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A BIOCHEMICAL STUDY OF THE ADENINE AND HISTIDINE REQUIRING MUTANT ADENINE-3 OF *Saccharomyces cerevisiae*

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The City University of New York.

1968

This manuscript has been read and accepted for the University Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

Studies of the ad_3 mutation in *S. cerevisiae* lead to the conclusion that the organism requires adenine because it cannot convert the adenine pathway intermediate AICAR to FAICAR. The block is due to defects in the enzymes which synthesize N^{10}formyl tetrahydrofolic acid (Jones and Magasanik, 1967a). The presence of a partial block in adenine biosynthesis prior to the production of AICAR was indicated by the reduced levels of Bratton-Marshal positive material in ad_3ad_{13} extracts. Neither the cause nor the location of the additional block in adenine biosynthesis could be determined. However, the results indicate that the block occurs before the formation of GAR. The possibility of an additional, partial block in the formylation of GAR to FGAR, as might be expected from the deficiency of $N^{5,10}$formyl tetrahydrofolic acid in ad_3, could not be eliminated.

Studies with a suppressor of the histidine requirement of ad_3 lead to the conclusion that this requirement of ad_3 is caused by inhibition of histidine biosynthesis by the AICAR which accumulates in this strain. Although the block in histidine biosynthesis is a partial one, it is sufficient to cause an absolute growth requirement for histidine. This block in histidine biosynthesis is prior to the synthesis of BBM III. However, it could not be

localized more precisely on the basis of accumulation
studies or by enzyme assay.

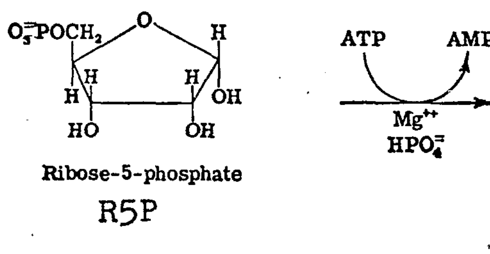
INTRODUCTION

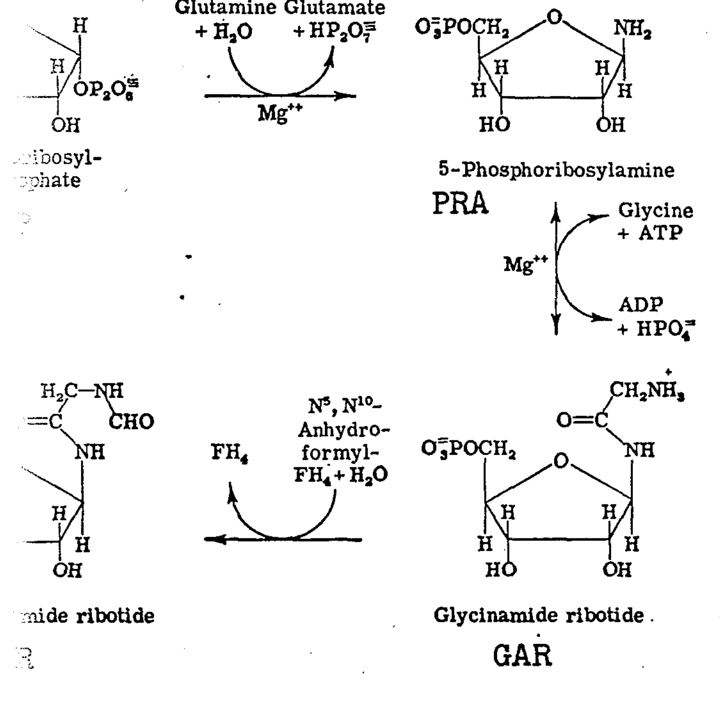
The biosynthesis of adenine was first studied in avian liver by Buchanan and his coworkers (Buchanan and Hartman, 1959). An elementary understanding of purine biosynthesis (Figure 1, Table 1) resulted from these studies. Adenine biosynthesis has also been studied in bacteria (Magasanik, 1962), Neurospora (Bernstein, 1961), Schizosaccharomyces pombe (Yeast Genetics Supp., 1966) and Saccharomyces cerevisiae (Dorfman, 1964; Silver, (1968). Purine biosynthesis has proved to be fundamentally the same in all of these organisms.

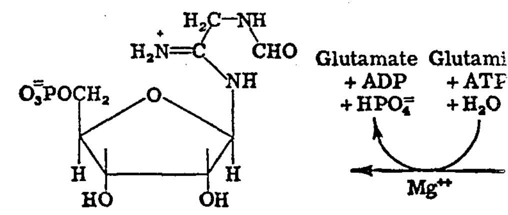
The biosynthesis of histidine was studied in Neurospora and, more extensively, in Salmonella by Ames and his coworkers. These studies were reviewed by Ames (1955) and Ames and Hartman(1963). Ames' methods were applied to a study of histidine biosynthesis in S. cerevisiae by Fink (1965), who showed that the histidine pathway in yeast was essentially the same as that in Neurospora and Salmonella (Figure 2).

Mutations in these microorganisms resulting in a requirement for one or the other of these two nutrients are indispensible tools for metabolic studies. A compound could be identified as a possible intermediate in adenine synthesis, for example, if it could substitute for adenine

Figure 1. Reaction steps and intermediates in purine biosynthesis







Formylglycinamidine ribotide

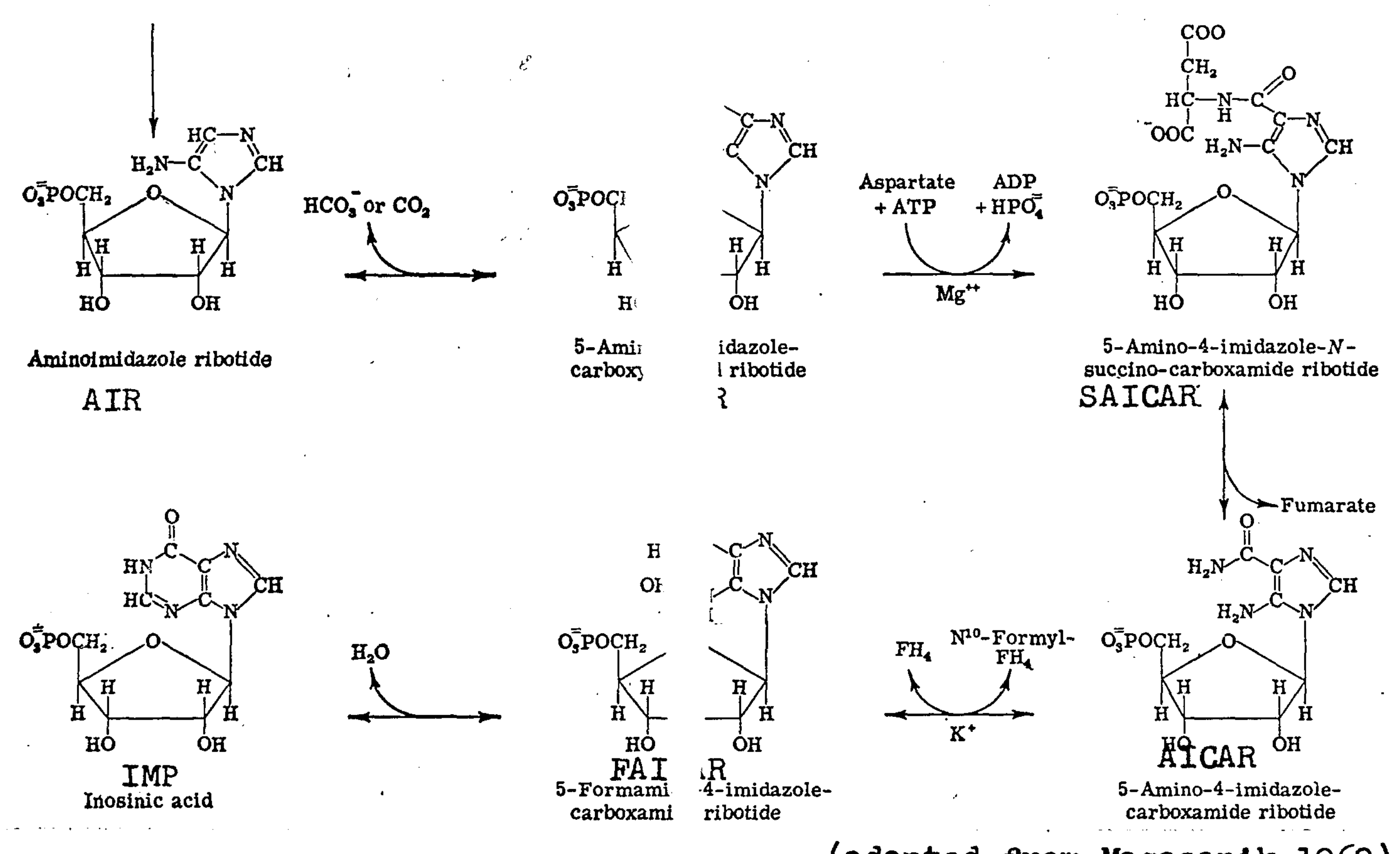
FGAM

ATP

K^+Mg^{++}

ADP
$+ HPO_4^=$

(adapted from Magasanik, 1962)



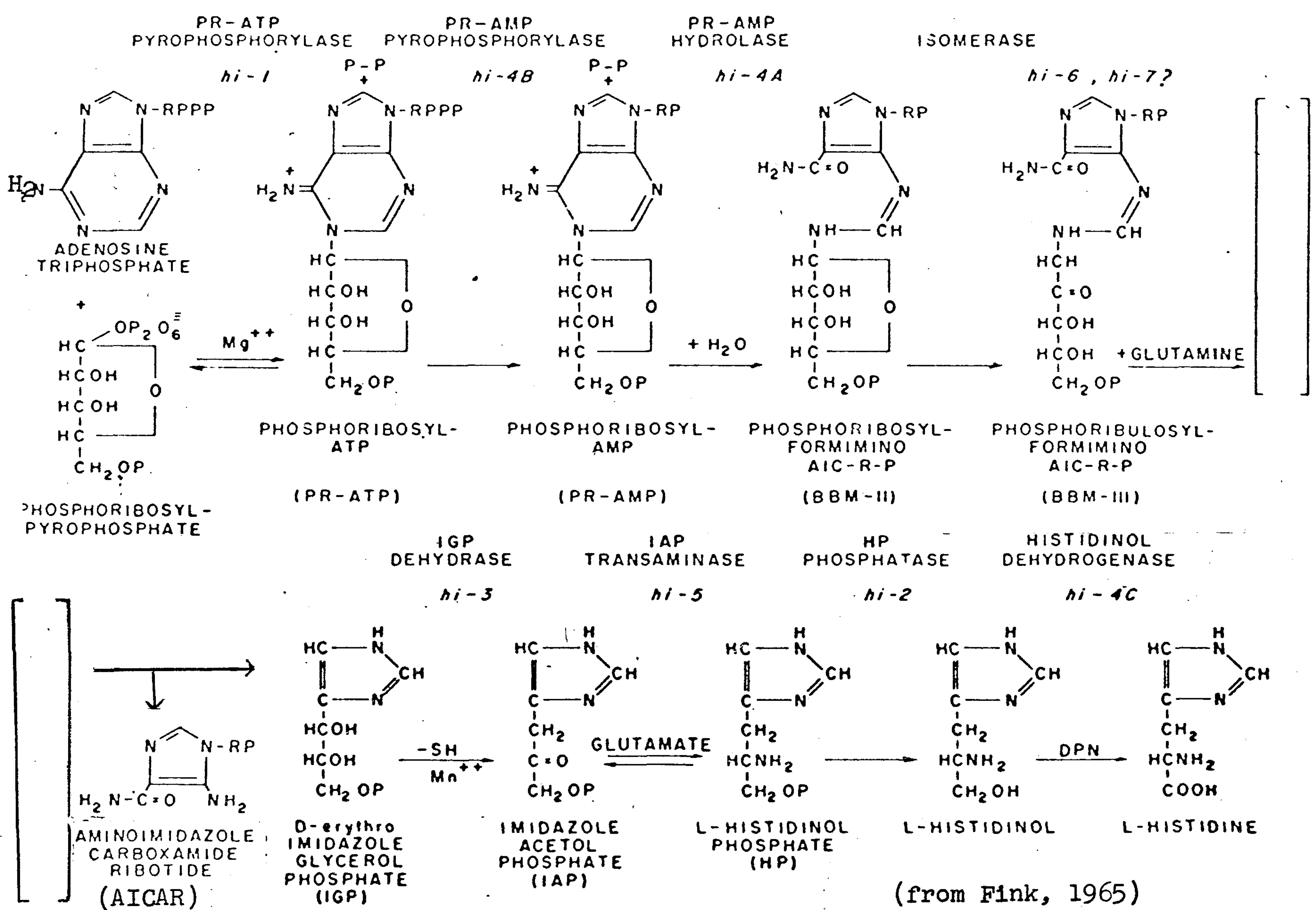
(adapted from Magasanik, 1962)

Table 1. The adenine pathway enzymes and the yeast strains blocked for these activities

Reaction	Trivial	Strain
R5P → PRA	Amidophosphoribosyltrans-ferase, phosphoribosylpyro-phosphateamidotransferase	?
PRA ⇌ GAR	phosphoribosyl-glycineamide synthetase	?
GAR → FGAR	phosphoribosyl-glycineamide formyltransferase	ad_4
FGAR ⇌ FGAM	phosphoribosyl-formylglycine-amidine synthetase	ad_6
FGAM → AIR	phosphoribosyl-aminoimidazole synthetase	ad_7
AIR ⇌ CAIR	phosphoribosyl-aminoimidazole carboxylase	ad_2
CAIR → SAICAR	phosphoribosyl-aminoimidazole succinocarboxamide synthetase	ad_1
SAICAR ⇌ AICAR AMPS ⇌ AMP	adenylosuccinate lyase (adenylosuccinase)	ad_{13}
AICAR ⇌ FAICAR	phosphoribosyl-aminoimidazole-carboxamide formyltransferase	?
FAICAR ⇌ IMP	IMP cyclohydrase (inosinicase)	?
IMP → AMPS	adenylosuccinate synthetase	ad_{12}

The blocks in the strains ad_4, ad_6, ad_7 were established by Silver (1968). The blocks of ad_2 and ad_1 were established by Dorfman (1964) and by Silver (1968). The blocks of ad_{12} and ad_{13} were established by Dorfman (1968).

Figure 2. Reaction steps and intermediates in histidine biosynthesis



(from Fink, 1965)

as a growth factor in an adenine requiring strain. Also, the accumulation of a mutant-specific compound in single strains has often led to the identification of that compound as an intermediate in the biosynthesis of the nutrient required by the strain.

Studies such as these support the notion of biochemical unity, that is, that "there are both broad and detailed similarities of composition and metabolic patterns among all living organisms" (Cohen, 1963). Differences and novel features among these systems, however, have been overlooked in the tendency to generalize. These differences often give the best insight into the essentials of the biosynthetic system and into evolutionary mechanisms themselves.

Studies of some of the atypical adenine and histidine mutants, in several organisms, have resulted in the elucidation of differences in the biosynthesis of adenine and histidine. For example, one finds occasional reports in the literature of mutants that show a relationship in their requirements for the two compounds. Although the two pathways share no unique, common intermediate, a metabolic relationship exists between them. AMP*, the end-product of adenine biosynthesis, when phosphorylated to ATP serves

*For explanation of abbreviations see Table 2.

