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NON-ALLELIC ADENINE-REQUIRING MUTANTS OF
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STUDIES ON THE BIOSYNTHETIC BLOCKS OF EIGHT NON - ALLELIC ADENINE - REQUIRING MUTANTS OF *SACCHAROMYCES CEREVISIAE*

by

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ABSTRACT

Studies on the biosynthetic blocks of eight
non-allelic adenine-requiring mutants of
Saccharomyces cerevisiae. by Julienne M. Silver

Advisor : Prof. Norman R. Eaton

The biosynthetic pathway of adenine in Saccharomyces cerevisiae has been studied with the aid of eight non-allelic adenine-requiring mutants artibrarily numbered ad_1, ad_2, ad_4, ad_5, ad_6, ad_7, ad_8 and ad_9.

The presumptive ordering of these mutants along the pathway has been made by comparisons of substances derived from C^{14}-glycine which accumulate in extracts of the mutants, as detected by thin layer chromatography.

The introduction of an ad_4, ad_5, ad_8 or ad_9 marker into either an ad_6 or ad_7 strain resulted in the loss of the characteristic accumulations of the latter two mutants, indicating that ad_6 and ad_7 were blocked relatively late in the pathway with respect to the above mutants. Since the chromatographic pattern of the ad_6ad_7 double mutant resembled that of the ad_6 parent, it was concluded that ad_6 was blocked earlier than ad_7. Using a similar approach, ad_8 was referred to a step in the pathway earlier than ad_4. The exact location of the ad_5, ad_8 and ad_9 blocks with respect to one another, could not be determined by this method.

The ad_6 and ad_7 mutants do not accumulate any imidazole compounds not found in the wildtype. In addition, these strains are blocked relatively late with respect to all other mutants tested except ad_1 and

ad_2. This suggested that ad_6 and ad_7 are blocked at steps immediately preceding ring closure and should therefore accumulate formylglycinamide ribotide (FGAR) or formylglycinamidine ribotide (FGAM). Extracts of the mutants were tested for the presence of these formylated intermediates and the presence of FGAR in both ad_6 and ad_7 was confirmed by chromatographic and enzymatic procedures. Since ad_7 was shown to be blocked at a step later than ad_6, the ad_7 block was placed at the conversion of FGAM to aminoimidazole ribotide (AIR) and the ad_6 block at FGAR to FGAM.

Double mutant studies suggested that ad_4, ad_5, ad_8 and ad_9 were blocked before the formylation of glycinamide ribotide (GAR) to FGAR. Tests for the presence of formylated purine precursors in these mutants were negative, confirming the results obtained from double mutant studies.

Based on their ability to accumulate C^{14}-glycine-labeled materials and on the absence of FGAR, the mutants ad_4 and ad_5 were tentatively assigned to a step in the pathway after the incorporation of glycine but before the incorporation of formate, although there is only one reported step which would correspond to such criteria (Buchanan, 1960). However only ad_4 was found to accumulate GAR confirming that this mutant is indeed blocked at the conversion of GAR to FGAR. The presence of GAR could not be demonstrated in extracts of ad_5, however, some GAR was found in extracts of the ad_4ad_5 double mutant. Because of these conflicting observations it was not possible to localize the ad_5 block.

Since crude extracts of ad_8 and ad_9 show comparatively little accumulation of C^{14}-glycine-labeled materials, these mutants were tentatively assigned to steps in the pathway preceding that at which glycine is incorporated i.e. prior to the conversion of phosphoribosylamine (PRA) to GAR. Enzymatic assays confirmed the absence of GAR in extracts of these mutants. Although ad_8 and ad_9 are blocked early in the pathway, it is unlikely that these mutants can not carry out the conversion of ribose-5-phosphate to phosphoribosylpyrophosphate (PRPP), since this compound is also required for the synthesis of histidine, tryptophan and uridine which are not required by these mutants for growth.

Of all the mutants studied only ad_1 and ad_2 accumulate imidazoles not found in the wildtype. Spectrophotometric analyses of Bratton-Marshall chromophores in extracts indicated that ad_2 was block at the conversion of AIR to 5-amino-4-imidazole-carboxylic acid ribotide (CAIR) and ad_1 at the conversion of CAIR to 5-amino-4-imidazole-N-succinocarboxamide ribotide (SAICAR). Further evidence that ad_2 is blocked before ad_1 is provided by the observation that the introduction of an ad_2 marker into an ad_1 strain results in the elimination of an imidazole characteristic of the ad_1 mutant.

The data presented in this study lead to the following assignment of gene-enzyme relationships:

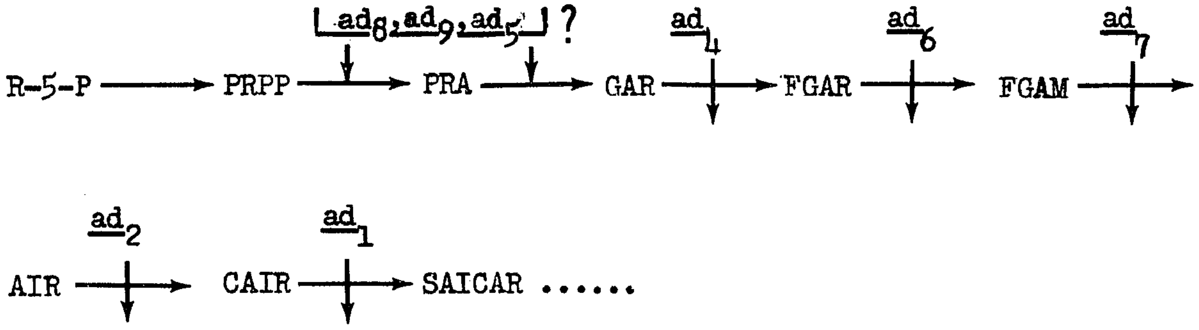


TABLE OF CONTENTS

LIST OF TABLES

Page No.

LIST OF FIGURES

LIST OF FIGURES (cont.)

LIST OF FIGURES (cont.)

LIST OF FIGURES (cont.)

CHAPTER I

INTRODUCTION

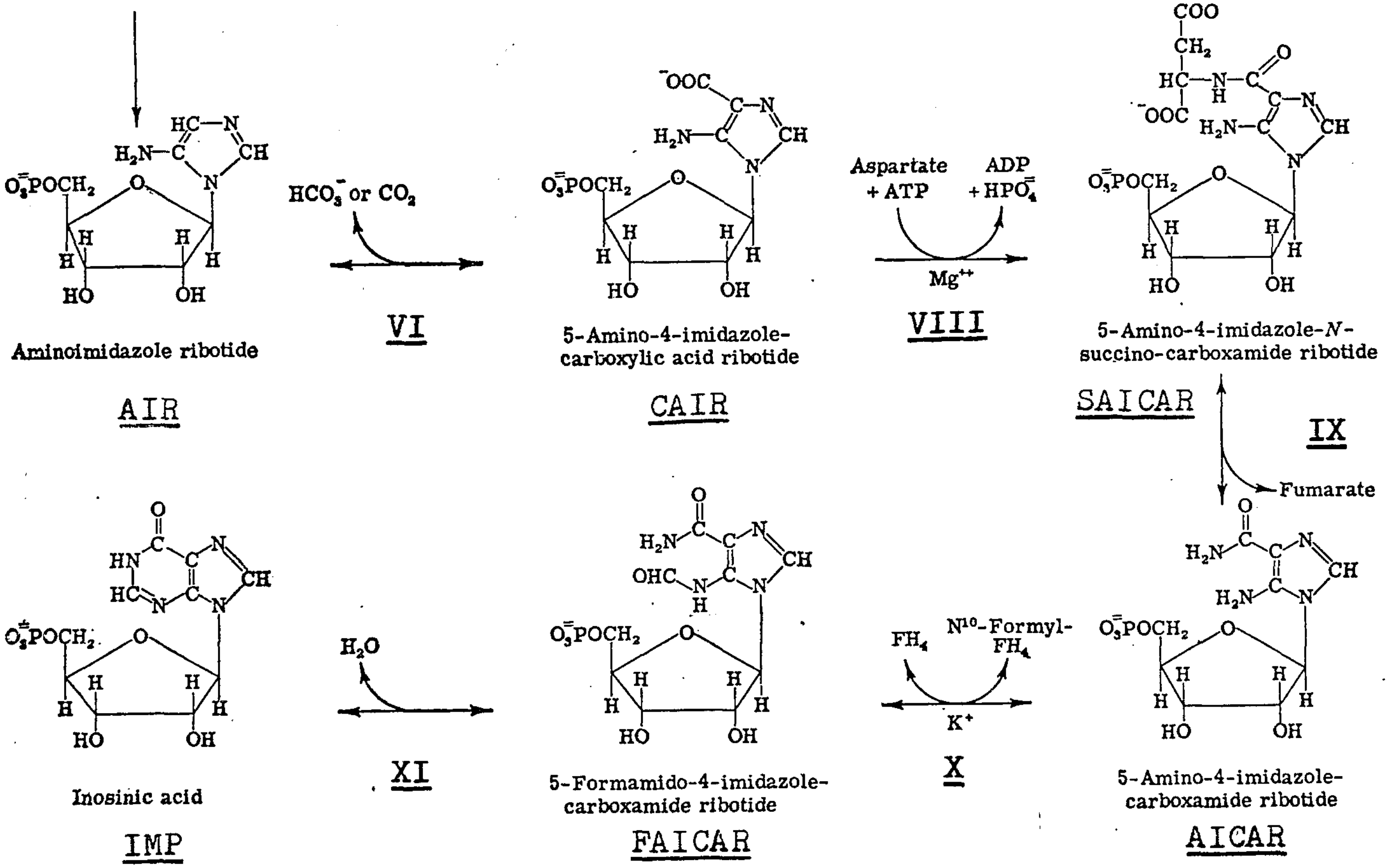
The purine biosynthetic pathway as it occurs in the avian liver system has been the subject of recent reviews by Buchanan (1960) and Buchanan and Hartman (1959). The reaction steps and intermediate compounds are shown in Fig 1 and the enzymes are listed in Table 1.

The synthesis of purines by bacteria (Magasanik, 1962), and normal and neoplastic mammalian tissues (Bennet _et al_, 1956; Moore and Lepage, 1957; Tomisek _et al_, 1958) appears to bear a remarkable similarity to the pathway described in the avian liver system. However, recent work indicates that there are alternative reactions for some of the steps. For instance, it has been shown that there are two independent amidotransferases both active in the formation of 5-phosphoribosylamine (step II, Fig 1). Glutamine and PRPP* are substrates for one of the enzymes, while ribose-5-phosphate and ammonia are substrates for the other. Both enzymes occur simultaneously in such divergent forms as _Escherichia coli B_ (LeGal _et al_, 1967a), Ehrlich ascites tumor cells, (Herscovics and Johnstone, 1964) and in wheat germ preparations (Kapoor and Waygood, 1962), and both appear to be sensitive to feedback inhibition by the end products of the purine pathway, AMP and GMP (LeGal _et al_, 1967a).

Although the role of glutamine as the donor of purine nitrogen atoms 3 and 9 (Fig 2) has been emphasized (Buchanan, 1960), Kapoor and Waygood (1962) demonstrated that the PRPP-amidotransferase of wheat germ embryos accepts the amide group of asparagine more readily than

*see list of abbreviations, Table 2.

Fig. 1. Reaction steps and intermediates in purine biosynthesis (from Dorfman, 1964a).


Aminoimidazole ribotide
AIR
HCO_3^- or CO_2
VI
5-Amino-4-imidazole-carboxylic acid ribotide
CAIR
Aspartate + ATP
ADP + $HPO_4^=$
Mg^{++}
VIII
5-Amino-4-imidazole-*N*-succino-carboxamide ribotide
SAICAR
IX
Fumarate
5-Amino-4-imidazole-carboxamide ribotide
AICAR
FH_4
N^{10}-Formyl-FH_4
K^+
X
5-Formamido-4-imidazole-carboxamide ribotide
FAICAR
H_2O
XI
Inosinic acid
IMP

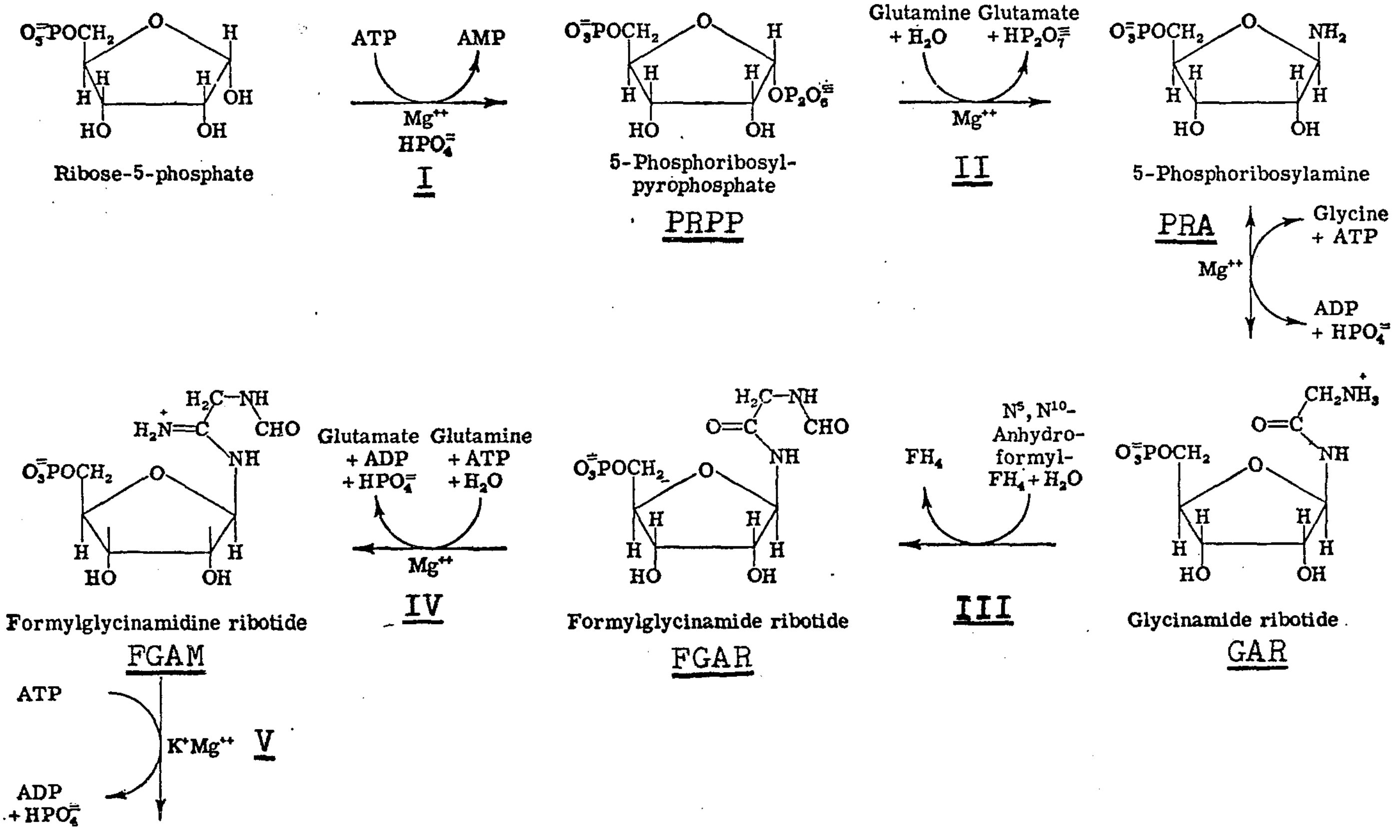

Ribose-5-phosphate
ATP
AMP
Mg++
HPO4=
I
5-Phosphoribosyl-pyrophosphate
PRPP
Glutamine + H2O
Glutamate + HP2O7≡
Mg++
II
5-Phosphoribosylamine
PRA
Glycine + ATP
Mg++
ADP + HPO4=
Glycinamide ribotide
GAR
FH4
N5, N10-Anhydro-formyl-FH4 + H2O
III
Formylglycinamide ribotide
FGAR
Glutamate + ADP + HPO4=
Glutamine + ATP + H2O
Mg++
IV
Formylglycinamidine ribotide
FGAM
ATP
ADP + HPO4=
K+Mg++
V

Table 1

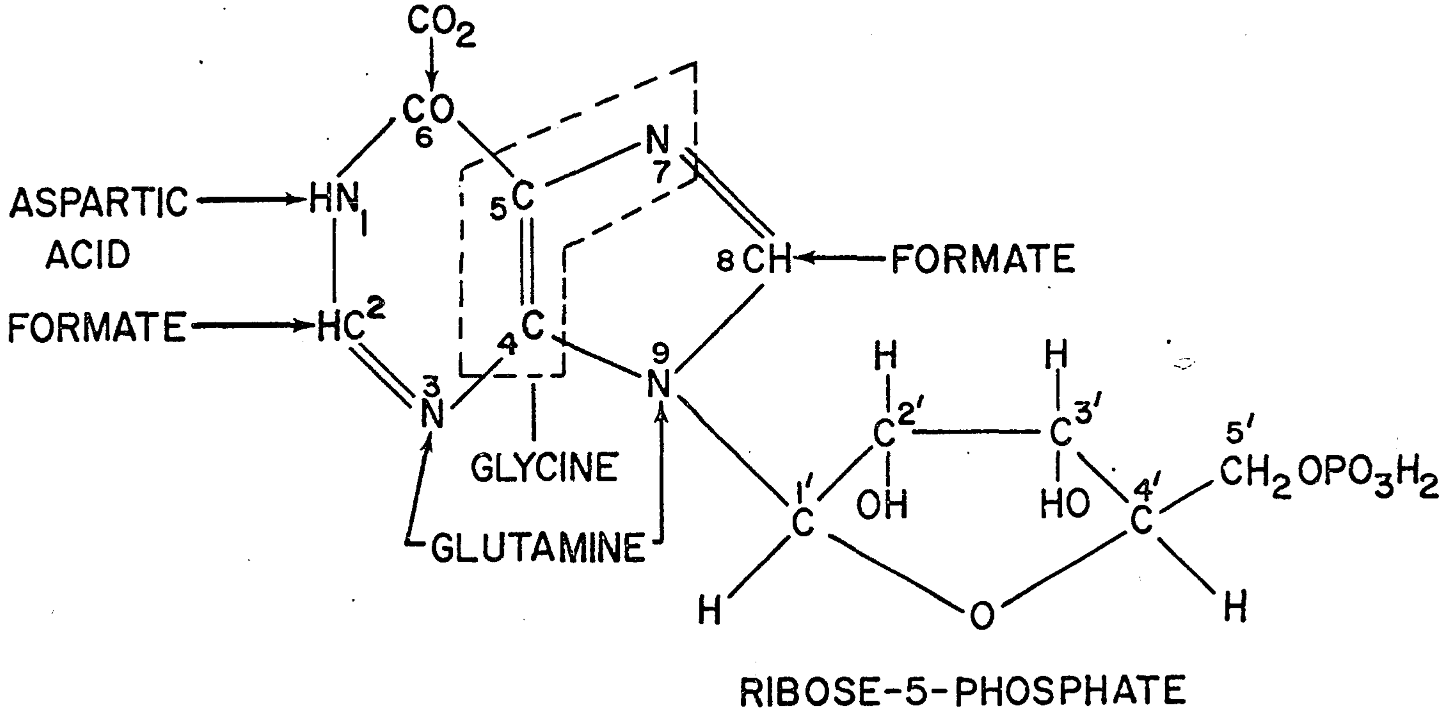
The enzymes catalyzing purine bioxynthesis (from Dorfman, 1964a)

Reaction	Systematic name	Trivial name	E.C. No.
5-RP → PRA	Ribosylamine-5-phosphate:pyrophosphate phosphoribosyltransferase (glutamate amidating)	Amidophosphoribosyltransferase phosphoribosylpyrophosphate-amidotransferase	2.4.2.14
PRA ⇌ GAR	5'-phosphoribosylamine: glycine ligase (ADP)	phosphoribosyl-glycine-amide synthetase	6.3.1.3
GAR → FGAR	5'-phosphoribosyl-N-formylglycineamide: tetrahydrofolate 5, 10-formyltransferase	phosphoribosyl-glycineamide formyltransferase	2.1.2.2
FGAR ⇌ FGAM	5'-phosphoribosyl-formylglycineamide: L-glutamine amido-ligase	phosphoribosyl-formylglycineamidine synthetase	6.3.5.3
FGAM → AIR	5'-phosphoribosyl-formylglycineamiding cycloligase (ADP)	phosphoribosyl-aminoimidazole synthetase	6.3.3.1
AIR ⇌ CAIR	5'-phosphoribosyl-5-amino-4-imidazolecarboxylate carboxylase	phosphoribosyl-aminoimidazole carboxylase	4.1.1.21
CAIR → SAICAR	5'phosphoribosyl-4-carboxy-5-aminoimidazole: L-aspartate	phosphoribosyl-aminoimidazole-succinocarboxamide synthetase	6.3.2.6
SAICAR ⇌ AICAR AMPS ⇌ AMP	adenylosuccinate AMP-lyase	adenylosuccinate lyase (adenylosuccinase)	4.3.2.2

Table 1 (cont.)

