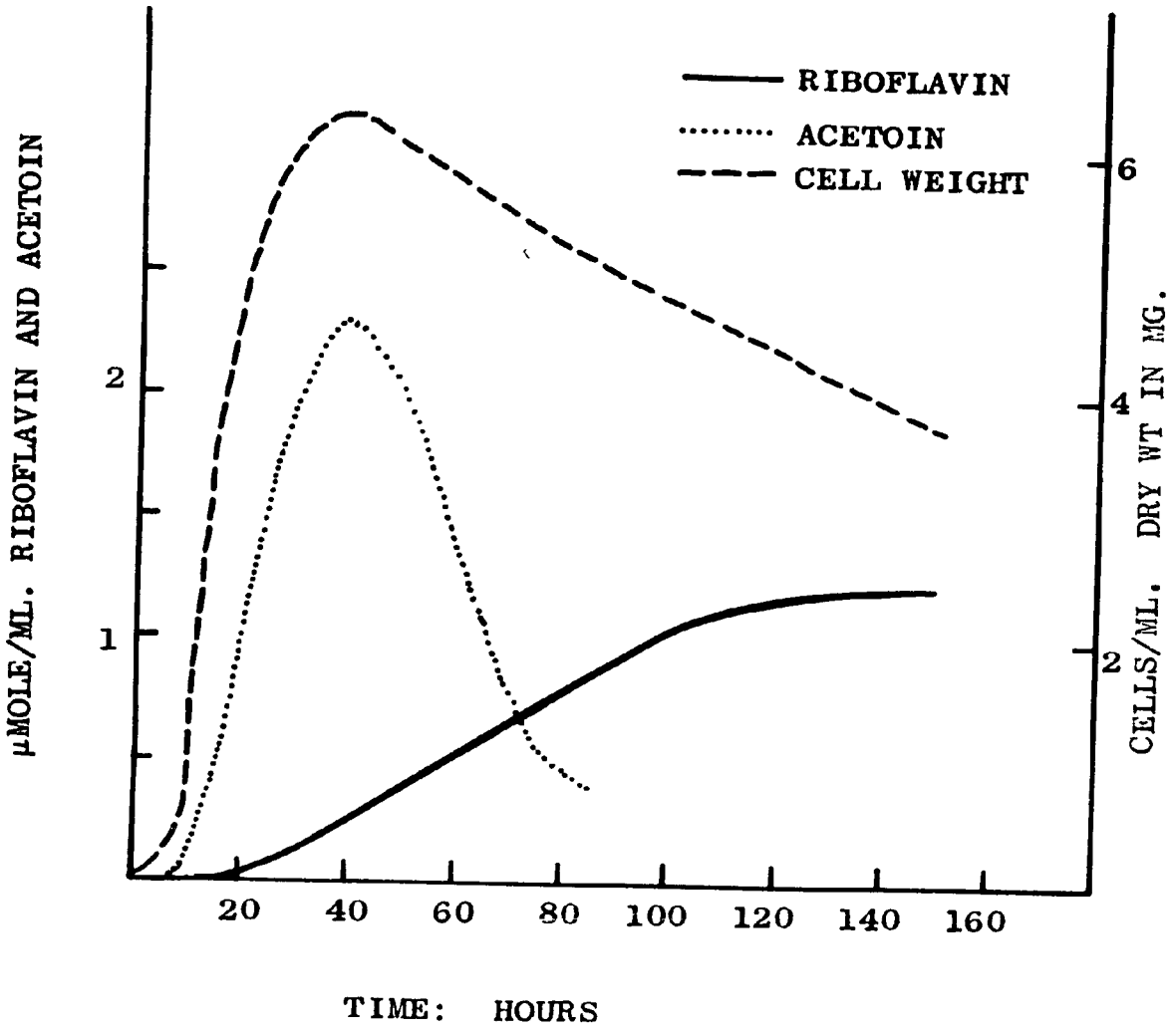


FIG. 5 The Growth, Riboflavin and Acetoin Production of E. Ashbyii.

Inoculum 24 hour old - 5%

Strain - North regional labs - Y1363

Grown at 30° in undefined medium

See text for details.

making riboflavin at a maximal rate would be more advisable, and for that Dr. D. H. Hayes studied various media and found that the following one was suitable:

Yeast extract	2 gms.
Bacto Peptone	5 gms.
Corn Steep Liquor*	20 ml
Glucose	40 gms.

The final pH of the medium was 5.6.

Cells were grown with good aeration. Small volumes (below a liter) were grown on a reciprocating shaker in flasks with a capacity of about 5 times the volume of medium used.

For larger quantities, the growth was carried out in large carboys immersed in a water bath maintained at 30° and stirred and aerated by a fast stream of air through a sintered glass bubbler. The air flow was in the order of 2 liters of air per minute per liter of growth medium. Foaming was minimized by addition of small amounts (about 10 ml/15 liters) of corn oil to the medium.

Collection of Mycelium - Centrifugation was less practical than filtration as the mycelium did not pack very well and it was more practical to filter larger volumes. The mycelium was filtered through Whatman's No. 1 filter paper for small quantities but diaper cloth was quite adequate when dealing with large quantities and provided much faster rates of filtration.

* A centrifuged 25% (w/w) solution of spray dried corn steep liquor - A. E. Staley Manufacturing Co., Decatur, Illinois.

The mycelium packed very well on a Buchner funnel with suction and could be sucked dry of most of the media. The pad could then be washed thoroughly with several portions of cold isotonic saline. This served to cool the mycelial pad. Washing with water after the saline wash caused the release of large amounts of ultraviolet-absorbing material and therefore was not employed.

The mycelial pad was easily pealed off the cloth or paper and weighed. The usual yield in 36 hours was 20-30 gm. of wet mycelium per liter in the undefined medium and about 1/3 as much in the synthetic medium. This represented 4 times the weight after drying in a dessicator or an oven and letting the dry material equilibrate with room air; or when the mycelial pads were spread out over a filter paper directly and allowed to dry in room air overnight.

Preparation of Extracts -

a. Sand Grinding - The mycelial pad was ground for 10-15 minutes with 2 weights of washed and ignited sand in a mortar in an ice bath with the gradual addition of 1 volume of 0.05 M phosphate buffer at pH 7.4, and the mixture was centrifuged at 1000 X g for 10 minutes. The precipitate was reground with 1 volume of the same phosphate buffer and centrifuged. These combined supernates were termed "sand-grinding extract". The extract had a volume about equal to that of the added buffer, a pH of approximately 7.2 and a protein content in the order of 5-10 mg. protein/ml.

b. Acetone Powder Extract - Acetone powder was made by homogenizing the mycelial pad 1 minute in the Waring Blendor at high speed with 5 volumes of acetone chilled to -15°. The mixture was filtered on a Buchner funnel and the resulting pad reextracted with 2 volumes of the cold acetone in the blendor for 15 seconds. The powder was worked to a loose consistency on filter paper, and put in a vacuum desiccator at 0° over P_2O_5 a few days to remove the last traces of acetone. The completely dry powder was stored in the deep freeze.

The powder was extracted by stirring at 0° with 5 volumes of 0.05 M potassium phosphate buffer, pH 7.4, and removing the debris by centrifugation.

c. Nossal Extract - Such extracts were prepared by shaking 1 volume each of mycelial pad, buffer and Superbrite microglass beads in the Nossal apparatus (36). Three shakings for 20 seconds were necessary to disrupt the cells. The tubes were cooled in ice between each shaking.

d. Waring Blendor Extract - It is possible to extract the pad by blending in the Waring Blendor with 2 volumes of buffer and one-half weight of sand. Sand seems to be necessary, very dilute extracts being obtained without it. It was necessary to homogenize for long periods to break the mycelial wall (twice at top speed for 1 minute each, cooling the container in ice between).

For large scale preparation of compound X, extraction of the mycelial pad was repeated two more times.

The second and third extracts showed approximately 0.5 and 0.25 of the activity of the first extract in converting guanine to compound X.

e. Alcohol Extract - Extracts of the mycelial pad with hot ethanolic solutions were used as a source of the non-protein material. The pad was extracted by boiling with 4 volumes of 75 per cent ethanol for 5 minutes and centrifuging. The alcohol extracted mycelial material in contrast to the normal mycelium, packed very well upon centrifugation. A second extraction was carried out and the combined supernates were lyophylized. The yield was about 5.5 gm. per 100 gm. wet mycelial pad.

f. Boiled Juice - The mycelial pad was suspended in 2 volumes of 0.05 M phosphate buffer raised to 95° in a boiling water bath, kept at that temperature 5 minutes, cooled and filtered. The filtrate was stored in the frozen state.

Enzymes -

Crystalline lactic dehydrogenase from rabbit muscle was kindly supplied by Dr. Paul A. Srere. Xanthine Oxidase as the ammonium sulfate paste was purchased from Nutritional Biochemical Co. Urease was purchased from Fisher Scientific Company in commercial powder form.

Guanase: Rat liver was extracted with 2 volumes of cold distilled water and centrifuged 20 minutes at 12,000 X g. The ammonium sulfate fraction between 0.4 and 0.6 saturation (37) was used without further purification. It showed very little xanthine oxidase activity and had no activity towards compound X.

II. Analytical Procedures:

Phosphate was determined according to the method of Fiske and SubbaRow (38) in the proportions to obtain a final volume of 5 ml. The orcinol test was run by the method of Kerr et al (39) decreasing the volume to 5 ml. The Pauly reaction was run according to MacPherson (40). The phenol reaction was carried out by the procedure of Folin and Ciocalteu (41) in a final volume of 1.2 ml. The uric acid reaction was that of Benedict and Franke (42) as modified by Brown (43). Both 2,6-dioxy-4,5-diamino pyrimidine and 2,4,5-triamino-6-oxy-pyrimidine were found to give quite strong reactions.

Periodic Acid oxidations - Oxidation by periodic acid was by the spectrophotometric procedure of Dixon and Lipkin (44). Several standards normally were used. These included AMP, riboflavin and FMN. The oxidations generally were carried out in the Beckman cell and followed directly at 227 mμ either at room temperature or at 50° (the latter being necessary to obtain reasonable rates of periodate utilization with FMN). In some cases the reactions were carried out at 0° in a 10-fold more concentrated solution, and aliquots were taken periodically, diluted with water and read in the Beckman at 227 mμ.

In all cases the oxidations were carried out in buffer, (usually 0.2 M phosphate, pH 7.4,) and compared to solutions containing buffer alone. Buffer and periodate were run simultaneously. The calculation of periodate uptake was based

on the drop in optical density in the unknown solutions as compared to that of the adenosine-5'-phosphate standard. The concentration of the standard AMP was determined from its optical density at 260 mμ, using a molar extinction coefficient of 16,000. One mole of AMP utilizes 1 mole of periodate, causing a decrease in the optical density at 227 mμ equal to 10 X 10^3 at pH 7.4.

Assay for Compound X - A reasonably rapid assay for compound X was developed on the basis of its strong adsorption to Dowex-1 ion exchange resin after treatment with 1 N mineral acid at 100° for one hour. This treatment hydrolyzes all known guanine derivatives to guanine, which adsorbs only very poorly to Dowex-1 resin.

The procedure was as follows:

1. The reaction was carried out with C^{14}-guanine as described elsewhere. The protein-free solution was heated with N HCl for 1 hour at 100°, then cooled and neutralized to a phenolphthalein end-point.

2. The neutralized solution was transferred quantitatively to a Dowex-1-formate column with the dimensions, 0.78 cm^2 X 5 cm. The column was drained between washings.

3. The column was washed with 200 ml of 0.05 M ammonium formate adjusted to pH 4.4 with HCOOH. The eluate was collected and 1 ml aliquot plated and counted.

4. The column was next washed with 50 ml of molar ammonium formate, pH 4.4, and the eluate was collected.

One ml of this was plated and counted.

5. The total counts in the molar ammonium formate fraction represent compound X plus a small contamination with guanine amounting to some 0.4% of the guanine. Therefore 0.4% of the total counts obtained in the 0.05 M ammonium formate fraction were subtracted from this figure. The corrected value was taken to be the incorporation of labeled guanine into compound X.

It is evident that such an assay is not sensitive because of the blank and is therefore suitable only when the incorporation of guanine into X is more than 1 or 2 per cent.

Attempts to determine the concentration of compound X by the phenol reagent assay rather than by measuring C^{14} failed because of the formation of a heavy white precipitate with the molar ammonium formate upon adding alkali.

Later it was found that 0.1 M formic acid could be used instead of M ammonium formate. Then the eluate was suitable for determinations with the phenol reagent. However, the blank was high, being about 2 mμmole of guanine-equivalents per ml. The radioactivity blank was the same as that obtained with molar ammonium formate.

Later the dependability of the assay was increased further by using 0.02 M ammonium formate, pH 5.5, for the preliminary elution of guanine and xanthine. Control experiments with the columns used showed that the radioactivity reached background levels within the first 100 ml of

0.02 M ammonium formate, pH 5.5, while compound X was not eluted with 400 ml.

When 0.02 M ammonium formate, pH 5.5, was used for the preliminary wash and 0.1 M formic acid was used to elute compound X, the assay was found reliable with widely varying amounts of extract and salts.

Riboflavin Determination - Riboflavin was determined by its optical density at 445 mμ, using a millimolar extinction coefficient of 11.1 (35). The amounts of FAD and FMN in the media were negligible and very low in extracts after incubation.

For more accurate determinations the solutions were saturated with Na_2SO_4 and extracted with 1/2 volume of n-butanol. Almost all the 445 mμ-absorbing material appeared in the first extraction. The second extraction contained only negligible quantities. Total flavin was determined by suspending the whole mycelia in N HCl and heating in a boiling bath for 45 minutes before saturating with Na_2SO_4 and extracting with butanol.

Tests for Reducing Groups - The ferric chloride-ammonia test, the phosphomolybdate-sodium hydroxide test and the 2,6-dichlorophenol indophenol test were carried out as described by Bendich and Clements (45).

Assay of Lactic Acid - L-Lactic acid was assayed using crystalline rabbit muscle lactic dehydrogenase kindly supplied by Dr. Paul Srere. The reaction was run according to Horn et al (46) i.e., in the presence of excess enzyme and

DPN in bicarbonate-semicarbazide buffer at pH 10.5 and following the reduction of DPN by the increase in optical density at 340 mμ. Total lactic acid was determined by the method of Barker and Summerson (47).

Carbon 14 Determinations - Carbon 14 was measured in infinitely thin layers with a gas flow thin end-window counter provided with an automatic changer. A standard was counted with each run and whenever necessary, the counts were corrected to the standard. Self-absorption was usually negligible. However, in rare cases when large amounts of material were used, various amounts were plated and the values plotted and extrapolated to zero weight.

Determination of Amino Acids - A quantitative measure of the amino acids was obtained by cutting out the ninhydrin-developed spots [sprayed with 1 per cent ninhydrin and 1 per cent 2,6-lutidine in 95 per cent ethanol and developed at 60° for 30 minutes in an atmosphere saturated with water vapor (48)] from paper chromatograms, eluting the paper with 70 per cent ethanol and measuring the color at 540 mμ.

Determination of Guanine in presence of compound X - Since guanase and xanthine oxidase do not act on compound X, singly or in combination, the breakdown of compound X to guanine can be followed spectrophotometrically with these enzymes by following the rise in the optical density at 290 mμ (49). A value of 7.25 for the change in the millimolar extinction was used.

The sample to be analyzed was diluted in 0.1 M $K\text{-}PO_4$ buffer, pH 7.4, and its absorbancy at 250, 260 and 290 mμ determined. A mixture of guanase and xanthine oxidase was added to both the sample and the blank, and readings taken at 290 mμ until no further change occurred. The reading was corrected for the dilution due to the addition of the enzyme solution.

III. Compounds:

Unless otherwise stated the compounds used were commercial products.

Guanine-8-C^{14} and some guanine-2-C^{14} were gifts from Dr. A. J. Guarino, originally purchased from Volk Radiochemical Company, Chicago. Another batch of guanine-2-C^{14} was purchased from the same company. N^2-methylguanine and 7-methylguanine were gifts from Dr. Gertrude B. Elion.

Ureidopropionic acid and 5-methylhydantoin were prepared from potassium cyanate and DL-alanine by the procedure of Boyd and Dakin (50,51).

IV. Miscellaneous Methods:

Titration of Salicylate Inhibition - As a typical example of the titration of the inhibitors, a salicylate inhibition experiment is described below. Similar experiments were performed with the other inhibitors.

To a series of 125 ml Erlenmeyer flasks were added 20 ml of the basic medium of Padlington et al (35) containing 0.1 per cent peptone and 1 per cent glucose. Then to each

flask was added 2 ml of a solution containing 12.5 mg of citric acid and 12.5 mg. of succinic acid brought to a pH of 5.5-6. Salicylate was added to give final concentrations of 0-500 mg salicylic acid/ml.

Each flask was inoculated with 1 ml of a two day old culture of E. ashbyii. A flask was filtered immediately and used as a zero time control. The rest were incubated at 30° for 68 hours with shaking, filtered on a buchner funnel, washed with 0.9 per cent NaCl and sucked dry. The mycelial pad was allowed to dry in the open for several hours and weighed. The optical density of the medium at 445 mμ also was determined. For finer titration the experiment was repeated exactly using the same range of concentration but with more flasks in the region of differential inhibition. Table I lists the results of this later titration.

A Typical Inhibition Experiment Using Labeled Guanine - Guanine-2-C^{14} or guanine-8-C^{14} was incubated in the presence of the inhibitor with cultures of E. ashbyii and the reaction mixture was examined for possible labeled intermediates.

Such an experiment using salicylate as the inhibitor is described in detail.

The following were added to 50 ml erlenmeyer flasks:

10 ml of medium (reference 35).

0.5 ml of citrate solution, pH 5.5 (equivalent to 6.75 mg citric acid).

0.5 ml of succinate solution, pH 5.5 (equivalent to 6.75 mg succinic acid).