Table 2. Abbreviations used in text

Abbreviation	Definition
AICA	5-Amino-4-imidazole carboxamide
AICAR	either or both of the following two compounds
AICARiboside	5-Amino-4-imidazole carboxamide riboside
AICARibotide	5-Amino-4-imidazole carboxamide ribotide
AIR	Aminoimidazole ribotide
AMP	Adenine 5' monophosphate
ATP	Adenine 5' triphosphate
BBM	any compound that releases BM positive material on mild acid hydrolysis; or either or both of the following two compounds
BBM II	Phosphoribosyl formimino AICARibotide
BBM III	Phosphoribulosyl formimino AICARibotide
BM	Bratton-Marshal
CAIR	5-Amino-4-imidazolecarboxylic acid ribotide
"Compound III"	equivalent to BBM III
FAICAR	5-Formamido-4-imidazole carboxamide ribotide
formylTHFA	various forms of formyltetrahydrofolic acid
FGAR	Formyl glycinamide ribotide
GAR	Glycinamide ribotide
IG	Imidazole glycerol
IGP	Imidazole glycerol phosphate
IMP	Inosine 5' monophosphate

Table 2. (continued)

Abbreviation	Definition
PR-ATP	Phosphoribosyl ATP
PR-AMP	Phosphoribosyl AMP
PRPP	5-Phosphoribosyl pyrophosphate
R5P	Ribose 5' phosphate
SAICAR	either or both of the following two compounds
SAICARiboside	5-Amino-4-imidazole-N-succinocarboxamide riboside
SAICARibotide	5-Amino-4-imidazole-N-succinocarboxamide ribotide
SC	Synthetic complete media

as a catalytic substrate for histidine biosynthesis. The ATP is first bound to PRPP, and then released several steps later as AICAR, an adenine pathway intermediate. Thus no adenine is actually consumed for histidine biosynthesis provided the by-product AICAR can be converted by the organism to adenine (Magasanik and Karibian, 1960). (Figure 3.)

Shedlovsky and Magasanik (1962) found a mutant in _E. coli_ which required either adenine or histidine for optimal growth. Upon further investigation this mutant was found to have a decreased ability to synthesize AICAR and IGP from "Compound III" (Figure 3), as shown by the accumulation of this intermediate in the mutant. When histidine was added to the medium the level of "Compound III" was reduced. This suggested that the histidine pathway was repressed and that there was a sparing effect by the histidine on the utilization of adenine for histidine biosynthesis. When adenine was added to the medium, sufficient histidine could be made without depleting the adenine pool. In the absence of both nutrients the mutant apparently could not make sufficient histidine to shut off histidine biosynthesis, resulting in a depletion of the adenine pool because the block in histidine biosynthesis occured before AICAR formation. A similar phenomenon was observed in _Salmonella_ (Hartman, _et al_, 1960). The growth

Figure 3. Purine nucleotide interconversions and histidine biosynthesis

Nucleic acid

AMP

AMPS

$HOOC-H_2C-\overset{H}{C}-COOH$

NH

GMP

DPN+

GTP, Asp

IMP

ATP, NH_3

TPNH

ATP

Nucleic acid

Hist

PRPP

"C"

BBM III

"compound III"

NH_3

AICAR

HISTIDINE

IGP

SAICAR

Protein

(from Dorfman, 1964)

of certain histidine mutants in Salmonella, partially blocked prior to the formation of IGP, can be stimulated by adding adenine to the histidineless medium. By contrast, however, mutants of S. cerevisiae blocked prior to the production of IGP (e. g. hi_1, hi_4, hi_7) have not been reported to show an adenine effect.

The ad_9 mutant of S. pombe has an absolute requirement for both histidine and adenine. A study of this strain by Whitehead et al (1966) showed that AICAR-transformylase activity is missing and the mutant, therefore, accumulates AICAR. AICAR was shown to inhibit an early histidine pathway enzyme. In contrast, mutants in E. coli and Neurospora, which accumulate AICAR have no histidine requirement (Gots, 1953; Magasanik and Karibian, 1960 and Bernstein, 1961). The preceding studies have revealed differences in the relationship between adenine and histidine biosynthesis in these organisms.

Adenine-3 mutations in S. cerevisiae are known to have double requirements for adenine and histidine (Roman, 1956). A fine structure study of the cistron showed the double requirement to result from all single, point mutations in the ad_3 gene (Jones, 1964). The accumulation of AICAR in this mutant was reported by Dorfman (1964). Strains of ad_3 were shown to have AICAR-transformylase

activity (Jones and Magasanik, 1967b), but to lack the ability to synthesize N^{10}formylTHFA, the cofactor needed for this reaction (Jones and Magasanik, 1967a). The histidine enzymes needed for AICAR and IGP synthesis were shown to be present in ad_3 extracts (Klopotowski *et al*, 1960). Suppressors of the histidine requirement of the ad_3 mutation in *Saccharomyces* are known (Luzzati, 1965). Unlike the suppressor of the histidine requirement in *S. pombe*, double adenine mutants consisting of ad_3 and a second mutation blocked before AICAR formation in adenine biosynthesis (e.g. ad_3ad_6 in *S. cerevisiae*), still require histidine.

In the light of these studies it was of interest to determine the basis for the double requirement in *S. cerevisiae*, and to compare the relationship between the two pathways in this organism to that of the other organisms described above. The results of this investigation follow.

MATERIALS AND METHODS

Yeast Strains

Strains of *Saccharomyces cerevisiae* used in these experiments were obtained from the sources shown in Table 3. Nomenclature and symbols follow the conventions established at the Carbondale Yeast Genetics Conference (1963). The suppressor designations, however, are those assigned at the laboratory from which they derive. Strain number 1 (a ad_3) was used throughout this study because of its good viability in crosses and its absolute requirement for adenine and histidine.

Media and Growth Conditions

The media used were those described by Roman (1956), Fogel and Hurst (1963) and Hurst and Fogel (1964). Liquid yeast extract peptone dextrose medium (YEPD) was used for growing cells for enzyme assay. Solid YEPD, fermentation media, a series of synthetic media missing one or more nutrilites, and potassium acetate sporulation media, were used for genetics experiments. Stock cultures were maintained at 4°C on storage slants of solid YEPD.

Liquid synthetic complete (SC) was used for accumulation studies (Wickerham, 1946). For certain experiments the amounts of adenine and histidine were varied from none to 1000 times the 10mg/ml of each prescribed by Wickerham (1946).

Table 3. Yeast strains used

Strain number	adenine-histidine (genotype)	phenotype	Source	number
1	ad_3	a ad hi	D. D. Hurst & S. Fogel	Z3414-2A
2	ad_3	α ad hi	derived	7 x 1
3	ad_3	αad hi ur	Roman	
4	ad_3PR_{40}	α ad ur	derived	1
5	ad_3PR_{51}	αad ur	derived	1
6	ad_3S_9	a ad	derived	7 x 1
7	ad_3S_9	α ad me tr	M. Luzzati	
8	ad_3PR_7	a ad le	E. W. Jones	C105-11C
9	ad_3FR_{13}	a ad ur hi	E. W. Jones	C103-5A
10	ad_3PR_2	a ad le	E. W. Jones	C104-2D
11	ad_3PR_5	a ad le	E. W. Jones	C107-1D
12	ad_3PR_3	a ad le	E. W. Jones	C108-1B
13	ad_3PR_4	a ad le	E. W. Jones	C109-1B

Table 3. (continued)

Strain number	adenine-histidine (genotype)	phenotype	source	number
14	ad_1	a ad le	D. D. Hurst & S. Fogel	A5779B
15	ad_1	α ad le	D. D. Hurst & S. Fogel	A5715C
16	ad_2	α ad	D. D. Hurst & S. Fogel	315
17	ad_2	a ad	H. Roman	RB
18	ad_4	a ad tr	R. Mortimer	M
19	ad_4	α ad le	D. D. Hurst & S. Fogel	F1394-8B
20	ad_5	a ad ur tr arg	B. Dorfman	M/C30 5 BZ
21	ad_5	α ad ur arg	H. Roman	RB
22	ad_6	a ad tr	R. Mortimer	M
23	ad_6	α ad	B. Dorfman	A 64 B
24	ad_6	α ad le thr tr arg	D. D. Hurst & S. Fogel	A5476A
25	ad_7	a ad	R. Mortimer	M
26	ad_7	α ad	R. Mortimer	M
27	ad_8	α ad le	D. D. Hurst & S. Fogel	A5415D

Table 3. (continued)

Strain number	adenine-histidine (genotype)	phenotype	source	number
28	ad_9	a ad ur tr	D. D. Hurst & S. Fogel	S2649B
29	ad_9	α ad ur tr	D. D. Hurst & S. Fogel	A5646B
30	ad_{12}	a ad le	B. Dorfman	A2035B
31	ad_{12}	α ad le	B. Dorfman	A2033C
32	ad_{13}	a ad ly	B. Dorfman	A473/4C
33	ad_{13}	α ad ur	B. Dorfman	A473/2A
34	hi_1	α hi me	G. Fink	M31
35	hi_2	α hi ad tr ur le	G. Fink	M32
36	hi_3	α hi tr	G. Fink	M33
37	hi_{4B}	α hi ad	G. Fink	E594
38	hi_{4A}	α hi ad	G. Fink	E588
39	hi_5	α hi	G. Fink	M35
40	hi_6	α hi ad thr ty tr le ur arg	G. Fink	M36

Table 3. (continued)

Strain number	adenine-histidine (genotype)	phenotype	source	number
41	hi_6	α hi	derived	40 x 1
42	hi_7	α hi	G. Fink	M37
43	ad_3hi_1	ad hi	D. D. Hurst & S. Fogel	Z3414Z
44	ad_3hi_3	ad hi	derived	36 x 1
45	ad_3hi_5	ad hi	derived	39 x 1
46	ad_3hi_6	ad hi	derived	40 x 1
47	ad_3hi_7	ad hi	derived	42 x 1
48	ad_3ad_1	ad hi	D. D. Hurst & S. Fogel	A5636A
49	ad_3ad_2	ad hi	N. R. Eaton	1894 D
50	ad_3ad_4	ad hi	derived	19 x 1
51	ad_3ad_6	ad hi	D. D. Hurst & S. Fogel	A5450B
52	ad_3ad_{13}	ad hi	derived	33 x 6
53	$ad_3ad_6hi_1$	ad hi	N. R. Eaton	1894D

Table 3. (continued)

Strain number	adenine-histidine (genotype)	phenotype	source	number
54	$ad_3ad_{13}S_9$	a ad	derived	33 x 6
55	ad_1S_9	ad	derived	35 x 6
56	ad_2S_9	ad	derived	37 x 6
57	ad_6S_9	ad	derived	41 x 6
58	hi_1S_9	hi	derived	34 x 6
59	hi_3S_9	hi	derived	36 x 6
60	hi_5S_9	hi	derived	39 x 6
61	S_9	+	derived	36 x 6
62	wild type	+	derived	39 x 1

soluble low molecular weight components of the cell.

Colorimetric Tests

The following tests were carried out without modification: the n-butanol extraction modification of the periodate oxidation procedure for IGP (Ames, 1957); the orcinol reaction for pentose sugars (Dische, 1953; the microbiuret test for proteins (Zamenhof, 1957); the Fiske and Subbarow reaction for phosphate (Fiske and Subbarow, 1925).

Total phosphate was determined by adding 0.2 ml of concentrated sulfuric acid to a test tube containing dry sample. The tube, capped with a glass marble, was placed directly on a heating coil for 15 minutes after which 0.1 ml 30% H_2O_2 was added and heated as before (P. O. Milch, pers. comm.). The Fiske and Subbarow reaction was used to measure the phosphate released.

Ribose-5-phosphate and potassium phosphate served as standards for the phosphate determinations. The IGP, synthesized and purified by the method described by Ames (1957), was used as a standard for the assay of IGP. Ribose, R5P and AICARiboside were standards for the orcinol reaction, and bovine serum albumin served as standard for the microbiuret test.

Several variations of the Bratton-Marshal (BM) reaction for arylamines were used (Bratton and Marshal,

1939). The following modification of the method of Lukens and Buchanan (1959) was used routinely to assure the detection of SAICARiboside and SAICARibotide: to 0.8 ml of sample in an ice bath, 0.3 ml of 10 N H_2SO_4 was added, followed by 0.1 ml of 0.1% sodium nitrite; after 3 minutes incubation 0.1 ml of 0.5% ammonium sulfamate was added, followed 1 minute later by 0.1 ml of 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride. The reaction mixture was allowed to stand at room temperature for 30 minutes and the absorbance was determined at 500 to 530 mμ. Under these conditions AICAR, at a concentration of 1 μmole per ml, has an absorbance of 12.5 at 540 mμ. This value was computed from the absorbance of AICARiboside standard BM chromophore after the AICARiboside had been eluted from a Sephadex G-10 column. The molecular weight was taken as 253. Calculations of the amounts of SAICAR are based on the determination for AICAR and therefore may not be a true quantitation of SAICAR. The BM reaction, carried out at room temperature to allow the detection of AICAR in the presence of SAICAR, followed the procedure described by Lukens and Flaks (1963). The bound BM (BBM) positive compounds, *i. e.*, those compounds which release BM-positive material upon mild acid hydrolysis, were detected by heating 0,8 ml of the sample in 0.2 N HCl at 100°C for 5 minutes, prior to

diazotization with nitrous acid. Extracts were first tested for the presence of acetylatable arylamines by adding 0.1 ml of 98% acetic anhydride to the sample and incubating at room temperature for 20 minutes prior to the BM reaction.

Absorption spectra in the UV and visible ranges of the compounds studied, and their assay derivatives, were measured either on a Hitachi-Coleman double beam recording spectrophotometer, model 124, or a Bechman DU Spectrophotometer.

Thin Layer Chromatography

Thin layer cellulose plates were prepared at a thickness of 0.5 mm. Samples of 5 to 25 µL were spotted 1.5 cm from the lower edge of the plate. After the spots dried the plates were developed by ascending chromatography in one or both of the following solvents: n-propanol: ammonia:water (7:1:2); chloroform:methanol:10% formic acid (3:3:1). The first was equilibrated overnight prior to use and the other was prepared immediately before use.

Substances on thin layer plates were examined by: UV fluorescence and quenching, ninhydrin spray for α-amino groups (Silver, 1968), and a BM spray for arylamines (Boruvka, 1964). The BM spray consisted of: 10 N H_2SO_4:

1% sodium nitrite (1:1 v/v); three minutes later 5% ammonium sulfamate; after one minute 1% N-(1-napthyl)-ethylenediamine dihydrochloride. The chromophore develops immediately.

The C^{14}-labeled compounds were located by radioautography.

Sephadex Column Chromatography

Sephadex G-10 was prepared by the rapid swelling technique to insure the removal of pentoses. A 2.5 by 23.0 cm column was prepared by slowly adding a slurry of the gel. A maximum of 10 ml of the preparation to be analyzed was absorbed onto the column. The material was eluted with 200 ml of distilled water. Fractions of 5 ml were collected and examined by UV absorbance or by the BM reactions. In certain experiments a column of 1.2 by 10 cm was used and all other volumes were reduced proportionately.

Ion Exchange Chromatography

Dowex-1-formate columns were used to isolate GAR and FGAR and were also used to isolate SAICAR and AICAR according to the method of LePage and Jones (1961). Dowex-1-formate was prepared from Dowex-1-chloride by washing the resin with 3 M NaCOOH until the supernate did not precipitate with 0.2 M $AgNO_3$. A 1 by 13 cm column

was charged with 25 volumes of 6 N HCOOH:1 M NaCOOH (1:1 v/v), followed by 100 ml of concentrated HCOOH, and was washed with distilled water until the eluant had a neutral pH. The sample, in 3 ml water, was added to the column. The column was then washed with 100 ml distilled water and then eluted with a gradient of 300 ml of 0.3 M HCOOH in a 500 ml mixing bottle and a reservoir of 1 liter of 4 M HCOOH. Fifty 5 ml fractions were collected and assayed by the BM reaction. Carbon-14 labeled compounds were detected by drying 50 µL from each fraction on a planchet and determining the radioactivity, as counts per minute, with a gas flow detector (Nuclear Chicago model D-47).