Reaction	Systematic name	Trivial name	E.C. No.
AICAR ⇌ FAICAR	5'-phosphoribosyl-5-formamido-4-imidazolecarboxamide: tetrahydrofolate 10-formyltransferase	phosphoribosyl-aminoimidazole-carboxamide formyltransferase	2.1.2.3
FAICAR ⇌ IMP	IMP 1, 2-hydrolase (decyclizing)	IMP cyclohydrase (inosinicase)	3.5.4.10
IMP → AMPS	IMP: L-aspartate ligase (GDP)	adenylosuccinate synthetase	6.3.4.4

Fig. 2. Origin of purine nucleotide atoms (from Buchanan and Hartman, 1959).


CO2
CO 6
ASPARTIC ACID
HN 1
FORMATE
HC 2
N 3
4 C
5 C
N 7
8 CH
FORMATE
N 9
GLYCINE
GLUTAMINE
H
C 2′
OH
H
C 3′
HO
C 4′
5′ CH2OPO3H2
C 1′
H
O
H
RIBOSE-5-PHOSPHATE

that of glutamine, and that carbamyl-phosphate was as active as glutamine as the source of N-9 in this plant.

In E. coli B, asparagine has little nitrogen donor activity in the PRPP-specific amidotransferase system (LeGal et al, 1967a) while glutamine and to a lesser extent carbamyl-phosphate are active. However, in this organism when PRA is formed directly from ribose-5-phosphate, ammonia is the preferred donor of N-9.

Alternative donors of nitrogen-3 (Fig 2), active in the conversion of FGAR to FGAM (step V, Fig 1), have also been found in E. coli B. These are formimino-glutamic and aspartic acids and formimino-glycine. The nitrogen donor activity of formimino-glutamic acid was similar to the activity of glutamine (LeGal et al,1967b). Thus, a variety of alternative or additional steps in purine biosynthesis may occur, and the further study of the pathway in other organisms would therefore be desirable.

The advantages of using mutants blocked at particular steps in an enzymatic pathway for the study of that pathway have been well documented. For instance, the early suggestion that AICA is an intermediate of the purine pathway, the further implication of glycine as a precursor of AICA and the role of folic acid in the utilization of AICA were all elucidated by the use of purine and PABA-requiring mutants of E. coli (Gots and Chu, 1952; Gots and Love, 1954). Confirmation of the role of glycine as a precursor of purines (Schulman et al, 1952) and the importance of folic acid derivatives (Greenberg, 1954) were demonstrated also in the pigeon liver system.

The availability of a series of adenine-requiring mutants of the yeast Saccharomyces cerevisiae, offers an opportunity to compare purine biosynthesis in this organism with that elucidated for other systems. Thirteen non-allelic adenine mutants arbitrarily numbered 1 through 13 have been reported (von Borstel, 1963). Of these only two, ad$_5$ and ad$_7$, are closely linked, and four, ad$_{10}$-ad$_{13}$, have not been mapped.

The mutant ad$_3$ has an absolute requirement for histidine as well as adenine (Roman, 1956; Dorfman, 1964) and appears to be functionally blocked at step X (Fig 1) since it accumulates AICAR (Mazlen, 1968). Adenine 12 accumulates inosine and is therefore thought to be blocked in the formation of adenylosuccinate synthetase, (Dorfman, 1968) and ad$_{13}$ is thought to lack adenylosuccinase activity since it accumulates SAICAR (Dorfman, 1968; Jones, unpublished; in Von Borstel, 1963).

Ad$_2$ and ad$_1$ form a red pigment as a result of the accumulation of AIR (Friedman and Moat, 1958; Smirnov et al, 1967). The enzymatic blocks of these mutants were tentatively placed at steps VII and VIII respectively, (Fig 1; Dorfman, 1964). The exact location of the blocks however, were not identified.

The adenine mutants ad$_4$ to ad$_9$ can utilize hypoxanthine for growth and do not accumulate any aryl-amines or imidazoles not found in the wild-type yeast. In addition, when any of these mutants (ad$_4$-ad$_9$) are introduced into either of the red mutants, ad$_1$ or ad$_2$, the resulting double mutant does not accumulate the red pigment characteristic of the ad$_1$ and ad$_2$ parent. These results suggest that ad$_4$, ad$_5$, ad$_6$, ad$_7$, ad$_8$ and ad$_9$ are early mutants blocked before ring closure (step VI).

Past attempts to locate the points in the pathway at which these mutants are blocked have been unsuccessful. The classical approach of feeding intermediates or accumulated products of mutants can not be used. Dorfman (1964a) tested ad$_1$-ad$_8$ for their ability to grow on SAICAR, AICA, AICAR and AMPS. With the exception of ad$_1$, which grew slightly on SAICAR, and ad_4, which grew at high concentrations of AICA, no growth of the mutants was obtained with any of the compounds tested. E. Jones (unpublished) found an ad_4 mutant which could grow on AICA provided a second mutation at another locus resulting in an altered "AICA permease" was also present.

These results indicate either that the intact yeast cells are impermeable to exogenous intermediates or their derivatives or that the cells do not possess the enzymes necessary to convert the riboside or free base derivatives to the corresponding nucleotide intermediates. Similar problems are found in the study of purine biosynthesis in bacteria (Magasanik, 1962).

Experiments with cell-free enzyme systems of yeast have also yielded ambiguous results (Reynolds, 1957). The activities of the yeast enzymes which control the early steps of the purine pathway are lower than those of the avian liver system. In vitro feeding experiments were therefore not considered to be a fruitful avenue of investigation.

Identification and isolation of the intermediates accumulating as a result of blocks early in the purine pathway are made difficult by the fact that these early intermediates do not give any characteristic colorimetric reactions, nor do they possess any characteristic spectrophotometric properties which would enable their detection or determination in crude extracts.

The above difficulties were largely circumvented in this study by the use of an approach which involved labeling the intermediates with radioactive precursors of the purine molecule (Fig 2). The products accumulating in the mutants were then examined by chromatographic techniques, and the results confirmed in some cases by enzymatic assays.

Table 2

List of Abbreviations

PRPP : 5-phosphoribosylpyrophosphate

PRA : 5-phosphoribosylamine

GAR : glycinamide ribotide

FGAR : formylglycinamide ribotide

FGAM : formylglycinamidine ribotide

AIR : aminoimidazole ribotide

CAIR : 5-amino-4-imidazole-carboxylic acid ribotide

SAICAR : 5-amino-4-imidazole-N-succino-carboxamide ribotide

AICAR : 5-amino-4-imidazole-carboxamide ribotide

FAICAR : 5-formamido-4-imidazole-carboxamide ribotide

IMP : inosinic acid

ATP : adenylic acid

EDTA : ethylenediaminetetraacetate

PGA : 3-phosphoglyceric acid

PABA : p-aminobenzoic acid

CHAPTER II

MATERIALS AND METHODS

Yeast stocks

The adenine requiring mutants used in this study were derived from strains originally supplied by Drs. S. Fogel, D. Hurst, R.K. Mortimer, and B. Dorfman and H. Roman. The phenotypes of the strains listed in Table 3 reflect only amino acid and nitrogenous base requirements. Alleles of markers other than adenine were not tested. Nomenclature and symbols for markers follow the convention of the Carbondale Yeast Genetic Conference (von Borstel, 1963).

Media

Yeast Extract Peptone (YEP) was used for general growth purposes, allele tests, crosses and for storage slants. The medium consists of 1% yeast extract, 1% bacto-peptone, 2% dextrose and 2% agar. The agar was omitted for liquid medium.

Synthetic Complete (SC) The following nutrilites were added to the basal medium of Wickerham (1946). All concentrations are given on a per liter basis: adenine 10 mg; uracil 10 mg; L-leucine 60 mg; L-isoleucine 30 mg; L-serine 10 mg; L-threonine 150 mg; phenylalanine 20 mg; L-tyrosine 50 mg; L-tryptophan 10 mg; L-methionine 10 mg; L-aspartate 20 mg; L-glutamate 20 mg; L-histidine 10 mg; L-arginine 10 mg; L-lysine 40 mg. The resulting SC medium was used for growing cells for accumulation studies. By omitting single nutrilites from this defined medium a drop-out series was obtained. This was used for testing phenotype and also as a selective medium.

Table 3

Yeast Strains

Strain	Ad Allele	Phenotype	Source
A5779B	ad_1	a ad leu	S.Fogel and D.Hurst
A5715C	ad_1	α ad leu	" "
315	ad_2	a ad leu	" "
RB	ad_2	a ad	H.Roman
AM	ad_3	a ad hi	"
AM	ad_3	α ad hi	"
M	ad_4	a ad tr	R.Mortimer
F1394-8B	ad_4	α ad leu	S.Fogel and D.Hurst
	ad_5	a ad ur tr arg	B. Dorfman
RB	ad_5	α ad ur arg	H. Roman
M	ad_6	a ad tr	R.Mortimer
A64B	ad_6	a ad	B.Dorfman
A5476A	ad_6	α ad leu thr tr arg	S.Fogel and D.Hurst
M	ad_7	a ad	R.Mortimer
M	ad_7	α ad	"
A373A	ad_7	a ad ur	B.Dorfman
A5415D	ad_8	α ad leu	S.Fogel and D.Hurst
S2649B	ad_9	a ad ur tr	" "
A5646B	ad_9	α ad ur tr	" "

Table 3 (cont.)

Strain	Ad Allele	Phenotype	Source
A5779D	ad_1ad_2	α ad thr tr arg	S.Fogel and D.Hurst
RB	ad_4ad_5	a ad tr	R.Boruvka
A5459C	ad_4ad_6	α ad ur leu	S.Fogel and D.Hurst
JS	ad_4ad_7	α ad	derived from crosses
Z1412-3A5438C	ad_4ad_8	a ad leu	S.Fogel and D.Hurst
A5467D	ad_5ad_6	a ad ur leu ϕala	" "
A5426B	ad_5ad_6	α ad tr	" "
BC-BB	ad_5ad_7	a ad	R.Boruvka
A5620C	ad_5ad_8	α ad	S.Fogel and D.Hurst
Z1410-2cA5445	ad_5ad_9	a ad tr	" "
JS-2AU	ad_6ad_7	a ad	derived from crosses
A5470B	ad_6ad_8	α ad leu tr	S.Fogel and D.Hurst
A5630A	ad_7ad_8	α ad tr	" "
A5509C	ad_7ad_9	α ad	" "

Potassium Acetate (KAC) medium of McLary et al (1959) was used for inducing the sporulation of diploids. The medium consists of 0.98% potassium acetate, 0.25% yeast extract, 0.1% glucose and 1.5% agar.

Methods

Allele Tests

Mutants of opposite mating type were inoculated as parallel streaks on YEP plates. After 24-48 hours at 30°C the growth on each of the plates was replica plated at right angles to one another on a third YEP plate, which was then incubated as above for an additional 24-48 hours. The cross-inoculated plate was then replica plated to adenine-less medium. Growth responses on the adenine-less plate were recorded are 24-48 hours.

Crosses

Double mutants were obtained from crosses made by cross-replicating streaks on YEP medium as above. After 24-48 hours wild-type diploids were selected by replica plating to adenine-less medium. The diploids were then sporulated on KAC medium. After digestion with snail enzyme, prepared by the method of Fogel and Hurst (1963), asci were dissected by micromanipulation (Johnston and Mortimer, 1959). The resultant ascospore cultures were allele tested with adenine testors by the technique described above, and scored for nutritional requirements by replica plating to a drop-out series.

Preparation of Extracts for Thin Layer Chromatography (TLC)

Two ml of a 24 hour starter culture were inoculated into SC medium containing 0.025 µC per ml of an appropriate, labeled precursor, e.g. C^{14}- U - glycine (60-116 µc per µM), C^{14}-formate (5-21 µc per µM), or carrier free P^{32}, as phosphoric acid (neutralized with KOH). After approximately 48 hours at 30°C with shaking (except in the case of ad_1, ad_2 and the double mutant ad_1ad_2, which were grown anaerobically) samples of the culture were plated on adenine-less medium to test for revertants, and the remainder was collected by centrifugation at 5000 x g for 10 minutes. Cultures having more than $1/10^6$ revertants were discarded. After two distilled water washes the cells were transferred to a precooled press with about 1 ml of water and crushed according to the method described by Eaton (1962). The frozen, crushed mass was thawed and centrifuged at 14,500 x g for 10 minutes, and the supernatant was recentrifuged at the same speed for 10 minutes. The supernatant volume was measured and 0.1 ml removed for a determination of total protein. Two volumes of 95% ethanol were added to the remainder and the suspensions centrifuged at 14,500 x g for 10 minutes. The supernatant was decanted and the pellet was washed with 3 ml of 70% ethanol. The two supernatants were pooled and lyophilized.

In other to standardize the amounts of materials spotted on chromatograms, the volume of distilledwater added to the lyophilized residues was determined on the basis of the protein content of the original extracts. For each 30 mg of protein in the original extract, 0.1 ml of distilled water was added to the residue. In general 10 ml of these solutions were then spotted on chromatograms.

Preparation of Extracts for Column Chromatography

Two ml of a 24 hour starter culture were inoculated into 2 or 4, 2.8 liter Fernbach flasks, each containing 1 liter of SC medium and into a 1 liter Ehrlenmeyer flask containing 200-400 ml of SC medium to which was added an appropriate labeled precursor at a concentration of 0.025 µc per ml. The cultures were grown with shaking for approximately 48 hours at 30°C. Samples were plated on adenine-less medium to determine the number of revertants. The cultures were pooled and collected by centrifugation at 5000 x g for 10 minutes. After two washes with distilled water, an amount of a chloroform:methanol mixture (2:1, vol/vol) equal to 1.5 volumes of packed cells was added to the pellets. Cells were extracted by mixing with a vortex mixer for 5 minutes, and the resulting suspension was centrifuged at 14,500 x g for 10 minutes. Three layers result from the centrifugation. The top (aqueous) layer of each was decanted and lyophilized. Best extraction was obtained when the volume of the cell pellet was between 4.5-5.5 ml. Incomplete mixture of the suspensions resulted in poor extraction.

The lyophilized residue was dissolved in 5 or 10 ml of distilled water, depending on the number of liters of cells extracted.

Demonstration of Feedback Inhibition

Mutants were grown to stationary phase in 1 liter of SC medium. They were then harvested under sterile conditions, washed twice with sterile water and separated into two equal batches based on net weight. These were then each resuspended in 200 ml of prewarmed and preaerated C^{14}-glycine-containing (5 µc) SC which was either adenine-less or contained 250 µg adenine per ml. Cell densities of the resultant suspensions were determined using a hemacytometer. Each had a cell density of about 3.7×10^8 cells per ml. The suspensions were incubated with shaking at 30°C for 1 hour and harvested. Deproteinized extracts for thin layer chromatography were then prepared as described above.

When extracts were to be analyzed by column chromatography, cells were grown in 4 liters of SC and inoculated into 400 ml of SC containing 10 µc C^{14}-glycine. The resultant suspension had a density of about 7.5×10^8 cells per ml. After shaking for 1 hour at 30°C the cells were harvested by centrifugation and the pellets extracted by the chloroform: methanol method described above.

Thin Layer Chromatography

MN 300 Cellulose powder (Brinkmann Instruments Inc.) was used throughout for the preparation of thin layer plates. One hundred eighty ml of distilled water were added to 30 g cellulose and the suspension was homogenized in a Waring Blender for 3 minutes. The mixture was immediately coated on clean, dry glass plates with an applicator (Model S-11, Brinkmann Instruments) which was adjusted to produce a layer of 0.5 mm thickness. All plates were dried at room temperature for 24 hours or longer before use. Variations in R_f's noted can probably be attributed to differences in the degree of hydration of the cellulose layer (Tomisek and Allan, 1964). Ten µl samples of extracts prepared as described above and suitable knowns were applied to thin layer plates with Drummond Disposal Micropipets (Bonus Labs.), using a Standard Labeling Template (number 25-09-20, Brinkmann Instruments).

The following solvent systems were used. The ratios are in volume proportions:

(1) n-propanol : NH_4OH : H_2O (70:10:20); (PAW)

(2) n-butanol : glacial acetic acid : H_2O (40:20:20); (BAW)

(3) isopropanol : H_2O : 10N HCl (66:33:1); (Iso-HCl)

(4) methanol : chloroform : 10% formic acid (3:3:1); (MCF)

(5) methanol : H_2O : conc. formic acid (80:15:5); (MFW)

Solvents 1,2, and 3 were allowed to equilibrate overnight before use. Solvents 4 and 5 were generally used after one hour. All chromatograms were developed by the ascending technique.

Radioactive compounds were located on chromatograms by applying the thoroughly dried chromatograms to Kodak No Screen Medical X-Ray Film (Eastman Kodak Co.) and incubating them for 14-20 days. Exposed films were developed in D19 developer (Eastman Kodak) for 6-8 minutes, rinsed in water and then cleared in Hypo (Eastman Kodak) for 30-60 minutes before washing in running water and drying.

Non-reducing sugars were located by the periodate method of Buchanan et al (1950). Phosphate compounds were detected by the method described by D. Waldi (1965). Imidazole compounds were localized with Pauly reagent (Ames and Mitchell, 1952), countersprayed with 5% Na_2CO_3, and amino acids with ninhydrin reagent (0.1 g of ninhydrin per 100 ml of n-butanol saturated with 1% acetic acid).

In other to standardize the R_f's, the data are presented as R_{gly}, defined as:

R_f of spot /R_f of glycine standard on the same plate.

Column Chromatographic Procedures

Deproteinized extracts of mutants were chromatographed on 10 x 100 mm columns of Dowex-1x8-formate (200-400 mesh) according to the gradient elution procedure of Lepage and Jones (1961). Fifty to 100 fractions of 5 ml each were collected at a speed of 1 ml per minute. Fifty µl aliquots of each fraction were plated on aluminum planchets which were then dried under an infra-red lamp, and the radioactivity of each residue measured with a Nuclear Chicago Gas Flow Detector (Model D47; Nuclear Chicago Corp.). Radioactive fractions were pooled and lyophilized.

When it was necessary to analyze in vitro reaction products by the anion exchange method described above, the reaction was stopped by removing the protein on Sephadex G-10 columns (10 x 100 mm). The columns were eluted with 25 ml of water. The protein, which comes off in the first 4 ml, was discarded. Subsequent fractions were collected, pooled and placed on Dowex-1-formate columns.

The extracts of ad_2 were partially purified on a Sephadex G25 column (15 x 60 mm). The column was eluted with distilled water; twenty-five 1 ml fractions were collected at a rate of 1 ml/ min.

Deformylation Procedures

Crude ethanol-deproteinized extracts of mutants were deformylated by 0.1N HCl at 100°C for 15 or 30 minutes (Warren and Buchanan, 1957). The reactions were stopped by placing vessels in ice. An equivalent volume of 0.1N NaOH was added to neutralize the material. The entire mixture was then lyophilized, residues were dissolved in distilled water and 10 µl samples were spotted on thin layer plates for analysis of the products.

Lyophilized radioactive materials isolated from crude extracts on Dowex columns were dissolved in water and deformylated as above. After neutralization the solutions were lyophilized. The residues were dissolved in distilled water and the products reisolated on the Dowex columns.

Dephosphorylation

Aqueous extracts were dephosphorylated in 0.001M Tris buffer, pH 8.0, using Bacterial Alkaline Phosphatase (Worthington Biochemical Corp.).