TABLE I

THE RESULTS OF THE SALICYLATE EXPERIMENT

Flask	mg Salicylic Acid (per ml)	Dry Weight, mycelium (mg)	OD_{445} Medium	OD_{445} units/ mg mycelium
1	0	21.8	.098	0.117
2	12.5	23.8	.081	0.085
3	25	24.8	.117	0.118
4	50	23.4	.080	0.085
5	75	19.8	.080	0.101
6	125	18.8	.070	0.093
7	175	18.6	.052	0.063
8	250	19.6	.042	0.053
9	300	12.4	.020	0.040
10	375	9.2	.007	0.019
11	425	7.4	.000	0.000
12	500	1.4	.000	0.000

0.75 ml of salicylate solution, pH 5.5 (equivalent to 4.5 mg salicylic acid).

2.5 μmoles of guanine-2-C^{14} or -8-C^{14} in 0.05 ml (specific activity 500,000 counts/minute/μmole).

Each flask was inoculated with a 24-hour culture of E. ashbyii and grown for 36 hours with shaking. The mycelial pad was filtered off and extracted 2 times with 5 ml of 70 per cent ethanol. The ethanol extract was evaporated to dryness in a vacuum, quantitatively spotted on 10 cm strip of Whatman 3MM paper and the papers developed in the isobutyric-NH_3 solvent (52). After drying, the papers were exposed to X-ray film (No-screen or Blue Brand Kodak X-ray films gave similar results).

The medium was shaken 30 minutes with 200 mg of charcoal. The charcoal was centrifuged, washed with water and extracted with 5 ml of 20 per cent pyridine. Pyridine was removed by extracting 3 times with 8 ml of chloroform. Two drops of N acetic acid and 2 ml of saturated silver acetate solution were added to the aqueous solution, and the resulting silver guanate precipitate was centrifuged off. To the supernate 0.2 ml of M NH_4Cl was added. The AgCl precipitate was centrifuged down and discarded and the supernate evaporated to dryness and spotted on a 5 cm strip of Whatman 3MM paper. The compounds were separated by isobutyric-NH_3 solvent, and the paper was dried and exposed to film. Any compound which would precipitate with guanine as a silver salt

would have been lost by this procedure. Nucleotides and nucleosides would be expected to be soluble.

Incorporation of Guanine-2-C^{14} into the Nucleic Acid Purines - E. ashbyii was grown at 30° for 44 hours in 1 liter of synthetic medium in the presence of 0.25 μmoles of guanine-2-C^{14} containing about 5 X 10^5 counts per minute per μmole. The mycelial pad weighed 10 gm. It was extracted three times with 20 ml of 70 per cent ethanol and dried. The dry, alcohol-extracted mycelial pad was placed in 20 ml of N H_2SO_4 in a boiling water bath for 1 hour. Potassium hydroxide was added to the sulfuric acid extract until the end point of phenolphthalein and 0.5 ml of N sulfuric acid and 5 ml of saturated solution of silver acetate were added. The suspension was left on ice overnight to allow complete precipitation of the purines. The crude silver purine precipitate was then dissolved in 15 ml of N HCl at 100°, cooled and the silver chloride centrifuged off. The supernate was adsorbed to a 0.74 cm^2 X 9 cm Dowex-50-H column and eluted with N HCl.

Both the adenine and guanine peaks were considered to be pure because they were separated cleanly, they migrated as single spots on paper in the isobutyric ammonia solvent, and they showed the expected ultraviolet absorption spectra. The specific activities of the isolated purines are shown in Table 3. (See results)

The Dowex-1 Separation of the Acid Soluble Nucleotides of E. Ashbyii - A four liter culture of E. ashbyii was grown

at 30° for 79 hours with shaking. At that time 13.5 μmoles of guanine-2-C^{14}, 530,000 counts per minute per μmole, were added and the growth continued for two hours. The cells were harvested, washed with saline and extracted 2 times with 100 ml portions of 5 per cent trichloroacetic acid at 0°. The TCA extract was extracted 4 times with 250 ml of ether, the solution concentrated under vacuum to 150 ml and neutralized to pH 6.7. The solution, containing a calculated total of 2450 optical density units at 260 mμ, was placed on a Dowex-1-Cl^- column 1 cm^2 X 100 cm and washed with water till the eluate was free of ultraviolet-absorbing material. The column was eluted using a linear gradient of 8 liters of 0.002 M NaCl in the mixture and 5 liters of 0.5 M NaCl-0.01 M NaHCOO buffer, pH 3 in the reservoir. Twenty ml fractions were collected, and the ultraviolet absorption of the effluent was recorded by the UV-recorder*. The tracing of the recorder is reproduced in Figure 6 using an optical density scale, rather than the per cent transmission of the recorder. From each, 0.5 ml aliquots were plated and counted. The results are shown in Table 2.

The peaks which were radioactive were acidified, adsorbed to charcoal and eluted from the charcoal with pyridine: ethanol: water (1:1:2) and the pyridine and ethanol extracted with chloroform. The remaining aqueous extract was made 1 N with respect to hydrochloric acid and placed in a

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* The author is indebted to Dr. A. J. Guarino for his help with these experiments.

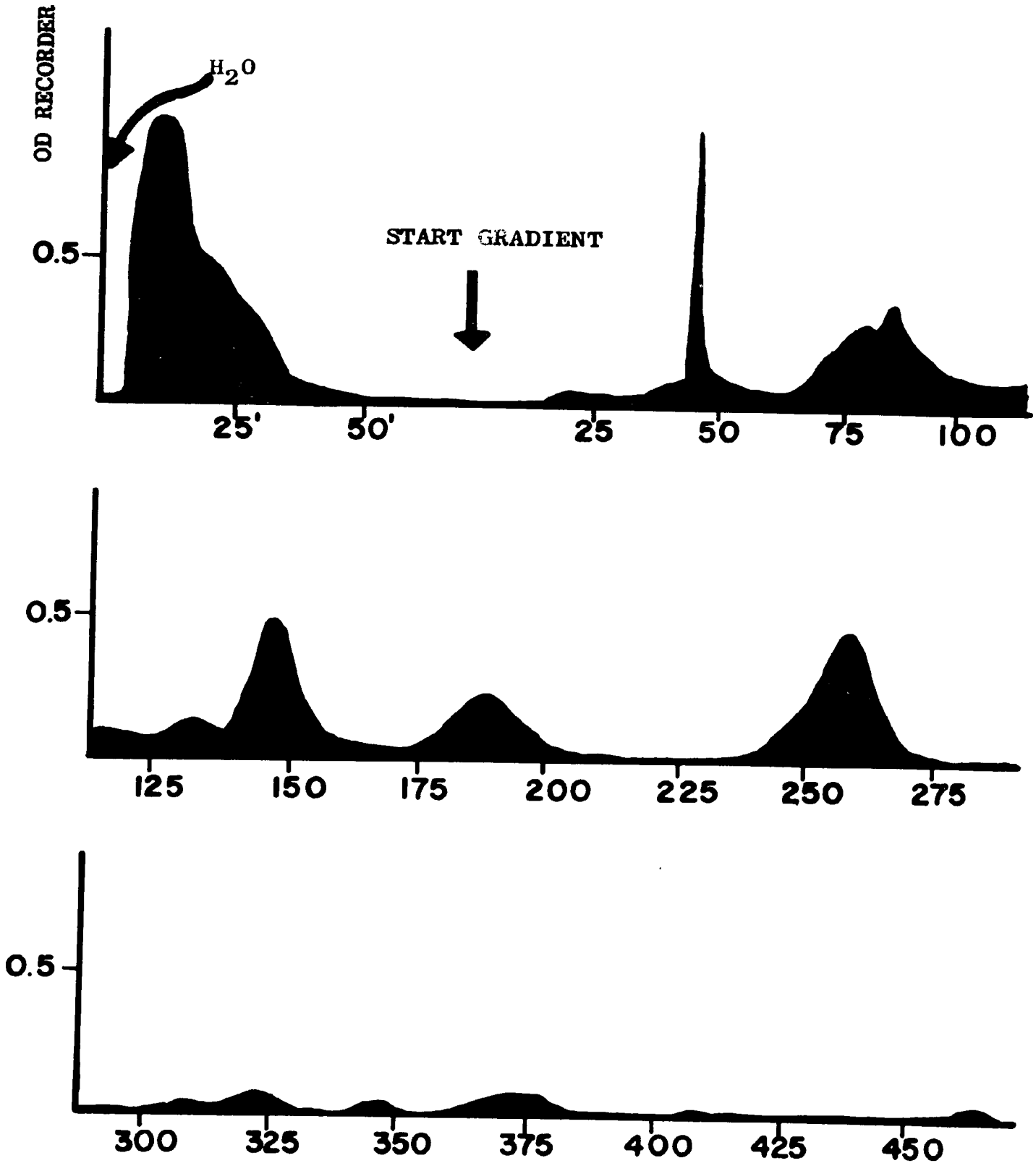


Figure 6. GRADIENT ELUTION OF ACID SOLUBLE NUCLEOTIDES OF E. ashbyii. See text for details.

TABLE 2

THE C^{14} CONTENT OF THE VARIOUS PEAKS OFF THE DOWEX-1 COLUMN

Tube #	c/m/.5 ml	Tube #	c/m/.5 ml	Tube #	c/m/.5 ml
6'	55	105	1	295	0
12'	117	115	17	315	0
21'	653	122	12	321	0
45'	0	130	0	335	0
20	0	135	0	347	0
35	2	145	0	357	23
44	65	165	0	374	69
60	4	175	24	384	1
71	0	185	239	400	0
79	0	205	11	420	1
83	9	255	4	467	0
90	0	280	0		

See Figure 6
See Text for Details

boiling water bath for 1 hour to hydrolyze any guanine glycoside linkage. The hydrochloric acid was evaporated off in the Rinco rotary evaporator with NaOH in the trap, and an aliquot of the solution spotted on paper and separated with the isobutyric acid-NH_3 solvent. These chromatograms were exposed to X-ray films for 14 days.

Attempts at Demonstrating Incorporation of Guanine-2-C^{14} into Riboflavin in Extracts - In a typical experiment 26 gm of mycelial pad was ground with sand along with 10 ml of 0.05 M potassium phosphate buffer, pH 7.4 and centrifuged at 3000 X g for 10 minutes. Ten ml of the extract were incubated with 0.2 ml of 2-C^{14}-guanine (1.14×10^6 counts per minute per μmole, 7.5 μmole per ml) and 0.5 ml of 0.1 M glucose-6-phosphate for 3 hours. At the end of the incubation time 2 volumes of alcohol were added and the supernate evaporated to dryness and hydrolyzed 1 hour at 100° in N HCl. This was carried out to hydrolyze the guanine glycosides. The riboflavin was separated on the benzyl alcohol-hydrochloric acid Hyflo column and analyzed. To the riboflavin fraction, enough carrier riboflavin (10-20 mg) was added to allow crystallization, and the resulting diluted riboflavin was crystallized from 75 per cent propanol, 10 per cent acetic acid and finally concentrated HCl. To follow the specific activity of the riboflavin a small aliquot was removed after each crystallization. The last few crystallizations, usually after the first or second, showed constant specific activity. The total incorporation of C^{14} into the riboflavin was

calculated from the specific activity and the total amount of riboflavin.

Paper Chromatography - The following solvents were used generally and are referred to in the text by their numbers. The ratios are volume/volume. Other special solvents are described in the appropriate places.

(1) Isobutyric acid: 0.5 M NH_4OH; 10:6 (52)

(2) Butanol:ethanol:water; 50:15:35: (20)

(3) Butanol:acetic acid:water; 2:1:1 - equilibrium mixture

(4) 5 Per cent K_2HPO_4 layered with isoamyl alcohol (53)

(5) Butanol saturated with 0.5 M NH_4OH (54)

(6) Saturated $(NH_4)_2SO_4$:acetate buffer:isopropanol; 79:19:2 (55)

(7) Ammonium acetate (Molar pH 3.8):ethanol; 40:60

(8) Ammonium acetate (Molar pH 8.4):ethanol; 40:60

(9) Ammonium acetate (Molar pH 8.4):ethanol; 40:60 saturated with sodium tetraborate

(10) Propanol:2M NH_4OH; 4:1

Paper Chromatography of Carboxylic Acids - Various solvents were tried. The basic solvents have the advantage of higher degree of sensitivity (56). With solvents containing ammonia a dark band appeared upon light spraying with 0.2 or 0.5 per cent bromcresol green in 95 per cent ethanol.

Since diethylamine solvents did not give this band it was used instead of ammonia. Less than 0.2 μmoles could be detected in a spot. The acid salts appeared as blue spots on a yellow background.

Among the various propanol and butanol solvents tried, propanol:water:diethylamine in the volume proportion of 85:15:1 was found to be quite suitable. The following are standard R_F values: acetic, 0.53; pyruvic, 0.61; lactic, 0.52; α-keto-glutaric, 0.36; succinic, 0.28; fumaric, 0.30; and malic, 0.18. Pyruvic, α-keto-glutaric and fumaric can be plainly seen on the paper as ultra-violet adsorbing spots before spraying.

The unknown was usually extracted exhaustively with ether from an acidified solution, the ether evaporated in a stream of N_2, and the unknown transferred to a single spot on the paper. All the acids mentioned above were run as standards.

The paper was developed in the solvent for 18 hours, which allowed the front to move about 30 cm, air dried and placed in an oven with a blower at 40° for 10 minutes.

If it was necessary to recover the acid, the paper was cut and the spot eluted with water. The indicator can be separated from the acid by a single run in the same solvent since bromcresol green moves to an R_F of approximately 0.85.

Charcoal Adsorption - Adsorption to charcoal was carried out by acidifying the solution with HCl to about pH 1,

adding charcoal (Norite SG-2, acid-washed and free of floating particles) and stirring for 30-60 minutes. The charcoal then was either centrifuged with a layer of ethanol at the surface or filtered using Whatman #5 discs. For elution a mixture of pyridine-ethanol-water in the volume proportions of 1:1:2 was normally employed. It has the distinct advantage over ethanol-NH_4OH-water mixtures of providing more quantitative elution of nucleotides and purines and especially of riboflavin and FMN. The pyridine was extracted from the eluate either by 3-4 extractions with 1 volume of chloroform or 5-6 extractions with 2 volumes of ether. The aqueous layer was then evaporated to dryness in vacuo.

In control experiments using purified compound X, the pyridine-ethanol mixture was found to cause about 40 per cent breakdown of compound X to guanine. More basic eluting agents therefore were sought in view of the stability of the compound in basic solutions. Compound X is eluted from 1 gm of charcoal by extracting twice with 20 ml of a solution of 0.05 M NaOH in 50 per cent ethanol in a yield of about 90 per cent. However, this procedure extracts almost none of the adsorbed riboflavin.

Ion Exchange Separations - Dowex-1 (usually in the formate form) was used for anion exchange separations. The resin was regenerated by passing 0.5-1 bed volumes of 90 per cent formic acid through a column and washing with deionized water until the pH of the effluent solution was above 5. Dowex-50 was regenerated by washing first with

0.5 M NaOH and then with 2 M HCl. Dowex-50-W (the analytical grade resin) was packed directly in the columns to be used and washed with 10-20 bed volumes of 0.5 N HCl. Regeneration was effected with 0.5 N NaOH followed by 2 M HCl.

Constant volume reservoir gradients, linear gradients and simple variation of eluents were used as required for separation.

Chlorine and Permanganate Oxidations - For chlorine oxidation, the compound was dissolved in 6 N HCl and warmed to 50°. Chlorine gas, generated by the reaction of potassium chlorate and concentrated HCl, was bubbled through the solution for 5 minutes. The flask was stoppered and the temperature maintained at 50-55° for 30 minutes. At the end of the incubation period, the flask was cooled in ice and the chlorine flushed out with a stream of nitrogen. The solution was evaporated to dryness in vacuo.

This procedure has an advantage over the usual ones in which potassium chlorate is added to a solution of the compound in HCl (57,58) since it does not introduce salts which might interfere with later chromatographic analyses.

Permanganate oxidation was carried out by the procedure of Heinrich and Wilson (59) using 50 per cent acetic acid as the solvent.

Neutral Hydrolysis of Compound X - The solution containing the compound was evaporated to dryness in vacuo in a

50 ml round bottom flask. Ten ml of the solution of the buffer or the reagent were added, the flask connected to a condenser and refluxed. The solution was evaporated under vacuum to 2 or 3 ml, transferred to a glass stoppered test tube, acidified with HCl and extracted 5-8 times with 2 volumes of ether. The ether layer was evaporated down in a stream of N_2 and chromatographed to detect organic acids. The aqueous layer was assayed to test for the breakdown of the compound.

Spectrophotometric Determinations of Ionizing Groups - To save material the determination of absorption spectra was not run in various buffers as is normally done, but instead two 5 ml solutions of the compound under investigation were acidified and alkalinized respectively with small amounts of concentrated HCl and 5 N KOH, delivered from very thinly drawn out capillary tubes. The pH of the solution after each incremental addition was determined with a Beckman pH meter before and after the determination of the spectra. Spectral changes were followed in the Cary Recording Spectrophotometer. Corrections have not been made for the changes in volume due to the addition of the acid or base. These volume changes were always less than 1 per cent.

Nitrous Acid Treatment - The nitrous acid treatment was a modification of the method of Devole (60), adding 0.1 volume of glacial acetic acid to a solution the compound in 0.2 M $NaNO_2$ which had been preheated to 50°. After incubation for 10 minutes, 0.5 volumes of 0.2 M urea was added

to destroy the excess of HNO_2. For more vigorous conditions deaminations were occasionally carried out using a final concentration of 3 N HCl instead of 1.5 N acetic acid.

Separation of Riboflavin from Guanine -

1. Attempts by paper chromatography. As both guanine and riboflavin are relatively insoluble in water both are expected to show tailing on most logical chromatographic solvents. In experiments where the guanine incorporation into riboflavin is studied, losses due to trailing of the riboflavin can be tolerated. It is easy to determine the total amount of riboflavin before the separation by the absorption of the solution at 445 mμ. However, in any solvent where guanine moves ahead of riboflavin no amount of trailing of the guanine, no matter how minor, can be allowed.