Analysis of Phosphorylated Intermediates

Extracts and isolated compounds were tested for the presence of esterified phosphate by treatment with alkaline phosphatase prior to chromatographic analysis. This procedure allows comparison of the compounds with available non-phosphorylated standards. The substance or mixture to be tested was incubated for one hour at room temperature with 0.5 mg of alkaline phosphatase in .001 M tris buffer, at pH 8 in a final volume of 1 ml. A control contained heat-inactivated alkaline phosphatase. Alkaline phosphatase activity was checked routinely by assaying its capacity to convert IMP to inosine.

Assay for Glycinamide Ribotide (GAR)

The assay, based on the conversion of GAR and IMP to FGAR and AICAR, was carried out as described by Nierlich and Magasanik (1965). The enzyme was prepared in this laboratory by J. Silver, from chicken liver by the method of Flaks and Lukens (1963).

AICAR-transformylase

The activity of AICAR-transformylase was assayed by measuring the rate of disappearance of AICAR as described by Flaks and Lukens (1963). The assay mixture, incubated at 30°C, contained per ml: 50 mμmoles AICAR; 100 mμmoles N^5formylTHFA; 10 μmoles phosphate buffer at pH7.6; 10 μmoles $MgCl_2$; and 8 mgms protein. The control contained no N^5formylTHFA. The crude enzyme extract was prepared by disrupting log phase cells in an Eaton press (Eaton, 1962), suspending the crushed cells in a volume of 0.1 M phosphate buffer, pH 7.6, equal to their wet weight in grams, centrifuging at 22,000g for 15 minutes and dialyzing the supernate 24 hours against 0.001 M solution of the same buffer.

Enzymatic Synthesis of AICAR from R5P and ATP

Enzyme activity was measured by a slight modification of the method of Kloptowski, et al (1960). One ml of assay mixture, incubated at 37°C, contained: 5 μmoles NaR5P; 2.5 μmoles K_2ATP; 50 μmoles $MgCl_2$; 5 μmoles potassium

3-phosphoglycerate; 7.5 μmoles glutamine; 50 μmoles phosphate buffer, pH 8.0; and 0.4 ml of extract containing approximately 3 mg protein. The reaction was stopped by addition of 0.5 ml 4 N perchloric acid. The perchloric acid was subsequently removed by addition of an equivalent amount of KOH. After centrifuging at 12,000 x g for 5 minutes the supernate was assayed for the presence of non-acetylatable BM- and BBM- positive compounds. Ribose-5P was omitted from the control. Cells were crushed as above and the crude extract was centrifuged for one hour at 144,000 x g in a spinco Model L ultracentrifuge, prior to dialysis.

Phosphoribosyl-ATP Pyrophosphorylase

The assay was carried out without modification as described by Fink (1965). K_2ATP was omitted from the control. Strains carrying the allele hi_{4B} were used for preparation of the extracts to prevent conversion of PR-ATP to BBM II (Figure 2). Preparation of the cell extract differed from that of Fink in that log phase cells were disrupted in an Eaton press and the 10,000 x g supernate was centrifuged for 1 hour at 144,000 x g.

Genetic Analysis

Crosses were made on solid YEPD and diploids were selected on appropriate dropout media.

Tetrad analysis involved sporulation of the diploid on potassium acetate agar (McClary, et al, 1959), and dissection of the ascospores according to the method of Johnston and Mortimer (1958). Snail enzyme used for digestion of the asci was prepared as described by Hurst and Fogel (1963). Phenotypes were verified by replica plating to appropriate synthetic media. The genotype was checked by complementation with the appropriate tester strains.

Selection of Partial Suppressors

The strain (3) ad_3ur was grown aerobically for 48 hours in 30 tubes containing 5 ml of YEPD. From each tube a volume containing approximately 10^8 cells was spread on to one histidineless and one adenineless plate. All clones growing on either of these media were tested for growth on adenineless, histidineless and adenineless-histidineless media. Clones that failed to grow on adenineless-histidineless medium but which grew on either of the other two media were further tested for the presence of a suppressor as indicated by its independent segregation from ad_3 in a cross to a wild type strain.

Materials

AICARibotide, which served as a standard in these studies, was obtained as a gift from Dr. H. T. Huang of International Mineral and Chemical Corporation. AICAR-

ibotide was also isoldated in our laboratory as a product of the in vitro enzyme assay.

The SAICAR used as a standard was obtained from ad_{13} extracts, known to accumulate SAICAR (Dorfman, 1968).

N^5formyl tetrahydrofolic acid was a gift of Dr. Ruegsegger of Lederle Laboratories. The formamidine used for the organic synthesis of IGP was purchased from Cyclo Chemical Corporation, Los Angeles

The alkaline phosphatase preparation containing 10 mg protein per ml with a specific activity of 28 was purchased from Worthington Biochemical Corporation.

Glycine-U-C^{14} and adenine-2-C^{14} were purchased from Nuclear Equipment Company and International Chemical and Nuclear Corporation, respectively.

Sephadex G-10 and the columns for chromatography were purchased from Pharmacia, Sweden.

Dowex-1-chloride resin was purchased from Baker.

Cellulose powder and equipment for preparing thin layer plates were purchased from Brinkman.

Kodak No Screen X-ray film was used for radioautography.

All other chemicals used were reagent grade.

RESULTS

The double nutritional requirement of ad_3 was first reported by Roman (1956). The absolute nature of the requirement for adenine and histidine and the absence of a sparing effect by excess amounts of either nutrient was demonstrated by Dorfman (1964) and by Luzzati (1965). These growth experiments were repeated, with the results shown in Figure 4 and Table 4. Strains of ad_3 grown to stationary phase in either adenineless or in histidineless media have an absorbance of 0.03 at 650 mμ, which is much less than the absorbance of 0.57 observed in SC medium. Therefore, virtually no growth can occur in the absence of either nutrient.

Basis for the Adenine Requirement of ad_3

Strains carrying ad_3 were initially found as white papillations on red colonies of ad_1 or ad_2 (Roman, 1956). Strains of ad_2 and ad_1 are unable to convert AIR to CAIR and CAIR to SAICAR respectively (Dorfman, 1964; Silver, 1968). This suggested that the white double mutants of ad_3ad_1 and ad_3ad_2 are blocked earlier than the production of AIR in adenine biosynthesis.

Extracts of ad_3 contain a BM positive, non-acetylatable arylamine. According to the adenine pathway described by Buchanan, (1960) compounds with these properties are

Figure 4. Growth of adenine and histidine requiring strains in media containing varied levels of these two nutrients

Growth was measured as absorbance of the culture at 650 mμ against a media blank in a Beckman DU spectrophotometer.

A. Growth of o———o ad_3 and •-----• ad_2. The top two curves are for growth in SC. The middle two are for growth in SC - 7.5γ/ml adenine. The bottom two are for growth in adenineless medium.

B. Growth of o———o ad_3 and •-----• hi_1. The top two curves are for growth in SC. The middle two are for growth in SC - 7.5γ/ml histidine. The bottom two are for growth in histidineless medium.

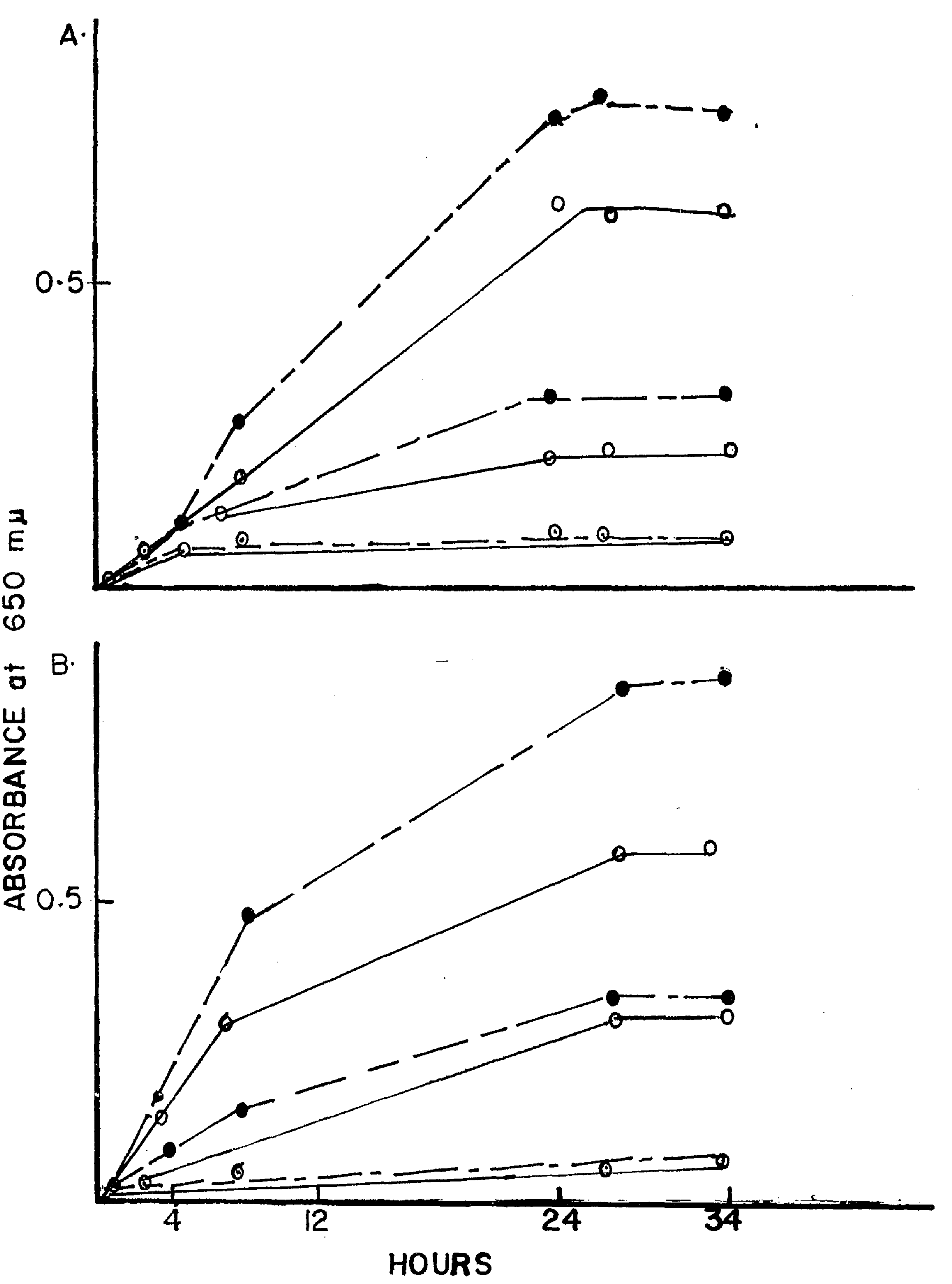

A·
0·5
B·
0.5
ABSORBANCE at 650 mμ
4
12
24
34
HOURS

Table 4. Growth of some adenine and histidine requiring strains in media containing varied levels of these two compounds

Strains	Absorbance at 650 mμ				
	SC-histidine	SC-7.5 γ/ml histidine	SC-adenine	SC-7.5 γ/ml adenine	SC[a]
ad_3 (1)[b]	0.03	0.29	0.03	0.19	0.57
hi_1 (34)	0.02	0.31	0.80	0.80	0.86
ad_2 (17	0.75	0.75	0.01	0.27	0.75

[a] SC contains 10 γ/ml each of adenine and histidine.

[b] (Strain number)

The strains were grown aerobically at 30°C to stationary phase in the media specified. The absorbance of the culture was read against a medium blank in a Beckman DU spectrophotometer.

formed at or after the formation of AIR. Chromatography of an extract of ad_3 in a chloroform:methanol: 10% formic acid (3:3:1) solvent yields four separate BM positive spots with R_f values of 0.29, 0.35, 0.52 and 0.7 (Table 5). If the extract is treated with alkaline phosphatase prior to chromatography only two spots with R_f values of 0.52 and 0.7 remain. The two remaining compounds show a visible increase in their reaction with the BM spray. These observations demonstrate the presence, in ad_3 extracts, of two phosphorylated arylamines with R_f values 0.29 and 0.35 and their unphosphorylated derivatives with R_f values 0.52 and 0.70 (Table 5).

The extract of ad_3 and alkaline phosphatase-treated extract of ad_3 were co-chromatographed with the standard ribotides and ribosides of AICAR and SAICAR on cellulose thin layer plates in one or more solvent systems. Each spot was found to correspond to one of the four standards in all solvents and to be unresolved from the standards upon two dimensional chromatography. The R_f values of these compounds in two of the solvents used are summarized in Tables 5 and 6. Several other solvents were also used and gave results consistent with those detailed above.

Table 5. R_f values of BM positive compounds chromatographed in chloroform:methanol:10%formic acid (3:3:1) solvent

Preparation	R_f values:			
	0.29	0.35	0.52	0.70
ad_3 extract (1)[a]	+	+	+	+
alkaline phosphatase treated ad_3 extract	-	-	+	+
AICARibotide standard	+	-	-	-
AICARiboside standard	-	-	+	-
AICA standard	-	-	-	+[a]
ad_{13} extract (33) standard SAICAR	-	+	-	+
alkaline phosphatase treated ad_{13} extract SAICARiboside standard	-	-	-	+
ad_3S_9 (6)	-	-	-	+

[a] Approximate R_f

[b](Strain number)

The presence (+) or absence (-) of a BM positive compound at a given R_f was based on a minimum of six separate experiments. The R_f values varied by less than 0.03. Freshly prepared solvent was used. Co-chromatographs were run in all combinations.

Table 6. R_f values of BM positive compounds chromatographed in n-propanol:ammonia:water (7:1:2) solvent

Preparation	R_f values:			
	0.02	0.06	0.47	0.53
ad_3 extract (1)[a]	+	+	+	-
alkaline phosphatase treated ad_3 extract	-	+	+	-
AICARibotide standard	+	-	-	-
AICARiboside standard	-	-	+	-
AICA standard	-	-	-	+
ad_{13}extract (33) standard SAICAR	-	+	-	-
alkaline phosphatase treated ad_{13} extract SAICARiboside standard	-	+	-	-
ad_3S_9 (6)	-	+	-	-

[a] (Strain number)

The presence (+) or absence (-) of a BM positive compound at a given R_f was based on a minimum of six separate experiments. The R_f values varied by less than 0.03. The solvent was equilibrated overnight prior to use. Co-chromatographs were run in all combinations. The compounds were detected by BM spray.

The ad_3 extract and standards were analyzed by ion-exchange chromatography on Dowex-1-formate (Figure 5). The composition of the eluant fractions was determined by thin layer chromatography. The AICARiboside and one BM positive component of ad_3 extract were removed together in the water wash. The SAICARiboside was eluted earlier than, but overlapped with, the AICARibotide peak. This compound peak contains the majority of the BM-positive substances accumulated by ad_3. The amount of SAICARibotide was too low to detect.

Partial resolution of the BM positive components of ad_3 extracts was also obtained by use of G-10 Sephadex columns (Figure 6). As one would predict from their molecular weights of 368 and 332 respectively, standard SAICARiboside eluted slightly before, but formed a compound peak with AICARibotide. AICARiboside with a molecular weight of 253 was eluted several fractions later. The ad_3 accumulation products consistently corresponded to the standard compounds. When the compound peak from the G-10 column, containing the suspected AICARibotide and SAICARiboside, was treated with alkaline phosphatase the suspected AICARibotide component disappeared from the mixture leaving the suspected SAICARiboside unaltered and a component corresponding to the AICARiboside standard (Figure 6).