Colorimetric Tests

AIR was detected by the Bratton-Marshall test for aryl-amines (Bratton and Marshall, 1939) as modified by Flaks and Lukens (1963), after treatment of the preparation with 10% acetic anhydride for 20 minutes. Potassium phosphate, 1.33M, adjusted to pH 1.4 with KOH and containing 20% trichloroacetic acid (Flaks and Lukens, 1963) was used to stop reactions and to acidify the solutions prior to assays for AIR or CAIR.

AICAR was detected by the modification of the Bratton-Marshall test described by Nierlich and Magasanik (1965). A solution of 15% trichloroacetic acid in 1N HCl was used for acidification of filtrates.

Protein was determined by the micro-biuret method of Zamenhoff (1957). Crystalline Bovine Albumin (Nutritional Biochemicals Corp.) served as the standard.

Phosphate was determined by the method of Fiske and Subbarow (1925). Total phosphate was measured after heating 0.5 ml samples for 90 minutes at 150°C in conc. H_2SO_4. For inorganic phosphate, samples were added to a mixture of 0.2 ml conc. H_2SO_4 and 0.1% H_2O_2 which had been previously heated at 150°C for 15 minutes and then cooled. Disodium ribose-5-phosphate (Sigma Chemical Co.) and KH_2PO_4 served as the organic and inorganic standards respectively.

Ribose was detected, after hydrolysis of samples in 1.2N HCl at 100°C for 40 minutes, by the method described by Hurlbert et al (1954). The orcinol (Fisher Scientific Co.) was recrystallized 3x from benzene before use. Ribose-5-phosphate served as the standard.

In Vitro Assays

FGAR was assayed by a method similar to that of Flaks and Lukens (1963). The FGAR is converted to AIR in the presence of L-glutamine and ATP, and the AIR formed assayed.

Enzymes were prepared as follows: a 24 hour starter culture of ad_2 was inoculated into 4L of YEP medium. The cultures were incubated at 30°C without aeration i.e. without shaking. Log phase cultures were harvested, washed twice and crushed in a precooled Eaton press. Extracts prepared under these growth conditions are white as compared to extracts of aerobically grown ad_2 which are red. The frozen crushed pellet was suspended in 0.01M potassium phosphate buffer, pH 7.4, containing 2M KCl, 5×10^{-4}M EDTA, and 5×10^{-4}M L-glutamine. The mixture was centrifuged 2x at 14,500 x g for 10 minutes. Supernatants were brought to 12 ml and centrifuged at 100,000 x g for 1 hour in a Spinco Model L Ultracentrifuge. The supernatant was dialyzed overnight in the cold against two changes of the liters each of the above buffer. Protein concentration of the dialysate was determined by the microbiuret method cited above.

FGAR was isolated as follows: four liters of SC medium and 200 ml of SC containing 5 uc of C^{14}-glycine were inoculated with a 24 hour starter culture of ad_6. An extract was prepared and this was applied to a Dowex-1-formate column. FGAR was eluted as described by Lepage and Jones (1961). Fractions containing the ribotide were pooled and lyophilized. The lyophilized residue dissolved in distilled water and the FGAR assayed in the following reaction mixture: 2.5 µM potassium ATP, 6.0 µM $MgCl_2$, 10.0 L-glutamine, 10.0 µM potassium, 18.6 mg crude dialyzed ad_2 extract, 10.0 µM potassium phosphate buffer, pH 7.4, containing 0.5 µM EDTA and 0.5 µM L-glutamine. The total volume per tube was 1.36 ml. Tubes were inclubated for zero or 60 minutes at 30°C and placed in ice and the reaction was stopped by the addition of 0.2 ml of 1.33M potassium phosphate buffer, pH 1.4, containing 20% TCA. After centrifugation at 14,500 x g for 15 minutes, the supernatants were acetylated and AIR determined.

GAR was assayed by the procedure of Nierlich and Magasanik (1965) with slight modifications. A 15-30% ethanol fraction of chicken liver was prepared according to the method of Flaks and Lukens (1963). The lyophilized 15-30% ethanol precipitate was stored at -15°C and was dissolved in buffer immediately before use.

CHAPTER III

Functional Blocks of AD_6 and AD_7

Glycine is a direct precursor of carbons 4 and 5 and nitrogen 7 of the purine molecule (Fig 2; Buchanan, 1960). As a preliminary approach to the localization of the enzymatic blocks of adenine mutants, one can characterize their ability to accumulate C^{14}-glycine into precursors of adenine.

Extracts of adenine mutants ad_6 and ad_7 grown in C^{14}-glycine exhibit marked accumulations of C^{14}-glycine-labeled materials. A heavy spot <u>A</u> ,(Fig 3) is characteristic of the TLC pattern of ad_6. Similarly, heavy accumulations at spots <u>A</u>, <u>B</u>, and occasionally at <u>C</u> (Fig 4) are characteristic of the TLC-pattern of the ad_7 mutant.

The presumptive purine intermediates observed on TLC plates, appear to be ribosides for the most part, rather than ribotides, which are the true intermediates Treatment of an ad_6 extract with alkaline phosphatase before chromatography did not alter the R_{glys} of most spots (Fig 5). Phosphate sprays and TLC profiles of P^{32} incorporation by ad_6 confirm that much of the accumulations observed might represent riboside derivatives of the intermediates.

That the dephosphorylation of the intermediates must occur *in vivo* rather than as a result of the extraction procedure, is indicated by the fact that the addition of Na fluoride to the crushing medium (16.7 µM per ml), which is reported to inhibit phosphatases (Flaks and Lukens, 1963), did not result in a marked alteration of the chromatographic patterns. Furthermore, extracts made by the chloroform-

Fig. 3. TLC-autoradiogram of an extract of C^{14}-glycine-labeled ad_6.

Solvent: PAW.

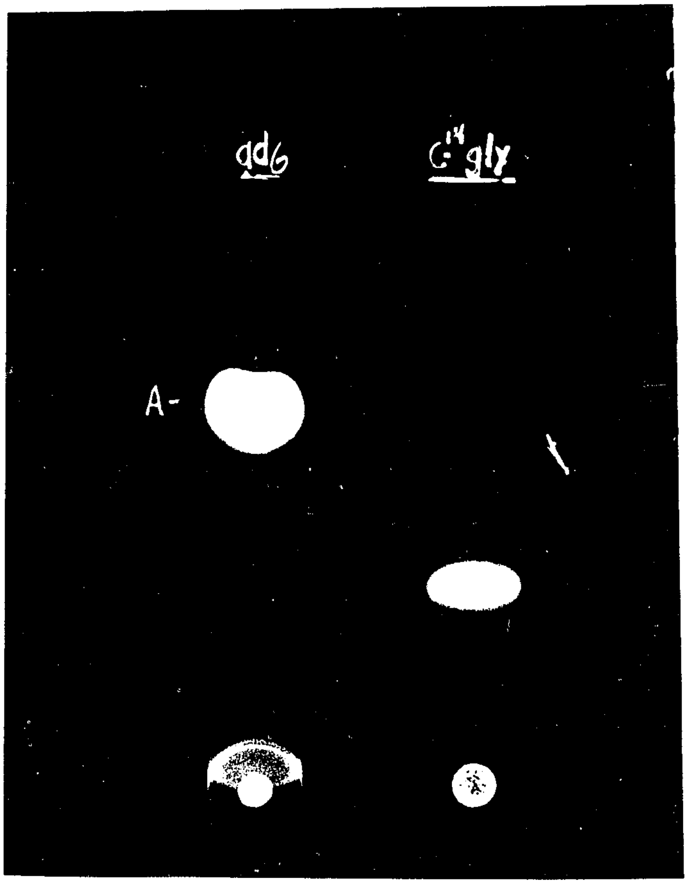

ad6
C^{14} gly
A-

Fig. 4. TLC-autoradiogram of an extract of C^{14}-glycine-labeled ad_7.

Solvent: PAW

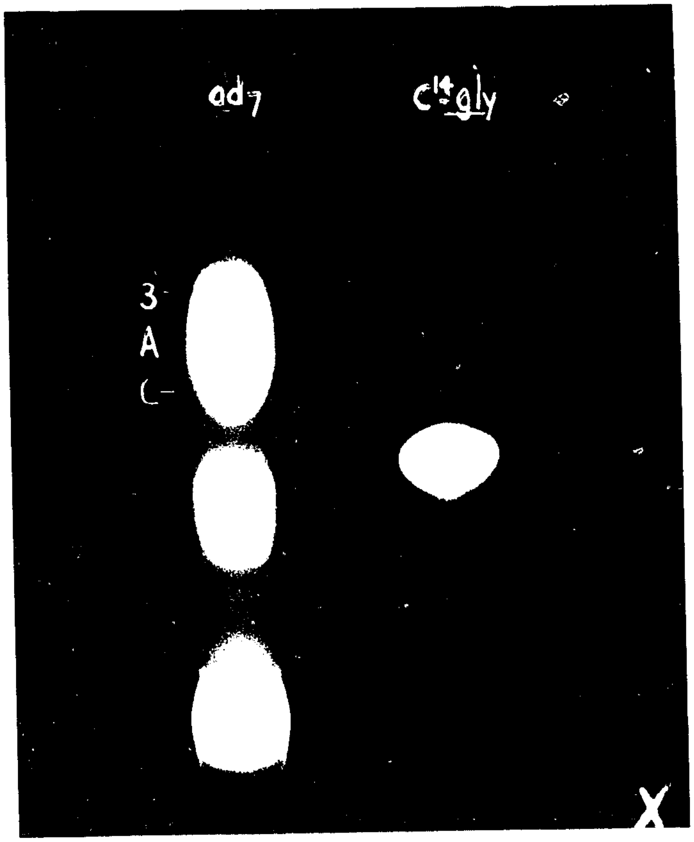
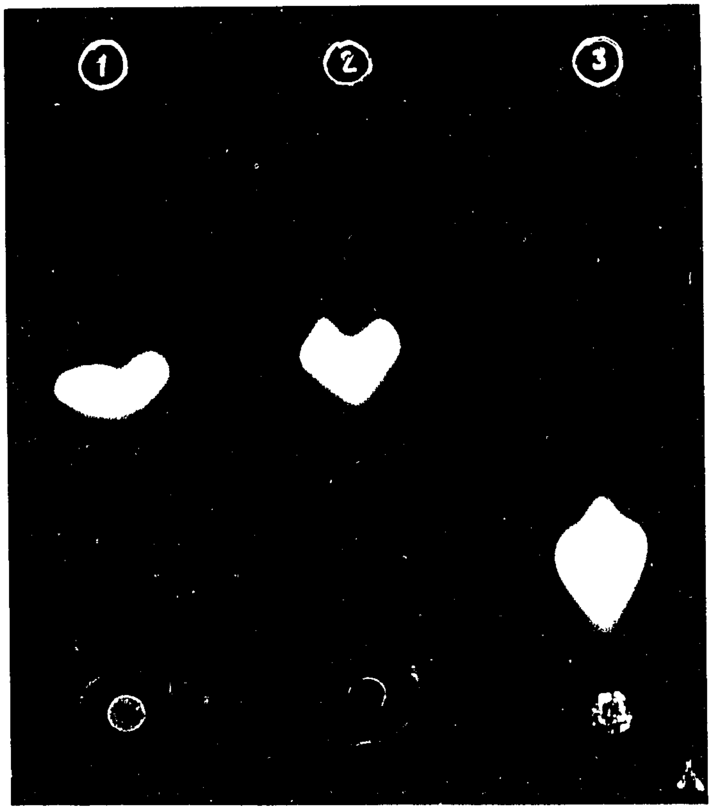

ad7
C14gly
3
A
C
X

Fig. 5. TLC-autoradiogram of an alkaline phosphatase-treated extract of C^{14}-glycine labeled ad_6. (1) untreated extract (2) alkaline phosphatase-treated extract (3) C^{14}-glycine standard. Solvent: PAW.


1
2
3
A

methanol method, which does not allow for in vitro enzyme activity, showed chromatographic profiles similar to extracts of cells prepared by crushing and deproteinizing with ethanol.

Regardless of this dephosphorylation, the accumulation by the mutants, ad_6 and ad_7 of characteristic substances derived from C^{14}-glycine, allows the tentative assignment of the enzymatic blocks of these mutants, to a step in the pathway after that at which glycine is incorporated into the molecule (Fig 1).

If these accumulated substances do indeed represent derivatives of intermediates of the purine pathway they should be subject to feedback inhibition by the end product of the pathway. Burns (1964) demonstrated that adenine, probably after conversion to the ribotide, inhibits the formation of AIR in an ad_2 mutant of S. cerevisiae. Experiments were therefore designed to test for feedback inhibition by adenine of C^{14}-glycine-labeled material accumulated by ad_6 cells.

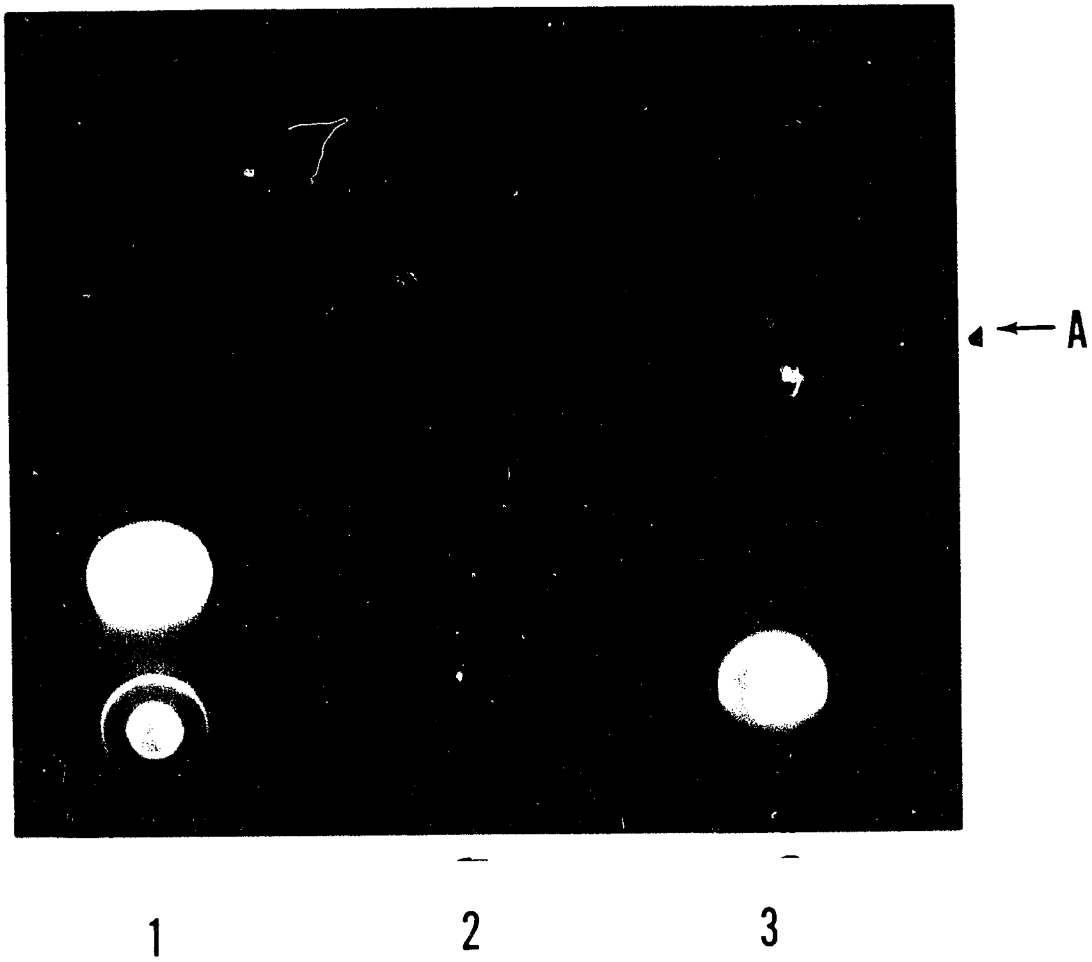
In the results shown in Fig 6, although incorporation of the label is light, since the cells were incubated in the C^{14}-medium for only one hour, the inhibition by adenine of the accumulation of C^{14}-labeled material into the characteristic ad_6 pattern is clearly apparent. The identify of the heavily labeled characteristic spot A of ad_6 is thus confirmed as an adenine intermediate, and by analogy, the heavily labeled spots characteristic of the ad_7 mutant may also be adenine intermediates.

Disappearance of the characteristic ad_6 spot upon introduction into the ad_6 strain of an additional adenine marker would constitute further evidence that the spot represents an intermediate of the adenine pathway. If the ad_7 accumulations behave similarly, one may conclude

Fig. 6. TLC-autoradiogram illustrating the effect of adenine on the accumulation of C^{14}-glycine-labeled material by ad_6.

(1) represents the C^{14}-glycine standard (2) an extract from cells incubated in the presence of adenine and (3) an extract from cells incubated in the absence of adenine. (A) indicates the characteristic spot of ad_6.

Solvent: PAW.


A
1
2
3

that they too are intermediates of the pathway. Furthermore, the TLC pattern of the resulting double mutant should correspond to the TLC pattern of one or the other singly deficient parent. This pattern, which should represent the products accumulated by the parent with the earlier enzymatic block, could thus be used to order the mutants along the pathway.

Using this approach, the relative positions of ad_6 and ad_7 mutations with respect to mutants ad_4, ad_5, ad_8 and ad_9, and with respect to one another was ascertained. The data on the various pairwise combinations of adenine mutants are presented below.

The TLC pattern of the ad_6ad_7 double mutant in both the PAW and the BAW solvent systems resembles that of the ad_6 parent rather than that of the ad_7 parent. In the PAW solvent (Fig 7), the ad_6ad_7 double mutant has a heavy accumulation in spot <u>A</u>, which is also present in, and characteristic of, the ad_6 mutant. The ad_7 parent has two or three heavy or moderately heavy spots which migrate to approximately the same area in this solvent.

In the BAW solvent (Fig 8), the ad_6ad_7 double mutant lacks the compound spots characteristic of the ad_7 parent but has instead one heavy spot similar to the spot at <u>A</u> in the ad_6 parent. Since the introduction of an ad_6 marker into an ad_7 strain results in a double mutant pattern like that of the ad_6 parent, one may conclude that ad_6 is blocked earlier than ad_7.

As seen in Figs 9 and 10, the patterns of C^{14}-glycine incorporation of the ad_5ad_6 and the ad_5ad_7 double mutants resemble that of the ad_5 single mutant and are lacking the heavily labeled spots characteristic

Fig. 7. A comparison of the TLC-patterns of extracts from C^{14}-glycine-labeled ad_6, ad_7 and the double mutant ad_6ad_7, resolved in the PAW solvent system.

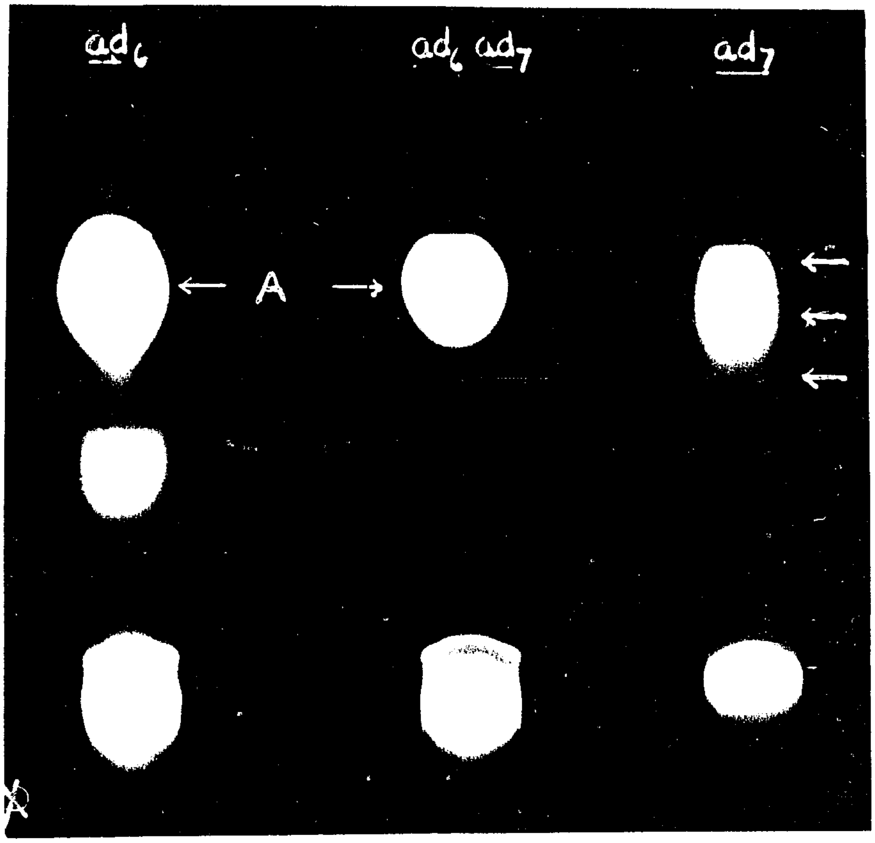

ad6
ad6 ad7
ad7
A

Fig. 8. A comparison of the TLC-patterns of extracts from C^{14}-glycine-labeled ad_6, ad_7 and the double mutant ad_6ad_7, resolved in the BAW solvent system.