Few of the solvents tried separated guanine and its derivatives, guanosine and GMP, from riboflavin well enough. Solvent 7 separated riboflavin rather well from the guanine series; however, since it suffers from the disadvantage that riboflavin moves slower than guanine, it is therefore prone to contamination by trailing.

Several solvents containing benzyl alcohol and butanol were made to overcome these problems. It was intended to utilize the high solubility of riboflavin in butanol and in benzyl alcohol. However, none of the combinations seemed to result in a good separation.

2. Partition columns for guanine and riboflavin. The partition coefficients were determined for the distribution

of riboflavin and guanine between benzyl alcohol and N HCl in the presence of acid-treated alcohol extracts of E. ashbyii. The riboflavin was measured by the optical density at 445 mμ and guanine by its C^{14} activity. The values obtained were 2.1 for riboflavin and 0.054 for guanine.

These properties were considered as making the separation of guanine and riboflavin on partition columns quite reasonable.

Hyflo Supercel was prepared for a column by mixing in a mortar 20 gm of Hyflo with 10 ml of N HCl saturated with benzyl alcohol. The mixture was flooded with benzyl alcohol saturated with HCl and the slurry packed in a 1.5 cm^2 column under air pressure. This formed a column 18 cm high.

In a small beaker, 25 mg of an alcoholic extract of E. ashbyii were mixed with: (1) 0.2 ml of 2-C^{14}-guanine containing 390,000 counts/minute/μmole, (2) 0.4 ml of N HCl saturated with benzyl alcohol and riboflavin and containing 15.1 OD_{445} units of riboflavin, and (3) 1 gm of Hyflo Supercel.

The mixture was flooded with 2.5 ml of benzyl alcohol saturated with N HCl, transferred to the top of the column and elution under pressure was started with benzyl alcohol saturated with N HCl. Figure (7) shows the results of the experiment.

The fraction between 17 and 30 ml contained 94.5 per cent of the riboflavin and 0.11 per cent of the guanine.

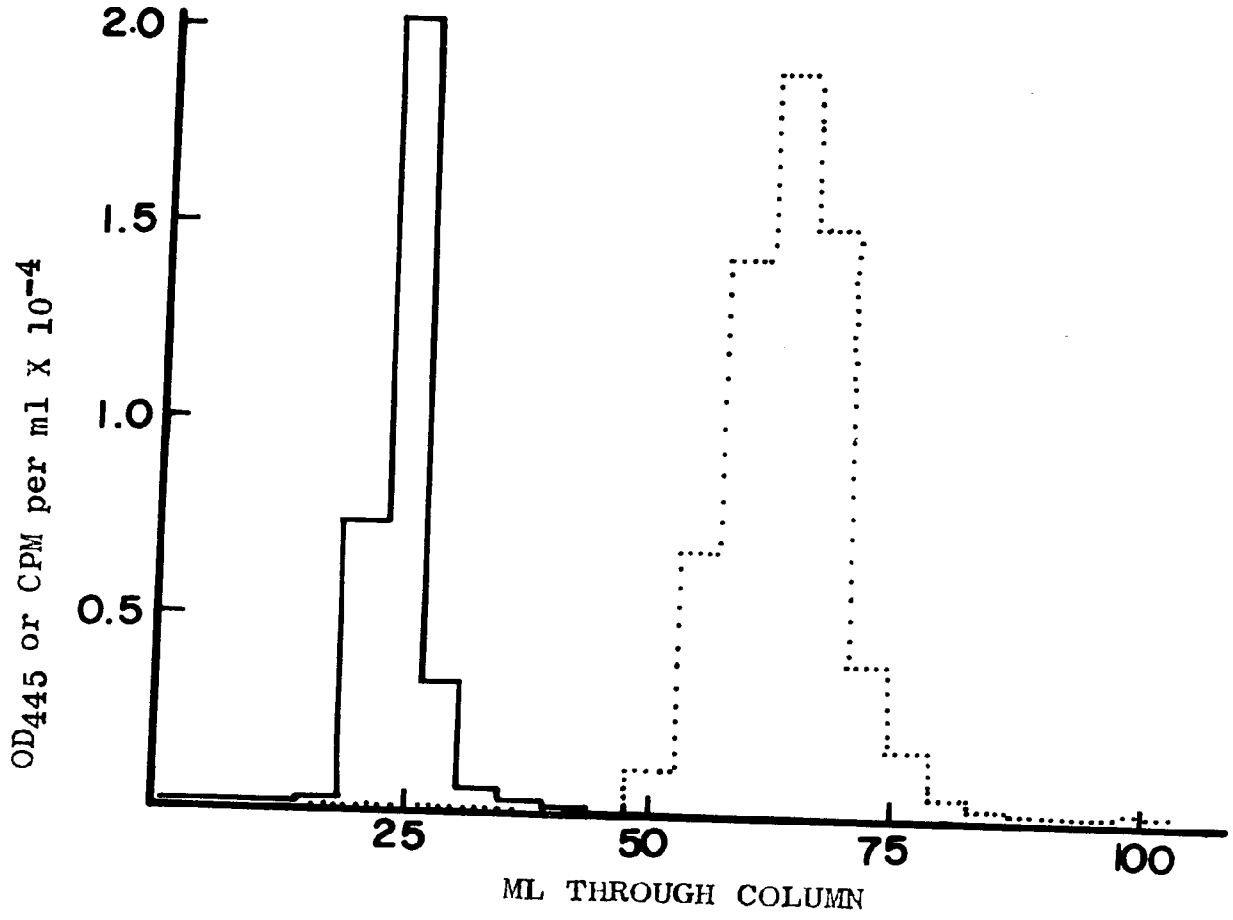


Figure 7. **THE SEPARATION OF GUANINE AND RIBOFLAVIN ON A PARTITION COLUMN.**
See text for details.

Solid line = OD_{445}

Dotted line = counts per min. per ml X 10^{-4}

This separation has an advantage in that the elution of riboflavin can be followed visually by watching for the yellow color and is complete within 1 hour. However, the preparation and packing of the columns is rather tedious and it would be difficult to use for routine analysis of large samples.

3. Attempts at separation using ion exchange methods. Attempts at separating riboflavin and guanine using Dowex-1 and borate in alkaline solution failed in spite of the fact that riboflavin complexed with the borate and adsorbed to Dowex-1 and most of the guanine did not. When the borate was removed in order to elute riboflavin, about 1 per cent of the starting guanine was found in the riboflavin fraction. Furthermore, a large loss occurred in the riboflavin fraction presumably because of its high sensitivity to light in alkaline media.

Dowex-50 does not separate the riboflavin and guanine well (see Figure 9). Perhaps this is due to the strong nonionic adsorbability of riboflavin to the resins.

4. Purification of riboflavin. This procedure finally was adopted for riboflavin purification from reaction mixtures. Riboflavin was extracted from the acid-hydrolyzed solution (1 N HCl 100°, 1 hour - this treatment hydrolyzes all known guanine derivatives to guanine and does not degrade riboflavin to any extent.) by 4-5 portions of 1/5 volume of benzyl alcohol. To the benzyl alcohol extract 4 volumes of ether were added, and the solution was extracted

3-4 times with 0.2 volumes of water. The extract was evaporated *in vacuo*. The residue dissolved in N HCl and fractionated using the partition columns (described p.39). The riboflavin fraction was freed from benzyl alcohol by diluting with ether and extracting with water and dried in vacuum. The residue was dissolved in hot 75 per cent propanol to a concentration of about 2 mg riboflavin/ml. A small fraction was removed from the solution and diluted with water for the OD_{445} measurement and determination of C^{14} activity. The riboflavin crystallized out upon cooling the propanol solution and was separated from the mother liquor by centrifugation. Crystallization from propanol was repeated 2 or 3 times taking aliquots each time. The riboflavin crystals were dissolved in 10 per cent acetic acid at 100° and allowed to crystallize by cooling to room temperature. This crystallization was repeated 2 or 3 times. The final crystallizations (1-3 times) were done by dissolving the crystals in a minimum volume of 6 N HCl and diluting with 10-20 volumes of water according to the method of Adams *et al* (61).

IV. Preparation of Compound X:

Incubation - For the large scale preparation of compound X, 15 liter batches of *E. ashbyii* were grown for 36 hours inoculating with 800 ml of 24 hour old cultures. The yield per batch was 250-330 gm of damp mycelia. The $OD_{445\ m\mu}$ of the media ranged from 2.7-4.0. The saline washed mold was shaped into square cakes, wrapped in aluminum foil, quickly

frozen by pressing it between dry ice discs, and stored in the deep freeze. When needed 100 gm portions of the mold were chiselled into approximately one cubic centimeter blocks and extracted three times with 200 ml portions of cold 0.05 M $K\text{-}PO_4$ buffer, pH 7.4, in the Waring Blendor.

Each of the extracts was incubated separately in a 2000 ml Erlenmeyer flask with 10 ml M $K\text{-}PO_4$ buffer, pH 7.4, 250 μmoles of guanine suspended in 5 ml water, and 500 mg of glucose. The incubation was carried out for 2.5 hours on the reciprocating shaker without temperature control. The temperature of the solution, however, maintained itself at 32-33°.

Protein Precipitation - At the end of the incubation time the three solutions were pooled and 25.5 ml of 70 per cent $HClO_4$ were added. The precipitated protein was filtered off over a Buchner funnel using Whatman #1 paper and about 5 gms of Hyphlo super cell filter aid. The filtrate was stored in the refrigerator.

Acid Hydrolysis and Salt Removal - When 6 such incubationswere accumulated, they were pooled and evaporated in the Rinco rotary evaporator to a volume of 1200 ml. (This gives a final acid concentration of 1 M). The solution was placed in a boiling water bath for 1 hour. The acid-hydrolyzed solution was cooled in an ice bath and to it 115 gm of $Ba(OH)_2.8H_2O$ (equivalent to the phosphate present) and 60 gm of solid KOH were added. After the complete solution of the solid hydroxides, 5 M KOH was added dropwise until

the solution gave a distinct red color with phenolphthalein. (The pH was 9 with the glass electrode.) The voluminous precipitate of $BaHPO_4$ and $KClO_4$ was filtered off on a Buchner funnel and washed with 200 ml of cold water and this filtrate carried through separation on Dowex-1 columns.

First Dowex-1 Separation - The filtrate was relatively salt free and was passed through a column of Dowex-1 formate, 20 cm^2 X 22 cm. The solution was washed into the column with about 100 ml of water and with 10 liters of 0.02 M ammonium formate, pH 4.4. Compound X was then eluted with 0.1 M formic acid. Most of the compound appeared in the first two liters (see Figure (8)). The peak containing compound X was evaporated in the Rinco evaporating apparatus to about 20 ml.

Dowex-50 Separation - To the concentrated solution 1 ml of concentrated HCl was added and the solution was passed through a 5 cm^2 X 22 cm column of Dowex-50-W-H and eluted with N HCl. The peak between 1050 and 1950 ml contained compound X (followed by U.V. spectra).

Charcoal Adsorbtion - To the solution containing compound X 4 gm of charcoal were added and the mixture stirred for 1 hour with a magnetic stirrer. The charcoal was collected by centrifugation and washed with 200 ml of water. It was eluted with two 100 ml portions of 0.05 M NaOH in 50 per cent ethanol by shaking each time for 1 hour on the reciprocating shaker. The charcoal was filtered off on a sintered glass filter of medium porosity.

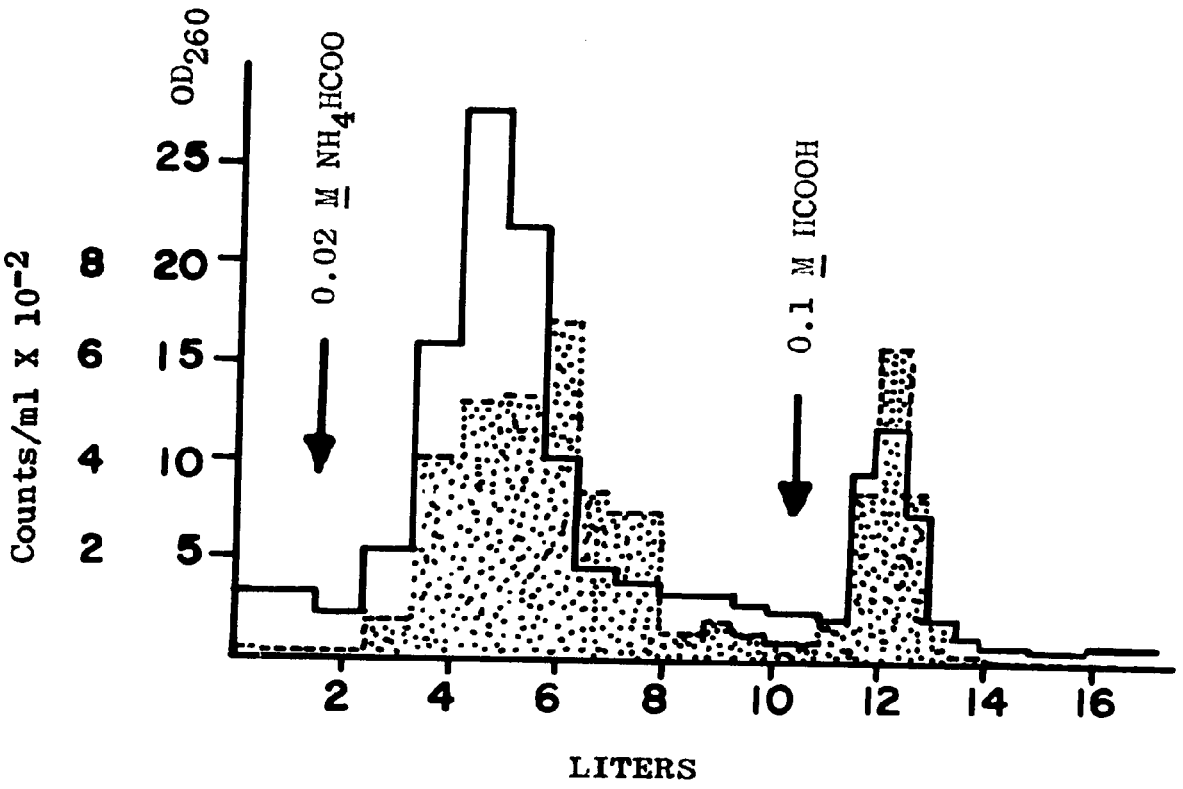


Figure 8. THE DOWEX-1 SEPARATION OF COMPOUND X FROM LARGE SCALE INCUBATION MIXTURE.

See text for details. Solid line represents optical density at 260 mμ; the dotted area represents counts of C^{14}.

Silver Precipitation - The filtrate from the charcoal was evaporated to 80 ml and neutralized to pH 4.5 with glacial acetic acid. 40 ml of saturated solution of silver acetate were added with mixing and the solution left in the refrigerator for 4 hours to complete the precipitation. The silver precipitate was centrifuged, washed with 200 ml of water containing 5 ml of saturated silver acetate, and dissolved by adding 5 ml of N HCl and heating at 70° for 15 minutes.

Second Dowex-1 Separation - The supernate was neutralized to phenolphthalein and adsorbed to a 3 cm^2 X 11 cm column of Dowex-1-formate and washed into the column with 20 ml of water. The column was washed with 1 liter of 0.02 M ammonium formate, pH 4.4. Compound X was eluted with 0.1 M HCOOH (approximately 7300 OD_{260} units).

The peak containing compound X was evaporated in the Rinco apparatus to about 1 ml and lyophilyzed. About 80 mg of the solid were collected from the flask. The compound was homogeneous as judged by paper chromatography in 4 solvents.

Crystallization - The solid remaining in the flask was dissolved in 0.2 ml of 90 per cent formic acid and crystallized by adding 1.2 ml of water and storing overnight in the refrigerator.

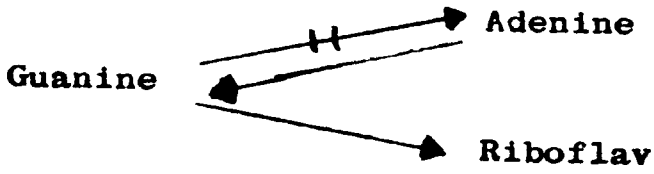
Paper chromatography of the crystalline material gave single UV spots in 4 solvents. The compound was recrystallized by adding 90 per cent formic acid to dissolve the wet crystals, then diluting the solution with water.

RESULTS

I. Whole Cell Experiments:

Incorporation of Guanine-2-C^{14} into the Nucleic Acid purines - This experiment was designed to test whether guanine was converted to adenine by the mold. Guanine-2-C^{14} was incubated with the mold 44 hours in a synthetic medium, and the purines of the nucleic acids isolated in pure form and assayed for radioactivity (see page 29). The results of these experiments are shown in Table 3. These results indicate that in this mold, as in yeast cells and in the mammal, guanine is not incorporated to any significant extent into adenine nucleic acid derivatives.

As guanine is converted to riboflavin but not to adenine in the organism, it seems reasonable to assume that guanine is a more direct precursor of riboflavin than adenine.



This is in agreement with the results of Brown et al (62) who found that purines stimulated riboflavin production in E. ashbyii in the order of: guanine > xanthine > adenine > hypoxanthine > uric acid. In Candida flareiri these same workers have found that while xanthine and guanine stimulated riboflavin production adenine and hypoxanthine did not (63). Furthermore, in the same organisms under their conditions

TABLE (3)

Specific Activities of the Nucleic Acid Purines

	OD_{260}	μMole/ml	c/m/ml	c/m/μMole
Adenine	3.63	.282	55	195
Guanine	2.28	.285	15,000	58,000

See text for details

68 per cent of the added C^{14}-guanine and only 16 per cent of the added C^{14}-adenine was found in riboflavin (64).