Figure 5. Elution of Bratton-Marshal positive compounds from a Dowex-1-formate column

A. Standard BM positive compounds. These compounds were run independently.

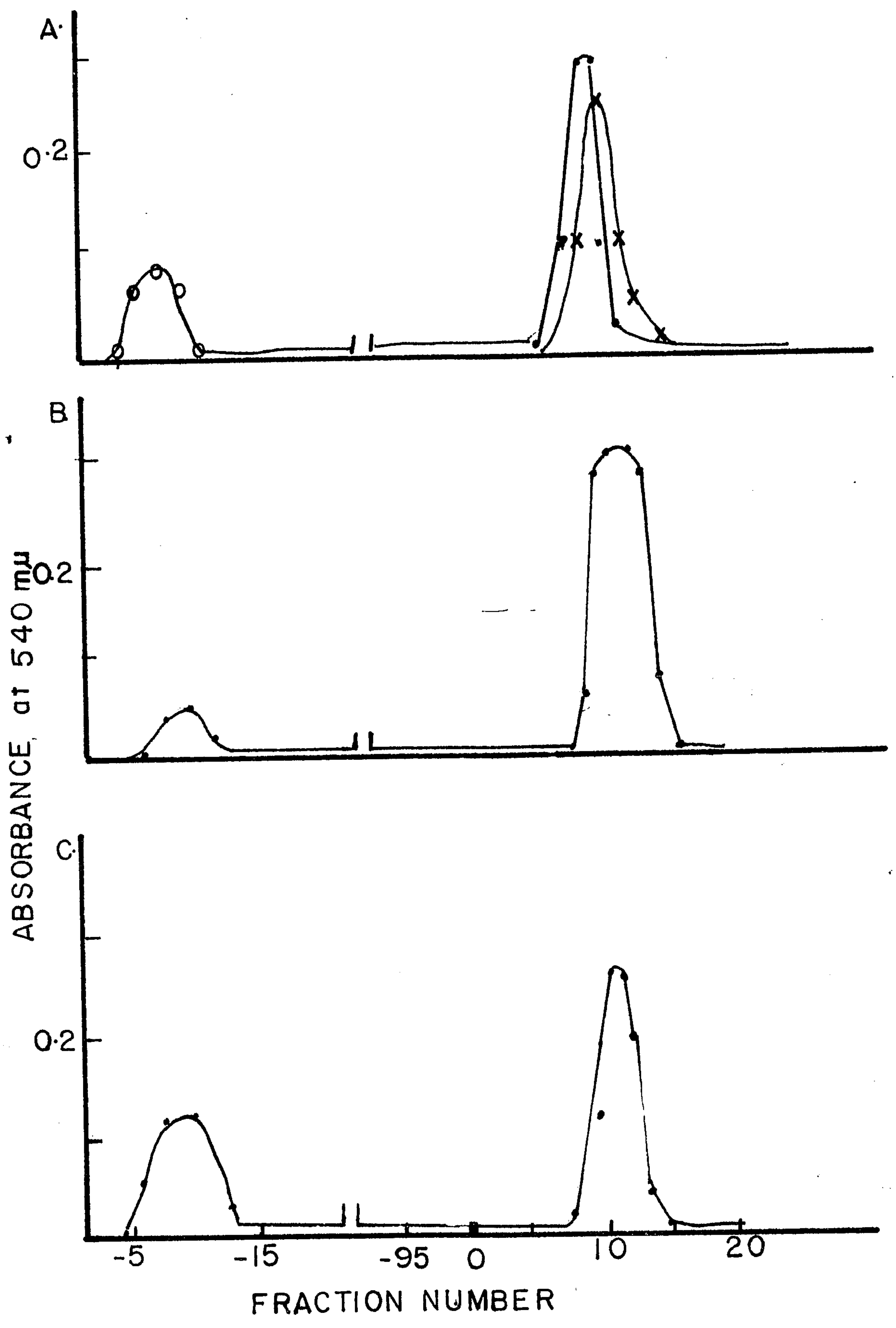
o——o AICARiboside

•——• SAICARiboside

x——x AICARibotide

B. Adenine-3 extract (Strain 1).

C. An alkaline phosphatase treated ad_3 extract.



A·
0·2
B
0.2
C·
0·2
ABSORBANCE at 540 mμ
-5
-15
-95
0
10
20
FRACTION NUMBER

Figure 6. Elution of Bratton-Marshal positive compounds from a Sephadex G-10 column

A. Standard BM positive compounds. These compounds were run independently.

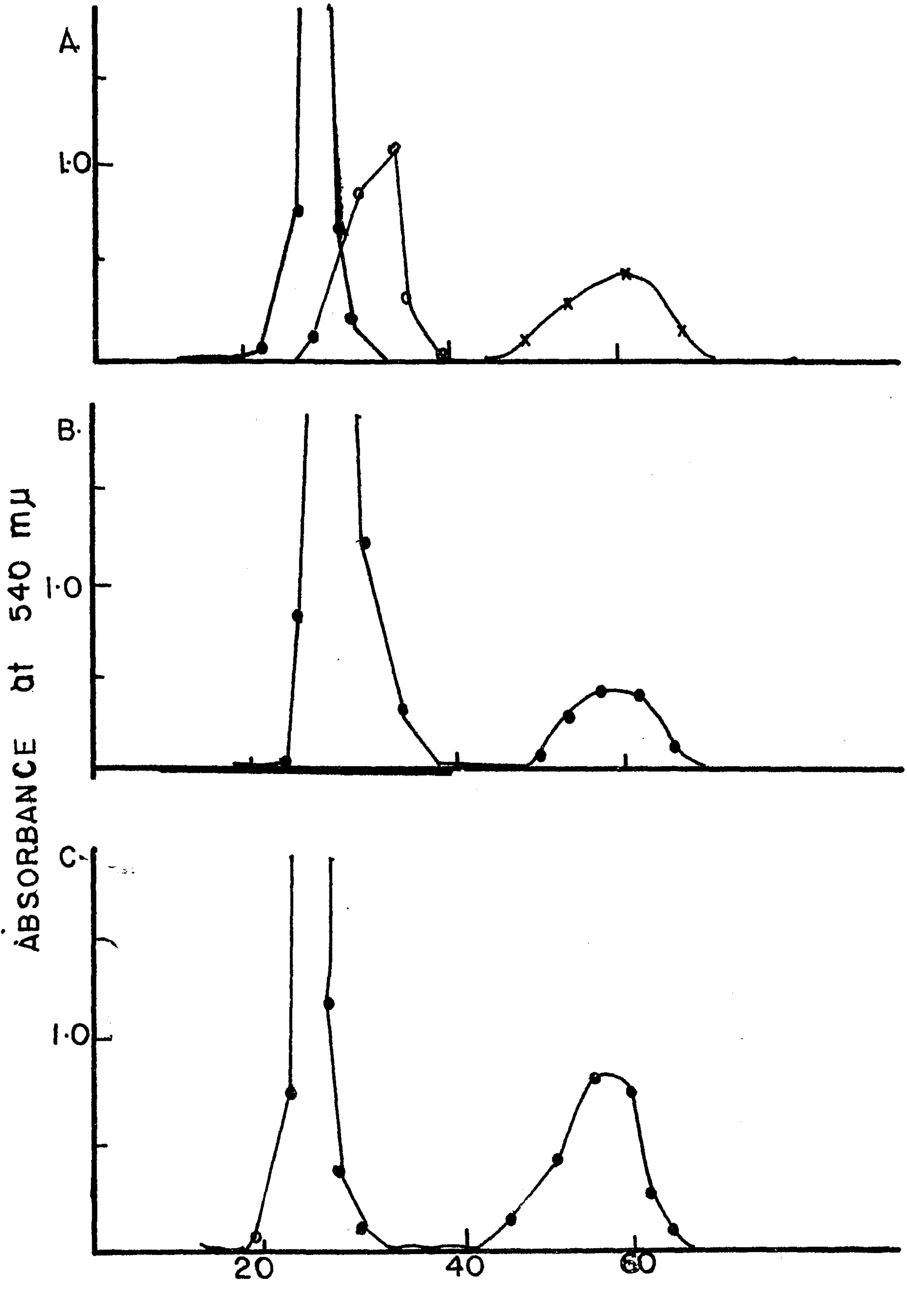
x———x AICARiboside

●———● SAICARiboside

o———o AICARibotide

B. Adenine-3 extract (Strain 1).

C. An alkaline phosphatase treated ad_3 extract.



A.
1·0
B·
1·0
C·
1·0
ABSORBANCE at 540 mμ
20
40
60
FRACTION NUMBER

The UV-spectra and the spectra of the chromophore of the BM reaction of the suspected riboside components of ad_3 extract were determined, and are compared in Table 7 with the standards and with published values. SAICARiboside from ad_{13} and the equivalent compound from ad_3 had an absorbance maximum of the BM chromophore varying from 530 to 550 mμ, which was lower than the literature value of 560 mμ reported for this compound (Lukens and Buchanan, 1959). The UV-absorbance maximum for AICAR and SAICAR was 265 mμ, also somewhat lower than the published value (267 mμ) for these two compounds (Lukens and Buchanan, 1959; Lukens and Flaks, 1963). The reason for the discrepancy with the previously reported values is not clear. However, in this study, spectra of ad_3 BM positive compounds correspond exactly with the standard compounds, as illustrated in Figures 7 and 8.

The BM-positive compounds from ad_3 extracts corresponding to SAICARiboside and AICARiboside contained a ratio of ribose:arylamine of 1:1. A total phosphate determination on these compounds failed to detect any phosphate. AICARibotide and the small amount of SAICARibotide which accumulates in ad_3 were converted to the corresponding ribosides by treatment with alkaline

Table 7. Absorbtion maxima of the BM chromophores and UV-spectra of BM positive compounds

Substance	UV-spectra			Visible spectra BM chromophore
	pH 1 shoulder (mμ)	pH 1 maximum (mμ)	pH 8 maximum (mμ)	maximum (mμ)
SAICARiboside:				
published value[c]	244-254	269	267	560[a]
ad_3 compound	245	265	265	530-550[a]
standard	245	265	265	530-550[a]
AICARiboside:				
published value[d]	245-255	269	267	540[b]
ad_3 compound	245	265	265	540[b]
standard	245	265	265	540[b]

[a] BM reaction carried out in an ice bath.

[b] BM reaction carried out at room temperature.

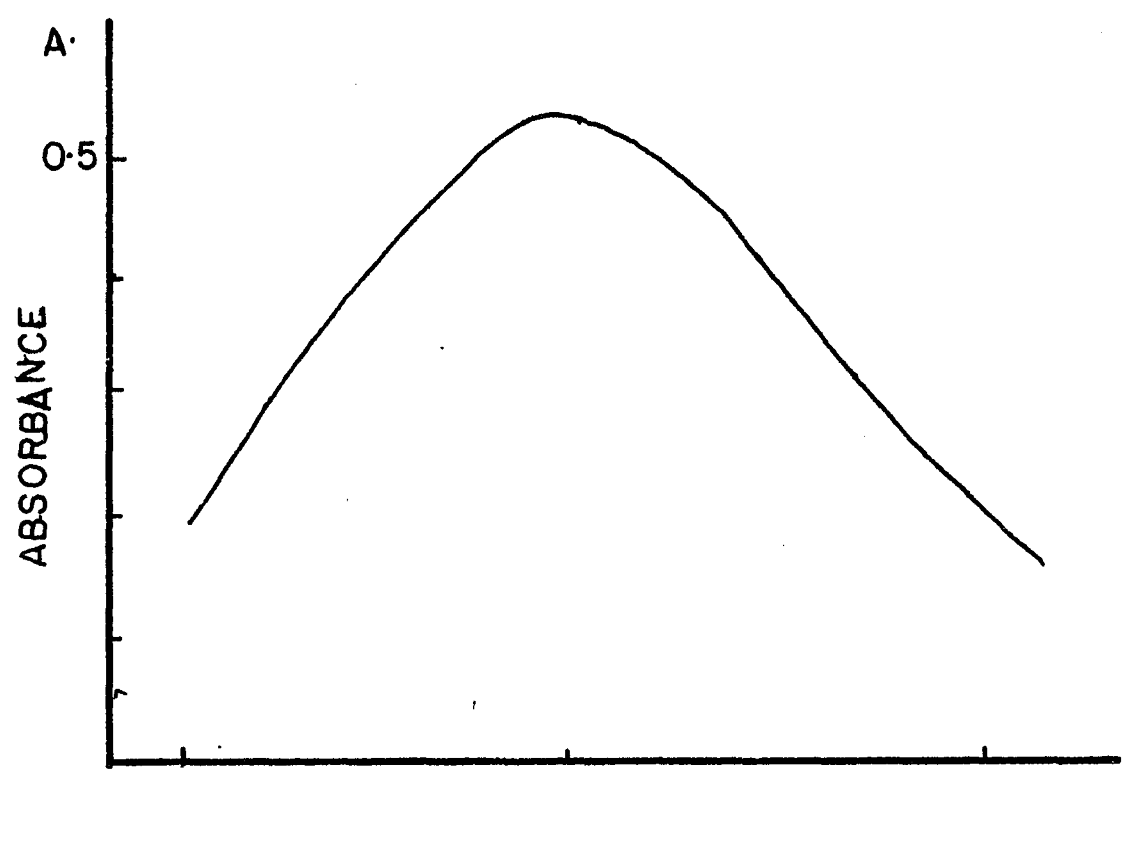
[c] Lukens and Buchanan (1959)

[d] Lukens and Flaks (1963)

Figure 7. Spectrum of colored derivatives of SAICAR and AICAR produced by the Bratton- Marshal reaction.