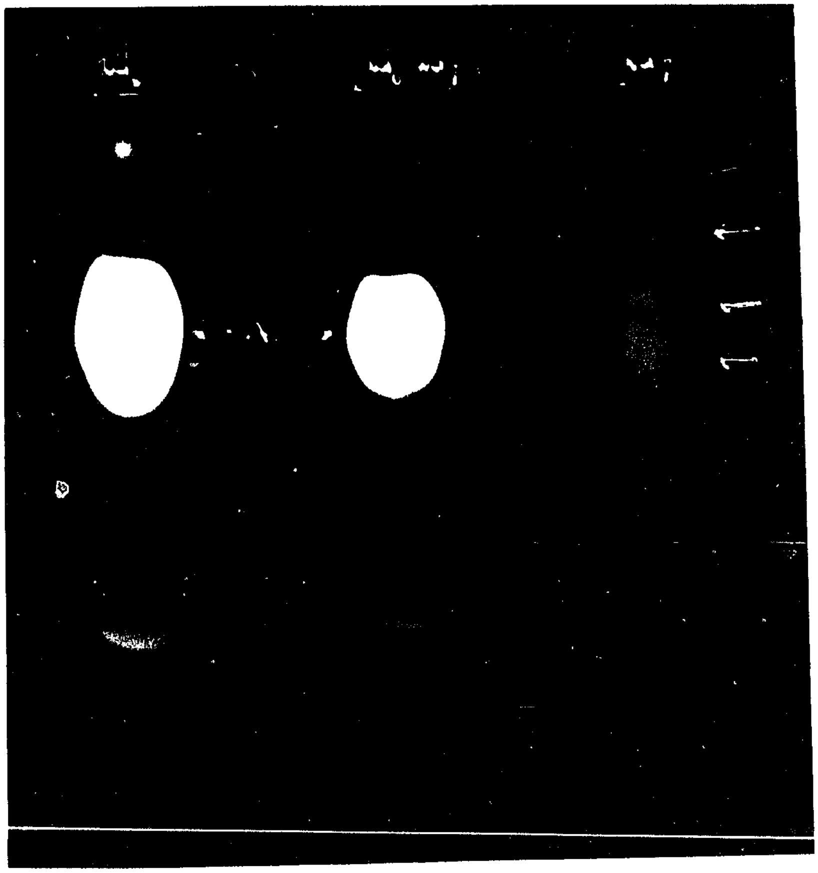


Fig. 9. TLC-comparison of extracts from C^{14}-glycine-labeled ad_5, ad_6 and ad_5ad_6.
The arrow indicates the location of the characteristic accumulation of ad_6.
Solvent: PAW.

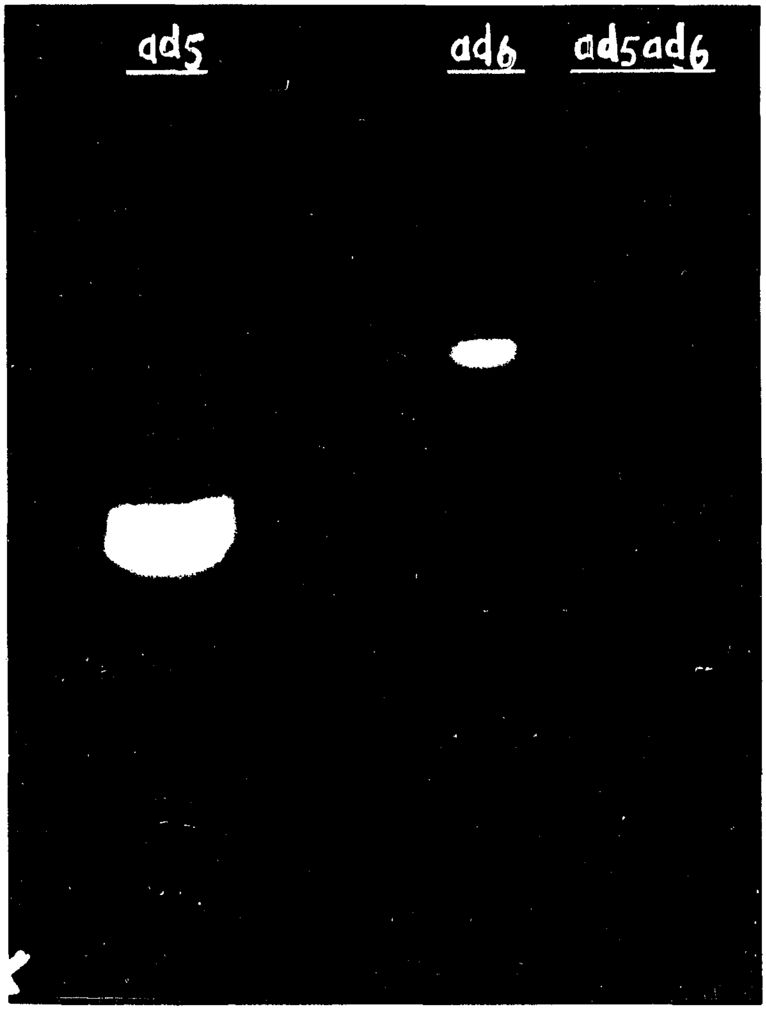
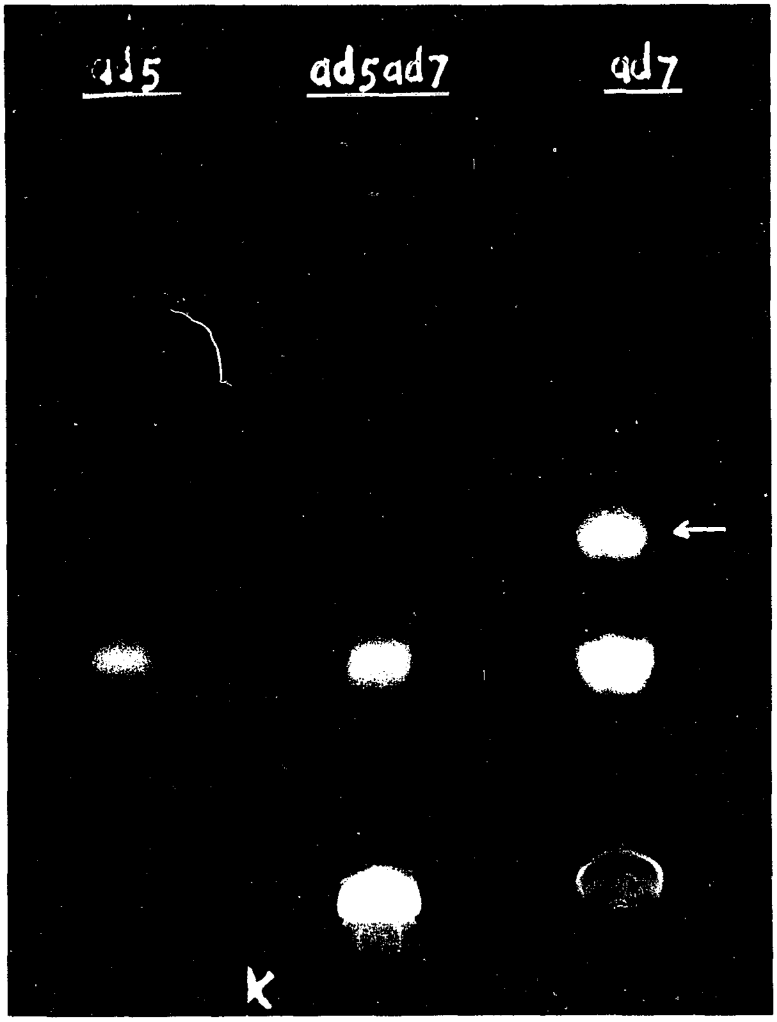

ad5
ad6
ad5ad6

Fig. 10. TLC-comparison of extracts from C^{14}-glycine labeled ad_5, ad_7 and ad_5ad_7.
The arrow indicates the location of the characteristic accumulations of ad_7.
Solvent: PAW.


ad5
ad5ad7
ad7
K

of ad_6 (Fig 9) or of ad_7 (Fig 10). These results indicate that the ad_5 block is before that of ad_6 or ad_7.

Glycine incorporation by ad_4, ad_6 and the double mutant ad_4ad_6 are compared in Fig 11. The characteristic heavy ad_6 accumulation disappears upon introduction of the ad_4 mutation. Consequently ad_4 must have the earlier block. Since, as shown above, ad_6 is blocked before ad_7, ad_4 must be blocked before ad_7 as well.

Comparisons of the double mutants ad_6ad_8 and ad_7ad_8 with the single mutants ad_6, ad_7 and ad_8 are shown in Figs 12 and 13. The characteristic ad_6 and ad_7 spots are lacking in the double mutants, indicating that ad_8 is blocked before ad_6 and ad_7.

Studies of double mutants carrying an ad_9 marker are somewhat ambiguous because of the leaky nature of the ad_9 mutant (see page 121). However, it was possible to demonstrate that the introduction of an ad_9 mutation into an ad_6 strain does result in the complete loss of the characteristic ad_6 spots (Fig 14). The ad_9 mutational block is therefore at an earlier step in the pathway than that of ad_6.

In summary, the TLC studies of extracts of C^{14}-glycine-labeled ad_6 and ad_7 indicate that they are blocked after glycine has been incorporated into the ribotide molecule (step III, Fig 1). Comparisons of C^{14}-glycine-labeled single and double mutant extracts showed that ad_6 and ad_7 are blocked relatively late in the pathway with respect to adenine mutants ad_4, ad_5, ad_8 and ad_9. In addition, the presence of an ad_6 marker in an ad_6ad_7 double mutant, will prevent the accumulation of label into TLC spots characteristic of the ad_7 parent, indicating that the ad_6 block is earlier than that of ad_7.

Fig. 11. TLC-comparison of extracts from C^{14}-glycine labeled ad_4, ad_6 and ad_4ad_6.
The arrow indicates the location of the characteristic accumulation of ad_6.
Solvent: PAW.

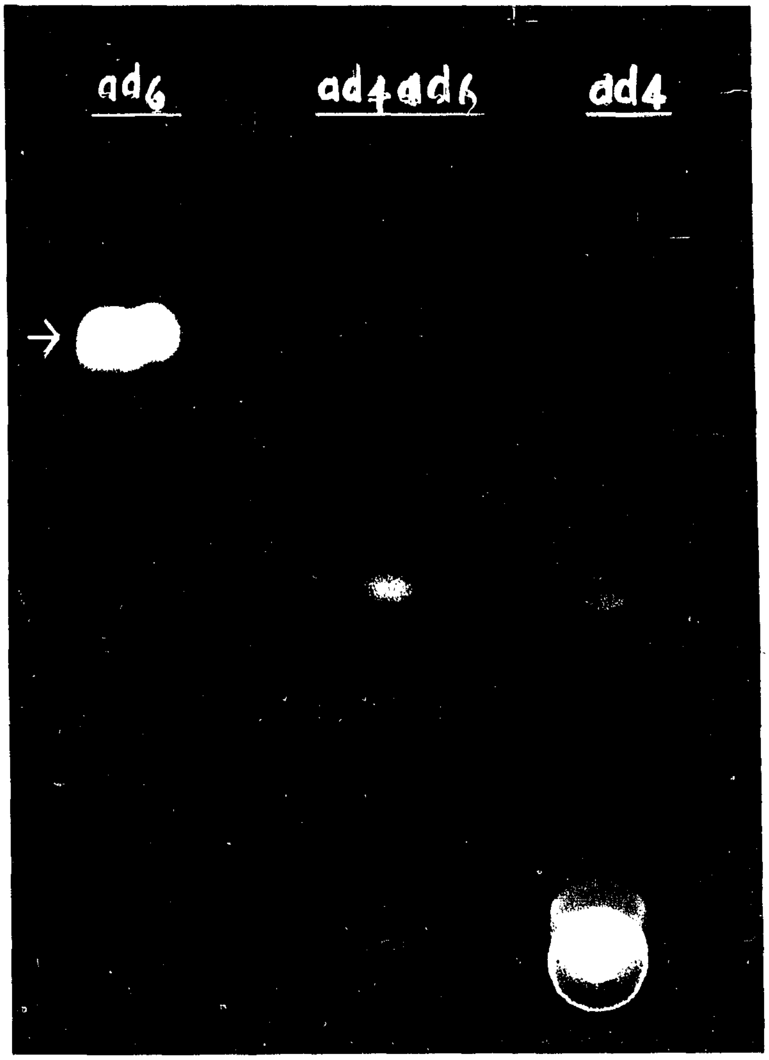

ad6
ad4ad6
ad4

Fig. 12. TLC-comparison of extracts from C^{14}-glycine-labeled ad_6, ad_8 and ad_6ad_8.
The arrow indicates the location of the characteristic accumulation of ad_6.
Solvent: PAW.

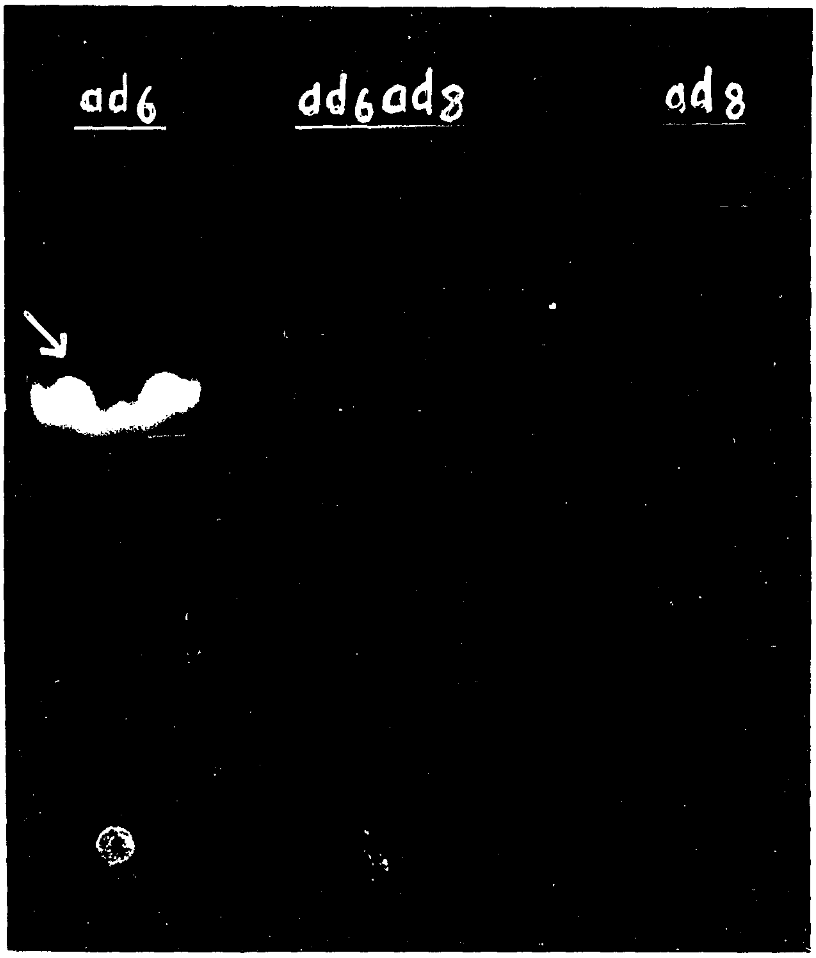

ad6
ad6ad8
ad8

Fig. 13. TLC-comparison of extracts from C^{14}-glycine-labeled ad_7, ad_8 and ad_7ad_8.
The arrow indicates the location of the characteristic accumulations of ad_7.
Solvent: PAW.

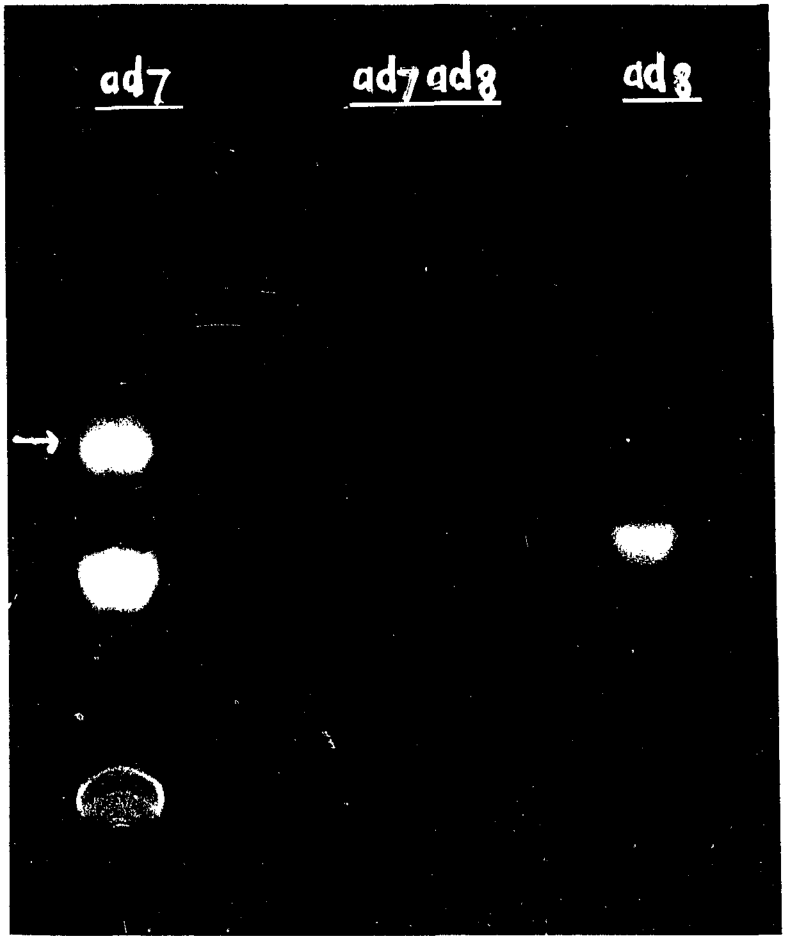
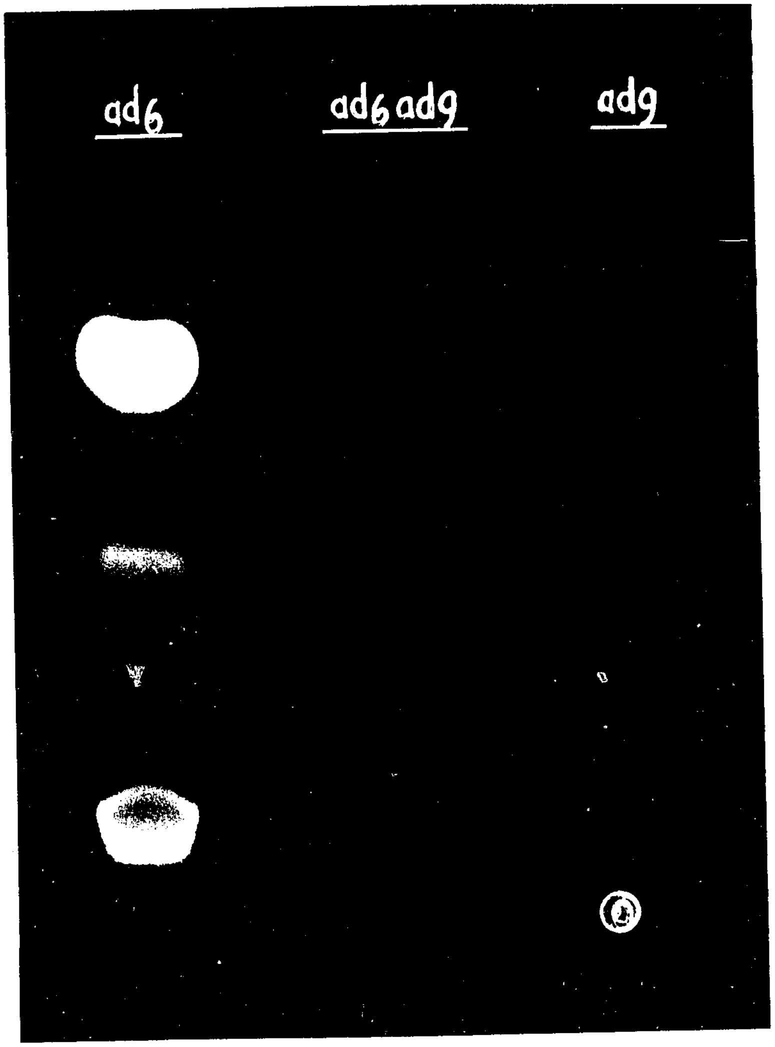

ad7
ad7 ad8
ad8

Fig. 14. TLC-comparison of extracts from C^{14}-glycine-labeled ad_6, ad_9 and ad_6ad_9.
Solvent: PAW.


ad6
ad6 ad9
ad9

The ad_6 and ad_7 mutants do not accumulate any imidazole compounds not found in the wildtype, and they must therefore be blocked before ring closure (step VI, Fig 1). However, they do occur relatively late with respect to the other mutants tested. This suggests that they may be blocked immediately preceding ring closure (Fig 1, steps V and VI). Additional information on the location of the blocks of ad_6 and ad_7 was obtained by the study of the incorporation of C^{14}-formate.