The acid-soluble Nucleotides of E. ashbyii - It was felt that if intermediates in riboflavin production accumulate in cells, they might be found in the acid-soluble nucleotide fraction. To study such a possibility, E. ashbyii cells were grown for 22 and 81 hours in the presence of guanine-2-C^{14} and their acid-soluble nucleotide fractions analyzed as is described in detail in the Experimental section for the 81 hour experiment. (See page 29)

In neither case was there any indication of a non-guanine radioactive compound. As intermediates in riboflavin synthesis would be expected to be labeled with guanine-2-C^{14}, the further identification of the acid-soluble nucleotides was not attempted.

Experiments using doubly-labeled guanine - The work of McNutt (18) and others established that a purine precursor loses its 8 carbon, and the remainder is incorporated as a unit into riboflavin. It was therefore logical to assume that some intermediates in riboflavin synthesis would be purines derivatives which have lost their C-8. More accurately these would be expected to be 4,5 diaminopyrimidine derivatives.

To look for such compounds guanine-2-C^{14} and guanine-8-C^{14} were used in parallel experiments. It was expected that intermediates would contain C^{14} in the experiments where guanine-2-C^{14} was used but not in those in which guanine-8-C^{14} was employed.

Cells were grown in the presence of 2-C^{14}- or 8-C^{14}-guanine under various conditions. The mycelial pads were extracted with hot 70 per cent ethanol, the C^{14} constituents of the extracts separated by paper chromatography and the paper exposed to X-ray film. We were interested in compounds which became labeled by 2-C^{14}-guanine and not 8-C^{14}-guanine. In our experience it is possible to detect on film some 1000 c/m (in terms of the gas flow counter) or about 0.001 μcuries of C^{14} in a spot under the conditions used. As the specific activity of the guanine used was 500,000 c/m/μmole, we expected to be able to detect the incorporation of 5 millimicromoles of guanine into such intermediates. Cells of different ages and cells in which riboflavin synthesis had been inhibited were used in these experiments.

Cells of different ages. Under our conditions of growing E. ashbyii (5 per cent inoculum and vigorous shaking), the production of riboflavin follows a lag period of some 6-12 hours although the cells are growing quite well. (See figure 5). It was thought that the younger cells might be accumulating intermediates, or that small amounts of intermediates could be present in older cells which are actively synthesizing riboflavin. But the results with cultures which were respectively 9, 11 and 36 hours old failed to show measurable accumulation of intermediates which have lost the C-8 from the purine ring.

Experiments with inhibitors. Inhibitors which would decrease the rate of riboflavin biosynthesis differentially over growth were used. Several such compounds have

been reported. Iron (65), uracil (9), and 4,5,6-triamino-2-oxypyrimidine (8) are examples. Compounds which might be expected to interfere with the metabolism of "acetate" were tried as possible riboflavin inhibitors since the work of Plaut (15) suggested that an acetate-like fragment was involved in the synthesis of the aromatic ring of riboflavin.

Fluoroacetate which is known to substitute for acetate in the formation of citrate inhibits the oxidation of acetate by yeast very selectively (66). We tried to inhibit riboflavin synthesis with fluoroacetate but added citrate to the medium hoping to reduce the fluoroacetate effect on energy metabolism.

Salicylate has been found to inhibit the synthesis of CoA in E. coli (67,68,69). This was also tried as an inhibitor of riboflavin. However, since coenzyme A is involved in the tricarboxylic acid cycle in the formation of citrate and succinate, these were added to the medium in an effort to overcome the general effect of such an inhibitor.

In these experiments the growth of the organism, as measured by dry weight, and the production of riboflavin were first titrated against various concentrations of the inhibitor. A concentration which showed the largest differential in inhibiting riboflavin production over growth was used. The titration of the inhibitors and the conduct of the inhibition experiments were carried out essentially as is described for salicylate in the Experimental section. Results of these titrations as well as the concentrations used in each case are listed in Table 4.

TABLE 4

THE INHIBITION OF RIBOFLAVIN SYNTHESIS AND OF GROWTH WITH VARIOUS INHIBITORS

Inhibitor	CONCENTRATION M		
	1/2 Growth Inhibition	1/2 Riboflavin Synthesis Inhibition	Used
Uracil	>0.02	0.01	0.015
Fluoroacetate*	0.0004	0.00004	0.0004
Salicylate**	0.003	0.0013	0.003
Ferric Iron	0.0025	0.0006	0.0025
4,5,6-Triamino-2-oxypyrimidine	-	-	0.009

* In the presence of 0.0054 M citrate.

** In the presence of 0.0027 M citrate and 0.0045 M succinate.

In none of these experiments was it possible to demonstrate the accumulation of a non-riboflavin compound labeled with 2-C^{14}- but not 8-C^{14}-guanine in either the cells or the medium. It may be appropriate to restate that an accumulation of 5 millimicromoles would have been detectable under the conditions of our experiments.

The failure to detect any such compounds in the inhibitor studies can be explained by one of these possibilities:

1. All these inhibitors, by chance, exerted their action in the early stages of the biosynthesis of riboflavin; prior to loss of the C-8 from the purine or at such a point after the ring opening that the equilibrium of the several reactions was in favor of the reformation of the purine.

2. These inhibitors were in part unspecific, and their primary action was to decrease the rate of synthesis of purines. The purines which were being synthesized were preferentially utilized for growth rather than for riboflavin synthesis.

II. Experiments with Extracts:

Attempts at Demonstrating Incorporation of Guaine-2-C^{14} into Riboflavin in Cell-free Extracts - These experiments were carried out with crude extracts prepared by grinding the mycelial pads with sand. Details of the incubation conditions are given under Experimental. The guanine

had a specific activity of 1.14 million c.p.m. per μmole and incubation time was 3 hours. At the end of the reaction, the riboflavin was isolated on a partition column, carrier was added and the mixture recrystallized to constant specific activity.

In no case were more than a few thousand counts incorporated. This represents less thant 2-5 millimicromoles. Such low incorporation might very well be due to the presence of whole cells in such extracts and even if it reflects real cell free incorporation, it is so low that the system is very difficult to work with and such low incorporation was considered negative.

Negative results were also obtained when the extract was fortified with DPN, PGA, diacetyl, PRPP, ATP, glycine, TPN, glucose-6-phosphate dehydrogenase, and alcohol extracts of the mycelia or several combinations of these additions. Again negative results were obtained with Nossal extracts, Waring Blendor extracts or acetone powder extracts.

Demonstration of the Biosynthesis of a New Guanine Derivative in Extracts of E. Ashbyii - In the course of the attempts to demonstrate the incorporation of guanine-2-C^{14} into riboflavin in cell-free extracts, a new guanine compound was uncovered. A protein-free filtrate of the incubation mixture was hydrolyzed in N HCl at 100° for 1 hour to breakdown the guanine nucleotides and nucleosides to guanine. Aliquots of the hydrolysate were placed on paper and separated in several solvents and the papers exposed to X-ray

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films. After development of the film, a new radioactive spot, not identical with guanine or xanthine, could be demonstrated. This separation was clear with either solvents 2 or 3.

When such a hydrolysate was placed on a Dowex-50-H column, three distinct radioactive peaks could be demonstrated (see Figure 9).

1. The first radioactive peak - The first C^{14} peak had xanthine like spectra and was not separable from xanthine by paper chromatography in 9 solvents. It was oxidized with xanthine oxidase to give a product which had the ultraviolet absorption spectrum of uric acid.

2. The third radioactive peak - This peak had ultraviolet absorption spectra identical with guanine and moved with guanine in solvents 1,2, and 3. It therefore was assumed to be guanine and was not studied further.

3. The second radioactive peak - The second radioactive peak was purified by paper chromatography with solvents 1,2 and 3 in that order, at which point it was pure enough to determine its ultraviolet absorption spectra. These were guanine-like but showed some differences from guanine which will be discussed under ultraviolet spectra.

This compound will be referred to henceforth as compound X. The production of this compound under various conditions was studied. A large amount of it was isolated and tentatively identified as N^2-(2-propiono)guanine (see figure 20).

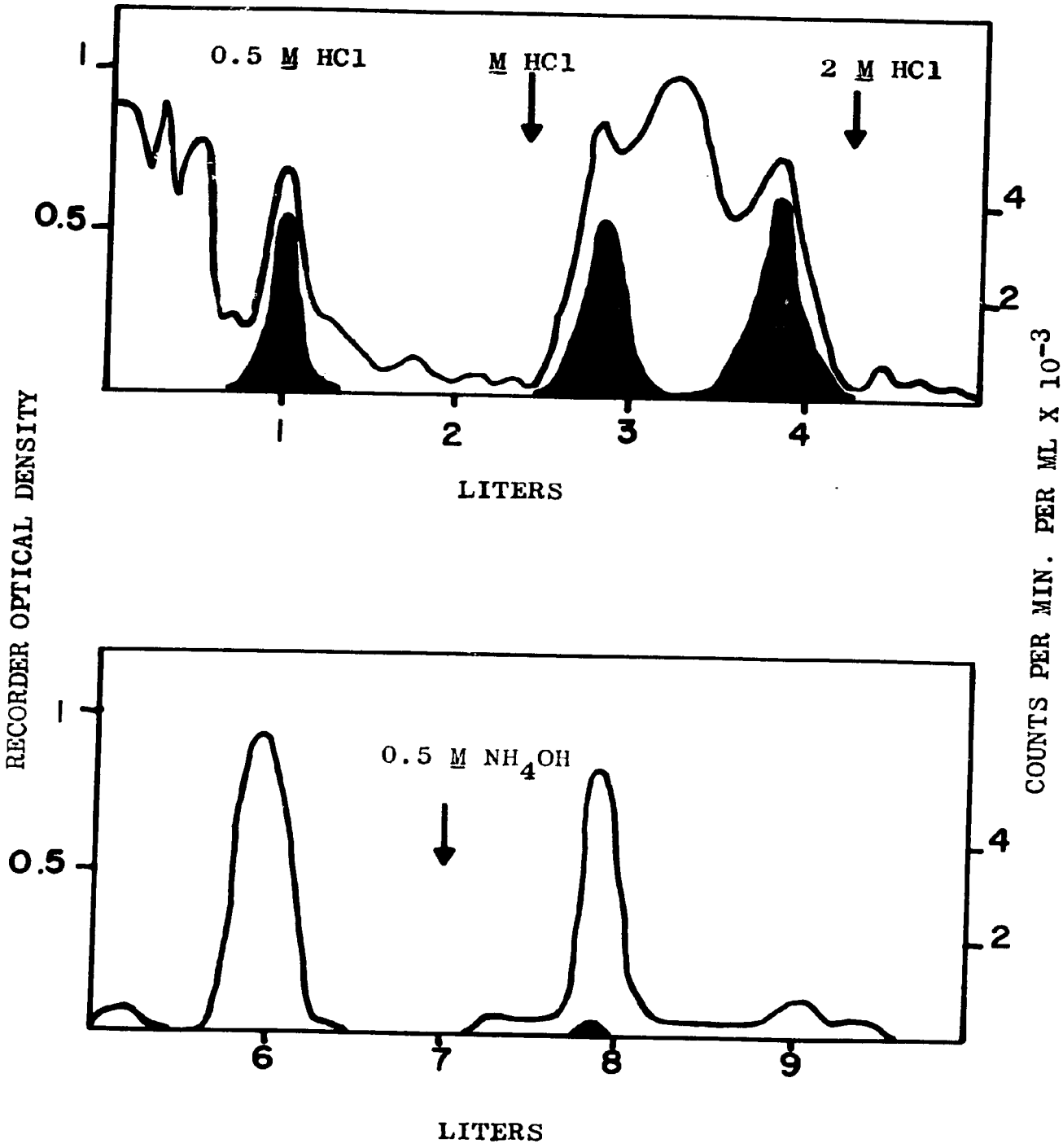


Figure 9. The Separation of an Incubation Mixture on Dowex-50-H.

Dark area = carbon-14

Line = ultraviolet absorption

Preliminary Experiments on the Production of Compound X - To accumulate large quantities of the compound it was necessary to study the best conditions for its synthesis. Only preliminary experiments in this direction were run as it was felt that it would be easier to study the biosynthesis of the compound after its structure was established. The results are noted briefly below. All these preliminary runs were with crude sand extracts.

pH and time studies - The most efficient incorporation of labeled guanine into the compound X occurs at around pH 7. The incorporation drops sharply with lower pH values and more slowly when the pH is raised. The incorporation reaches a maximum in about 150 minutes at 32°.

Concentration of guanine - Figure 10 shows the rate of incorporation of guanine into compound X with increasing concentration of guanine. There is a sharp, more than proportional increase in the incorporation up to a concentration of about 0.25×10^{-3} M guanine and then a slower increase with higher concentrations.

The non-linear increase at lower concentrations can be explained by assuming that the competing reactions are more efficient at lower concentrations. The break in the curve at the 0.25×10^{-3} M guanine may be due to the saturation of the solution with guanine at that pH and protein concentration.

Cells of different ages - Six 500 ml conical flasks containing 100 ml of medium each were inoculated with 5 ml

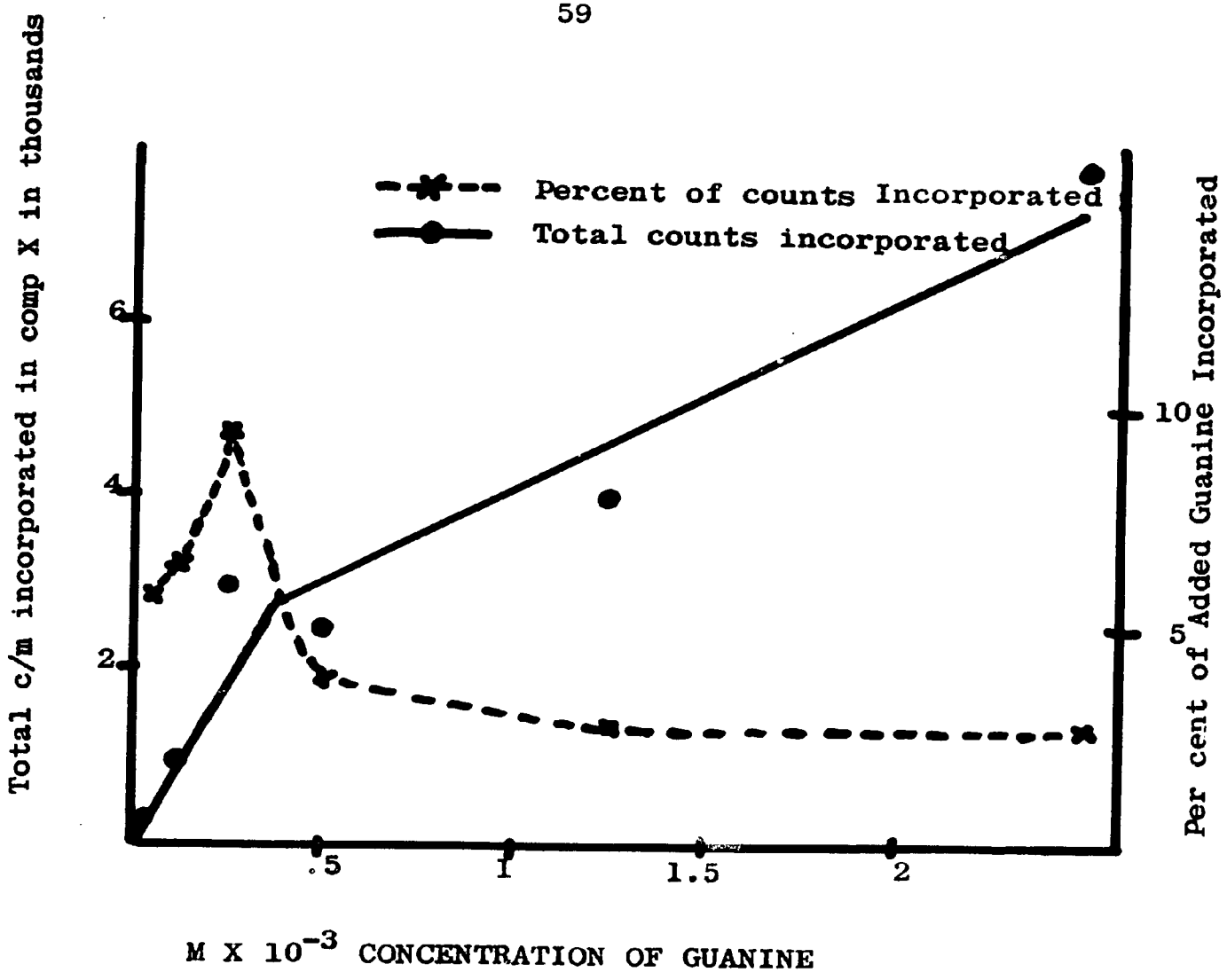


FIGURE 10. THE EFFECT OF THE CONCENTRATION OF GUANINE ON THE INCORPORATION OF C^{14} INTO COMPOUND X. Each tube contained 1 ml of sand extract, adjusted to pH 7.7 with K_2HPO_4, 7.5 mg of glucose, the indicated amount of guanine-2-C^{14} (1.3×10^5 c/m/μmole) and water to a total volume of 1 ml. Incubation 1 hr. at 30° on the reciprocating shaker.

each of a 24 hour old inoculum and grown for various periods of time.

The cells from a flask were harvested and weighed, and the rate of synthesis of riboflavin was determined. Extracts were made from each of these and tested for the production of compound X.

Figure 11 is a scatter diagram showing the relationship between the rate of riboflavin synthesis in whole cells of various ages and the incorporation of guanine into compound X in the corresponding sand extracts. Although the number of points is small, the correlation is obvious. This, however, may have little meaning as the rate of riboflavin synthesis is correlated with the rate of growth and with changes in the age of the cells, the pH of the medium, etc. (See figure 5).

Aeration - Aeration generally increased the rate of production of the compound. The effect of aeration was more dramatic with some extracts than with others.