A. SAICARiboside: Identical spectra were obtained for both SAICARiboside standard and SAICARiboside from ad_3 extracts.

B. AICARiboside: Identical spectra were obtained for both AICARiboside standard and AICARiboside from ad_3 extracts.


A
0·5
ABSORBANCE

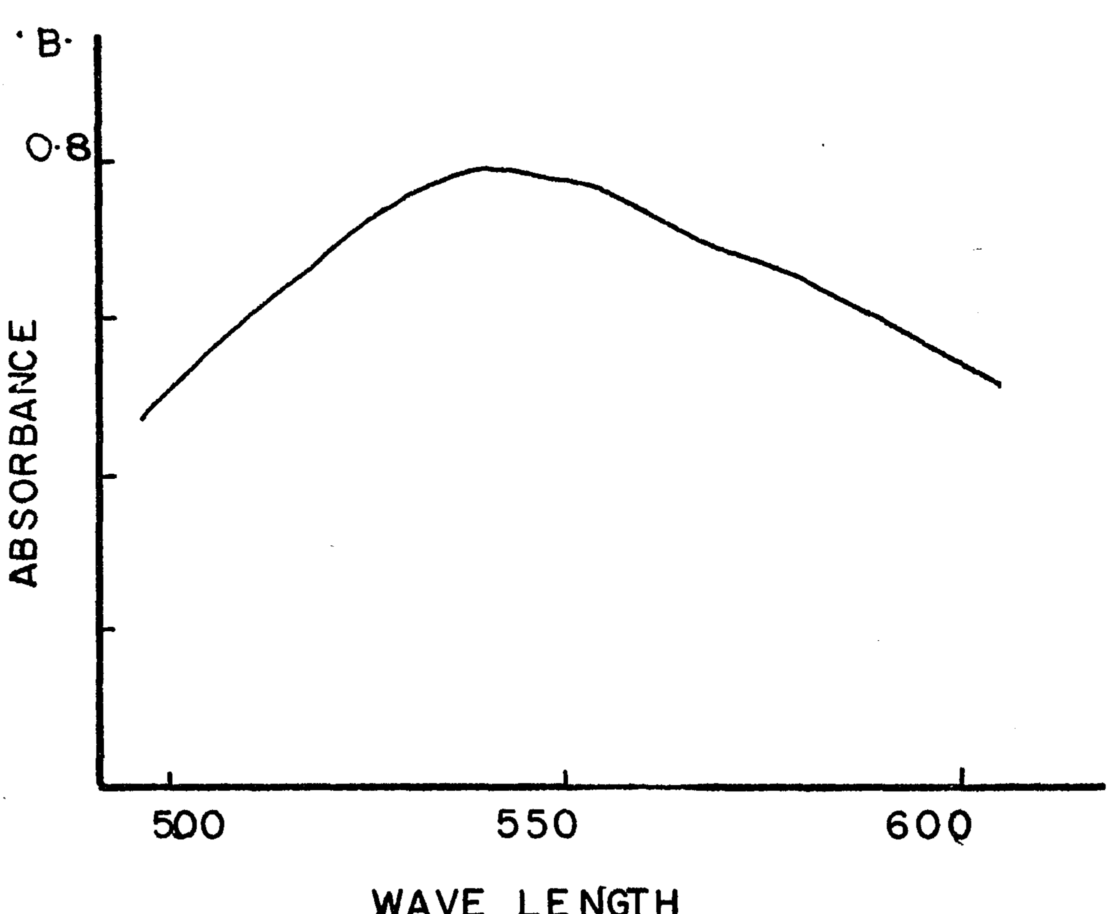
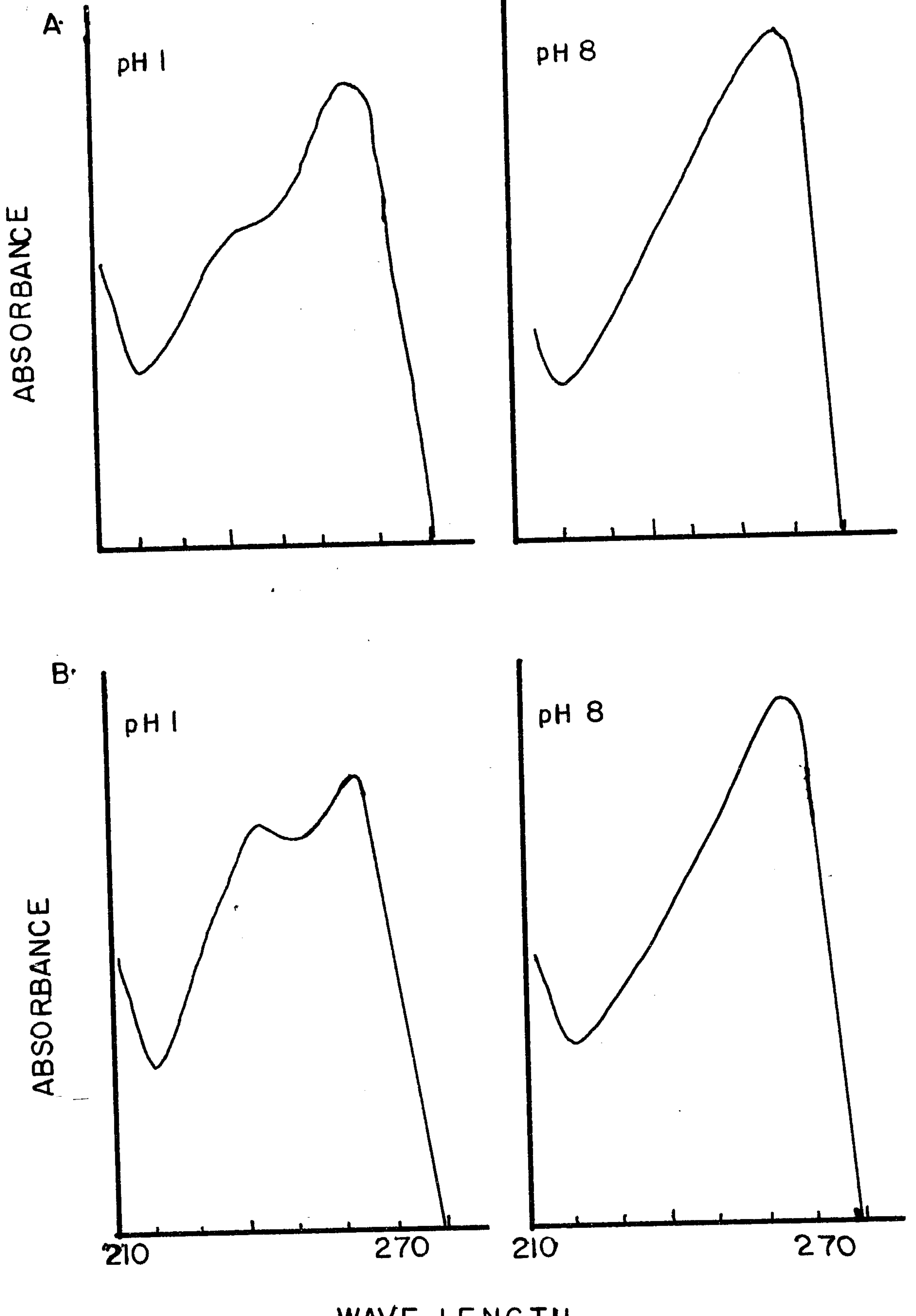

B
0·8
ABSORBANCE
500
550
600
WAVE LENGTH

Figure 8. Ultraviolet spectra of SAICAR and AICAR at pH 1 and pH 8

A. SAICARiboside: Identical spectra were obtained for both SAICARiboside standard and SAICARiboside from ad_3 extracts.

B. AICARiboside: Identical spectra were obtained for both AICARiboside standard and AICARiboside from ad_3 extracts.


A.
pH 1
pH 8
ABSORBANCE
B.
pH 1
pH 8
ABSORBANCE
210
270
210
270
WAVE LENGTH

phosphatase, and were identified as such by all of the above procedures.

In summary, the BM positive compounds accumulating in ad_3 mutants have been identified as the adenine pathway intermediates SAICARibotide and AICARibotide and their corresponding ribosides. These observations are in agreement with those of Dorfman (1964).

It is possible that BBM, a histidine pathway intermediate, rather than AICAR, accumulates _in vivo_ in ad_3 and that the presence of AICAR in extracts of ad_3 is therefore an artifact of the extraction procedure. To eliminate this possibility the following experiment was done. Extracts of strains containing hi_7, which accumulate BBM (Fink, 1965), were compared to extracts of ad_3 (Table 8). No free BM positive compound was present in hi_7 extracts. The data presented in Table 9 clearly show that the BBM compound is stable during the extraction procedure. Extracts of ad_{13} which were prepared under the same conditions as ad_3 extracts contain only SAICAR (Table 8). These results exclude the possibility that AICAR in ad_3 is a non-enzymatic breakdown product of the SAICAR formed via the adenine biosynthetic pathway.

The accumulation of AICAR and SAICAR in ad_3 strains demonstrated that adenine biosynthesis is blocked at the

Table 8. BM and BBM content of cell extracts of ad_3, hi_7 and ad_{13}

	Bratton-Marshal				
Source of Extract	Total amount[a]	Free amount[a]	Free %total	Bound amount[a]	Bound %total
ad_3 (1)[b]	0.56	0.56	100	0.00	0
hi_7 (42)	0.36	0.02	7	0.34	93
ad_{13} (33)	12.00	12.00	100	0.00	0

[a]Amount in µmoles/gram wet weight.

[b](Strain number)

The total BM value was measured by the ice bath assay after five minutes hydrolysis in dilute acid at 100°C. The values in the table are the means of at least three experiments. The µmoles/ml BM positive material were computed from the absorbance at 540 mµ with 12.5 absorbance units equivalent to 1 µmole/ml.

conversion of AICAR to FAICAR. But a block in adenine biosynthesis at the formylation of AICAR to FAICAR cannot account for the observation that ad_3ad_1 and ad_3ad_2 double mutants are white rather than red. Since the red pigment is a polymer of AIR (Smirnov _et al_, 1967), lack of pigment in the double mutants suggests that ad_3 strains are also blocked before the synthesis of AIR. It will be recalled that AICAR is a by-product of histidine biosynthesis in addition to being an intermediate of adenine biosynthesis. AICAR could therefore still accumulate as a by-product of histidine biosynthesis, even if none were generated by the adenine pathway. Thus, if there is a complete block in adenine biosynthesis prior to AIR formation, AICAR in ad_3 could originate exclusively from the histidine pathway. A strain with the genotype ad_3hi_1, blocked at the first step in histidine biosynthesis (Fink, 1965), should, therefore, accumulate no AICAR. Extracts of a strain with the genotype ad_3hi_1 do, however, accumulate AICAR (Table 9). It should be noted, on the other hand, that the amount is less than 6% of that in the strain containing the ad_3 marker alone.

These results would still be consistent with a complete block in adenine biosynthesis if the hi_1

Table 9. BM positive material in strains blocked at different points in adenine and histidine biosynthesis

Source of extract:	BM positive material	
	µmoles gram wet weight	percentage[a]
ad_3 (1)[b]	0.56	100
ad_3hi_1 (43)	0.03	6
ad_3hi_6 (46)	0.03	6
ad_3ad_4 (50)	0.25	45
$ad_3ad_6hi_1$ (53)	0.00	0

[a] The value for ad_3 was taken as 100.

[b] (Strain number)

The BM reaction was carried out in an ice bath to insure the detection of SAICAR. The µmoles/ml were computed from the absorbance at 540 mµ, using 12.5 absorbance units equivalent to 1 µmole/ml.

marker were leaky. If all of the AICAR were in fact derived only from the histidine pathway, an ad_3ad_4 strain, also blocked at the conversion of GAR to FGAR (Silver, 1968), should have the same level of AICAR as a strain containing the ad_3 marker alone. However, the ad_3ad_4 strain accumulates half as much BM positive compound as strains containing only the ad_3 marker. An extract of a strain with the genotype $ad_3ad_6hi_1$, which is blocked early in both histidine and adenine biosynthesis, accumulates no BM positive compound. Both pathways, therefore, contribute to the BM positive material accumulating in ad_3. The evidence which follows further demonstrates that the adenine pathway is not completely blocked prior to the synthesis of SAICAR.

The presence of C^{14}-SAICAR in strains of ad_3 grown in glycine-U-C^{14} was established by the failure to resolve the BM positive, C^{14}-positive peak isolated from a Dowex-1-formate column (Figure 9) by two dimensional chromatography. The presence of C^{14}-SAICAR in these extracts can only be explained if adenine biosynthesis in ad_3 is not completely blocked prior to the production of AIR.

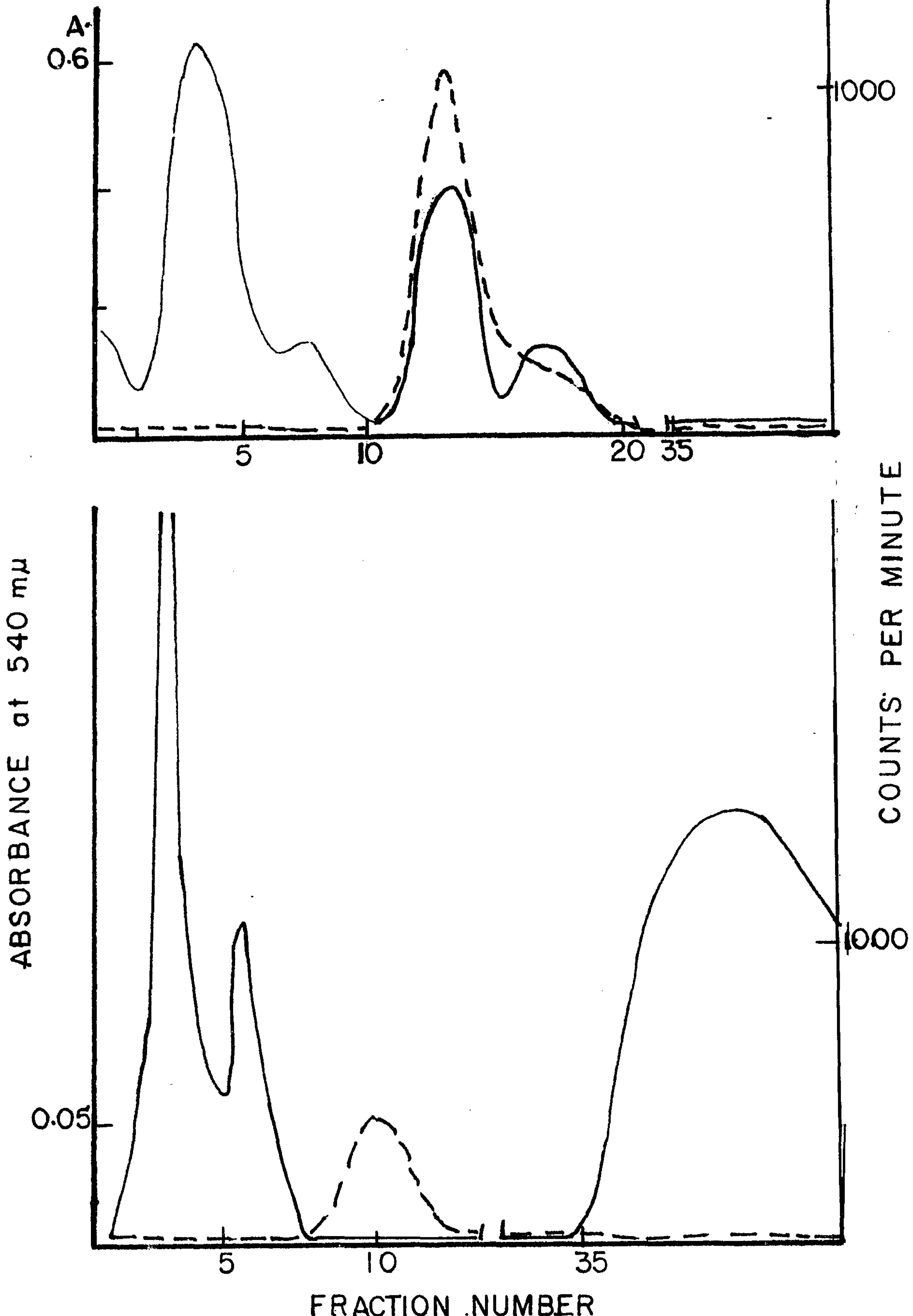
Figure 9. Elution from Dowex-1-formate of C^{14}-labeled and BM positive compounds from ad_3 and ad_3ad_6 extracts grown in media containing glycine-U-C^{14}

A. Extracts from ad_3 (Strain 1).

B. Extract from ad_3ad_6 (Strain 51).

______ counts per minute.

------ BM reaction.



A
0.6
1000
5
10
20
35
ABSORBANCE at 540 mμ
COUNTS PER MINUTE
1000
0.05
5
10
35
FRACTION NUMBER

These results show that the adenine pathway in ad_3 is not completely blocked prior to AICAR formation as was suggested by the lack of red pigment in ad_3ad_2 strains. However, it is possible that the adenine pathway in ad_3 is partially blocked prior to the production of AIR. Such a partial block might not allow sufficient quantities of AIR and CAIR to accumulate in the ad_3ad_2 and ad_3ad_1 double mutants to form red pigment. If there is a partial block prior to AIR synthesis in ad_3 then the quantity of BM positive compounds accumulating from the adenine pathway in ad_3 should be less than the amount of intermediate accumulating in a mutant not blocked prior to AIR formation. A comparison was therefore made between ad_3 and ad_{13}, which is blocked at the conversion of SAICAR to AICAR and not before this step (Dorfman, 1968). (Table 10). Strains of ad_{13} accumulate at least 24 times more BM positive material than ad_3. Furthermore, the strain ad_3ad_{13} accumulates only 4% of the BM positive material that accumulates in ad_{13}.

Therefore, studies on the amount of BM positive material accumulating in ad_3, compared to other strains, indicate that the mutant has a partial block prior to the synthesis of SAICAR and AICAR in addition to a block at the formylation of AICAR to FAICAR. The presence of

Table 10. Comparison of BM positive material accumulating in strains of ad_3, ad_{13} and ad_3ad_{13}

Source of extract:	µmoles/gram wet weight assayed for: Total BM[a] (1)	AICAR[b] (2)	SAICAR (1)-(2)
ad_3 (1)[c]	0.50	0.25	0.25
ad_{13} (33)	12.00	0.00	12.00
ad_3ad_{13} (52)	0.47	0.08	0.39

[a]Total BM was assayed in an ice bath.