Formate is incorporated at step IV in the pathway (Fig 1). Mutants blocked at later steps would be expected to accumulate intermediates labeled by both C^{14}-glycine and C^{14}-formate. As shown in Fig 15 and 16, the C^{14}-glycine-labeled intermediates accumulated by ad_6 and ad_7 are also strongly labeled by C^{14}-formate. Moreover, the ad_6 C^{14}-formate and C^{14}-glycine profiles are similar in four different solvents (Table 4), and a mixture of extracts from glycine and formate labeled cells has a TLC pattern which is identical to the TLC pattern of either extract alone (Fig 17). This supports the hypothesis that these mutants are blocked after the addition of formate to the molecule.

Studies on the relative order of the ad_6 and ad_7 mutations cited above further suggest that ad_6 is blocked at step V (Fig 1) and ad_7 at step VI. These mutants should therefore accumulate FGAR and FGAM respectively. Some FGAR might also be expected to accumulate in the ad_7 mutant because it is a precursor to FGAM and because of the spontaneous breakdown of FGAM (Lukens and Flaks, 1963).

Fig. 15. TLC-comparison of extracts from C^{14}-glycine-labeled and C^{14}-formate-labeled ad_6. (a) gly-ad_6 : C^{14}-glycine-labeled extract (b) form-ad_6 : C^{14}-formate-labeled extract (c) C^{14}-gly : C^{14}-glycine standard.

Solvent: PAW.

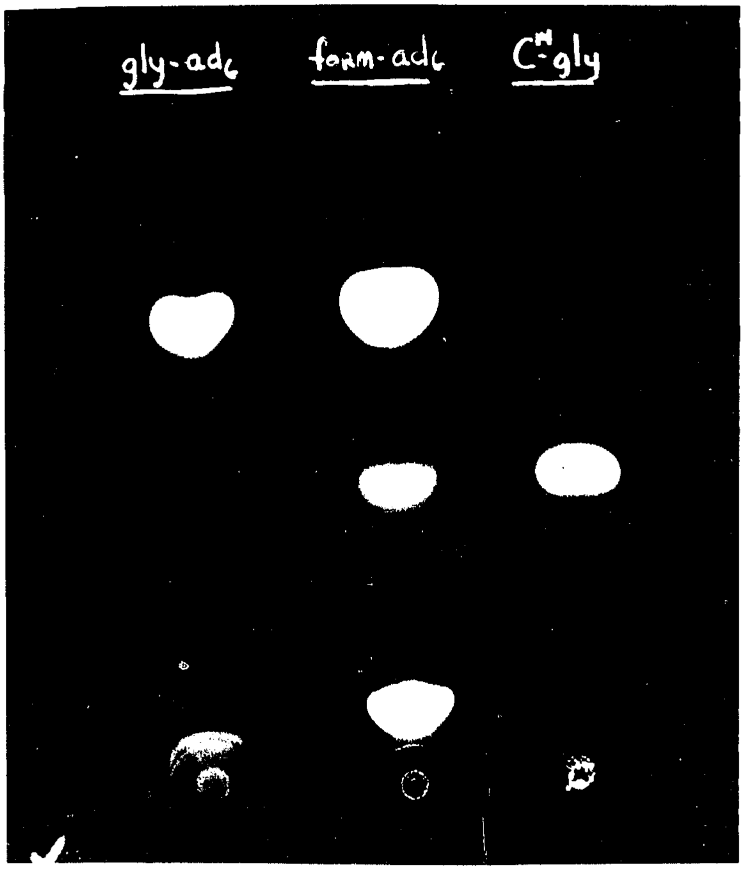

gly-ad$_6$
form-ad$_6$
C^{14}-gly

Fig. 16. TLC-comparison of extracts from C^{14}-glycine-labeled and C^{14}-formate-labeled ad_7. (a) gly-ad_7 : C^{14}-glycine-labeled extract (b) form-ad_7 : C^{14}-formate-labeled extract (c) C^{14}-gly : C^{14}-glycine standard.

Solvent: PAW.

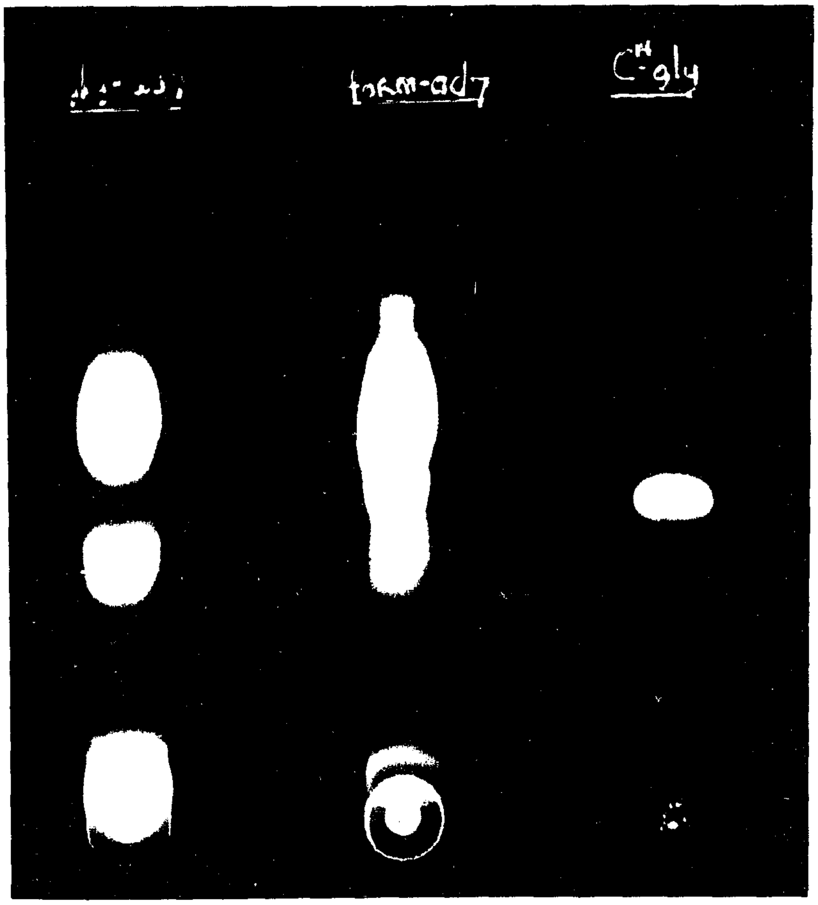

form-ad7
C^{14}-gly

TABLE 4

Comparison of C^{14}-glycine and C^{14}-formate-labeled ad_6 extracts using TLC.

R_{gly}*

Solvent: PAW

C^{14}-glycine	a	b	C^{14}-formate		
0.14± .02	MH	(10)	0.15± .02	MH	(4)
0.27± .03	MH	(13)	0.25± .03	MH	(3)
-			0.31±	VL	(1)
0.94± .06	M-VH	(13)	0.87± .08	MH	(4)
1.05± .04	L-VL	(4)	1.04± .06	L	(4)
1.46± .11	VH	(14)	1.55± .10	VH	(4)
1.57± .03	VL	(2)	-		
1.88± .05	VVL	(3)	1.88± .00	VVL	(3)

Solvent: MFW

0.42	M	(1)	0.42	MH	(1)
0.63	M	(1)	0.64± .04	M-H	(2)
-			0.76	M	(1)
0.84	H	(1)	0.80	VH	(1)
0.96± .04	MH	(2)	1.03± .00	MH	(2)

TABLE 4 (cont.)

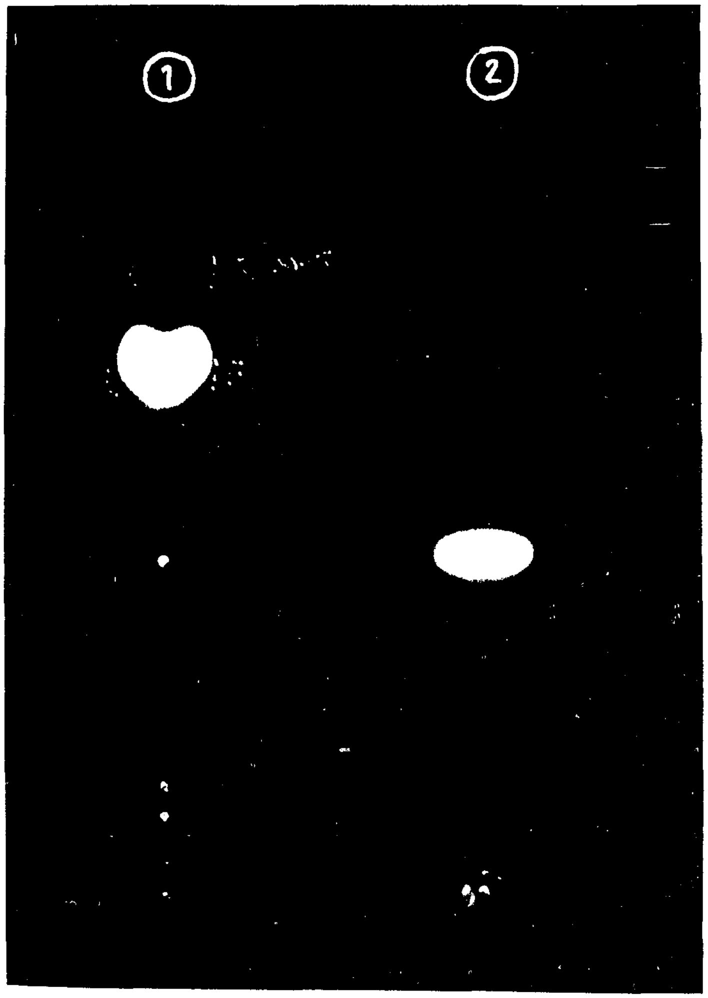
Solvent: BAW C^{14}-glycine			C^{14}-formate		
0.10± .00	LM	(3)	0.17± .03	M	(2)
0.26± .03	LM	(5)	0.31	L	(1)
0.38	ML	(1)			
0.45± .00	L	(2)	0.42± .01	M	(2)
0.57± .01	L	(3)	-		
0.68± .01	M	(3)	0.68± .01	M	(2)
0.82± .01	LM	(3)	0.92	M	(1)
0.98± .01	MH	(3)			
1.09± .05	VH	(5)	1.16± .03	VH	(3)
Solvent: MCF					
0.08± .03	M	(3)	-		
0.22± .01	H	(5)	-		
0.42± .02	L-M	(3)	-		
0.61	MH	(1)	0.61± .00	MH	(2)
0.71± .02	M-L	(2)	0.72± .02	M	(2)
0.98± .05	VH	(5)	1.03± .05	VH	(2)
1.08	H	(1)	1.16± .04	VH	(2)

(a) letters refer to qualitative estimate of the degree of incorporation of the label as follows: VVL: trace; VL: very light; L: light; M: moderate; H: heavy; VH: very heavy.

(b) the number in brackets indicates the number of times this spot was observed.

* see materials and methods

Fig. 17. Cochromatography of C^{14}-glycine-labeled and C^{14}-formate-labeled extracts of ad_6, in the PAW solvent system. (1) Represents the mixed ad_6 extracts, (2) the C^{14}-glycine standard. All other markings may be disregarded.


①
②

FGAR, which is ninhydrin negative, can be deformylated by mild acid hydrolysis to GAR, which is ninhydrin positive (Warren and Buchanan, 1957). An extract of C^{14}-glycine-labeled cells treated in this way, is compared in Fig 18 with an untreated extract. With deformulation the heavily labeled unique accumulation of ad_6, A disappears, with the concomitant appearance of a heavily labeled ninhydrin positive band, B at a different R_{gly}.

Similar treatment of an extract labeled with C^{14}-formate, results in the appearance of a strongly ninhydrin positive band at B (Fig 18) with the simulataneous loss of the characteristic C^{14}-labeled material. This ninhydrin positive band is not labeled. These results are consistant with the hypothesis that ad_6 accumulates FGAR.

Additional characterization of the intermediates accumulated by ad_6 and ad_7, and confirmation of the above results was accomplished by the use of Dowex-1-formate columns. Extracts of ad_6 and ad_7 labeled with C^{14}-glycine were analyzed for the presence of GAR and FGAR by the procedure of Lepage and Jones (1961), which allows clear separation of these two intermediates. In contrast to thin layer chromatography which, for the most part, permitted resolution only of the ribosides, the anion-exchange method separates the ribotides.

Both the ad_6 and ad_7 extracts have a radioactive peak in the region reported to be that in which GAR is eluted (peakI; Figs 19 and 20). In some instances, peak I was resolved into two components (Fig 21). Extracts of both mutants also have a C^{14}-peak in the region described as that in which FGAR is eluted (peak II). Among all of the mutants tested and the wildtype strain, the material appearing in the

Fig. 18. TLC-autoradiogram of C^{14}-glycine-labeled and C^{14}-formate-labeled extracts of ad_6 before and after deformyation. Deformylation was carried out by mild acid hydrolysis in 0.1 N HCl. (1) Represents the C^{14}-glycine-labeled untreated control, (2) C^{14}-glycine-lageled extract, hydrolyzed 15 minutes, (3) C^{14}-glycine-labeled extract, hydrolyzed 30 minutes, (4) C^{14}-formate-labeled extract, hydrolyzed 15 minutes, (5) C^{14} -formate-labeled untreated control, (A) is the location of the characteristic ninhydrin negative intermediate which accumulates in ad_6 cells, and (B) is the location of the ninhydrin positive band which forms on deformylation of an ad_6 extract.

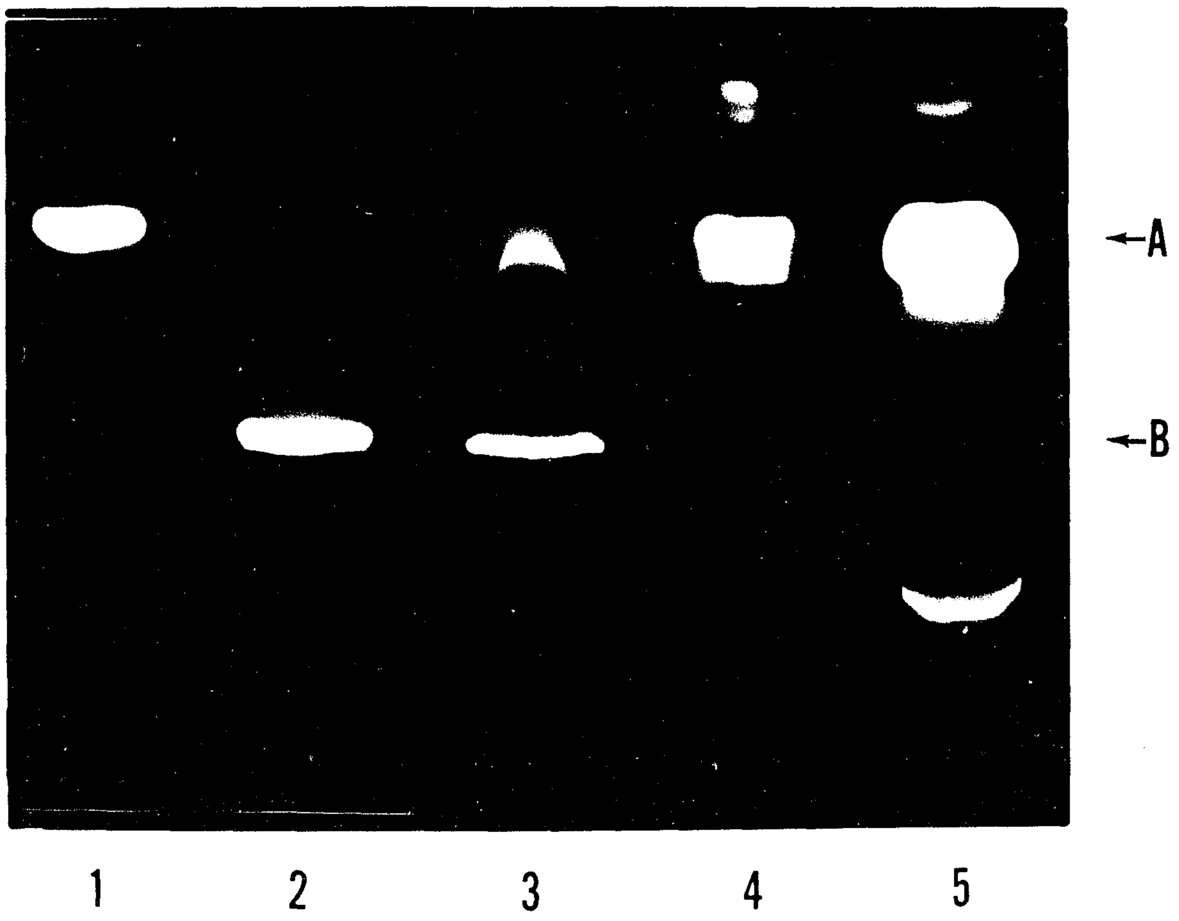
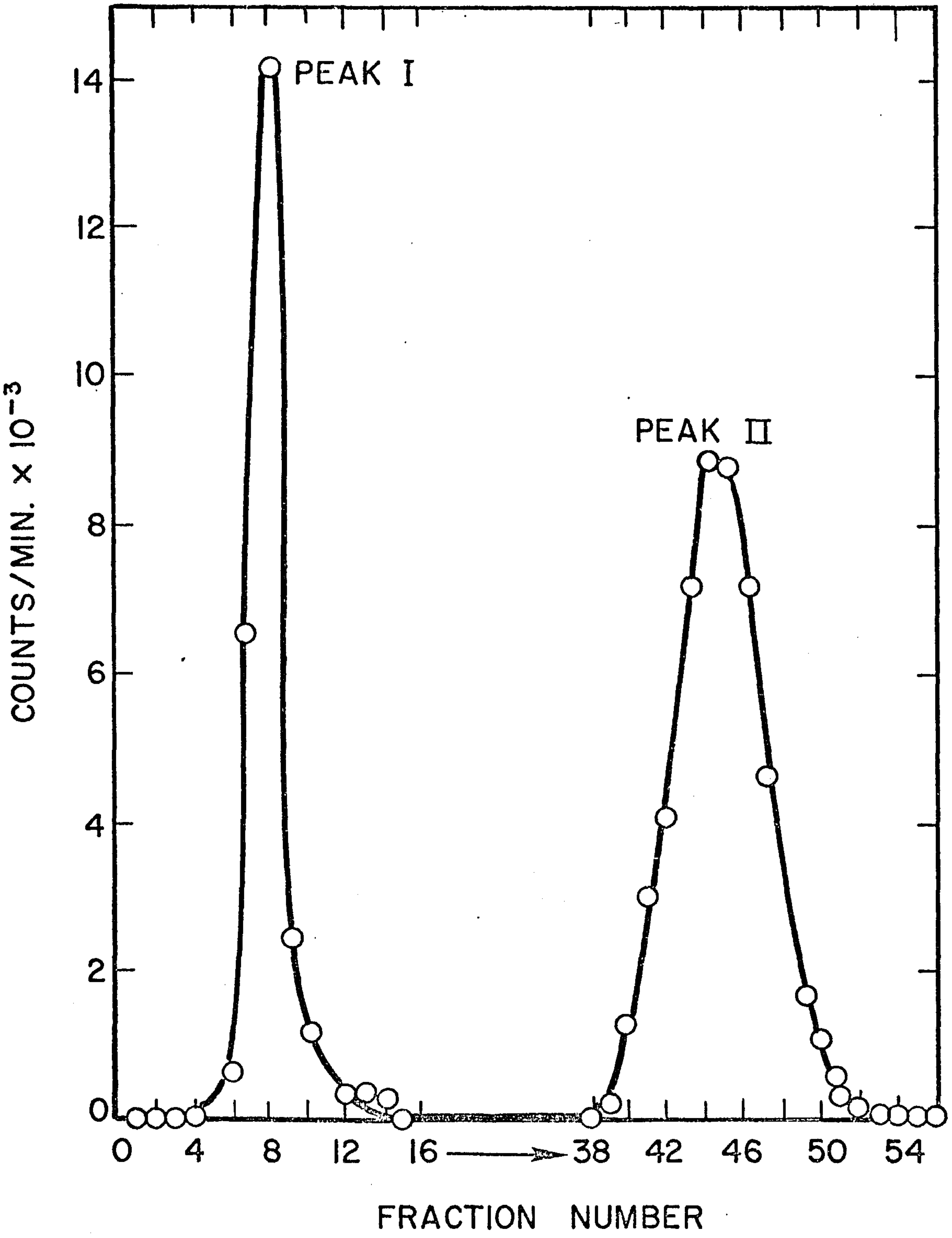

←A
←B
1
2
3
4
5

Fig. 19. Ion-exchange chromatography of ad_6. The extract was made from cells grown in SC containing 10 μC of C^{14}-glycine.