Acetone powder extracts and dialysis - No significant incorporation of guanine into Compound X could be detected in acetone powder extracts or dialyzed sand extracts. Addition of "alcohol extracts" or "boiled juice" to the dialyzed extracts did not restore the incorporation.

Dilution of the incorporation - The addition of GMP (10 fold the concentration of guanine-2-C^{14}) or of guanosine, (30 fold of the guanine) caused very little dilution of the incorporation of guanine into Compound X.

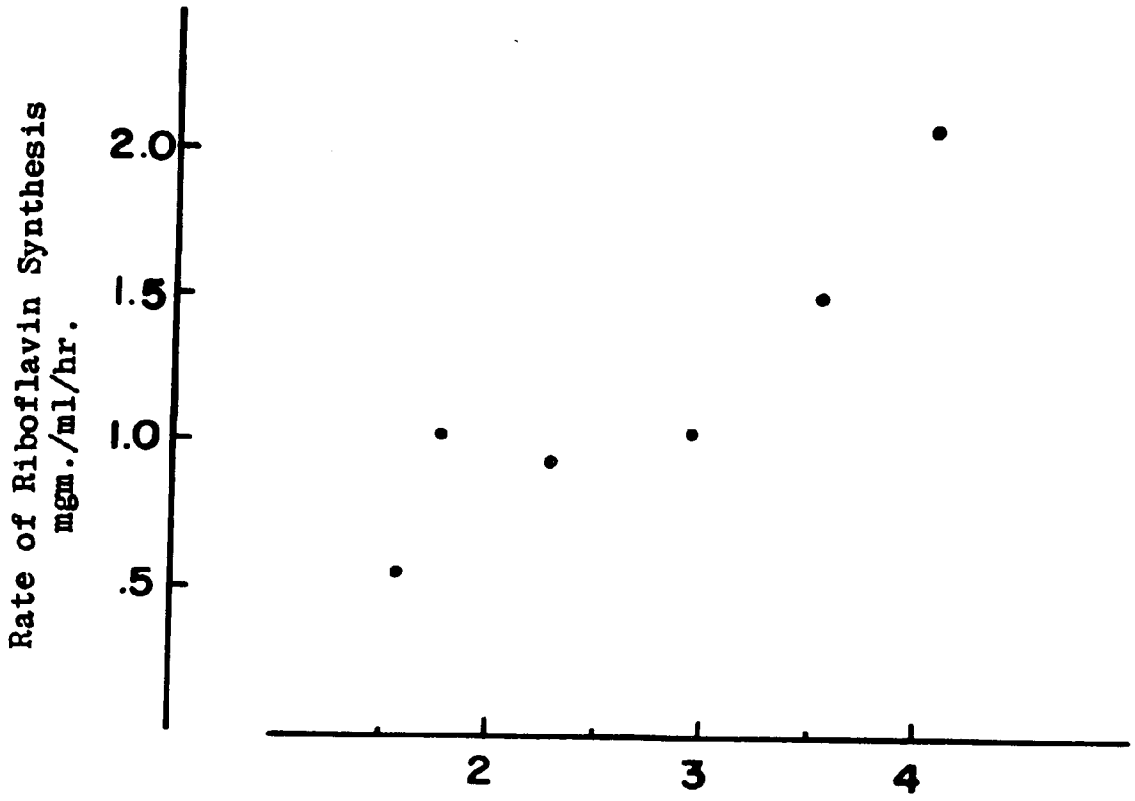


Figure 11 THE CORRELATION BETWEEN RIBOFLAVIN SYNTHESIS AND INCORPORATION OF GUANINE INTO COMPOUND X.

The rate of riboflavin synthesis calculated from a plot of time against riboflavin production.

The rate of formation of compound X in extracts determined by incubating 2 ml sand extract, 100 μmole K_2HPO_4, 0.6 μmole guanine-2-C^{14} (2.5×10^5 cpm per μmole) and 12 mg of glucose in a total volume of 2.4 ml for 1 hour at 30° and assaying for incorporation of C^{14} into compound X.

III. Physical and Chemical Properties of Compound X:

Crystallization - Figure 12 shows crystals of compound X in a microscope field. These crystals are sparingly soluble in water but more soluble in dilute acid and alkali. The melting point was 300-305° with decomposition.

Salt formation - Compound X forms water-insoluble salts with silver and cupric ions but water-soluble salts with barium and calcium. Figure 13 shows that the silver precipitation of compound X follows a curve reminiscent of a dissociation curve with a midpoint around pH 3.5. The silver salt of compound X, like that of guanosine and in contrast to the salt of guanine, is soluble in M NH_4OH.

Ultraviolet Absorption Spectra - The ultraviolet absorption spectra of compound X were run in the Cary Recording Spectrophotometer. Figure 14 shows the spectra and Table 5 summarizes the data. The spectra at the pH values given are for the different ionic forms.

The ultraviolet absorption spectra of a purine derivative usually furnish a powerful tool for deciding the point of substitution on the ring (70-73). However, it may be dangerous to assume that the substitution of a purine nitrogen with a 2-propionate group would affect the absorption spectra in the same way as substituting the same nitrogen with a methyl group or by glycosidic linkage to a sugar. The electronegativity of the substituent and the possibilities of conjugation with the ring influence the spectra considerably. For example, the spectra of 9-

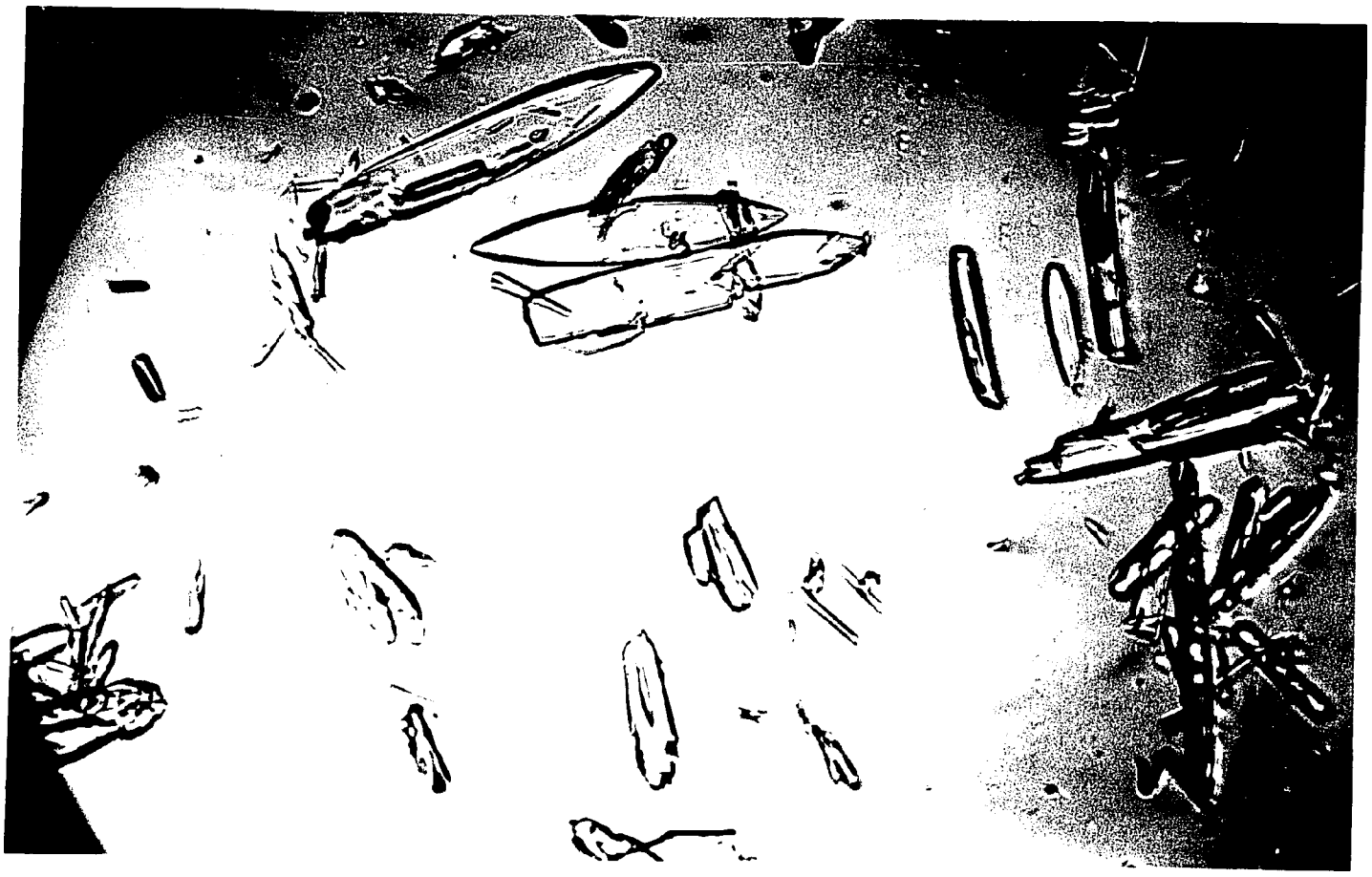


FIGURE 12 - CRYSTALS OF COMPOUND X

See Experimental for details.

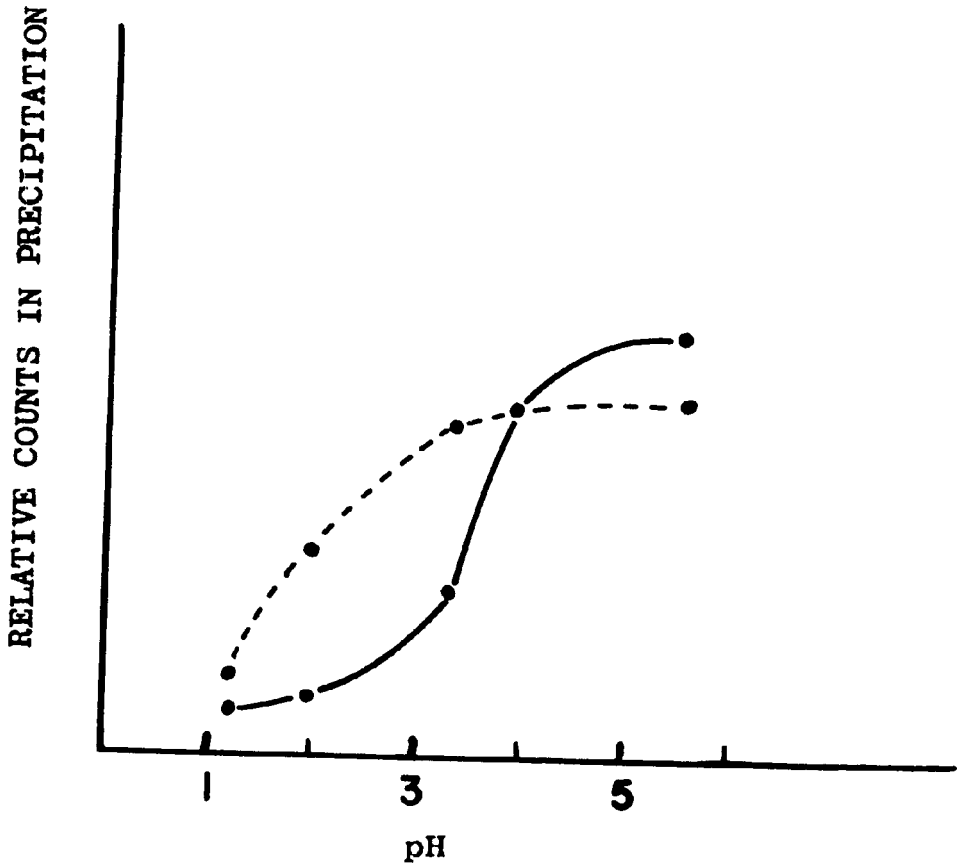


Figure 13. THE SILVER PRECIPITATION OF GUANINE AND COMPOUND X.
Solid line, compound X.
Dotted line, guanine.
The solutions were adjusted with respect to pH and to 3.5 ml volume. To each 1 ml of 10% $AgNO_3$ added, left in ice box overnight, centrifuged and the precipitate dissolved in 2 times 2 ml 0.5 M HCl and assayed.

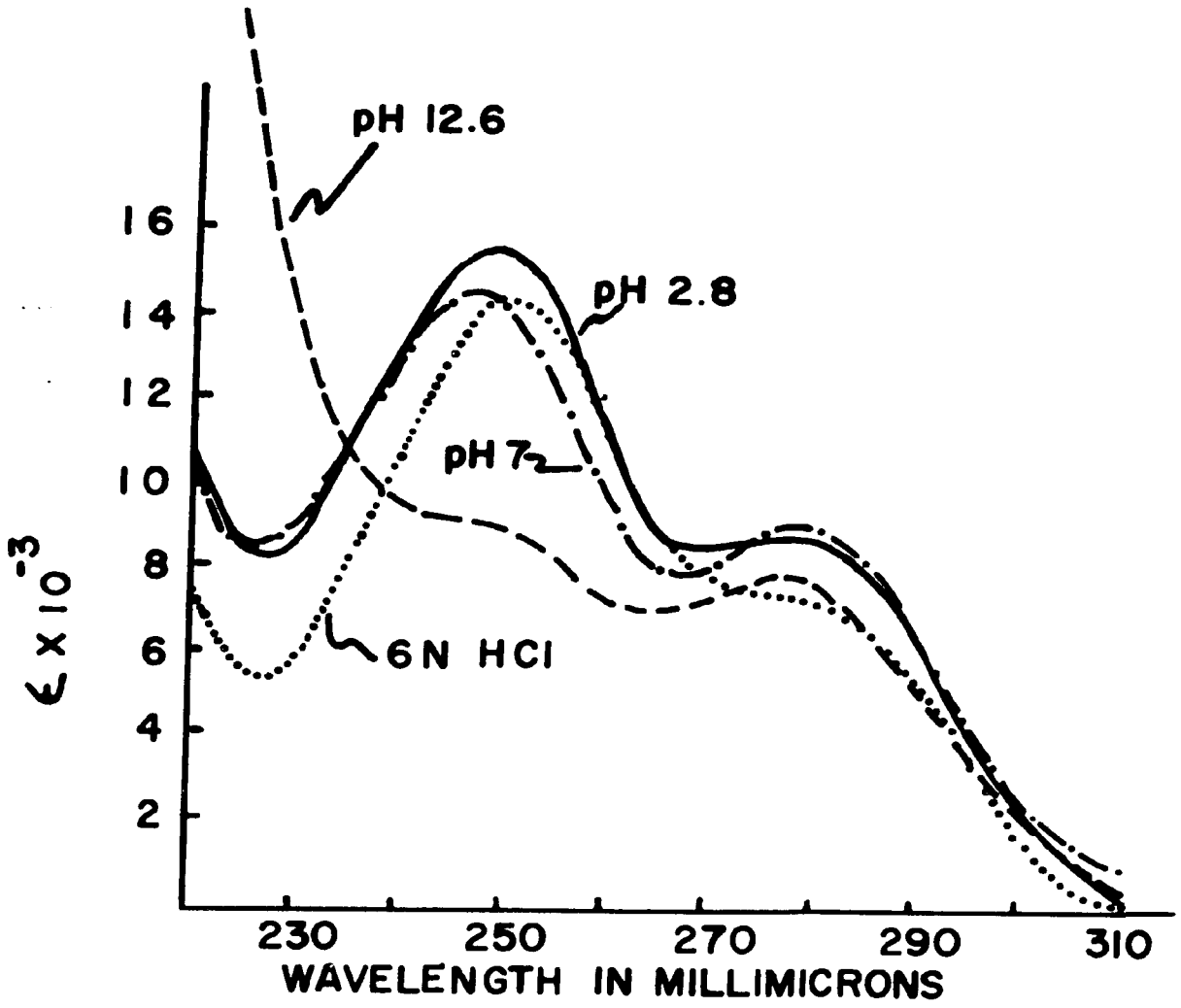


Figure 14. The Ultraviolet Absorption Spectra of Compound X.

TABLE 5

THE ULTRAVIOLET ABSORPTION SPECTRA OF COMPOUND X

Solution	λMax.	∈Max. X 10^{-3}	λMin.	∈Min. X 10^{-3}
6 N HCl	252, 277*	13.4, 6.9	227	5.0
pH 0.4	250, 277	14.3, 7.6	227, 270	4.4, 7.4
pH 2.3	249, 278	15.1, 8.3	227, 272	8.0, 8.0
pH 7	247, 278	14.6, 8.2	227, 267	7.8, 7.2
pH 12.6	245*, 277	8.7, 7.4	264	6.7

* Shoulder only

benzylguanine are vastly different from those of 9-phenylguanine (74). Even subtle changes in the nature of the substituent can effect the spectra perceptibly thus N^2-methylguanine and N^2-ethylguanine show definitely distinguishable spectra (75).

The ultraviolet absorption spectra of compound X are compared with those of some known guanine derivatives in Figures 15-17. The spectra of these compounds are all very similar in weakly acidic solutions, (e.g. pH 1). However, they exhibit large differences in alkaline or strongly acidic solutions.

By spectral titration, see Figure 18, compound X has apparent pK_a values of: 1.4, 3.2, and 10.2. Table 6 summarizes the dissociation constants of various guanine derivatives. The probable assignment of the dissociation constants to the groups in compound X is discussed in the following paragraphs:

The group with a pK' of 10.2 - probably corresponds to the group with a dissociation constant of 9.2 in guanine. The dissociation of the two groups show very similar effects on the spectra. The -CO-NH- group in position 1-6 generally is assigned the pK' 9.2 (79).