[b]AICAR was assayed at room temperature. The values in the table are based on a minimum of two experiments.

[c](Strain number)

this partial block can explain the lack of pigment in ad_3ad_2.

In an attempt to determine the enzymatic basis for the accumulation of AICAR in ad_3, a modification of the Flaks and Lukens (1963) assay for AICAR-transformylase was developed. Using N^5-formylTHFA as a cofactor, dialized extracts of an adenine-independent strain, convert AICAR to FAICAR as indicated by the disappearance of AICAR under the assay conditions. Dialyzed extracts of ad_3 did not have any AICAR-transformylase activity under these conditions (Figure 10). Since ad_3 extracts added to the wild type assay mixture had no effect on the activity of the wild type enzyme, the absence of activity in ad_3 preparations was not the result of an inhibitor of AICAR-transformylase in this strain. On the basis of these observations it was suggested (Mazlen and Eaton, 1967) that an enzyme defect at this step results in the inability of ad_3 strains to formylate AICAR and therefore prevents synthesis of adenine.

Using N^{10}formylTHFA and the strain of ad_3 used throughout these studies Nagy (pers. comm.) demonstrated the presence of AICAR-transformylase activity. Jones and Magasanik (1967b) reported the presence of AICAR transformylase activity in their strain of ad_3, and

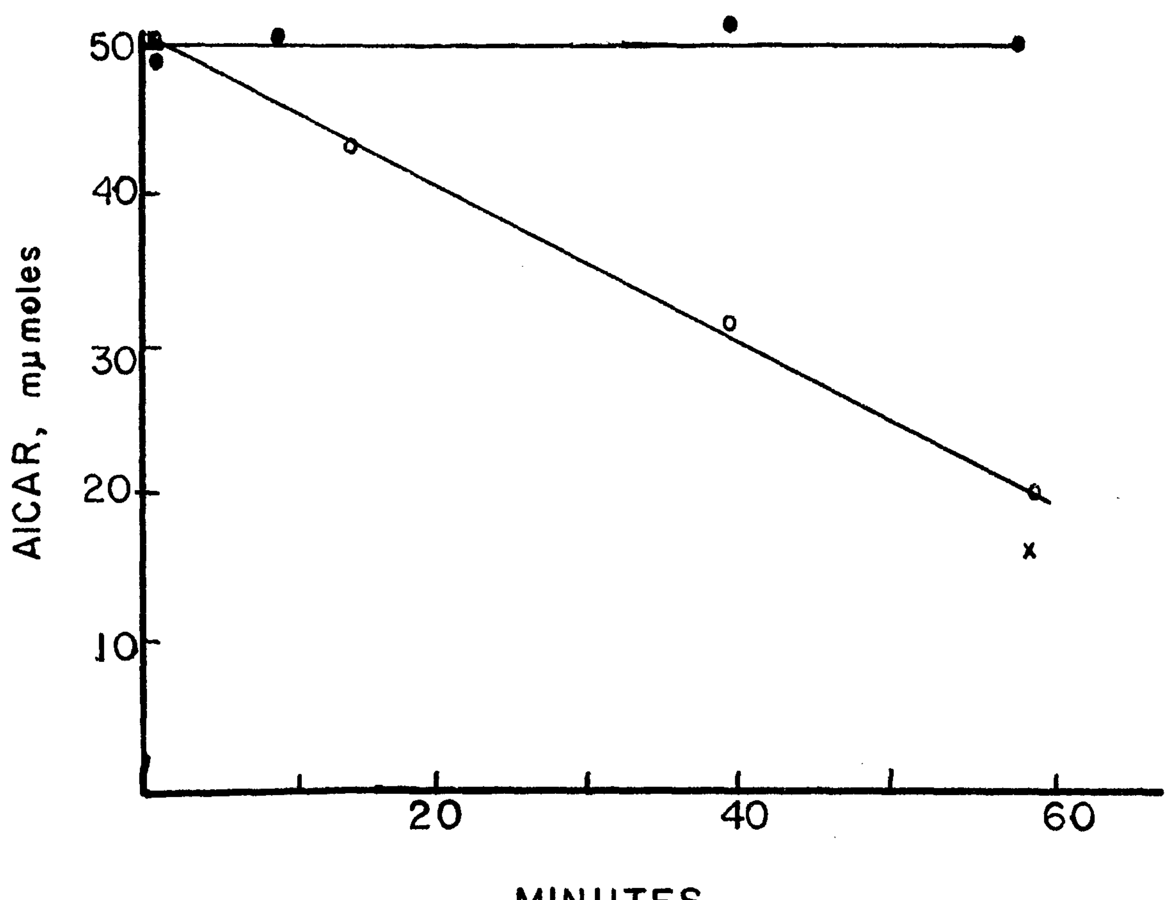
Figure 10. AICAR-transformylase activity in wild type and adenine requiring mutants of Saccharomyces.

The complete assay mixture contained per ml: 50 mμmoles AICAR, 100 mμmoles formyl tetrahydrofolic acid N^5formylTHFA, 10 μmoles phosphate buffer (pH 7.6), 10 μmoles $MgCl_2$, 8 mgm protein. Incubated at 30°C. AICAR was quantitated by Bratton-Marshal reaction using AICARiboside as standard. The control, without N^5formylTHFA (not shown), had no activity.

o———o adenine independent extract.

●———● ad_3 extract.

x———x adenine-independent extract plus ad_3 extract.


50
40
30
20
10
AICAR, mμ moles
20
40
60
MINUTES

Jones (pers. comm.) also confirmed the observation that no activity of AICAR-transformylase could be demonstrated in ad_3 using N^5formylTHFA as cofactor.

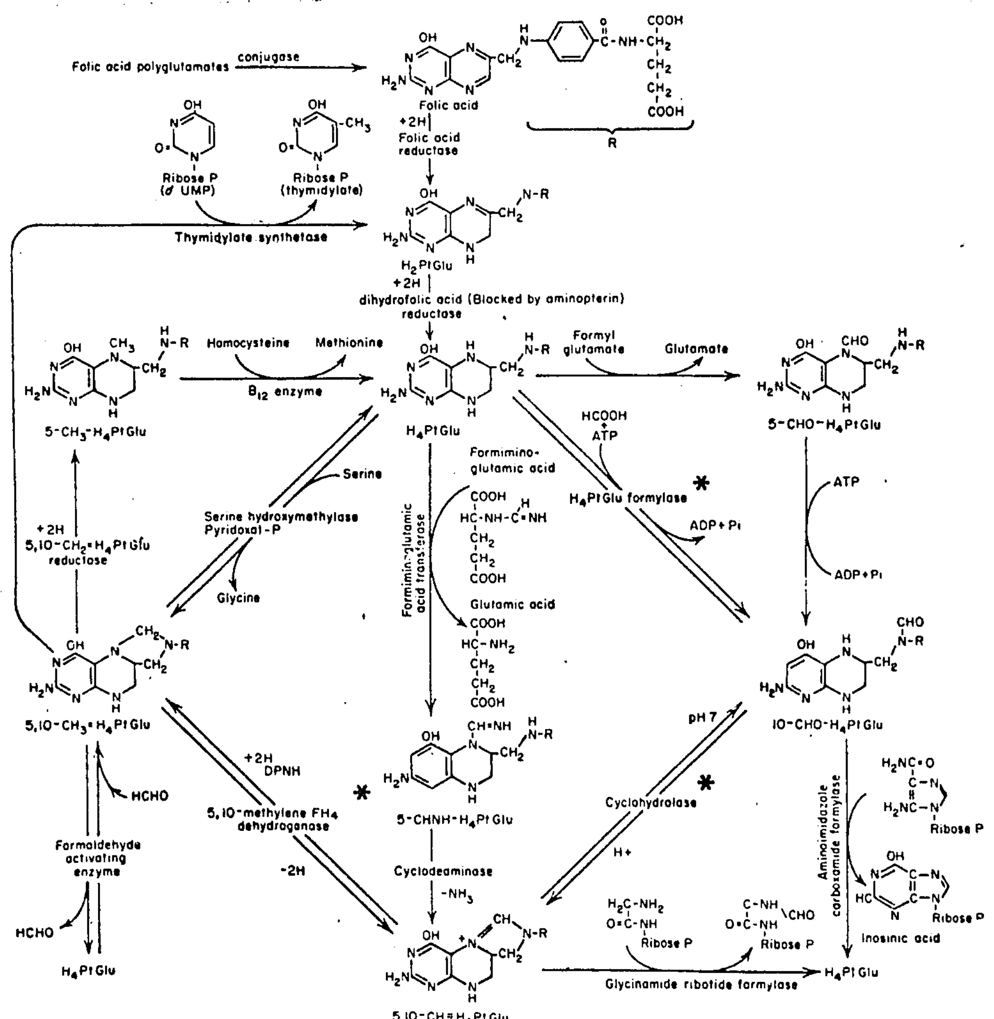
Since AICAR-transformylase in ad_3 requires N^{10}formylTHFA, and N^5formylTHFA cannot substitute for the N^{10}formylTHFA, as it can in wild type strains, the block at the conversion of AICAR to FAICAR in ad_3 appears to result from an inability to synthesize the N^{10}formylTHFA cofactor. This hypothesis was supported by the observations of Jones and Magasanik (1967a), which demonstrated by direct enzymatic assay that ad_3 strains lack formyl tetrahydrofolate synthetase, cyclohydrase activity, and show 10-15% of the wild type activity for $N^{5,10}$methylene tetrahydrofolate dehydrogenase. These enzymes are necessary for the synthesis of N^{10}formylTHFA and $N^{5,10}$formylTHFA. The metabolic reactions of the folic acid cofactors are illustrated in Figure 11.

The presence of AICAR in extracts of ad_3 and the enzyme deficiencies described above lead to the conclusion that ad_3 requires adenine because of a defect of formylTHFA producing enzymes rather than a deficiency of an enzyme of the adenine pathway.

Figure 11. Metabolism of Folic Acid

•H_4PtGlu in this figure is THFA in the text.

* enzymes for which ad_3 (in *Saccharomyces*) is deficient (Jones and Magasanik, 1967a).



(from Stokstad and Koch, 1967)

The presence of an early block in adenine biosynthesis in ad_3 mutants, indicated by the data presented above (Table 9) can be explained by the deficiency of formylTHFA in this mutant. In avian liver (Hartman and Buchanan, 1959) the formylation of GAR to FGAR requires $N^{5,10}$formylTHFA. In Saccharomyces, as stated above, ad_3 lacks wild type levels of the enzymes needed to synthesize this cofactor (Jones and Magasanik, 1967a). Since the mechanism of adenine biosynthesis in Saccharomyces appears to be identical to that of avian liver, it is reasonable to hypothesize that ad_3, which also might require $N^{5,10}$formylTHFA to formylate GAR to FGAR, is blocked at this point in adenine biosynthesis. GAR should therefore accumulate in extracts of ad_3.

Adenine-3 extracts were assayed for the presence of GAR. A Dowex-1-formate column which separates GAR from FGAR was used to examine extracts of ad_4 (ad_4 is known to accumulate GAR, Silver, 1968), ad_3 and wild type strains grown in glycine-U-C^{14}. All of the chromatographed extracts of glycine-U-C^{14} grown strains had a peak of C^{14}-labeled material in the fractions reported by LePage and Jones (1961) to contain GAR. Although this peak in ad_3 and ad_4 proved to have a ninhydrin positive, C^{14}-labeled spot on thin layer radioautography, wild type strains gave similar results.

Since the results of the above study were ambiguous, the presence of GAR in an extract of ad_3 was assayed by the method of Nierlich and Magasanik (1965). Table 11 shows that GAR accumulates in ad_4, but not in ad_3. The possible presence of an inhibitor of the assay in ad_3 extracts was excluded by the observation that ad_3 extracts added to the ad_4 assay mixture had no effect on the detection of GAR in ad_4. It therefore appears, in contradiction to the above hypothesis, that GAR does not accumulate in ad_3.

If the partial block in adenine biosynthesis is prior to the formation of GAR as suggested by the preceding experiment, the double mutant ad_3ad_4 should accumulate less GAR than the ad_4 mutant. AICAR in ad_3ad_4 extracts, which would otherwise interfere with the determination of GAR, was removed by ion exchange on a Dowex-1-formate column. As a control the ad_4 extract was also subjected to the same ion-exchange chromatography. Fractions one through ten from the column, known to contain GAR and to exclude AICAR, were pooled and assayed for the presence of GAR. Under these conditions GAR was found in ad_4 extracts but was absent from ad_3ad_4 extract (Table 11). This evidence supports the contention that ad_3 is blocked prior to the formation of GAR in adenine biosynthesis. One would expect to find some GAR in ad_3ad_4 extracts, since the

Table 11. Assay for the presence of GAR in the extracts of strains of ad_3, ad_4 and ad_3ad_4

Source of extract	Presence of GAR	mμmoles of GAR converted to AICAR experiment 1	experiment 2
1. ad_3[a] (1)[d]	-	0	0
2. ad_4[a] (19)	+	33	40
3. ad_3ad_4[a](50)	-	0	0
4. ad_4[b]	+	150[c]	133
5. ad_4[b] + ad_3[a]	+	144	-

[a] Fraction 1-10 from Dowex-1-formate column.

[b] Crude extract prepared by chloroform-methanol procedure.

[c] Average of two determinations.

[d] (Strain number)

The presence (+) or absence (-) of GAR was assayed by the method of Nierlich and Magasanik (1965). The amount of GAR converted to AICAR was calculated from the absorbance at 540 mμ, with an absorbance of 12.5 equal to 1 μmole/ml.

adenine pathway is not completely blocked prior to AICAR synthesis. The failure to detect GAR in the ad_3ad_4 extract may therefore reflect limitations of the method.

The above results show that the adenine pathway is partially blocked prior to the synthesis of GAR. However, it was not possible to identify precisely the position of this block. A possible explanation of this early partial block will be discussed in the following section.

A partial block at the formylation of GAR to FGAR in ad_3 is suggested by the deficiency in $N^{5,10}$formylTHFA synthesis in extracts of this strain. This block could go undetected in the presence of the additional early block in the adenine pathway. If, for example, the reduced levels of GAR synthesized could be metabolized with the available cofactor no GAR would accumulate. On the other hand, there is no direct evidence that the formylation of GAR to FGAR requires $N^{5,10}$formylTHFA in *Saccharomyces* as this reaction does in avian liver.

Basis for the Histidine Requirement of ad_3

The adenine requirement of ad_3 results from a deficiency of N^{10}formylTHFA and possibly $N^{5,10}$formylTHFA (Jones and Magasanik, 1967a). There is no formylTHFA requirement for histidine biosynthesis. Thus, the

metabolic basis for the adenine requirement of ad_3 leads to no obvious explanation of the histidine requirement of this mutant.

Fink (1965) was able to associate all of the histidine loci of Saccharomyces, except ad_3, with particular steps in histidine biosynthesis. The histidine mutants are listed in Figure 2 with their characteristic accumulation products and their enzyme defects. The fact that AICAR, derived from the histidine pathway, accumulates in ad_3 (Table 9) suggests that the block in histidine biosynthesis might occur after the formation of AICAR and IGP. Extracts of ad_3 strains were compared chromatographically to extracts of the strains hi_2, hi_3 and hi_5 which are blocked in histidine biosynthesis after AICAR production. However, none of the intermediates which accumulate in these strains were present in extracts of ad_3. To determine whether the absence of these histidine intermediates resulted from a block prior to the production of AICAR and IGP, extracts from the strains hi_3 (which accumulates IGP), ad_3 and ad_3hi_3 were assayed for IGP by the periodate oxidation method (Ames, 1957) (Table 12). None accumulated in ad_3 and the amount of IGP accumulating in ad_3hi_3 was about one-third the amount accumulating in hi_3.