PEAK I
PEAK II
COUNTS/MIN. × 10^{-3}
0
2
4
6
8
10
12
14
0
4
8
12
16
38
42
46
50
54
FRACTION NUMBER

Fig. 20. Ion-exchange chromatography of ad_7. The extract was made from cells grown in SC containing 10 µC of C^{14}-glycine.

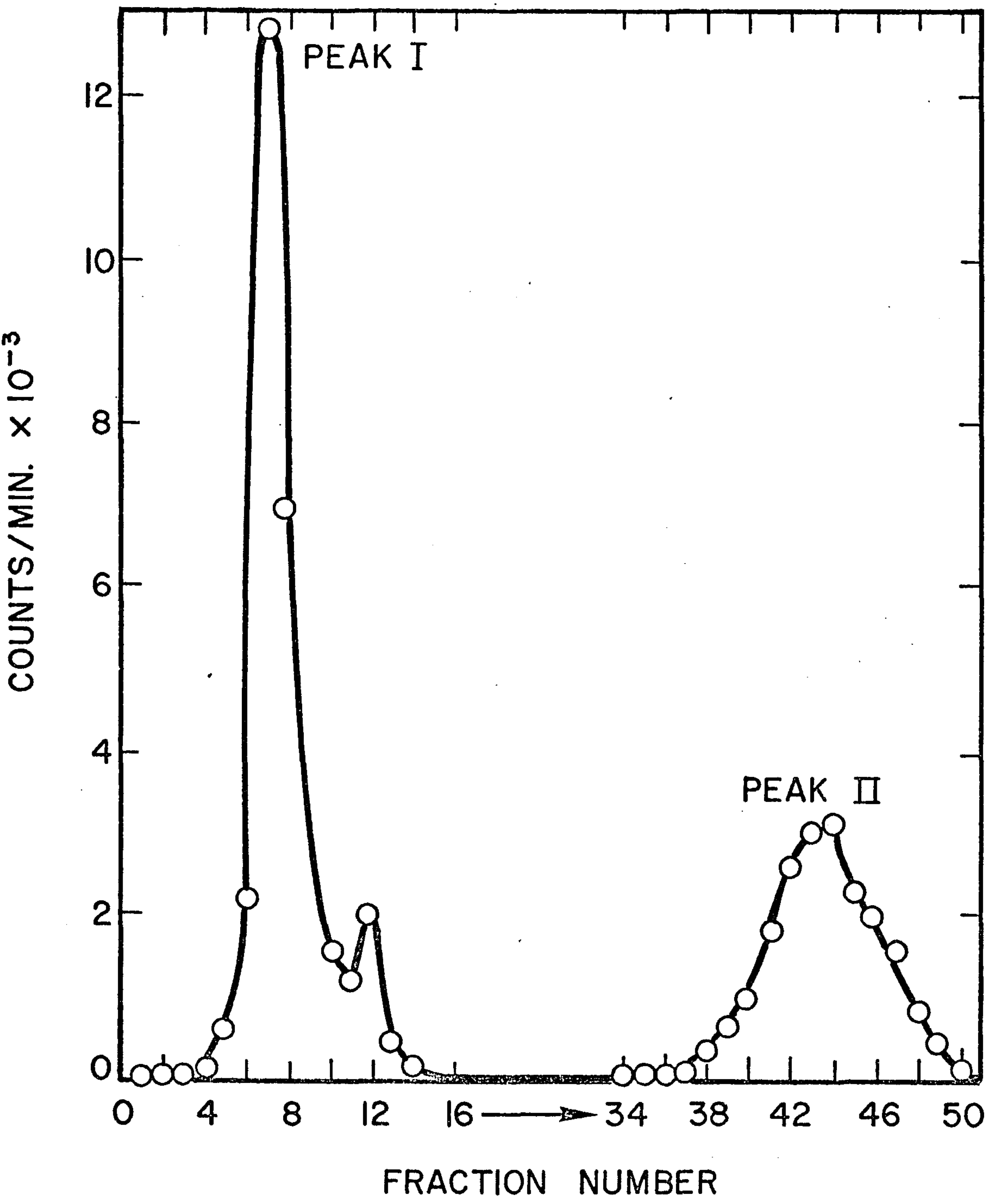
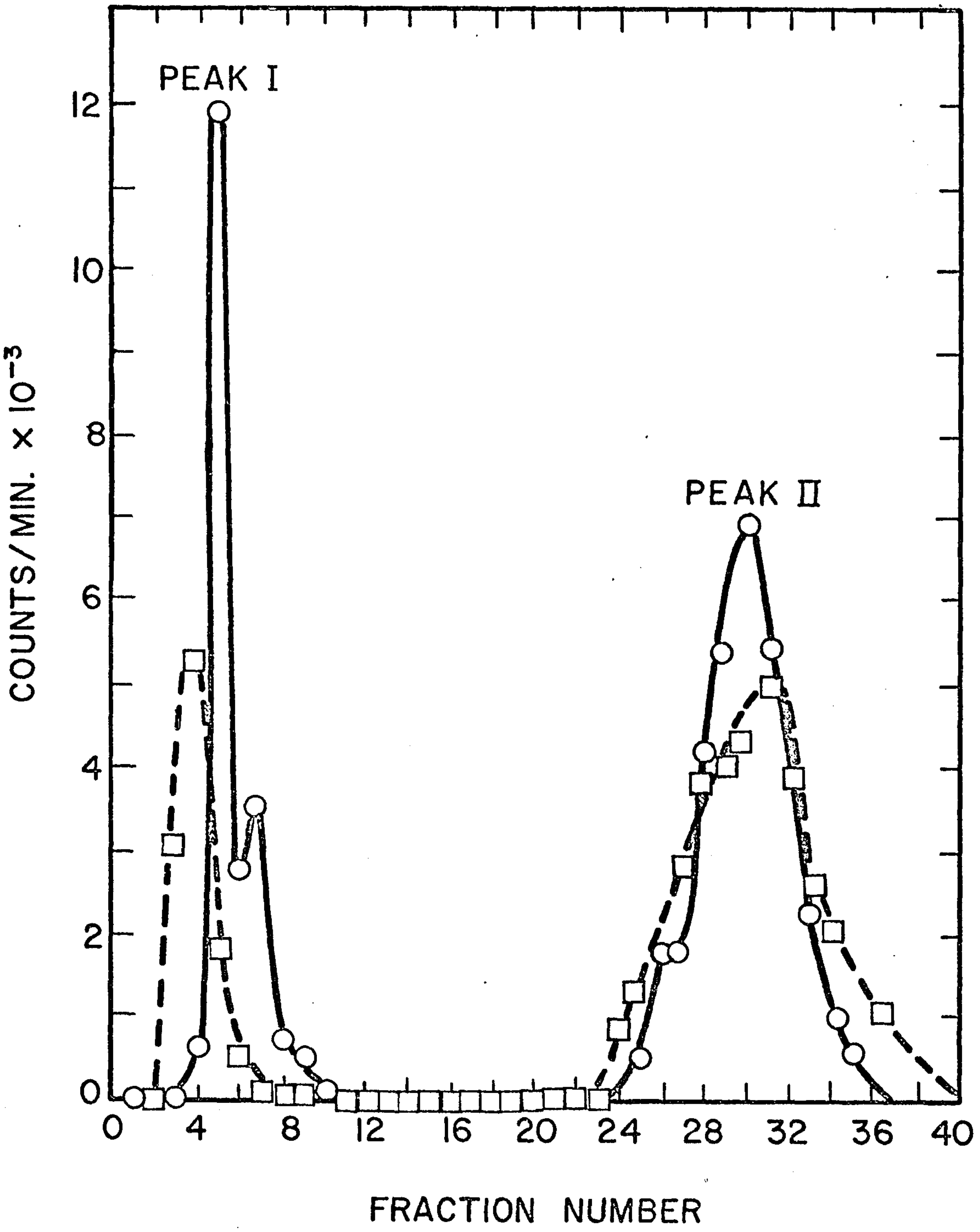

PEAK I
PEAK II
COUNTS/MIN. × 10⁻³
0
2
4
6
8
10
12
0
4
8
12
16 ⟶ 34
38
42
46
50
FRACTION NUMBER

Fig. 21. Ion-exchange chromatography of C^{14}-glycine-labeled and C^{14}-formate-labeled extracts of ad_6. The extracts were made from cells grown in SC containing either C^{14}-glycine (5 µC) of C^{14}-formate (5 µC). Each extract was chromatographed separately. O———O, glycine-labeled extract, □------□ formate-labeled extract.


PEAK I
PEAK II
COUNTS/MIN. × 10⁻³
0
2
4
6
8
10
12
0
4
8
12
16
20
24
28
32
36
40
FRACTION NUMBER

FGAR region was observed only in extracts of ad$_6$ and ad$_7$ (Figs 22-26).

In addition to FGAR ad$_7$ would be expected to accumulate FGAM, for which no elution pattern under these conditions has been described. It was therefore necessary to identify the C^{14}-material in peak II of the ad$_7$ profile.

On two trials, mixtures of an ad$_6$ and an ad$_7$ peak II could not be separated from one another when the mixture was rechromatographed on Dowex-1-formate (Fig 27). Furthermore, a mixture of peak II material from ad$_6$ and ad$_7$, subjected to the deformylation procedure discussed above, behaved identically to peak II material of ad$_6$ which had been subjected to the same treatment (Fig 28). Finally, when peak II material from each of the ad$_6$ and ad$_7$ mutants is lyophilized and compared by TLC, the R$_{glys}$ of the C^{14}-material from each mutant is similar in four different solvents (Fig 29 and Table 5). It seems unlikely that FGAR and FGAM should fail to separate in any of the four different solvent systems. One may conclude, therefore, that the C^{14}-labeled material in peak II of a chromatographed ad$_7$ extract is identical to that in peak II of a similarly treated ad$_6$ extract. It has not yet been possible to isolate FGAM from extracts of ad$_7$.

If these C^{14}-labeled peaks represent purine pathway intermediates, their presence in the ad$_6$ and ad$_7$ extract should be subject to feedback inhibition. The results shown in Fig 30 and 31 show that the material isolated as peak II from the ad$_6$ and ad$_7$ mutants is subject to feedback inhibition by adenine and therefore is indeed an intermediate in purine biosynthesis. No feedback inhibition of the accumulation of material eluted in the GAR region was obtained. The nature of the material

Fig. 22. Ion-exchange chromatography of ad_4. The extract was made from cells grown in SC containing 10 μC of C^{14}-glycine.

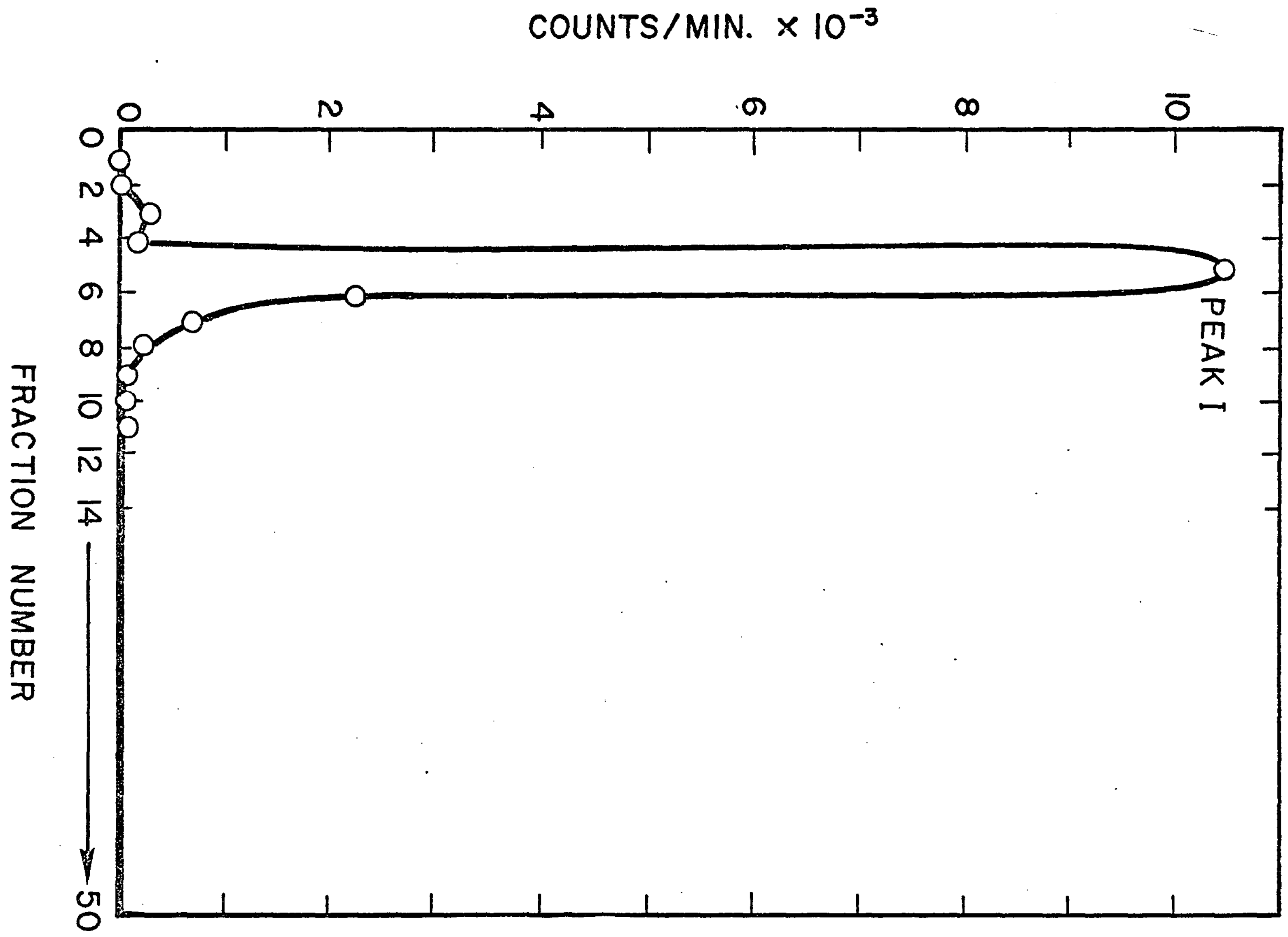

COUNTS/MIN. × 10^{-3}
0
2
4
6
8
10
PEAK I
FRACTION NUMBER
0
2
4
6
8
10
12
14
50

Fig. 23. Ion-exchange chromatography of ad$_5$. The extract was made from cells grown in SC containing 10 μC of C^{14}-glycine.

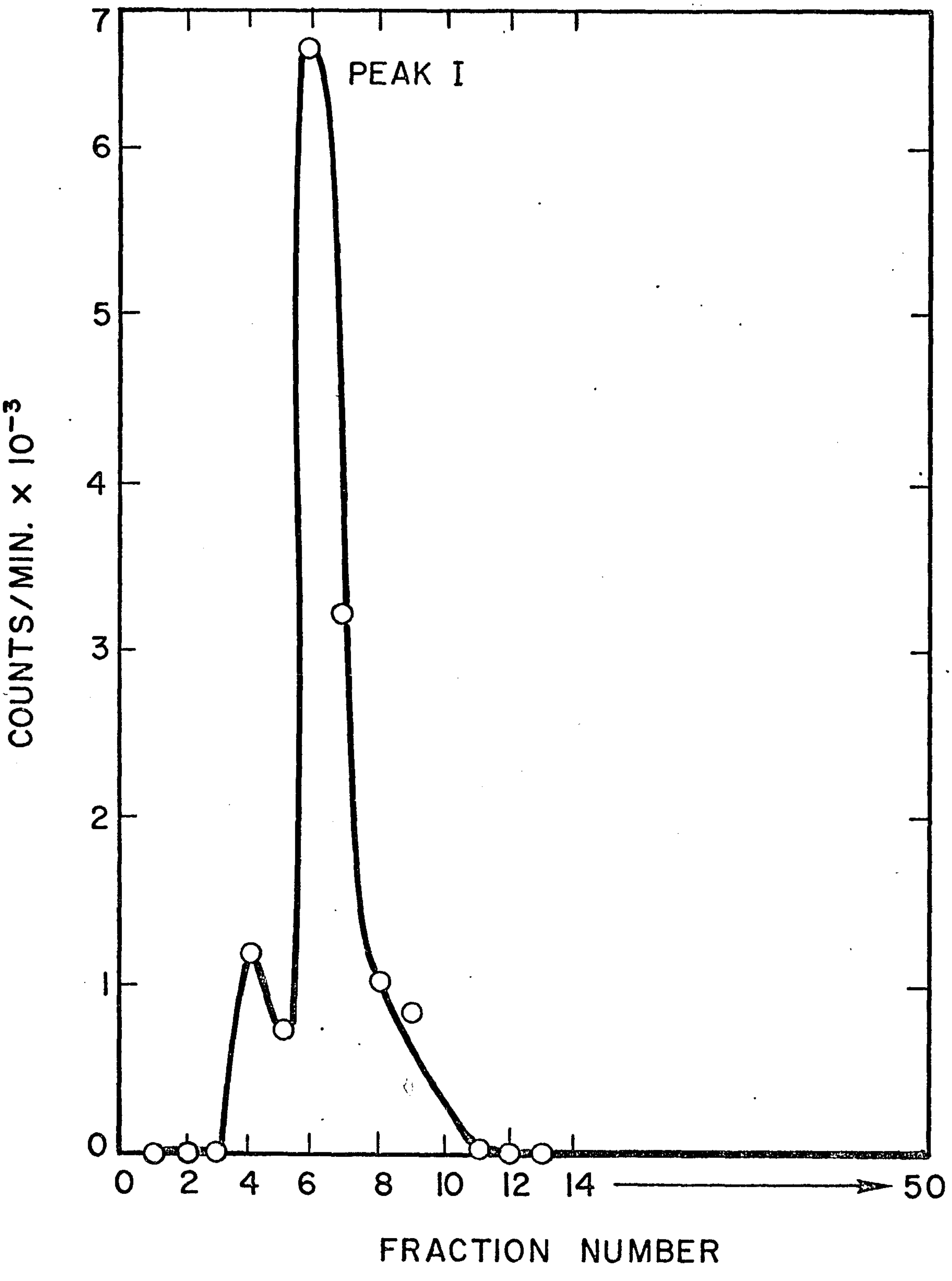

PEAK I
COUNTS/MIN. × 10⁻³
0
1
2
3
4
5
6
7
0
2
4
6
8
10
12
14
50
FRACTION NUMBER

Fig. 24. Ion-exchange chromatography of ad_8. The extract was made from cells grown in SC containing 5 µC of C^{14}-glycine.