The groups with pK' 3.2 and 1.4 - tentatively are considered to be the carboxyl and amino groups respectively. This assignment is preferred because:

1. The pK' of the amino group in guanosine has a value of 1.6. However, there is no reason to assume that a

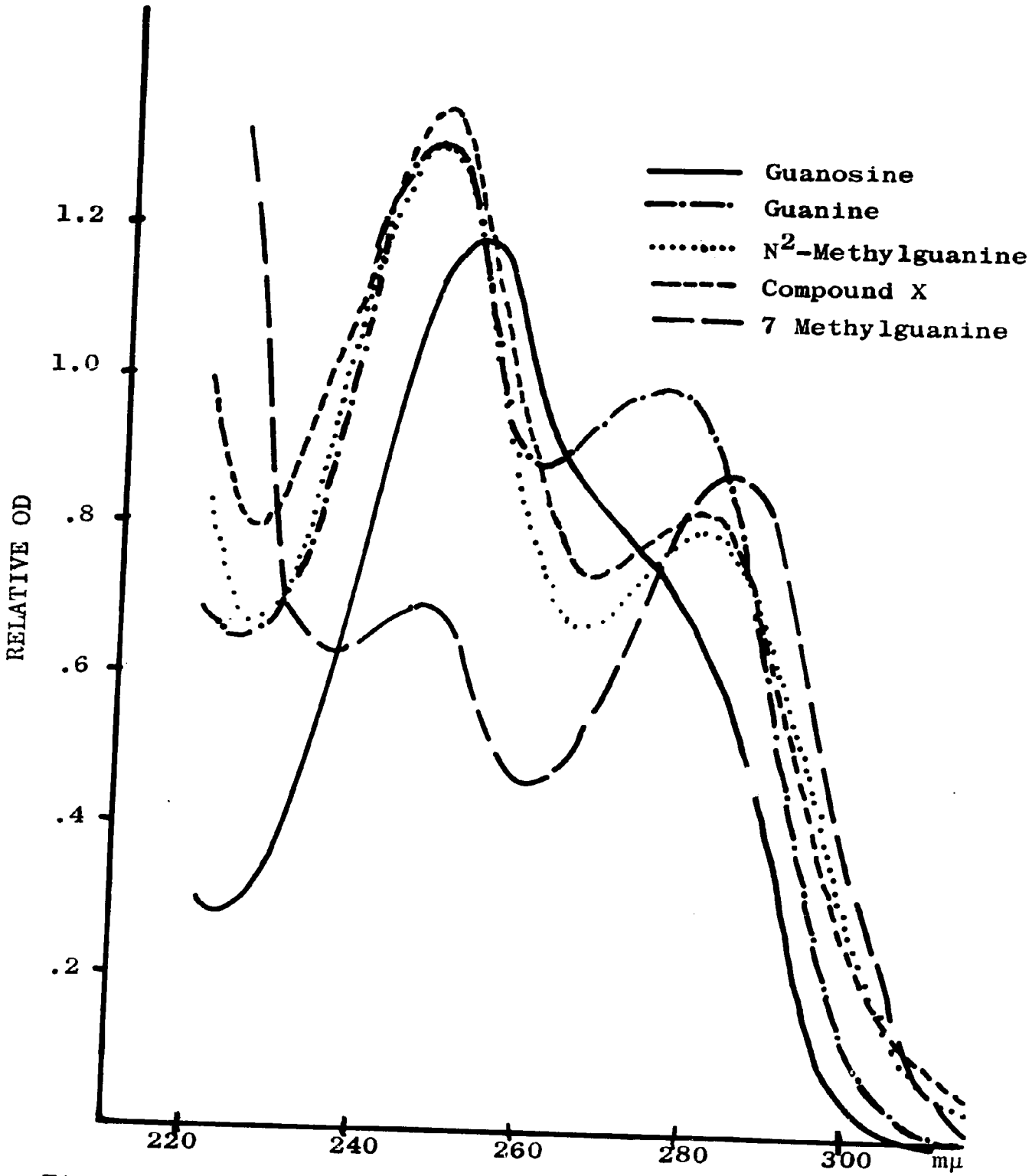


Figure 15. The Spectra of Certain Guanine Derivatives at pH 7. In all cases the OD_{260} of the compound at pH 1 is adjusted to 1.0.

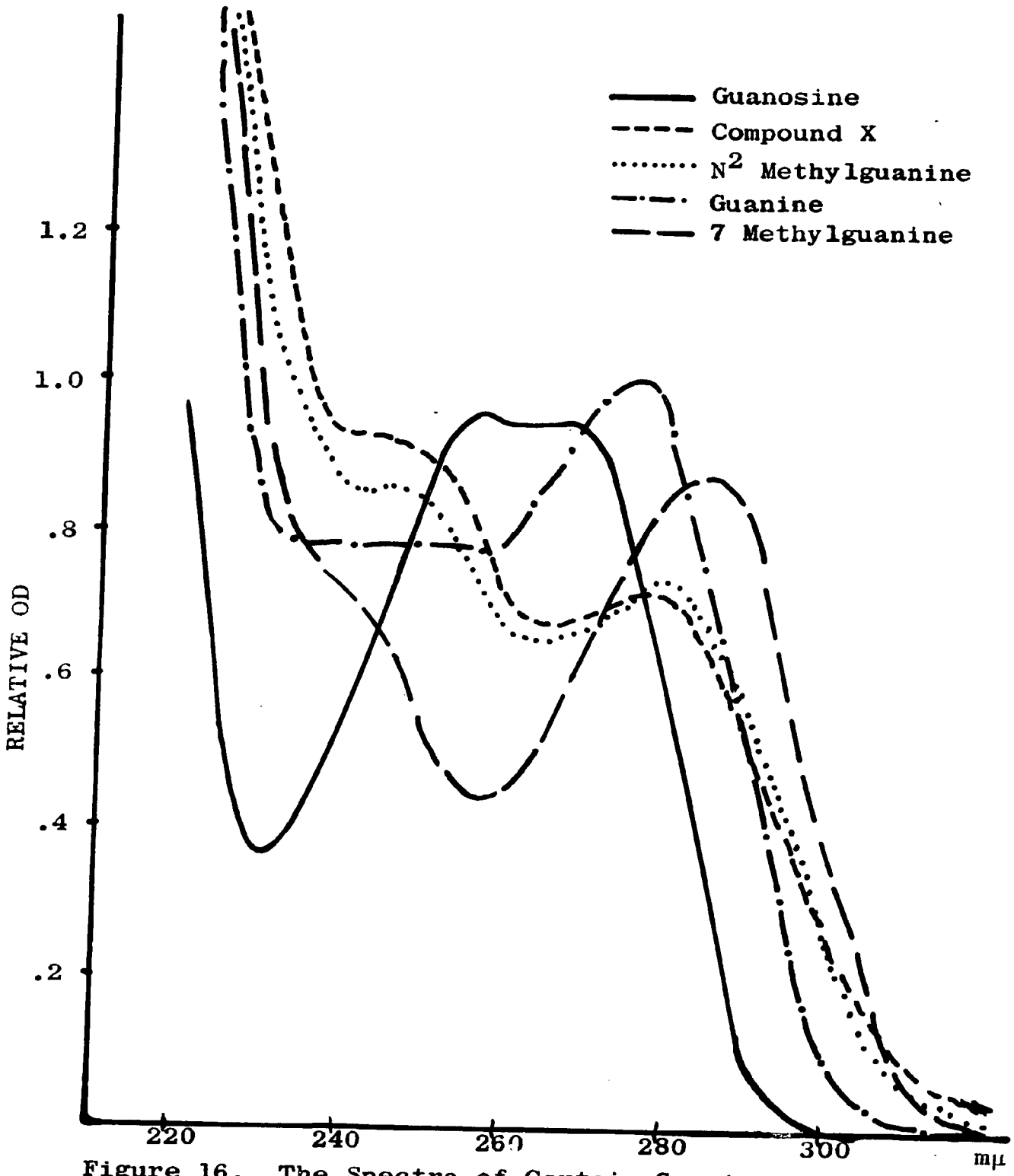


Figure 16. The Spectra of Certain Guanine Derivatives at pH 11. In all cases the OD_{260} of the compound at pH 1 is adjusted to 1.0.

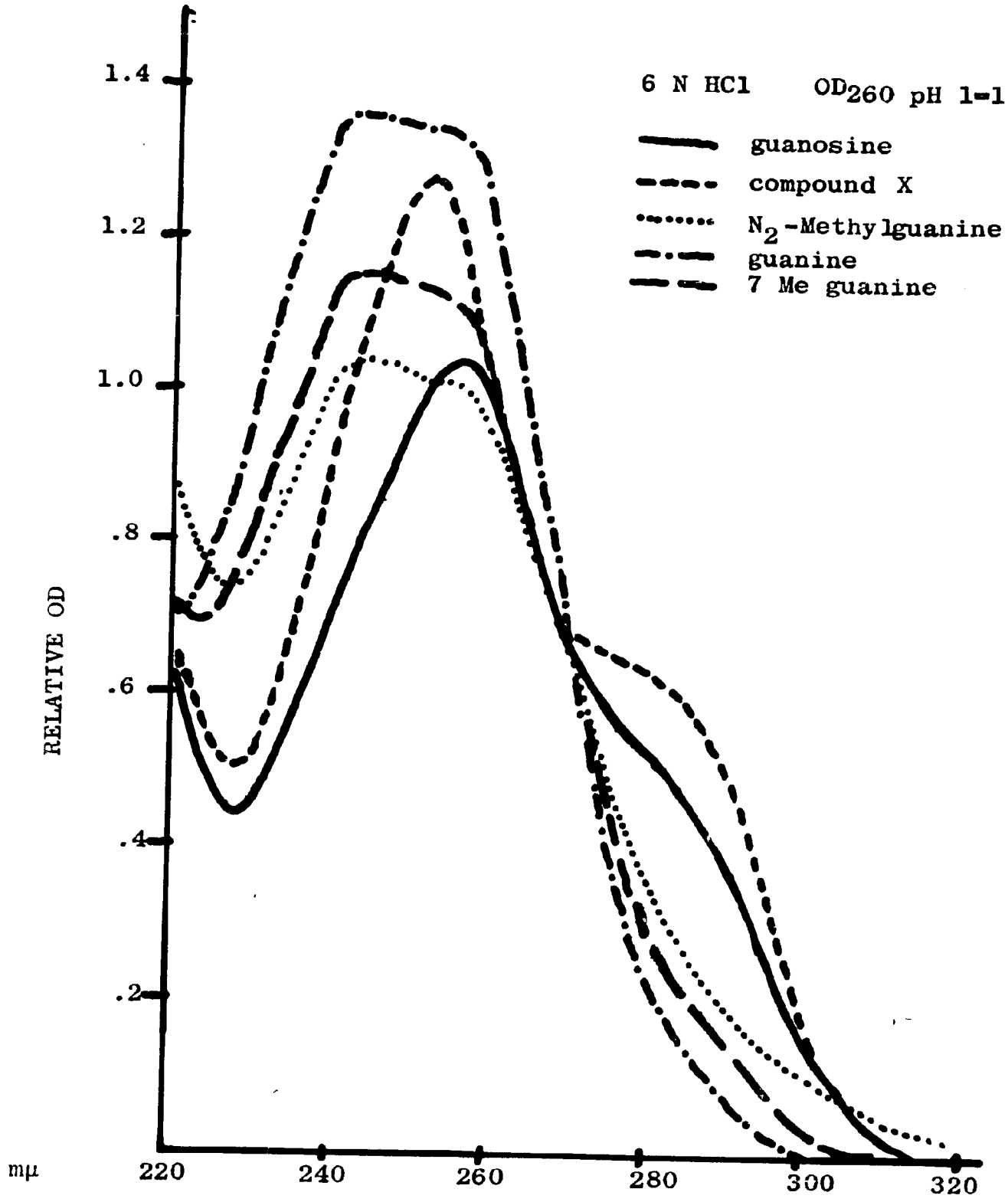


FIGURE 17. The Spectra of certain guanine derivatives in 6 N HCl. In all cases the OD_{260} of the compound at pH 1 is adjusted to 1.0.

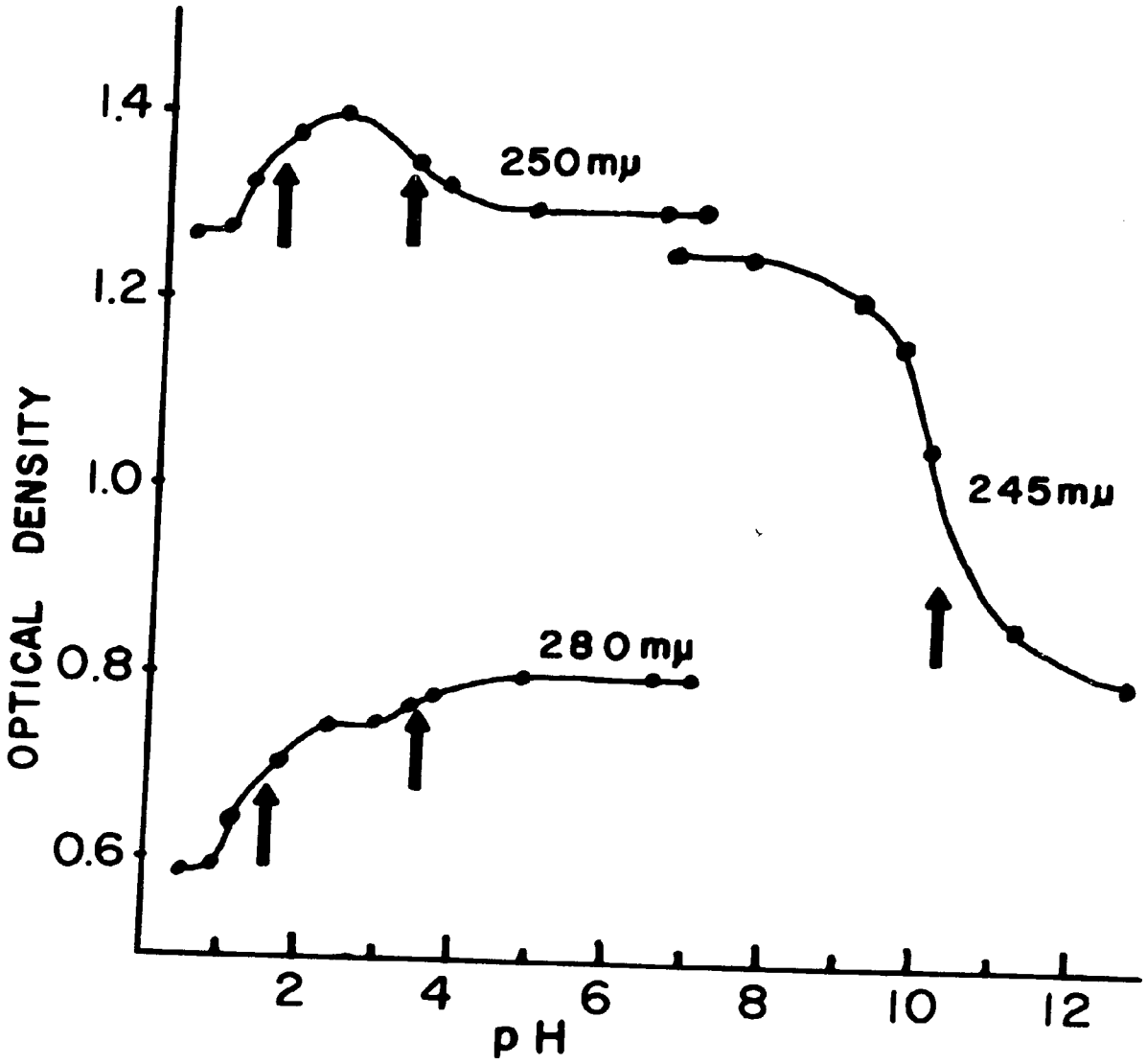


Figure 18. The Spectral Titration of Compound X.

The experiment is described in the Experimental Section.

TABLE 6

THE DISSOCIATION CONSTANTS OF THE AMINO AND HYDROXY GROUPS IN CERTAIN GUANINE DERIVATIVES

COMPOUND	(a) NH_2	(a*) -NH-CO-	REFERENCE
Guanine	3.3	9.2	76
N^2-Methylguanine	3.3	9.8	(b)
7-Methylguanine	3.7	9.3	(b)
Guanosine	1.6	9.2	77
Guanylic Acid (2', 3' phosphate)	2.4	9.3	77
Guanylic Acid (5' phosphate	2.3	9.4	78
Compound X	1.4	10.2	(b)

* Dissociation from the 1-6 position.

(a)Probable Assignment

(b)Determined spectrophotometrically. See Experimental section.

normal carboxyl group would have such a low pK.

2. The behavior of compound X on Dowex-1 points to a pK of about 3 for the carboxyl group. The compound remains adsorbed even at concentrations of 0.05 M ammonium formate, pH 4.4, but is eluted quite rapidly with 0.1 M formic acid (0.012 M formate ion, pH 1.9). This behavior would not be expected, if the carboxyl group had th lower pK since the compound still would have a large net negative charge at pH 1.9.

3. The infra red spectra of compound X lacked the 1845 cm^{-1} band characteristic of $-NH_3^+$ groupings (see infrared spectra).