Table 12. IGP accumulation

Extract	IGP µmoles/gram wet weight	Percent[a]
ad_3 (1)[b]	0.00	0
ad_3hi_3 (44)	0.04 ± .015[c]	35
hi_3 (36)	0.14 ± .030	100

[a] Amount accumulating in hi_3 taken as 100.

[b] (Strain number)

[c] Value ± standard deviation.

IGP was assayed by the method of Ames (1957). The amount of IGP was calculated from the absorbance of the assay product at 290 mu. AT that wavelength 1 µmole/ml is reported to have an absorbance of 6.90. These data are based on a minimum of four experiments.

These results suggest that a partial block in ad_3 prior to the formation of IGP in histidine biosynthesis allows the synthesis of limited amounts of histidine, insufficient for growth. AICAR, a by-product of this limited synthesis, accumulates because of a block in the adenine pathway. The observation that low amounts of histidine intermediates accumulate only when an additional block in histidine biosynthesis is present is consistent with the above suggestion that the histidine block is a partial one.

Additional experiments based on analysis of the accumulation of AICAR and IGP under histidine-repressed and derepressed growth conditions support the suggestion of a partial block prior to the formation of these compounds in histidine biosynthesis in ad_3. In order to determine the maximum potential activity of the histidine pathway enzymes it was necessary to examine the difference in accumulation between repressed and derepressed conditions. In this study strains containing ad_3, ad_3hi_3, hi_3 and ad_3S_9 (S_9 is a suppressor of the histidine requirement of ad_3) were grown for 48 hours in SC medium. The cells were harvested and the pellet was divided in half and resuspended in either histidineless medium or a medium containing a thousand times the level of histidine

present in SC, and incubated for one hour. The amount of IGP and AICAR accumulating under these conditions was assayed (Table 13). If the activity of the early histidine enzymes under these two growth conditions is the same in ad_3 as it is in ad_3S_9 and hi_3 then the levels of AICAR or IGP accumulating in them should be the same. The results, however, showed that less IGP was present in the strain ad_3hi_3 than in hi_3. In addition, less AICAR was present in ad_3 and ad_3hi_3 than in ad_3S_9. Thus, more AICAR and IGP can be made in strains with no block in histidine biosynthesis prior to IGP formation than in strains containing an ad_3 marker. These data support the view that the early histidine enzymes function less efficiently _in vivo_ in ad_3 than in strains where histidine biosynthesis is unimpaired prior to the synthesis of AICAR and IGP.

Under the conditions of these experiments the maximum amount of AICAR accumulating from the histidine pathway is 0.50 μmoles/gram wet weight (Table 10) which is equivalent to one-twenty fourth the amount of histidine (10 γ/ml in SC medium) that is actually needed for growth of this quantity of cells. This observation further supports the suggestion that ad_3 mutants have a partial block in

Table 13. Accumulation of AICAR and IGP under histidine-repressed and derepressed conditions

Extract	IGP[a]			BM compounds[a]		
	minus histidine (1)	plus[c] histidine (2)	(1)-(2)	minus histidine (3)	plus[c] histidine (4)	(3)-(4)
hi_3	0.175 ± .050[b]	0.073 ± .022	0.102	-	-	-
ad_3hi_3[d]	0.040 ± .014	0.037 ± .016	0.003	0.78 ± .15	0.67 ± .24	-0.01
ad_3S_9	-	-	-	1.98	0.93	1.05
ad_3	-	-	-	0.57	0.43	0.13

[a]Values in table are in µmoles/gram wet weight.

[b]Value ± standard deviation.

[c]SC plus 1000 times the normal level of histidine.

[d]No precise comparison can be made between the levels of IGP and AICAR, because the IGP assay could not be accurately standardized.

The amount of IGP and of AICAR was measured as described in Tables 12 and 11 respectively. Except for the strain ad_3S_9 the values in the table are the means of at least two experiments.

histidine biosynthesis prior to the synthesis of AICAR, sufficient to cause an absolute requirement for histidine in this strain.

In Salmonella, two loci, H and F, together convert BBM III to AICAR and IGP (Ames and Hartman, 1963). Since there is a partial block prior to IGP synthesis in ad_3, the possibility that ad_3 corresponds to a partial block at this step was examined. The absence of any BBM positive material from ad_3 extracts (Tables 8 and 14) demonstrates that ad_3 is not blocked at this step. Furthermore, if the partial block in histidine biosynthesis of ad_3 occurs at or after the conversion of BBM to AICAR and IGP, then equivalent amounts of BBM should accumulate in the strains ad_3hi_7 and hi_7, since hi_7 is blocked at either BBM II or BBM III (Fink, 1965). The amount of BBM accumulating in hi_7, however, was greater than that accumulating in ad_3hi_7 (Table 14). The reduced amount of BBM in ad_3hi_7 could not be the result of a "leaky" hi_7 in the ad_3hi_7 strain since the hi_7 locus in the strain ad_3hi_7 is a homoallele of the locus in the hi_7 strain used.

If hi_7 corresponds to BBM II rather than to BBM III the above data would suggest a block prior to BBM II. According to Fink (1965) hi_6 accumulates a BBM compound.

Table 14. BBM and free-BM positive compounds accumulating in the strains ad_3, ad_3hi_7, hi_6 and ad_3hi_6

	Bratton-Marshal				
Extract	Total[a]	Free amount[a]	%total	Bound (total minus free) amount[a]	Bound (total minus free) %total
ad_3 (1)[b]	0.50	0.50	100	0.00	0.00
hi_7 (42)	0.36	0.02	6	0.34	94
ad_3hi_7 (47)	0.45	0.35	78	0.10	22
hi_6 (41)	0.00	0.00	-	-	-
ad_3hi_6 (46)	0.03	0.03	100	0.00	0.00

[a] Amount in µmoles/gram wet weight

[b] (strain number)

The total BM determination was made after hydrolyzing the extract in HCl at 100°C for five minutes. All BM assays were done in an ice bath. The µmoles/ml of BM positive material was computed as in Table 11. Each value is the mean of at least two experiments.

In an attempt to resolve whether ad_3 is blocked prior to BBM II or BBM III, hi_6 and ad_3hi_6 extracts were assayed for the presence of a BBM compound (Table 14). A strain of hi_6 obtained from Fink, however, had no free BM or BBM compounds contrary to the results reported by him (Fink, 1965). The strain ad_3hi_6, which contained a homoallele of the hi_6 strain above, had only 6% of the free BM positive material present in ad_3 strains, suggesting that hi_6 may in fact be blocked before BBM II in histidine biosynthesis (Figure 2). The discrepancy with Fink's report that hi_6 accumulates a BBM compound may result from differences in growth conditions, preparation of cell extracts, or concentrations of cell extracts in Fink's and this laboratory.

The results of the above experiment are consistent with an hypothesis which places the partial block in histidine biosynthesis prior to the formation of BBM III and possibly prior to BBM II and contradicts the suggestion that ad_3 is blocked at the conversion of BBM III to AICAR and IGP. This partial block is apparently sufficient to cause an absolute requirement for histidine.

Of the first three steps of histidine biosynthesis a block at the first, which makes PR-ATP from PRPP and ATP, will not result in the accumulation of a characteristic

compound, since both ATP and PRPP are widely used cell constituents. PR-ATP and PR-AMP, which would accumulate if the block were at the second and third steps of histidine biosynthesis respectively, are not easily identifiable. An attempt was made, however, to determine whether either PR-ATP or PR-AMP accumulate in ad_3. The success of the approach used depended on the ability to demonstrate the presence of a C^{14}-labeled compound in ad_3, grown in media containing adenine-2-C^{14}, which was absent in wild type and ad_3S_9 strains grown in the same media. If such a compound were detected it might be found to correspond to a characteristic accumulation product of hi_{4B} or hi_{4A} which are blocked at PR-ATP and PR-AMP respectively. The crude extracts were chromatographed in several solvent systems and no C^{14}-labeled spot accumulated in ad_3 that was not also present in ad_3S_9 and wild type extracts. These inconclusive results made it impossible to make any further comparisons between ad_3 and hi_{4A} or hi_{4B}.

It can be concluded from these data that ad_3 can synthesize histidine only in quantity insufficient for growth in histidineless media. The deficiency in histidine biosynthesis results from a defect prior to the synthesis of BBM III. The precise step in histidine biosynthesis at which the partial block occurs, however, could not be ascertained by these studies.

An attempt by direct assay was made to determine whether the block in histidine biosynthesis of ad_3 resulted from reduced activity of one of the early histidine biosynthetic enzymes. The *in vitro* activities of ad_3 and wild type cell extracts were compared in an over-all assay which measures the synthesis of AICAR and IGP from R5P and ATP. No difference in activity between the wild type and ad_3-containing strains could be found. It should be noted that the activity was measured by the BM reaction for AICAR and that attempts to assay the IGP formed were unsuccessful.

The activity of six different enzymes are involved in this assay. Thus, it is possible that rate limiting enzyme under these *in vitro* assay conditions might mask an *in vivo* ad_3 deficiency that does not exist in wild type. The enzymes which catalyze the synthesis of BBM II from PRPP and ATP were therefore assayed separately. The activities in enzyme preparations of ad_3 were at least as high as that of wild type strains (Table 15). It was, therefore, not possible to demonstrate a deficiency in enzyme activity for the early steps of histidine biosynthesis by *in vitro* assay. This apparent contradiction of the results of the *in vivo* studies might be explained by the presence of an inhibitor in ad_3 strains which is lost upon dialysis. Alternatively, the discrepancy could be explained

Table 15. Activity of histidine biosynthetic enzymes and inhibition by histidine and AICAR of these enzymes

strain	reaction assayed	Addition			
		none	AICAR (x 10^{-4} <u>M</u>)[d]	histidine (10^{-4} <u>M</u>)[d]	histidine and AICAR (10^{-4} <u>M</u>)-2 x 10^{-4} <u>M</u>
hi_{4B}[a] (37)[b]	PRPP+ATP→ PR-ATP	44 ± 7[c]	47 ± 9	30 ± 3	31 ± 3
ad_3 (1)	PRPP+ATP→ BBM II	260	-	198	248
wild-type (62)	PRPP+ATP→ BBM II	215	-	140	130

[a]Several trials were done with this strain.

[b](Strain number)

[c]Values ± standard deviation.

[d]The amount of histidine or AICAR added.

Activity is computed as change in absorbance per hour per mg protein.

if a labile enzyme that is actually missing from ad_3 *in vivo*, were also lost from the wild type during the prepartion of the extract.

Role of AICAR

The mutant ad_9 of *S. pombe* requires both histidine and adenine and was shown by Whitehead *et al*, (1966) to accumulate AICAR. These workers demonstrated that AICAR inhibits the synthesis of BBM II from PRPP and ATP and that AICAR at non-inhibitory levels enhances the histidine inhibition of the first enzyme. The histidine requirement in *S. pombe*, therefore, is a secondary effect of the AICAR accumulation. It was of interest to see whether AICAR has a similar effect in *Saccharomyces*. No effect of AICAR on the enzymatic activities of extracts of ad_3, wild type or hi_{4B} strains of *S. cerevisiae* was detected (Table 15, Figure 12). AICAR also showed no synergistic affect with histidine, which inhibits the first reaction of histidine biosynthesis in *Saccharomyces* (Fink, 1965).

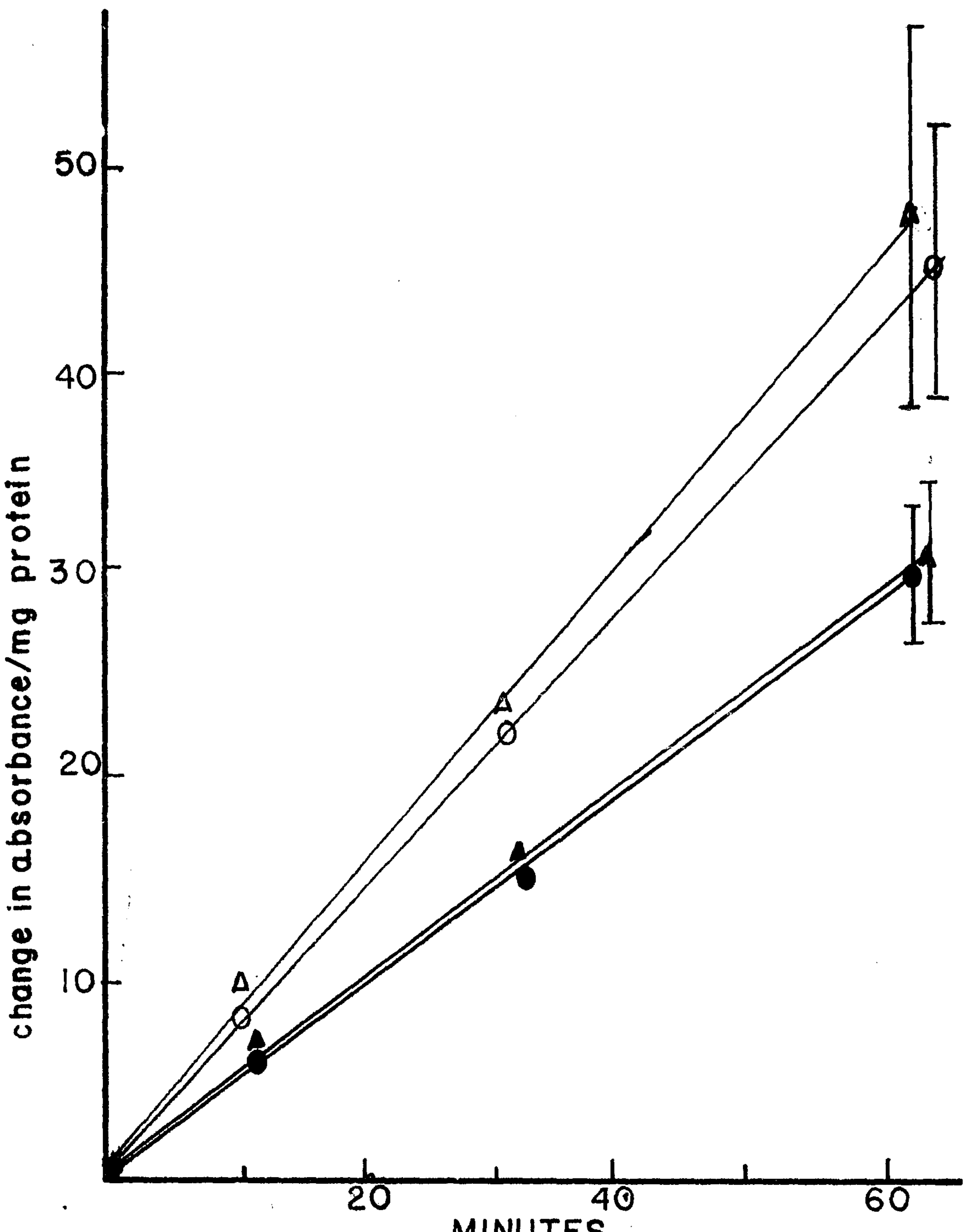
The ad_9 mutation of *S. pombe* differs from ad_3 in three major respects. The defect, resulting in AICAR accumulation, is a faulty AICAR-transformylase (Whitehead *et al*, 1966) and not an inability to form N^{10}formylTHFA. In addition, ad_3 has an earlier partial block in adenine biosynthesis, whereas ad_9 of *S. pombe* is not blocked prior to AICAR synthesis. Therefore, much more AICAR should

Figure 12. Synthesis of PR-ATP from PRPP and ATP by hi_{4B} extracts in the presence and absence of AICAR and histidine.