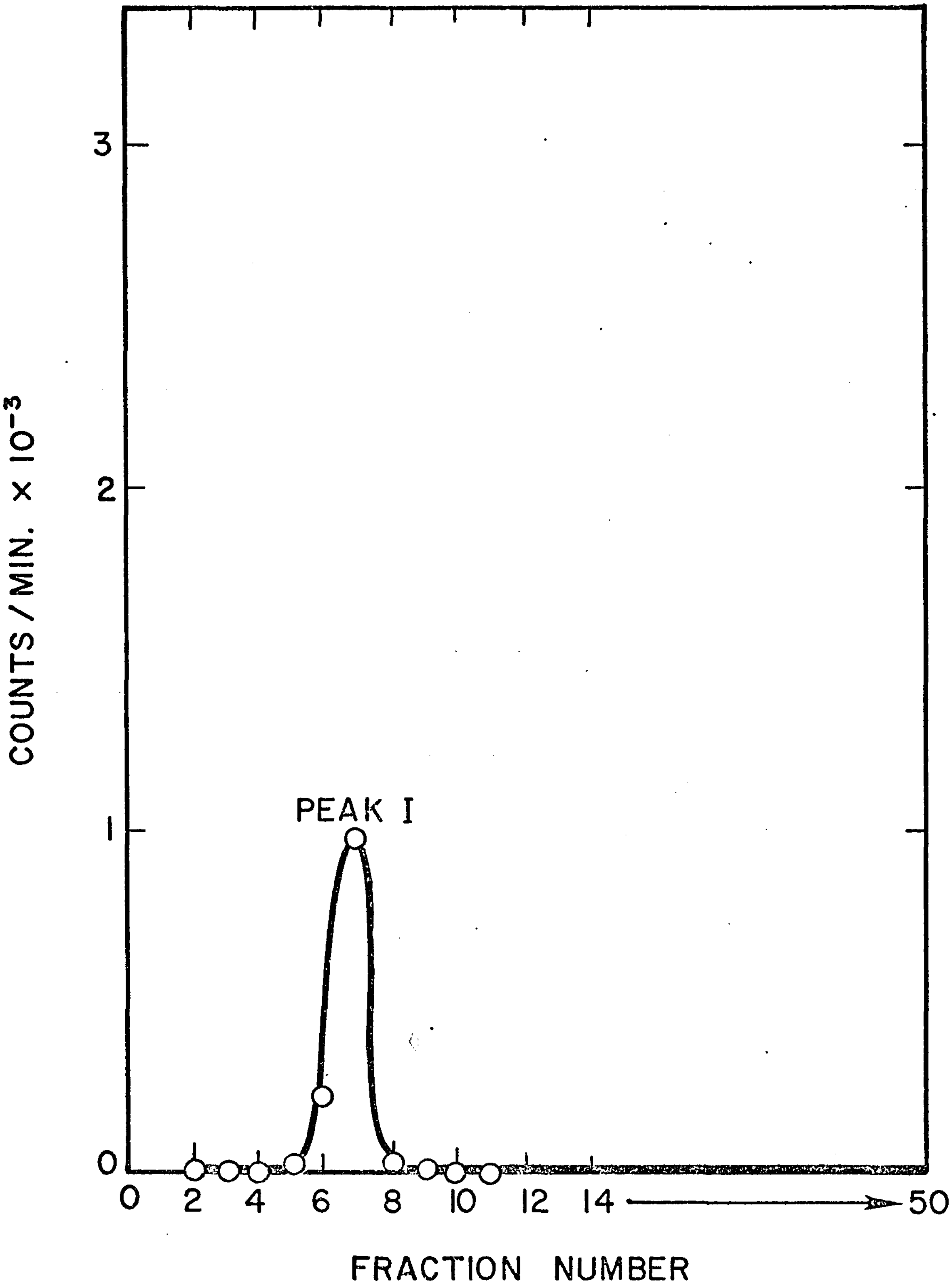

COUNTS / MIN. × 10⁻³
3
2
1
0
PEAK I
0
2
4
6
8
10
12
14
50
FRACTION NUMBER

Fig. 25. Ion-exchange chromatography of ad_9. The extract was made from cells grown in SC containing 5 µC of C^{14}-glycine.

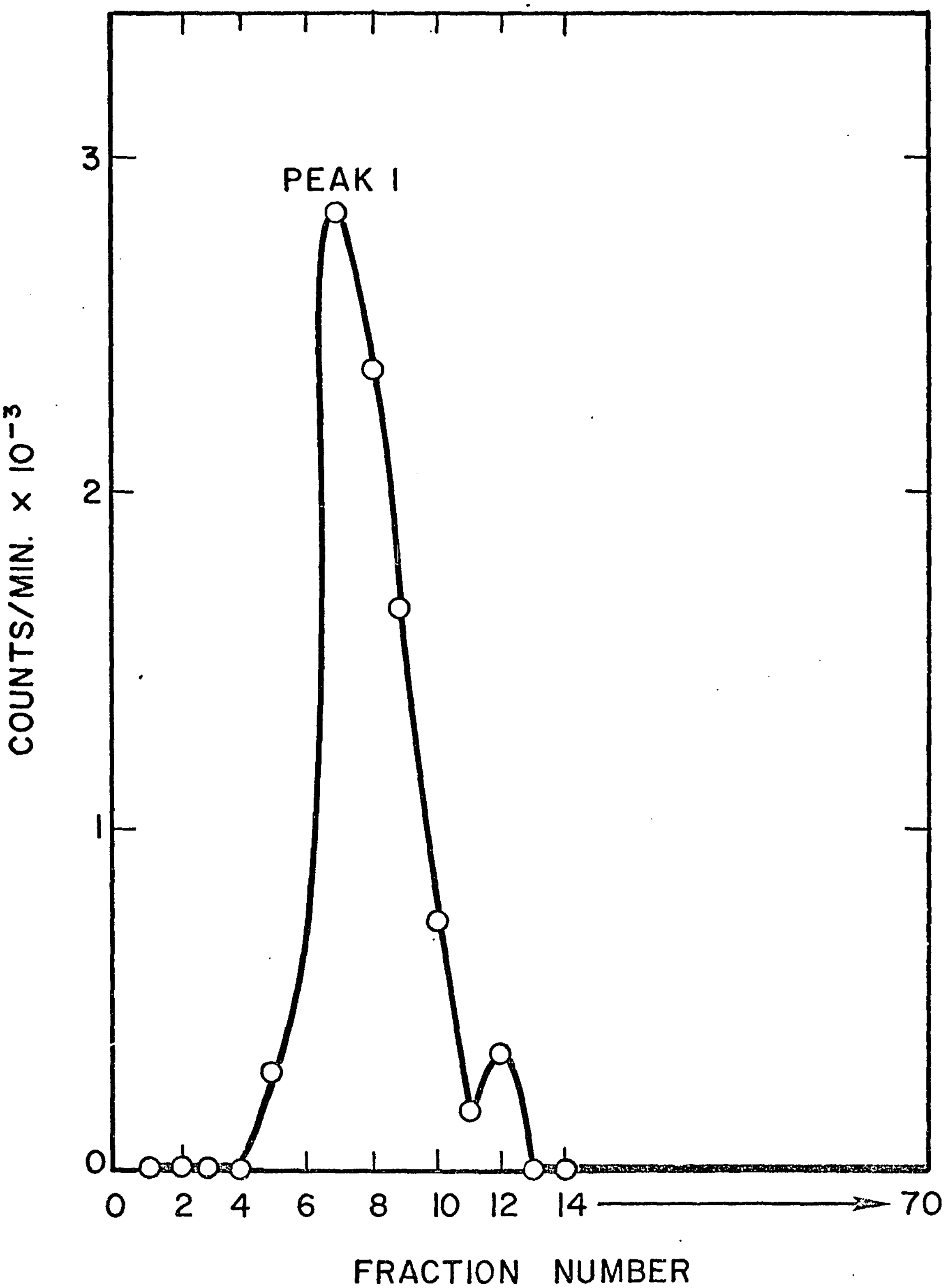

PEAK I
COUNTS/MIN. × 10^{-3}
3
2
1
0
0
2
4
6
8
10
12
14
70
FRACTION NUMBER

Fig. 26. Ion-exchange chromatography of the wildtype. The extract was made from cells grown in SC containing 10 µC of C^{14}-glycine.

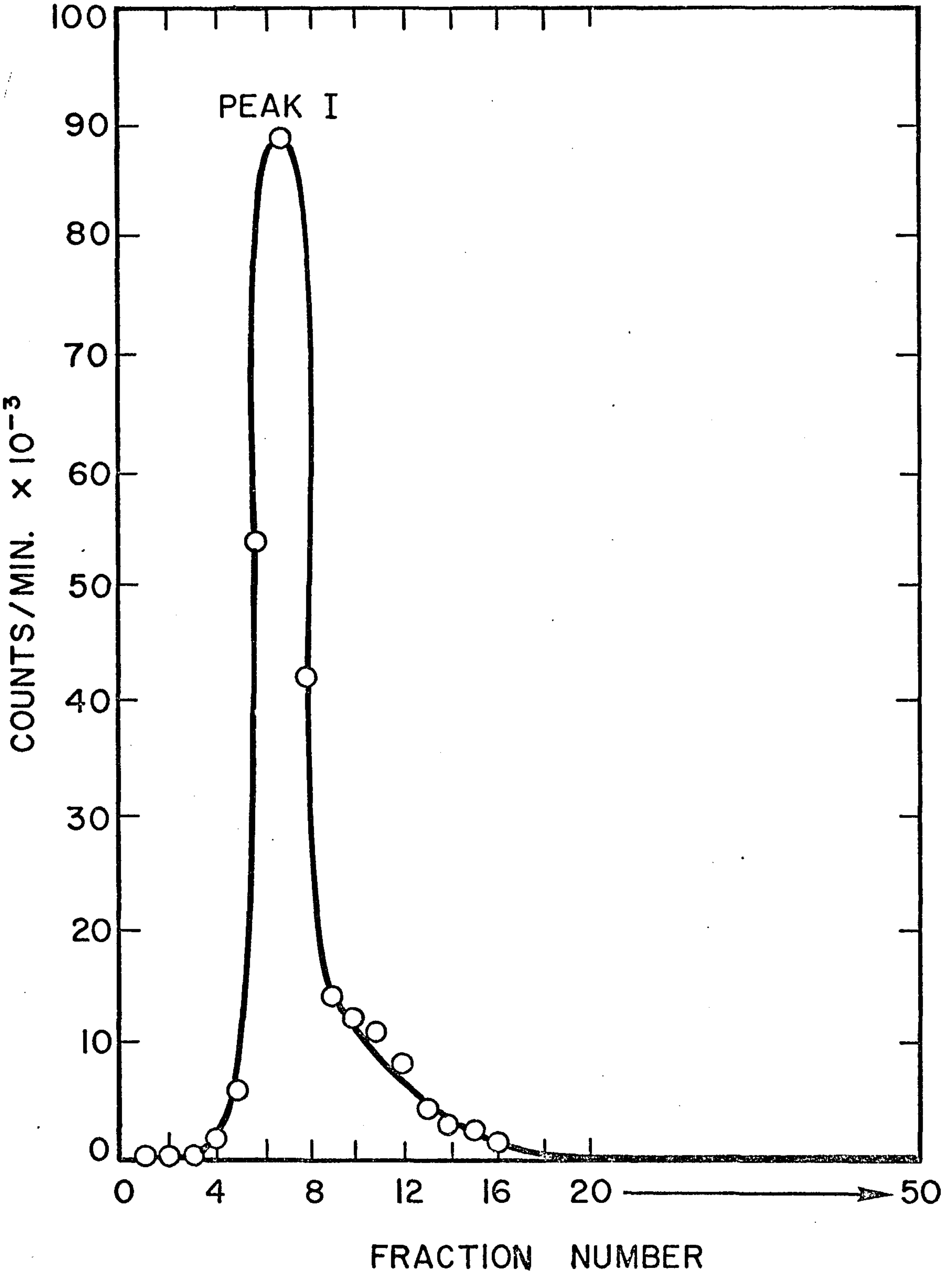
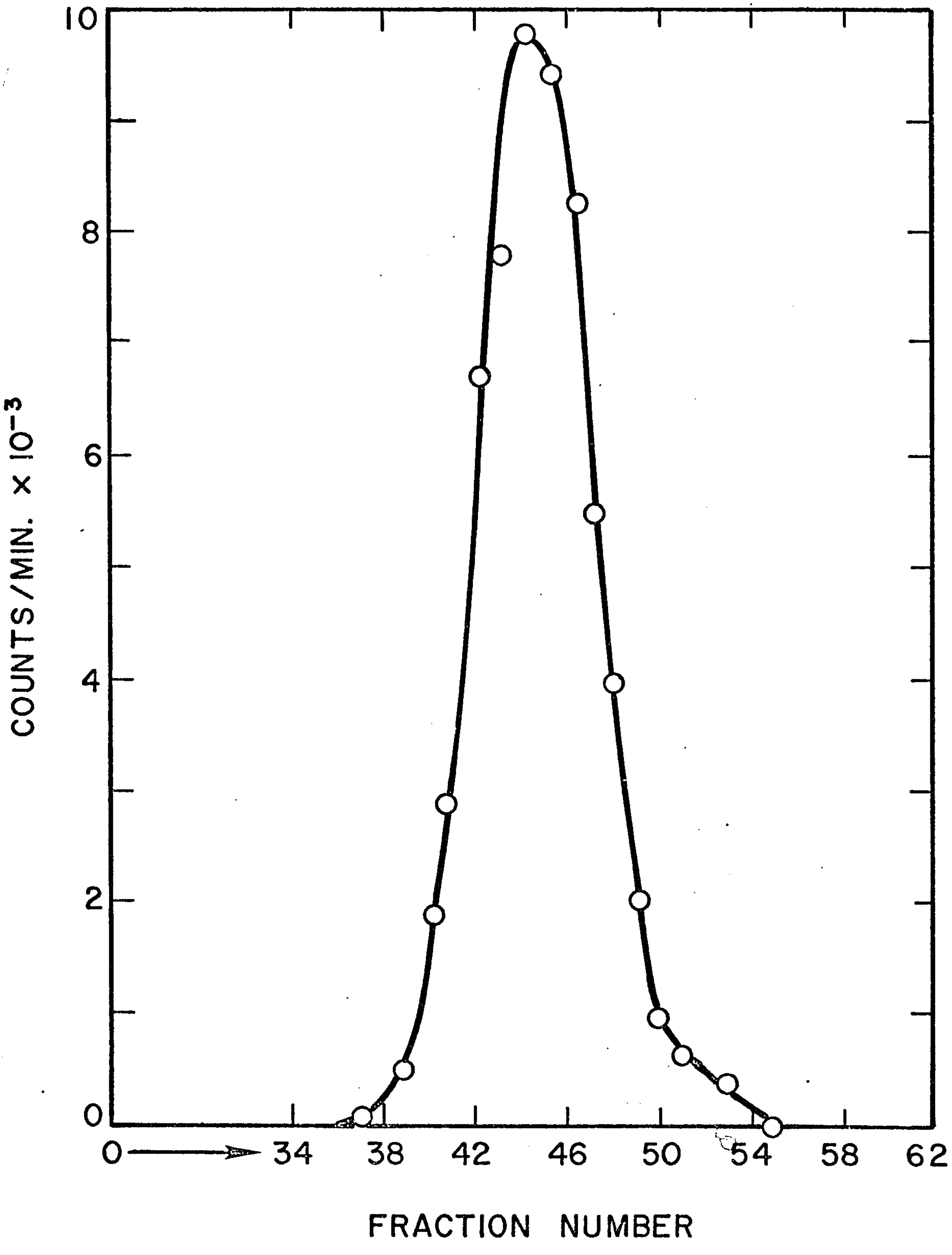

PEAK I
COUNTS/MIN. × 10⁻³
100
90
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
50
FRACTION NUMBER

Fig. 27. Ion-exchange chromatography of a mixture of peak II material from ad_6 and ad_7. C^{14}-glycine-labeled extracts of each mutant were chromatographed on Dowex-1X8-formate columns and peak II material isolated from each. The peak II materials from each mutant were pooled, lyophilized, dissolved in water and rechromatographed on Dowex-1X8-formate columns.



COUNTS/MIN. × 10^{-3}
10
8
6
4
2
0
0
34
38
42
46
50
54
58
62
FRACTION NUMBER

Fig. 28. Ion-exchange chromatography of deformylated peak II material. Peak II material labeled with C^{14}-glycine, isolated from ad_6 and ad_7 was deformylated by mild acid hydrolysis in 0.1N HCl and rechromatographed on Dowex-1X8-formate. (A) is the elution profile of deformylated peak II material from ad_6, (B) the elution profile of deformylated peak II from ad_7 and (C) the elution profile of a deformylated mixture of ad_6 and ad_7 peak II material.

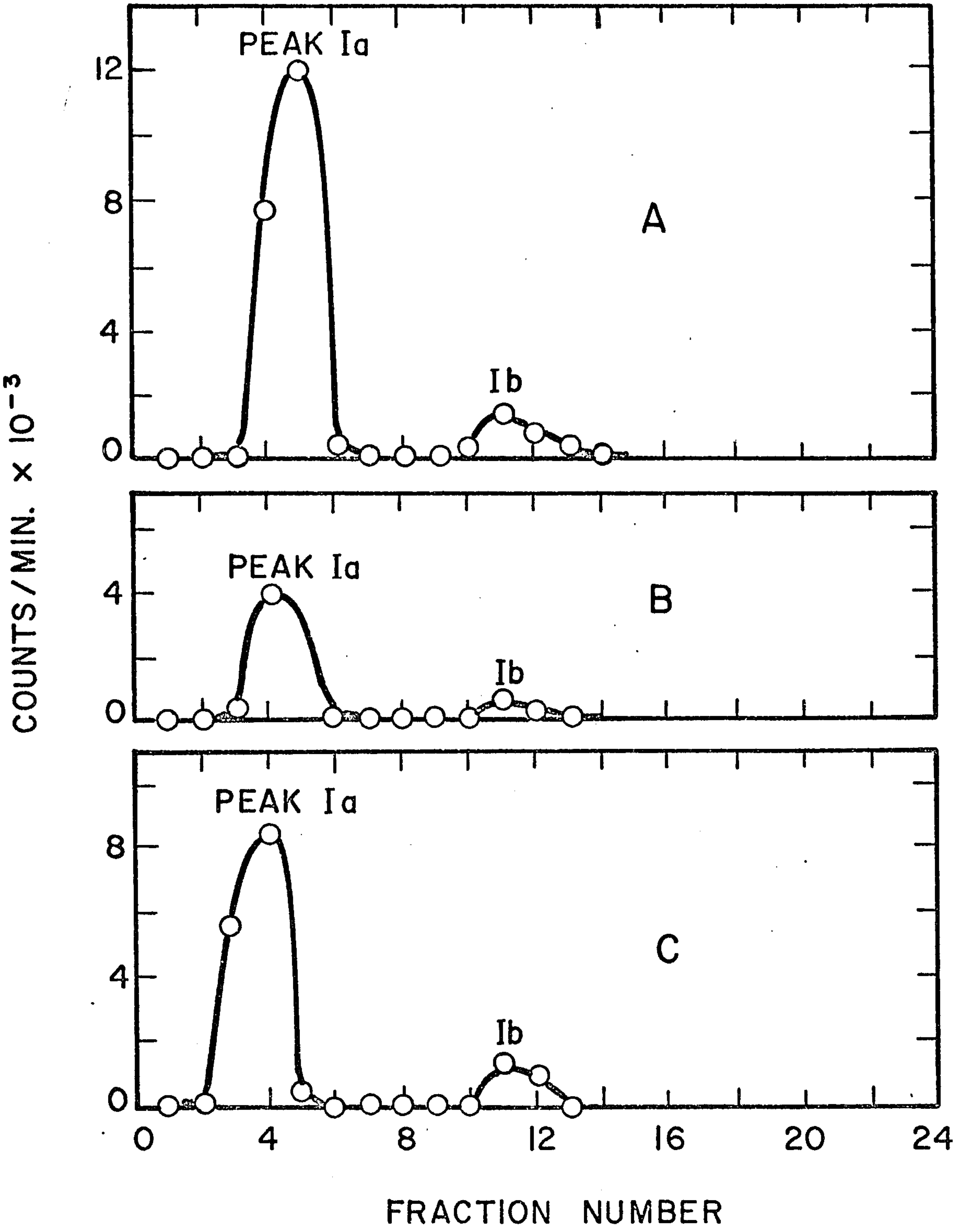

PEAK Ia
Ib
A
PEAK Ia
Ib
B
PEAK Ia
Ib
C
COUNTS/MIN. × 10^{-3}
0
4
8
12
0
4
0
4
8
0
4
8
12
16
20
24
FRACTION NUMBER

Fig. 29. TLC-comparison of C^{14}-glycine-labeled peak II material from ad_6 and ad_7. In solvents (A) Iso-HCl, (C) PAW and (D) BAW, (1) is peak II material from ad_6 (2) peak II material from ad_7 and (3) the C^{14}-glycine standard. In solvent (B) MCF, (1) is the C^{14}-glycine standard (2) peak II material from ad_6 and (3) peak II material from ad_7.