Infrared Spectra - Compound X crystallized from concentrated formic acid by dilution with water was used for the determination of its infrared spectrum. The spectrum was run in Nujol by the Chemistry Department, The University of Michigan. Figure 19 shows its spectrum. It resembles the infrared spectra of guanine (run simultaneously). One definite conclusion which can be drawn from these spectra is the lack of the peak at 1845 cm^{-1} associated with a charged amino group (such a peak also is found with N^2-methylguanine hydrochloride). This method of preparation (precipitation from concentrated formic acid and drying under high vacuum) should lead to the compound in its isoelectric condition. The compound prepared in this way had no significant amount of formate (see analysis). On the reasonable assumption that the compound is isoelectric the

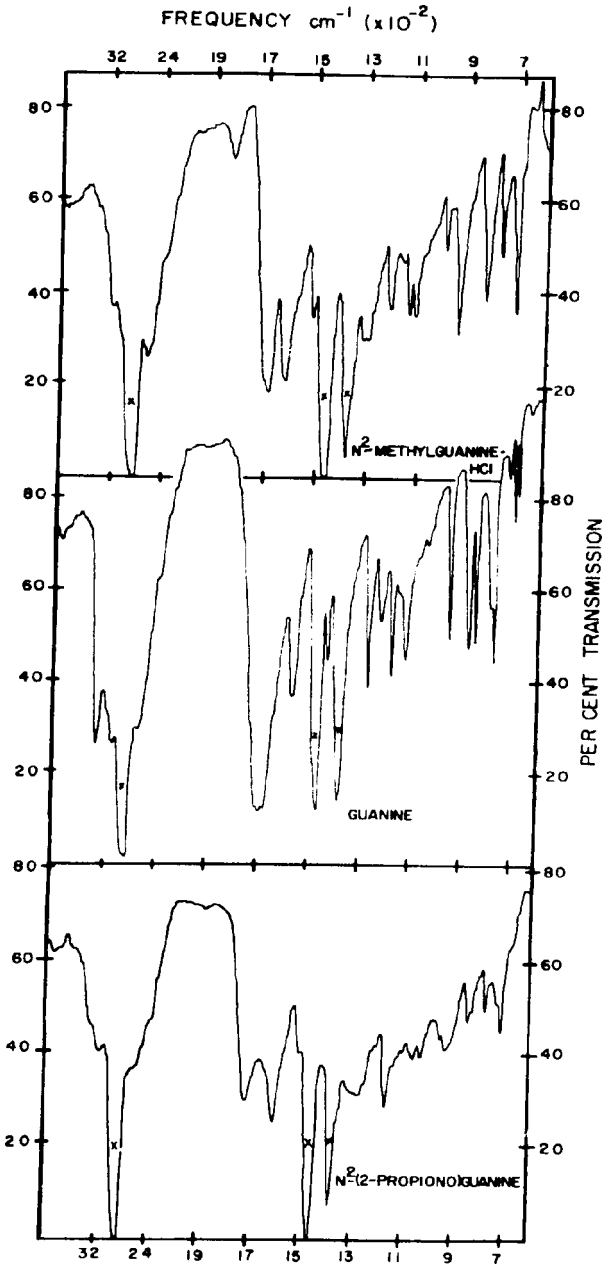


FIGURE 19. **The Infrared Spectra of Guanine, N^2-Methylguanine and Compound X in Nujol.** The peaks marked with X are Nujol peaks.

absence of a charged amino group suggests that the pK_a of its amino group is lower than the pK_a of its carboxyl group.

Fluorescent Spectra - Fluorescent spectra of compound X and of various other guanine derivatives were run in the Aminco-Bowman Spectrophotofluorometer at a concentration of 0.01 μmoles per ml in 0.1 M HCl*. The excitation wavelength was fixed in all cases at 285 mμ (maximum for guanine).

Table 7 shows the results. Clearly compound X shows lower fluorescence than guanine, whereas all other guanine derivatives which were tested showed a greater fluorescence than guanine. The position of the maximum is nearest to that of 7-methylguanine and guanine and resembles least the fluorescence of N^2-methylguanine which shows a double peak.

Paper chromatography - Table 8 shows the R_F values of compound X in various solvents. Guanine is included for comparison.

Various Reagents and Analysis - Purified compound X gave the following negative tests: organic phosphate, orcinol reaction, Bratton-Marshall reaction, Bratton-Marshall reaction after treatment with 6N HCl 1 hour at 100°, reduction with the $FeCl_3$-NH_3 reagent, reduction of 2,6-dichlorophenol indophenol and the Folin uric acid reagent reaction. In all these cases, a standard was added at the end of the reaction, or tested in the presence of X and shown to give the reaction.

The compound was not acted upon by guanase and xanthine oxidase, singly or in combination.

* The author is indebted to Dr. P. Pal for his help in this study.

TABLE 7

THE FLUORESCENCE SPECTRA OF CERTAIN GUANINE DERIVATIVES

Compound	λ Max. mμ	Relative Intensity
Guanine	355	7.4
Guanosine	380	13.6
7-Methylguanine	355	9.4
N^2-Methylguanine	365, 410	11.5, 14.4
Compound X	350	4.7

Excitation light 285 mμ, pH 1

See text for details.

TABLE 8

THE R_F VALUES OF COMPOUND X COMPARED WITH GUANINE

Solvent	No.	R_F Compound X	R_F Guanine
Isobutyric-NH_3	1	0.44	0.47
Butanol-Ethanol-Water	2	0.08	0.28
Butanol-Acetic Acid (Equilibrium)	3	0.34	0.11
Ethanol-NH_4acetate, pH 8.5	8	0.60	0.59
Ethanol-NH_4acetate, pH 8.4 - saturated with $Na_2B_4O_7$	9	0.65	0.63
77 per cent Ethanol	-	0.35	0.63
Propanol: Conc.NH_4OH:H_2O 60:30:10	-	0.21	0.17
Methylethyl ketone: Butanol: Conc. HCl: H_2O, 30:40:20:10	-	0.19	0.03
70 per cent Propanol, diethylamine 100:1	-	0.42	0.28
Propanol: 2N NH_4OH; 4:1	10	0.19	0.21

Periodic Acid Oxidation - Compound X did not take up any periodate at 0° in the dark over a period of 120 hours. Over this period FMN, riboflavin and adenosine-5-phosphate standards took up the theoretical amount of periodate. However, solutions of compound X as isolated on Dowex-1 formate columns did reduce periodate at 50° and at a rate comparable to that of FMN. Whether or not this uptake was due to contaminating material reacting with periodate at the high temperature of the reaction (80) (formate; example) is not known.

Reaction with Phenol Reagent - Compound X reacts with the Folin phenol reagent to give the same molar extinction coefficient as guanine. The reacting group in guanine is unknown. However, N^2-methylguanine, 1,3-dimethylxanthine and 2,4,5-triamino-6-hydroxypyrimidine were found to give the reaction, whereas guanosine, 7-methylguanine and 1,7-dimethylxanthine did not.

It seems from the above test that substituting the imidazol ring prevents the reaction.

Reaction with the Pauly Reagent - Compound X gives a positive reaction with the Pauly reagent. Table 9 describes the nature of chromophores formed by various purines with the reagent.

Acid Degradation of Compound X - Emil Fischer showed that upon refluxing guanine with 6 N HCl xanthine was formed (81). At higher temperatures the xanthine and guanine start to break down to glycine (position 4,5, and 7) and

TABLE 9

REACTION OF COMPOUND X AND VARIOUS XANTHINE AND GUANINE DERIVATIVES WITH THE PAULY REAGENT

Compound	λ Max. of Color	$\frac{OD_{Max.\ Pauly}}{OD_{260\ Original\ Comp.}}$
Guanine	440	0.72
Guanosine	417	0.27
N^2-Methylguanine	<350, 450	0.033
Compound X	460	0.80
1,7-dimethylxanthine	-	-
1,3-dimethylxanthine	420	0.18
Xanthine	425	0.65

Following the reaction, the spectra of the reaction mixtures were run in the Cary Recording Spectrophotometer between 350 and 650 mμ.

guanidinoimidazole (82), CO_2 (carbons 2 and 6), formic acid (carbon 8) and ammonia. Guanidinoimidazole will eventually yield glycine, ammonia, formic acid and CO_2. N-methylated xanthine derivatives form methylamine except when the substitution is on position 7. Then sarcosine (N-methylglycine) results. When compound X was refluxed for 60 hours in 6 N hydrochloric acid, xanthine, alanine, glycine and lactic acid were formed as hydrolysis products.

Identity of the xanthine - The ultraviolet absorption spectra of the solution after hydrolysis with hydrochloric acid were identical with the spectra of xanthine at various pH values. When the solution was neutralized and diluted and treated with xanthine oxidase, the resulting absorption spectrum was indistinguishable from that of uric acid. When this reaction was followed in the Cary Spectrophotometer recording the entire spectrum at intermediate times, all the curves crossed at two isobestic points characteristic of the conversion of xanthine to uric acid, suggesting that xanthine oxidase was acting on a single compound. The formation of uric acid as measured by the change in optical density at 290 mμ after the action of xanthine oxidase was 92 per cent of theory on the basis of the extinction coefficients of xanthine and uric acid. Table 10A compares the R_F value of authentic xanthine with the xanthine derived from acid hydolysis.

Identity of alanine and glycine - The acid-hydrolyzed solution of compound X was chromatographed on paper with

TABLE 10

CHROMATOGRAPHIC IDENTIFICATION OF THE PRODUCTS OF ACID HYDROLYSIS OF COMPOUND X

A. Ultraviolet-Absorbing Spots

Solvent	R_F Values		
	Xanthine	Hydrolyzed Compound X	Mixture
Isobutyric-NH_3 (#1)	.17	.16	.16
Butanol-acetic acid (#3)	.11	.11	.11
Butanol, ethanol, water (#2)	.37	.37	.37
Propanol, conc. NH_4OH, water; 60,30,10	.29	.29	.29
77 per cent ethanol	.34	.35	.34

B. Ninhydrin Spots

Solvent	R_F Values		
	Alanine.HCl	Glycine HCl	Hydrolyzed Compound X
77 per cent ethanol	.61	.31	.32,.62
70 per cent propanol, diethylamine, 100:1	.43	.36	.33,.42
Methanol, pyridine, water, 80,4,20	.50	.34	.34,.50
Butanol, acetic acid, water; 2,1,1 (fresh)	.43	.34	.33,.43

4 solvents, together with glycine and alanine as standards. Upon spraying with ninhydrin 2 spots developed corresponding in R_F and colors to the glycine hydrochloride and alanine hydrochloride (see Table 10B). The determination of the configuration of the alanine by the use of snake venom as the L-amino acid oxidase was found not too feasible because of the very slow reaction with known L-alanine.

Quantitation - Twenty-two μmoles of compound X refluxed for 60 hours in 6 N HCl led to the formation of (1) 9.3 μmoles of xanthine (by ultraviolet absorption), (2) 18.2 ± 0.9* μmoles of alanine, (3) 13.3 ± 0.2* μmoles of glycine, and (4) 1.7 μmoles of lactic acid (colorimetric) or 1.7 μmoles of L-lactic acid (lactic dehydrogenase).

The glycine which reflects the breakdown of the purine ring, and the xanthine add up to 22.6 μmoles (103 per cent recovery). The alanine and the L-lactic acid account for 19.9 μmoles (91 per cent recovery of the propionate residue).

Neutral Hydrolysis of Compound X - Upon boiling in neutral solution compound X breaks down to give guanine and L-lactic acid. This breakdown seems to occur at all pH values between 3 and 12. With different preparations of compound X the rate of the reaction was different suggesting that it might be catalyzed by an impurity. However, in contrast to the breakdown of 6-succinamino-purine and some of its derivatives (83), the addition of calcium or ferric ions did not increase the rate.

* Standard deviation of the mean. Glycine and alanine were determined by extracting the ninhydrin color from paper chromatograms. The ninhydrin data are based on 5 determinations from two different solvents.

Identification of Guanine - Guanine was identified by paper chromatography with solvent #2, by its ultraviolet absorption spectra at various pH values, by its reaction with guanase and xanthine oxidase and by its behavior on Dowex-50 columns. Finally carrier guanine was added to compound X which was highly labeled with C^{14}, the mixture was separated on Dowex-50, adsorbed to and eluted from charcoal, and separated successively in solvents 1, 2, and 3. The spots on paper corresponding to guanine were cut out and the radioactivity determined. The specific activities agreed with the dilution by carrier guanine, providing evidence that the breakdown product was guanine.

Identity of L-lactic acid - Lactic acid was identified by its R_F on paper in 85 per cent propanol:diethyamine; 100:1 (R_F 0.54 lactic acid 0.53), its reaction with crystalline lactic dehydrogenase and the specific colorimetric test.

In one experiment the lactic acid was extracted from the paper and measured by lactic dehydrogenase and DPN. A 2,4-dinitrophenylhydroazone was made from the product. It migrated on paper chromatograms to the same R_F values as the known 2,4-dinitrophenylhydrazone of pyruvic acid (2 solvents).

Quantitation - Table 11 shows the stoichiometry of two experiments. A near 1:1 relationship exists between formation of guanine and lactic acid as well as between total lactic acid and L-lactic acid. This provides evidence suggesting that all the lactic acid produced is the L-form.

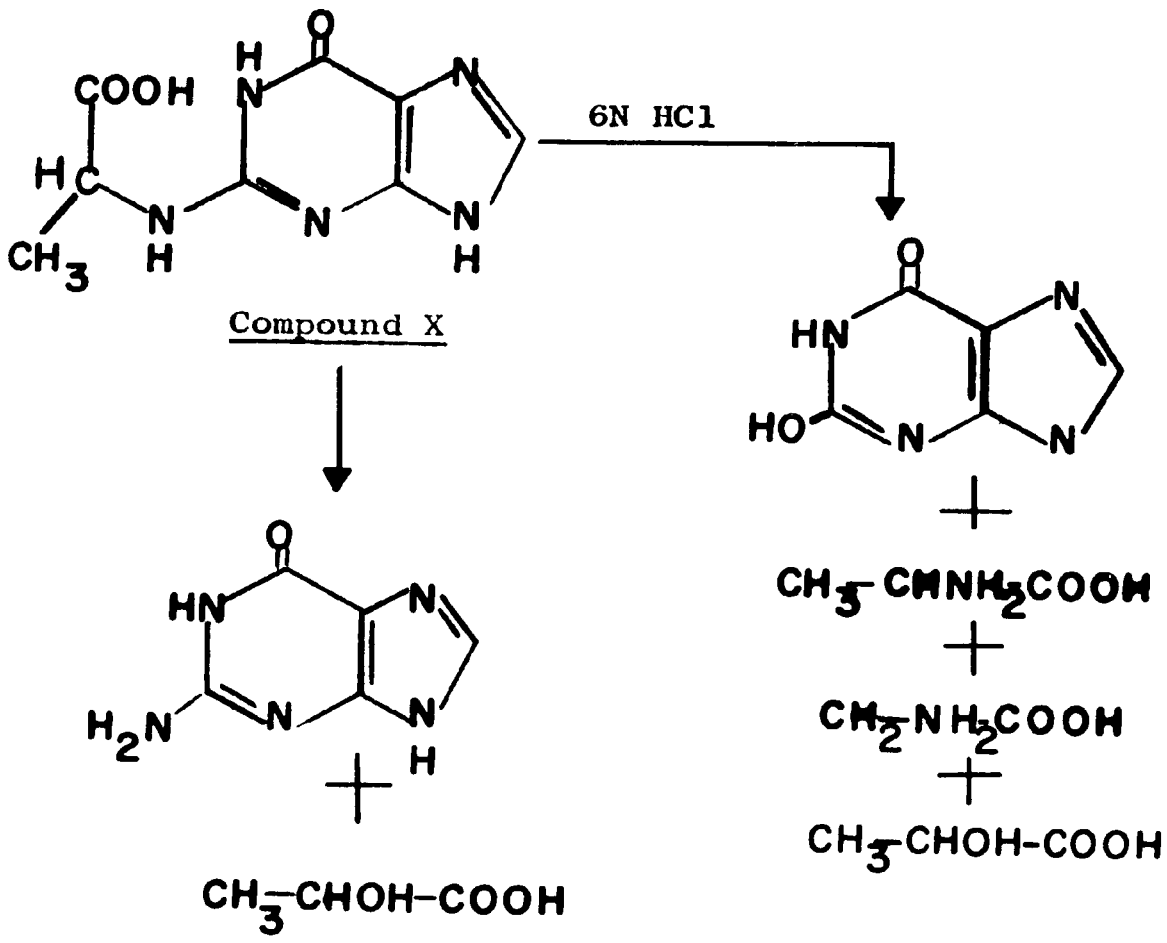


Figure 20. The Degradation of Compound X.

See text for details.

TABLE 11

STOICHIOMETRY OF NEUTRAL HYDROLYSIS

Exp.	Starting Material	Products		
	µmoles compound X/ml	µmoles guanine/ml	µmoles total lactic acid/ml (c)	µmoles L-lactic acid/ml (d)
1[a]	2.5	0.91	1.12	0.91
2[b]	2.1	-	1.21	0.99

(a) Refluxed 88 hours in 0.05 M $KHCO_3$.

(b) Refluxed 96 hours in water, pH 5.

(c) Determined colorimetrically.

(d) Determined with lactic dehydrogenase and DPN.

Chlorine and Permanganate Oxidations - Chlorine and permanganate oxidations of compound X were tried in an attempt to demonstrate the position of the propionyl residue on the guanine.

Chlorine oxidation of guanine leads to the formation of guanidine (C_2) and parabanic acid ($C_{4,5}$, and 8). In turn parabanic acid breaks down partially to urea (C_8)(57). Preliminary experiments on the oxidation of compound X with chlorine led to the formation of guanidine (identified by paper chromatography) only small amounts of urea and no parabanic acid. Attempts to demonstrate the formation of 5-methylhydantoin, which is the expected product if the propionic acid residue is on the imidazole ring, were inconclusive.

Permanganate oxidation of guanine is similar to chlorine oxidation since it leads to the formation of guanidine (C_2) and urea (C_8) (59). When compound X was oxidized with permanganate, guanidine was obtained, but no urea or ureidopropionic acid could be detected.

Analysis - The elemental analysis for carbon, hydrogen and nitrogen and the carboxyl analysis were performed on a crystalline sample of compound X dried at 30° in vacuo for 10 hours. Huffman Microanalytical Laboratories, High Count, Colorado performed the analysis.

The results and the expected values for a propionate derivative of guanine are reported in Table (12).

TABLE 12

THE ELEMENTAL AND CARBOXYL ANALYSIS OF COMPOUND X

Found	Calculated for: $C_8H_9N_5O_3.1/2\ H_2O$
C 40.96	41.3
H 4.51	4.31
N 29.35	30.2
O* 23.18	24.1
Carboxyl 20.5	19.4

* By difference

Reasonably good agreement between the reported and the expected values was observed if 0.5 moles of water of crystallization was assumed.