The assay mixture contained: 36 µmoles tris HCl pH 8.5, 6 µmoles $MgCl_2$, 0.19 µmoles PRPP, 4 µmoles K_2ATP, 0.10 ml cell extract, 0.05 ml water or inhibitor to a final volume of 0.50 ml. The K_2ATP was replaced by water in the blank. (Fink, 1965).

histidine (10^{-4}M)

(-)	(+)	
○	●	(-) AICARibotide
△	▲	(+) AICARibotide (2×10^{-4}M)


change in absorbance/mg protein
50
40
30
20
10
20
40
60
MINUTES

accumulate in ad_9 of S. pombe than accumulates in ad_3 of S. cerevisiae. Thus, if the effect of AICAR in S. cerevisiae and S. pombe were alike, levels of AICAR which inhibit the latter organism should be more than enough to inhibit the histidine enzymes of S. cerevisiae. No AICAR inhibition of these enzymes was found *in vitro*. Finally, in S. pombe partial suppressors of ad_9 which eliminate the requirement for histidine are actually adenine pathway mutants blocked before the production of AICAR (Whitehead, *et al*, 1966). In *Saccharomyces* none of the earlier adenine mutants relieve the histidine requirement of ad_3.

As in S. pombe, partial suppressors of ad_3 of *Saccharomyces* relieve the histidine requirement. No partial suppressors of the adenine requirement are known. A study of these partial suppressors was undertaken since they might elucidate the cause of the histidine requirement.

Partial suppressors isolate in this laboratory proved to be dominant and were unlinked to the ad_3 locus as demonstrated by the 5 to 4 ratio of parental ditype to non-parental ditype resulting from crosses of these suppressors with a wild type. Luzzati (1965) and Jones (pers. comm.) also isolated similar suppressors. Luzzati found a closely linked suppressor and Jones found an

unlinked recessive suppressor and one which suppresses either the adenine or the histidine requirement of ad_3 but not both. The characteristics of the various classes of suppressors of the ad_3 locus are summarized in Table 16. Luzzati's suppressor S_9 was used for further study because it had the best viability in crosses.

By the synthesis of the appropriate strains, the suppressor (S_9) was shown to be specific for the histidine requirement of ad_3 and to have no effect on wild type, other adenine or other histidine mutants (Table 17). It suppressed the histidine requirement of two heteroalleles of ad_3, one KCl remedial and another non-KCl remedial ad_3 allele showing that it is locus and not allele specific. Therefore, it cannot be classed as a supersuppressor since supersuppressors do not act on KCl remedial strains (Hawthorne and Friis, 1964). The suppressor S_9 was found to be linked to hi_5 at a distance of 13.6 centimorgans (Table 18).

Since the adenine requirement of the strain ad_3S_9 is unaffected by the suppressor, the ribotides and ribosides of AICAR and SAICAR should accumulate in this strain as they do in ad_3. However, ad_3S_9 accumulates SAICARiboside exclusively. The data on BM positive compounds accumulating in this strain are presented in Tables 4, 5 and 19.

Table 16. Properties of all known classes of suppressors of the ad_3 locus

Class	strain (genotype)	adenine-less	histidine-less	histidineless and adenineless	Dominance[a]	Linkage[b]	Source
1[c]	ad_3S_9 (7)	-	+	-	D	U	Luzzati
2	ad_3PR_7	-	+	-	R	U	Jones
3	ad_3FR_{13}	+	+	-	D	U	Jones
4	ad_3S_{15}	-	+	-	R	L	Luzzati

[a] D - dominant; R - recessive.

[b] L - linked; U - unlinked.

[c] Other strains in this class are: 4, 5, 6, 10, 11, 12 and 13.

Table 17. The effect of suppressor S_9 on the adenine and histidine requirement of strains carrying various adenine and histidine markers

Genotype	Strain number	Growth in the following media: adenineless	histidineless	adenineless histidineless
ad_3S_9	6	-	+	-
ad_3S_9	7	-	+	-
ad_1S_9	55	-	+	-
ad_2S_9	56	-	+	-
ad_6S_9	57	-	+	-
hi_1S_9	58	+	-	-
hi_3S_9	59	+	-	-
hi_5S_9	60	+	-	-
S_9(Ad+Hi+)	61	+	+	+

The strain ad_3 (6) will grow on adenineless media supplemented with 0.9 M KCl, whereas the strain ad_3 (7) will not.

Table 18. Analysis of a cross between strains containing the markers ad_3S_9 and hi_5

diploid: $\frac{a\ ad_3hi_5}{\alpha\ ad_3S_9tr}$

Genotype			Phenotype	Tetrad	
ad_3	S_9	hi_5	hi	type	number
1. -	S	+	+	parental ditype	16
-	S	+	+		
-	s	-	-		
-	s	-	-		
2. -	S	-	-	non-parental ditype	0
-	S	-	-		
-	s	+	-		
-	s	+	-		
3. -	S	-	-	tetratype	6
-	S	+	+		
-	s	-	-		
-	s	+	-		

Strains containing the other class 1 suppressors listed in Table 16 also accumulate exclusively SAICARiboside.

These results suggest that AICAR is removed in the presence of S_9. Since SAICARiboside is the exclusive BM positive accumulation product, and since the total amount of BM positive compound accumulating in ad_3S_9 is not less than that in ad_3 strains, it may be suggested that SAICARibotide is dephosphorylated to SAICARiboside in the presence of the suppressor. The decreased SAICARibotide concentration is followed by the conversion of AICARibotide to SAICARibotide by the enzyme adenylosuccinase, in order to establish equilibrium between the two ribotides. However, because no effect of AICAR could be demonstrated _in vitro_ it is possible that the suppressor is acting in some other, undetected manner and that the absence of AICAR is an irrelevant side effect of the suppressor. To determine whether the suppressor removes AICAR as outlined above, and also to determine whether the removal of AICAR is the basis for the suppression of the histidine requirement by S_9, ad_{13} which lacks adenylosuccinase activity was crossed to ad_3S_9 to form a strain with the genotype $ad_3ad_{13}S_9$. The ad_{13} allele should prevent the conversion of the AICAR synthesized by the histidine pathway to SAICAR, and since the original ad_3 allele is present, AICAR should accumulate. If S_9

suppresses the histidine requirement of ad_3 by some means other than the removal of AICAR to SAICARiboside, the strain $ad_3ad_{13}S_9$ should accumulate AICAR but remain histidine independent. The strain did, indeed, accumulate AICAR(Table 19). However, it was no longer histidine independent despite the presence of S_9 (Table 17). Complementation with all the histidine testers showed that no other histidine markers were present and the presence of S_9 in the strain was confirmed by growth on histidineless media of the diploid $ad_3/ad_3ad_{13}S_9$. It may be concluded that AICAR acts _in vivo_ to inhibit histidine biosynthesis. Since no effect of AICAR could be demonstrated _in vitro_ the actual effect of AICAR on the early steps of histidine biosynthesis remains to be established.

Table 19. BM positive compounds accumulating in ad_3, ad_3S_9, ad_3ad_{13} and $ad_3ad_{13}S_9$

Source of extract	Amount of BM material in µmoles/gram wet weight		
	Total free BM	SAICAR	AICAR
ad_3 (1)[a]	0.56	0.25	0.25
ad_3S_9 (6)	1.46	1.46	0.00
ad_3ad_{13} (52)	0.47	0.08	0.39
$ad_3ad_{13}S_9$ (54)	1.00	0.22	0.78

[a] (strain number)

Total BM was assayed in an ice bath. The amount of AICAR was determined by carrying out the BM assay at room temperature. The amount of SAICAR was calculated as the difference between these two determinations. The µmoles/ml of BM positive compound were calculated as described in Table 11.

DISCUSSION

The basis for the double requirement of the ad_3 mutation of Saccharomyces is not yet fully resolved. The BM positive accumulation product in ad_3 was shown to be AICAR and SAICAR by Dorfman (1964). These results appeared to contradict the observation that ad_3ad_2 double mutants were white. The results in this study showed, in agreement with Dorfman (1964), that the ribosides and ribotides of AICAR and SAICAR accumulate in ad_3 and, furthermore, that the AICAR accumulating is not a breakdown product of either BBM or SAICAR resulting from the extraction procedure.

The enzyme AICAR-transformylase which converts AICAR to FAICAR is inactive in ad_3 with N^5formylTHFA as the cofactor. AICAR-transformylase in wild type extracts, on the other hand, is active with this form of the cofactor (Figure 10). Nagy (pers. comm.) and Jones and Magasanik (1967b) showed activity of AICAR-transformylase in ad_3 extracts with N^{10}formylTHFA. The latter investigators also showed that ad_3 extracts lack THFA-formylase and cyclohydrase activity and have 10-15% of the wild type levels of $N^{5,10}$methyleneTHFA dehydrogenase (Figure 11). A single genetic lesion, ad_3, therefore appears to result in the deficiency of three enzymes. This is an unusual

observation and warrants further study. These enzymes are involved in the synthesis of formylTHFA. Demonstration of the deficiency of these three formylTHFA synthesizing enzymes is not sufficient, however, to explain the adenine requirement of ad_3, since a kinase exists in some organisms which converts N^5formylTHFA to N^{10}formylTHFA, and the absence of such an enzyme from wild type yeast has not been demonstrated. Both the observation that ad_3 extracts are deficient for these enzymes and the observation that in ad_3 N^5formylTHFA cannot substitute for N^{10}formylTHFA for the formylation of AICAR, as it can in wild type, lead to the conclusion that ad_3 requires adenine because of a defect in its ability to synthesize N^{10}formylTHFA.

Although the basis for the adenine requirement of ad_3 was established by the above observations, other effects of this mutation on adenine biosynthesis remained to be explained. The fact that the double mutant ad_3ad_2 is white rather than red suggested that ad_3 has a block prior to AIR formation in adenine biosynthesis, in addition to a block at the formylation of AICAR. Jones and Magasanik (1967b) stated that the deficiency of the three folic acid cofactor synthesizing enzymes listed above results in a deficiency of folic acid cofactors at

the formate level of oxidation, and suggested (Jones, pers. comm.) that yeast GAR-transformylase, which was not assayed, might require the $N^{5,10}$formylTHFA as does avian liver GAR-transformylase. However, no GAR was found in ad_3 extracts (Table 11) as would be expected on the basis of the above hypothesis. Strains of ad_3ad_4 also failed to accumulate detectable quantities of GAR, while ad_4 extracts blocked at the conversion of GAR to FGAR (Silver, 1968) contained GAR under the conditions used to assay the ad_3 and ad_3ad_4 preparations. It is possible that GAR accumulates in ad_3 and ad_3ad_4 at levels below the limits of detection of the method. The data, however, show that GAR accumulation in ad_3ad_4 is, at the least, less than that of ad_4. A block in adenine biosynthesis in ad_3, prior to the formation of GAR is suggested by this observation. One hypothesis which might account for a block prior to GAR formation in ad_3 will be discussed below.

The failure to detect GAR in ad_3 and ad_3ad_4 extracts is not sufficient evidence to eliminate the hypothesis that ad_3 strains are also blocked at the formylation of GAR.

However $N^{5,10}$formylTHFA can be synthesized as a by-product of histidine catabolism by the enzyme

cyclodeaminase (Figure 11). The absence of this enzyme from wild type yeast has not been demonstrated and it is possible that yeast can synthesize $N^{5,10}$formylTHFA and that ad_3 is therefore not blocked at the formylation of GAR.

The demonstration that the AICAR accumulation in ad_3 is not due to a defective AICAR-transformylase strongly suggests that mutants at another adenine-requiring locus in Saccharomyces remain to be isolated. One possible explanation why such a mutant has not been found is that most investigators have looked for strains containing new adenine markers as white papilations on colonies of ad_2 or ad_1 which are red. A mutant defective for AICAR-transformylase, however, would not be white if AICAR accumulation did not sufficiently block an early adenine pathway enzyme. Since there is no rapid screening technique for this mutation, its recovery remains a matter of a search for a forward mutation at this site. The availability of such a mutant would allow a test of the hypothesis that AICAR accumulation is responsible for the histidine requirement in ad_3 since, if this hypothesis were true, such a mutant would have an absolute requirement for histidine as does ad_3.

Accumulation of AICAR has been shown to be responsible for the histidine requirement of ad_3 (Table 19). The block in histidine biosynthesis in ad_3 does not completely prevent the synthesis of histidine. This block is sufficient, however, to cause an absolute requirement for histidine and is located prior to the formation of BBM III. Inhibition by AICAR of an early histidine enzyme could not be demonstrated *in vitro*, although the level of AICAR used in the inhibition study on the enzymes of *Saccharomyces* converting PRPP and ATP to PR-ATP was sufficient to inhibit *S. pombe* enzymes (Whitehead, *et al*, 1966).

Marver *et al* (1967) demonstrated that AICAR altered the derepression of the histidine enzymes in *Salmonella* from a simultaneous to a sequential derepression. It is, therefore, possible that AICAR might have an effect on the synthesis of the histidine enzymes in yeast. However, no difference was found between the histidine enzyme levels of wild type and those of ad_3.

The following hypothesis could account for the AICAR inhibition of histidine biosynthesis, as well as for the partial block in adenine biosynthesis prior to the formation of GAR. If AICAR partially inhibits the formation of PRPP from R5P and ATP by the enzyme 5-phosphoribose-pyrophosphokinase then less PRPP would

be available *in vivo* for the synthesis of uracil and tryptophan and for the ribosylation of the bases to mononucleotides. These reactions would compete effectively with the adenine and histidine biosynthetic enzymes for the reduced amounts of PRPP resulting in a decreased synthesis of these two compounds. The enzyme 5-phosphoribose pyrophosphokinase is active in ad_3 extracts. However, since the enzymes which compete with the adenine and histidine pathways *in vivo* for the PRPP were not supplied with substrate, no competition for the end-product PRPP could be detected. A test of this hypothesis has not been carried out. Such a system, showing preferential use of PRPP, is not without precedent. Rat liver shows a preferential use of PRPP by the uracil pathway in the presence of orotic acid resulting in a deficiency in adenine biosynthesis (Rajalakshmi and Handschumacher, 1968). It should be pointed out, however, that a sparing effect of uracil and tryptophan expected on the basis of this hypothesis could not be demonstrated.

The relationship between histidine and adenine biosynthesis in ad_3 is unlike that in any organism studied to date. *E. coli* has no histidine requirement when the adenine pathway is blocked at the formylation of AICAR. The AICAR which accumulates in *E. coli* is in

the form of the riboside and AICA, and these BM positive compounds are found in the media in which the strains have been grown (Magasanik and Karibian, 1960). It is possible that the absence of a histidine requirement in these strains of *E. coli* results from their ability to convert the AICARibotide to the riboside and AICA and to excrete these compounds.

No histidine effect has been reported for a strain of *Neurospora* which was shown by Bernstein (1961) to accumulate AICAR.

Adenine-3 shares with *S. pombe* mutant ad_9 a block at the conversion of AICAR to FAICAR. In both of these organisms the accumulation of AICAR is responsible for their histidine requirement. The cause of the block resulting in AICAR accumulation is different in the two organisms, and the effect of AICAR on histidine biosynthesis also appears to be different.

SUMMARY

Studies of the ad_3 mutation in _S. cerevisiae_ lead to the conclusion that the organism requires adenine because it cannot convert the adenine pathway intermediate AICAR to FAICAR. The block is due to defects in the enzymes which synthesize N^{10}formylTHFA (Jones and Magasanik, 1967a). The presence of a partial block in adenine biosynthesis prior to the production of AICAR was indicated by the reduced levels of BM positive material in ad_3ad_{13} extracts. Neither the cause nor the location of the additional block in adenine biosynthesis could be determined. However, the results indicate that the block occurs before the formation of GAR. The possibility of an additional, partial block in the formylation of GAR to FGAR, as might be expected from the deficiency of $N^{5,10}$formylTHFA in ad_3, could not be eliminated.

Studies of the suppressor of the histidine requirement of ad_3 showed that this requirement of ad_3 is caused by inhibition of histidine biosynthesis by the AICAR which accumulates in this strain. Although the block in histidine biosynthesis is a partial one, it is sufficient to cause an absolute growth requirement for histidine. This block in histidine biosynthesis is prior to the synthesis of BBM III. However, it could not be localized more precisely on the basis of accumulation studies or by enzyme assay.

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