BRIEF SUMMARY OF RESULTS

I. In vivo Experiments

A. Guanine was not incorporated into nucleic acid adenine.

B. There was no acid-soluble nucleotide, (except riboflavin derivatives) of E. ashbyii, which was a non-guanine compound and which was labeled from guanine-2-C^{14}.

C. Five inhibitors which caused a decrease of the rate of synthesis of riboflavin did not bring about the accumulation of intermediates in its synthesis.

II. Experiments with Extracts

A. Only very little incorporation of guanine-2-C^{14} into riboflavin was obtained.

B. A new guanine derivative was discovered.

C. Conditions which bring about the accumulation of the new derivative were studied.

D. A large scale preparation of the compound was developed. The compound was purified by ion exchange chromatography and crystallized.

III. The Structure of the New Guanine Derivative

A. The compound had ultraviolet, infrared and

fluorescent spectra similar to those of guanine.

B. It showed pK'_a values of 1.4, 3.2 and 10.2.

C. The compound was positive to the phenol and Pauly reactions, gave a negative test for phosphate and was negative with orcinol, periodate, nitrous acid, uric acid reagent, the Bratton-Marshall test and various reagents for reducing groups.

D. Boiling the new compound in neutral solutions yielded guanine and L-lactic acid in stoichiometric amounts.

E. Degradation of the compound in 6 N HCl yielded xanthine, alanine, glycine and L-lactic acid in stoichiometric amounts.

F. Chlorine degradation failed to yield definitive results.

G. The elemental analysis agreed with the structure: $C_8H_9N_5O_3 . 1/2\ H_2O$ or propionoguanine.

GENERAL DISCUSSION

The studies reported in this thesis have dealt with a search for possible intermediates in the conversion of C^{14}-guanine to riboflavin. No intermediates were found in studies with normal or inhibited cells. In a study of the possible formation of riboflavin in cell-free extracts, guanine was converted to a new guanine derivative, propionoguanine. although it is not known whether this new compound is an intermediate in riboflavin biosynthesis, a major part of this thesis is concerned with its formation, isolation, properties and structure.

A. Studies with Whole Cells - In E. ashbyii the synthesis of riboflavin is quantitatively a major pathway of purine metabolism. A culture will produce as much as 1 millimole of riboflavin per liter. This amount is at least as great as the quantity of purines in the nucleic acids. However, both normal and inhibited cells failed to show a detectable accumulation of riboflavin intermediates (as measured by labeling from guanine-2-C^{14}), even though 5 millimicromoles of an intermediate could have been detected. This is not an uncommon situation. Similar instances in which an intermediate in a major metabolic pathway occurs only in small amounts are well known. Obviously the accumulation under normal conditions is a function of a limiting

enzyme concentration, of K_m values and of the equilibrium constants of the several enzymes concerned.

The failure of these isotopic and chromatographic techniques to detect intermediates such as compounds G or V which Masuda (20) has reported to occur in E. ashbyii (compound G, 30 μmoles per liter and compound V, 3 μmoles per liter) perhaps may be explained on the basis of differences in strains.* In a subsequent experiment, we succeeded in isolating a small amount of compound V after a 3 hour incubation of the culture with guanine-2-C^{14}. The total radioactivity in the isolated material represented an incorporation of less than 1.4×10^{-4} μmoles of C^{14} guanine per liter of culture.

The use of inhibitors to accumulate intermediates may be a very logical approach, but this is true only if the inhibitors used are specific for the pathway under study and if the reactions to the intermediate are not in some way dependent on its normal synthesis. Often, as in the case of this work, it is almost impossible to tell whether an inhibitor is acting specifically on a certain pathway or is affecting it only through indirect means. Thus, the accumulation of an intermediate through the use of unstudied

* Dr. D. H. Hayes in our laboratory also has tried to repeat the work of Masuda with 4 different strains of E. ashbyii. In no case has he been able to detect compound G though he has found small quantities of V (84). From a consideration of his techniques it can be concluded that there could not have been more than 0.1 μmoles of compound G per liter in any of these strains. Furthermore, the quantity of compound V varied greatly from strain to strain. A 32 hour incubation of the culture with guanine-2-C^{14} brought about labeling of compound V.

inhibitors becomes a matter of trial and error. Even an inhibitor which is specific for a particular pathway does not necessarily have to lead to the accumulation of an intermediate in the pathway if equilibrium conditions are strongly against such an accumulation. It now is clear that we might have decided that these apparent inhibitions of riboflavin synthesis were on a level prior to purine by adding purine derivatives to determine whether the "inhibitions" were overcome.

B. Isolation and General Structure of Compound X (Propionoguanine) - Despite the fact that propionoguanine is a free base, its anionic character at pH 4 facilitates its separation from a crude reaction mixture and from the parent C^{14}-guanine. This property also has been used to develop a relatively simple assay for the compound. In addition, because of the carboxyl group, the silver salt of propionoguanine behaves very differently from those of the usual purines and purine derivatives. On the other hand, in hydrochloric acid solution it behaves on Dowex-50-H columns as a cation similarly to guanine.

The compound has been tentatively identified as N^2-(2-propiono)guanine on the basis of the presence of guanine and a threecarbon acid as component parts, the correspondence to the empirical formula, and measurement of equivalent weight by determination of the carboxyl group.

The evidence for the presence of guanine in the molecule is as follows:

(1) The ultraviolet, infrared and fluorescence spectra are all similar to guanine. (2) In extracts of the mold, the compound is labeled by both guanine-2-C^{14} and guanine-8-C^{14}. (3) Guanine, identified by chromatography, spectra, and enzymatic analysis, is formed from propionoguanine by neutral hydrolysis at 100°. (4) Xanthine and glycine are formed from the compound by hydrolysis with 6 N HCl at 100°.

The evidence for the propionic acid moiety and for its attachment to guanine through carbon 2 is: (1) The production of lactic acid from the compound on neutral hydrolysis, (2) the production of alanine from the compound by hydrolysis with 6 N HCl.

C. The Position of Attachment of Propionate to Guanine - The production of alanine from propionoguanine by hydrolysis with 6 N hydrochloric acid places the propionate residue on one of the nitrogen atoms: 1,2-amino,3 or 9. These positions all yield NH_3 on vigorous acid hydrolysis. Nitrogen atom 7 will be found as the amino group of glycine after strong acid hydrolysis. While some evidence is conflicting as to which nitrogen atom is substituted, the bulk of the evidence suggests attachment to the amino group. This evidence is summarized as follows: (1) The ultraviolet spectra of the compound at all pH values except in 6 N HCl is more like N^2-methylguanine than any known guanine derivative (see Figures 15 and 16). (2) A balance of the products of the hydrolysis in 6 N HCl excludes all other possibilities (see acid hydrolysis under "Results" and (3)

the inability to deaminate propionoguanine is strong evidence for substitution on the amino group of guanine. In support of this evidence are the following findings: (1) Propionoguanine and N^2-methylguanine are the only two guanine derivatives studied or found in the literature which show a significant elevation of the pK' of the -CO-NH-dissociation (see Table 6). It appears that the amino group and its electron-donating substituents in some manner interact with the 1-6 grouping to repress the ionization. Possibly the effect is via the ring or by spatial effects including hydrogen bonding. (2) Propionoguanine and N^2-methylguanine both show a suppression of the 1690 cm^{-1} band in the infrared. This band is assigned to the C-O bond stretching in position 6 on guanine (85). In turn the N-H stretching band which is very pronounced in guanine is almost completely suppressed in methylguanine and propionoguanine. (3) The reaction of the compound with the phenol reagent is evidence against attachment to one of the imidazole ring nitrogens (see Results).

Certain facts are difficult to reconcile with substitution on the amino-N of guanine. In general, our experiments tend to rule out substitution on nitrogen atoms 1,3, and 7. However, the following observations point to substitution on N-9 instead of on the amino group: (1) Both propionoguanine and guanosine show similar ultraviolet spectra under certain conditions. Each shows a peak at 250 mμ and a shoulder at 280 mμ in 6 N HCl. The ultraviolet spectra of

guanine, 7-methyl- and 2-methylguanine all show broad maxima at 260-270 mμ at this concentration of acid (see Figure 17). (2) The pK_a' of the amino group of propionoguanine is close to that of guanosine. Both of these compounds have amino group dissociations which are 1.5-2 pK units lower than the pK_a' of the amino group in guanine or N^2-methylguanine (see Table 6). (3) The preliminary results of the chlorine and permanganate oxidations show that guanidine is produced in the oxidation of propionoguanine. (4) The new compound, in contrast to N^2-methylguanine, gives a positive Pauly reaction. (5) The fluorescence spectrum of the compound does not resemble that of N^2-methylguanine which shows a double peak. Naturally these findings are disturbing as they tend to argue against the observations which place the propionic acid group on the amino group. However, the interpretation of such results as the protonation effect and fluorescence spectra is difficult. The results of the hydrolysis experiments are very strong in comparison to these findings.

Unfortunately the results of the chlorine and permanganate oxidations were not conclusive. These degradations should in theory yield derivatives of propionic acid which are characteristic of its point of attachment to the ring. If it is attached to a nitrogen on the imidazole ring, ureidopropionic acid and 5-methylhydantoin should be formed. If it is on the amino nitrogen or the 1 or 3 nitrogen N-(2-propiono)guanidine ought to be a product. Our inability

to detect these derivatives may be due, at least partially, to the low yields of products and the complication of side reactions. The degradation of certain guanine derivatives with chlorine is very poor and yields side products (86). A careful study of this degradation and a large scale run of propionoguanine will have to be made before the problem can be resolved. A study of the structure of the original derivative, which on acid hydrolysis leads to the formation of propionoguanine, or of its metabolism may provide evidence for the structure. Total synthesis, of course, would give an unambiguous answer.

Another problem which has not yet been resolved is whether the configuation of the 2-propionic acid moiety is D or L. Neutral hydrolysis of propionoguanine yields L-lactic acid, but it is not known whether an inversion has taken place during the hydrolysis. The configuration of the alanine liberated on acid hydrolysis is under investigation with stereospecific enzymes.

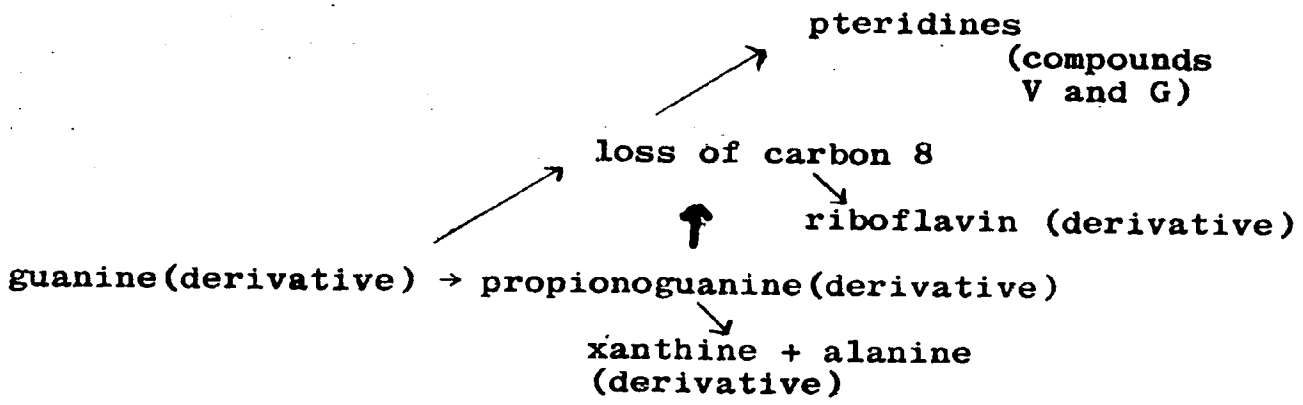
D. <u>The Role of Propionoguanine</u> - There is good indication that propionoguanine as isolated is an artifact of the preparation which involves hydrolysis in strong mineral acid. The original compound is probably a nucleotide or a nucleoside. Whether the original compound is an intermediate in riboflavin synthesis is unknown. Certainly the accumulation of a propionoguanine derivative in extracts but not in whole cells suggests that it may be an intermediate in either a synthesis or a degradation. The quantity of

this compound formed in the extracts is of the same order of magnitude as the quantity of riboflavin formed in whole cells. In fact a rather good correlation exists between the synthesis of riboflavin in various cultures and the synthesis of propionoguanine in extracts prepared from these cultures. This is circumstantial evidence that its original derivative is an intermediate in riboflavin biosynthesis.

The assignment of the propionic residue to the amino group could mean that the compound may be only an intermediate in the deamination of guanine to form xanthine or an intermediate in the amination of xanthine (derivatives) to guanine (derivatives). This would be analogous to the role of adenylosuccinate (87,88) and might be thought of more as an energy-conserving mechanism for deamination than are the reactions with guanase or as an alternative way of aminating xanthine.

However, the presence of propionoguanine in this particular system may well suggest a role in riboflavin synthesis. Albert has shown (89,90) that while a purine substituted on the 2 position with a hydroxy group very readily loses carbon 8, 2-amino purines are more stable. Brown et al. (91) found that purine riboside is even more labile in losing its carbon 8. It is quite possible that propionoguanine, or a derivative of it, by virtue of the substitution of the amino group (which causes profound inhibitions of both the acidic and basic dissociations of the guanine nucleus) may become more labile to the opening of the imidazole

ring. The possible role of the new compound in riboflavin synthesis as suggested in this discussion (and in xanthine formation) may be diagrammed as follows:



A propionoguanine derivative also would make a good precursor of a pteridine derivative which has a guanidine rather than ureido group as part of the structure. Of course, if the residue were on the imidazole ring, the propionate carbon atoms might be precursors for the carbon atoms in the aromatic ring of riboflavin.

Unfortunately a cell free system is not available to test the possible conversion of the new guanine compound or its derivative to riboflavin. It is possible that the compound could be tested with whole cells, but this might become very complicated. The final solution will require the isolation of the original propionoguanine derivative and the study of its stepwise metabolism on an enzymatic level.

Perhaps something might be said about the possible pathway of biosynthesis of propionoguanine or its derivative. Adenosine-5'-phosphate is synthesized by a series of reactions (93):

Inosinic acid + Aspartic acid $\xrightarrow{GTP}$ 6-succinoaminoadenylic acid

↓

adenylic acid + fumarate

Stadtman and coworkers (92) have demonstrated that acrylyl CoA reacts enzymatically with NH_4^+ to form ß-alanylCoA or with water to form lactoyl CoA. Therefore it is conceivable that acrylylCoA might react with a guanine derivative in a manner which is analagous to the fumarate-adenylate reaction, i.e.

AcrylylCoA + guanine(derivative) → N^2-(2-propionoCoA)-guanine (derivative)

↓

N^2-(2-propiono)-guanine (derivative) + CoA

These possibilities could be tested in the extract system.

E. Concluding Comment - This study was made for two purposes. One was to find intermediates in the synthesis of the vitamin, riboflavin, and eventually to be able to describe the stepwise enzymatic synthesis of this compound. The other was to approach the problem of devising a rational approach to the development of antimetabolites. Since most microorganisms must make their own riboflavin, it is reasonable to assume that an antagonist along this pathway would be very valuable. The latter has not yet been realized by these studies. However, it can be expected that intermediates in this pathway will be unique enough that synthetic antagonists can be devised which will specifically affect this pathway and not a biosynthetic path which is also common to the host cell. If a propionoguanine

derivative is an intermediate, antagonists to it obviously could be prepared.

A final remark may be included regarding the significance of the apparent overall synthesis of riboflavin of E. ashbyii and a number of other organisms. All of the 7 organisms listed by the American Type Culture Collection as over-producers of riboflavin as well as Clostridium acetobutylicum are spore-formers. It has been known for about 25 years that in E. ashbyii riboflavin production appears to be associated with the elaboration of spores . It is conceivable, therefore, that riboflavin production is somehow related to the process of sporulation. If this is so, an intimate knowledge of the biosynthetic pathway as well as the availability of specific antagonists for the pathway would provide a useful tool for the study of this interesting phenomenon.

SUMMARY

This research program was designed to isolate and identify intermediates in the biosynthesis of riboflavin. Our intention has been to use such intermediates to study the more intimate mechanism of the pathway by classical fractionation at the enzymatic level. Various techniques to accumulate intermediates were investigated. As a method of detecting intermediates either guanine-2-C^{14} which brings about labeling of riboflavin or guanine-8-C^{14} which does not label riboflavin was added to a culture medium. After incubation, the radioactive cultures were extracted, subjected to paper chromatography and examined for new C^{14} compounds which were not guanine derivatives by radioautographic techniques. We examined young cultures which form little riboflavin and might be expected to show accumulation of intermediates. We also studied a number of cultures after treatment with various compounds which appeared to inhibit riboflavin formation preferentially over growth. Unfortunately intermediates were not detected. This is explained in part by the realization that these inhibitors may have been depressing the synthesis of purines and remaining purines were siphoned into nucleic acids and growth rather than into riboflavin.

A similar explanation might hold in young cultures. It is also conceivable that the equilbria of the various

reactions involved between guanine and riboflavin would work against accumulation of intermediate compounds.

Extracts of E. ashbyii brought about the enzymatic conversion of C^{14}-labeled guanine to a new compound. After acid hydrolysis of the reaction mixture an apparent degradation product of the new compound was isolated in crystalline state after purification by chromatography and other techniques. By degradation studies, elemental analysis, carboxyl group determination, chromatographic properties, quantitative color tests, ultraviolet spectra at various pH values and infrared spectra, it has been concluded that the compound is N^2-(2-propiono)guanine. The position of substitution of the propionic on the guanine ring is equivocal, but the bulk of evidence favors the 2 amino group.

It seems possible that a derivative of propionoguanine is an intermediate in riboflavin synthesis since it accumulates in relatively large quantities in extracts and approximately in proportion to the rate of riboflavin synthesis by the original culture.

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