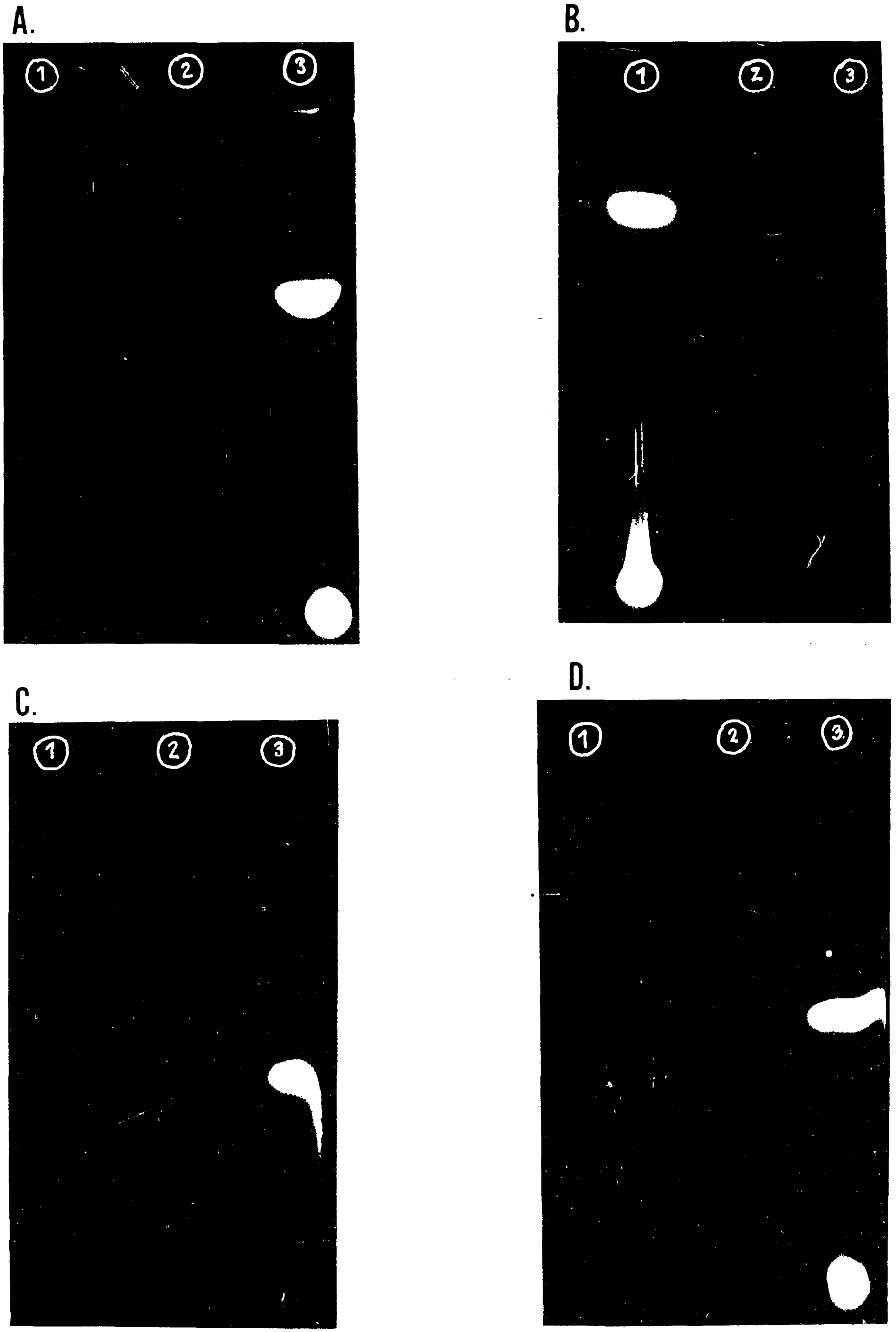

A.
①
②
③
B.
①
②
③
C.
①
②
③
D.
①
②
③

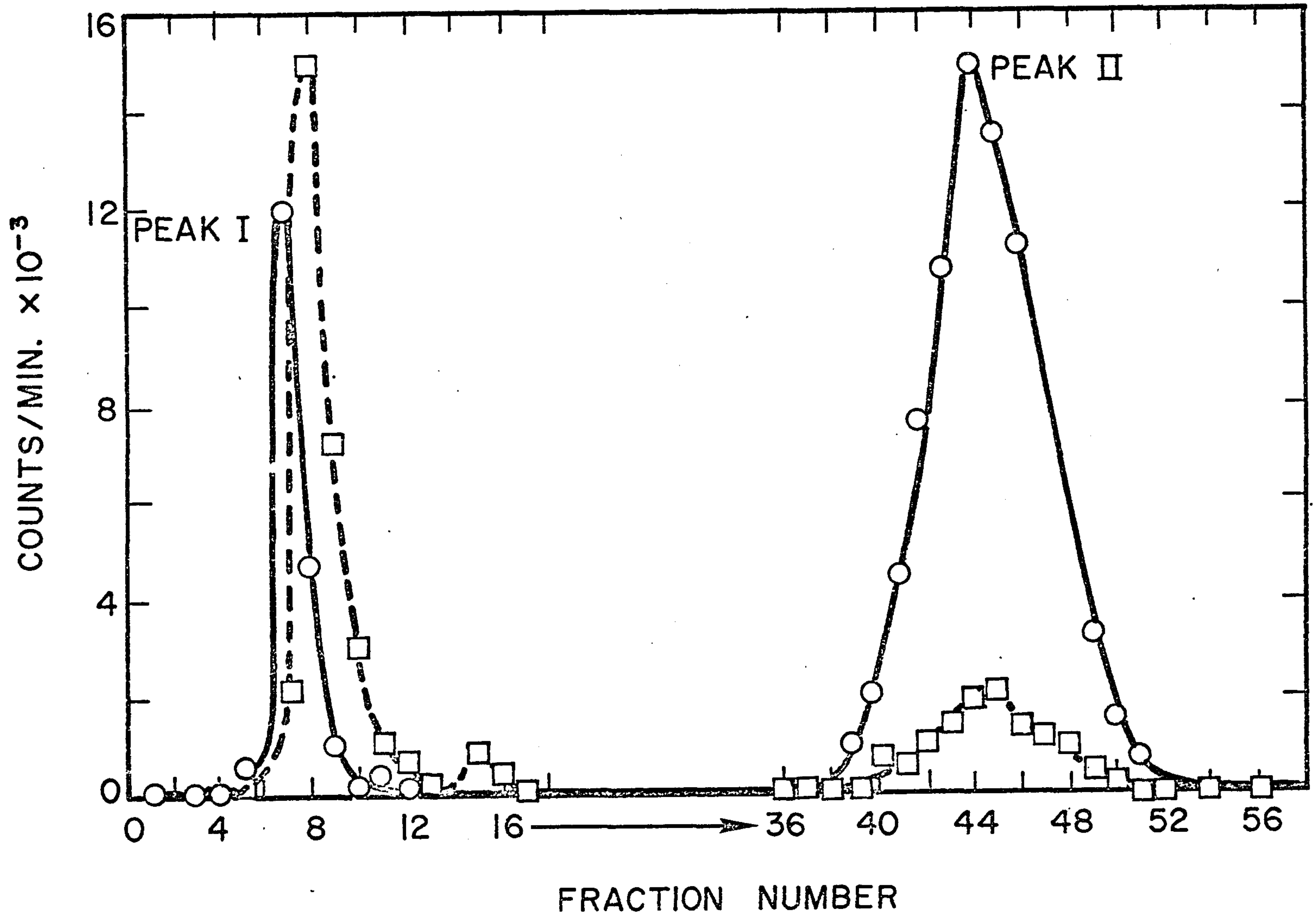
TABLE 5

TLC analysis of C^{14}-glycine-labeled material eluted as peak II from extracts of ad_6 and ad_7.

Solvent:	Rgly:							
	C^{14}		sugar		phosphate		ninhydrin	
	ad_6	ad_7	ad_6	ad_7	ad_6	ad_7	ad_6	ad_7
BAW	0.44	0.41	+	+	...	...	...	...
PAW	0.23	0.23	+	+	...	...	...	...
MCF	0.88	0.85	...	...	...	...	-	-
Iso-HCl	1.3	1.3	...	...	+	+	...	...

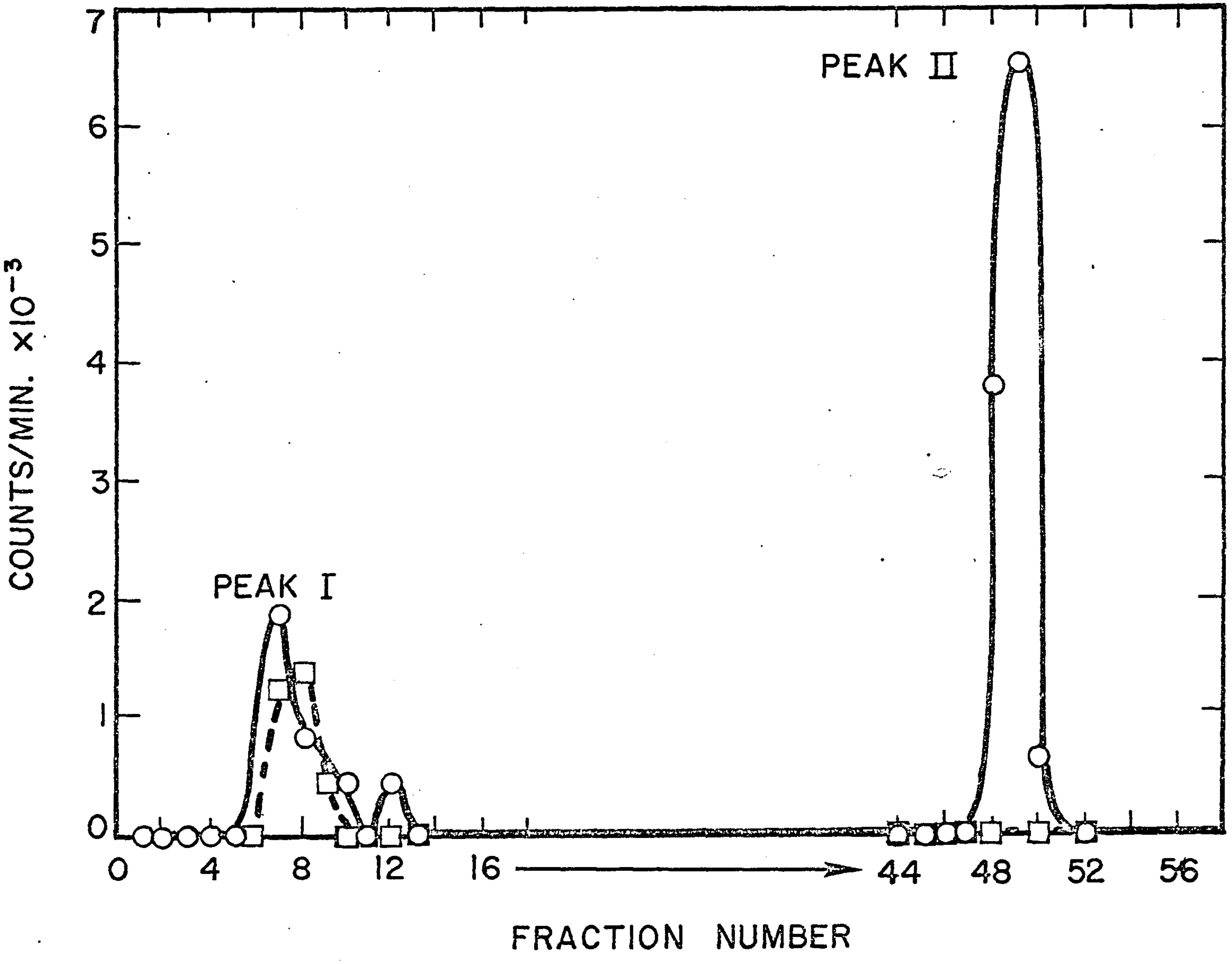
(1) The C^{14} on each plate was localized by autoradiography. The plates were subsequently sprayed with colorimetric reagents and the results indicated by a (+) or (-) sign.

Fig. 30. Feedback inhibition by adenine of the accumulation of peak II material in ad_6. Extracts were made from cells incubated in SC medium containing 10 µC of C^{14}-glycine and which was either adenine-less O———O, or contained 250 µg adenine per ml □-------□.



COUNTS/MIN. × 10^{-3}
16
12
8
4
0
PEAK I
PEAK II
0
4
8
12
16
36
40
44
48
52
56
FRACTION NUMBER

Fig. 31. Feedback inhibition by adenine of the accumulation of peak II material in ad_7. Extracts were made from cells incubated in SC medium containing 10 µC of C^{14}-glycine and which was either adenine-less 0———0, or contained 250 µg adenine per ml □-------□.


PEAK II
PEAK I
COUNTS/MIN. ×10⁻³
0
1
2
3
4
5
6
7
0
4
8
12
16
44
48
52
56
FRACTION NUMBER

eluted as peak I will be discussed elsewhere (see Chapt IV). Additional evidence that the intermediate isolated as peak II in FGAR is provided by the following observations: The material contains sugar and phosphate, is ninhydrin negative and is labeled by C^{14}-formate (Table 5, Fig 21).

When peak II of ad_6, peak II of ad_7 and a mixture of the two are deformylated, each gives rise to two new peaks (peak Ia and Ib, Fig 28). Peak Ia behaves chromatographically as GAR (Lepage and Jones, 1961); peak Ib is eluted somewhat later.

The formylated intermediate (native peak II) and the deformylated products (peak Ia and Ib) of the ad_6 and ad_7 mutants were compared on thin layer chromatograms. The chromatograms were developed in two different solvents (BAW and MCF) and sprayed with ninhydrin reagent. Both ad_6 and ad_7 native peak II materials were ninhydrin negative. One ninhydrin positive spot was observed in the chromatograms of peak Ia from both ad_6 and ad_7, and some ninhydrin positive material remained at the origin. Peak Ib material which is formed in relatively small amounts, gave a variable ninhydrin reaction and was not investigated further.

Radioautograms of native and deformylated peak II of ad_6, chromatographed in four different solvents, are shown in Figs 32 and 33. The native peak II and peak Ia showed coincident spots of C^{14}, phosphate and sugar. Peak Ib showed coincident spots of C^{14} and phosphate, however the sugar reaction of this peak was negative.

Consistent with the data above, which suggests that peak II is FGAR, is the fact that ad_6 peak II material can be converted to AIR by a crude enzyme preparation from ad_2 (Table 6). The activity of the

Fig. 32. TLC-autoradiograms of native and deformylated peak II material of ad_6 chromatographed in the PAW and the MCF solvent systems.

(A) PAW, (B) MCF, (1) native peak II material, (2) peak Ia material (3) peak Ib material (4) C^{14}-glycine standard.

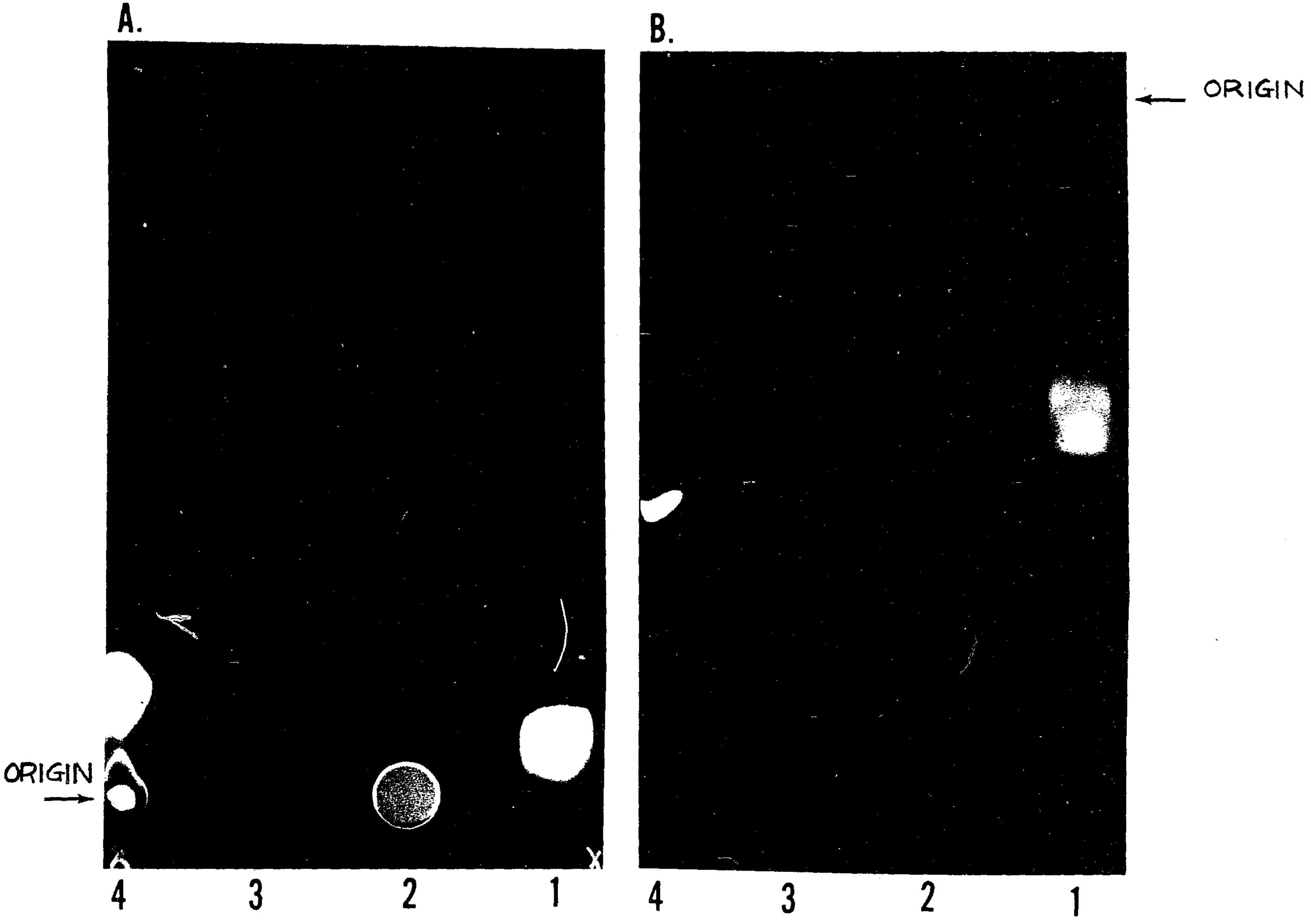

A.
B.
ORIGIN
ORIGIN
4
3
2
1
4
3
2
1

Fig. 33. TLC-autoradiograms of native and deformylated peak II of ad_6 chromatographed in the Iso-HCl and BAW solvent systems.

(A) Iso-HCl, (B) BAW, (1) native peak II material, (2) peak Ia material, (3) peak Ib material (4) C^{14}-glycine standard.

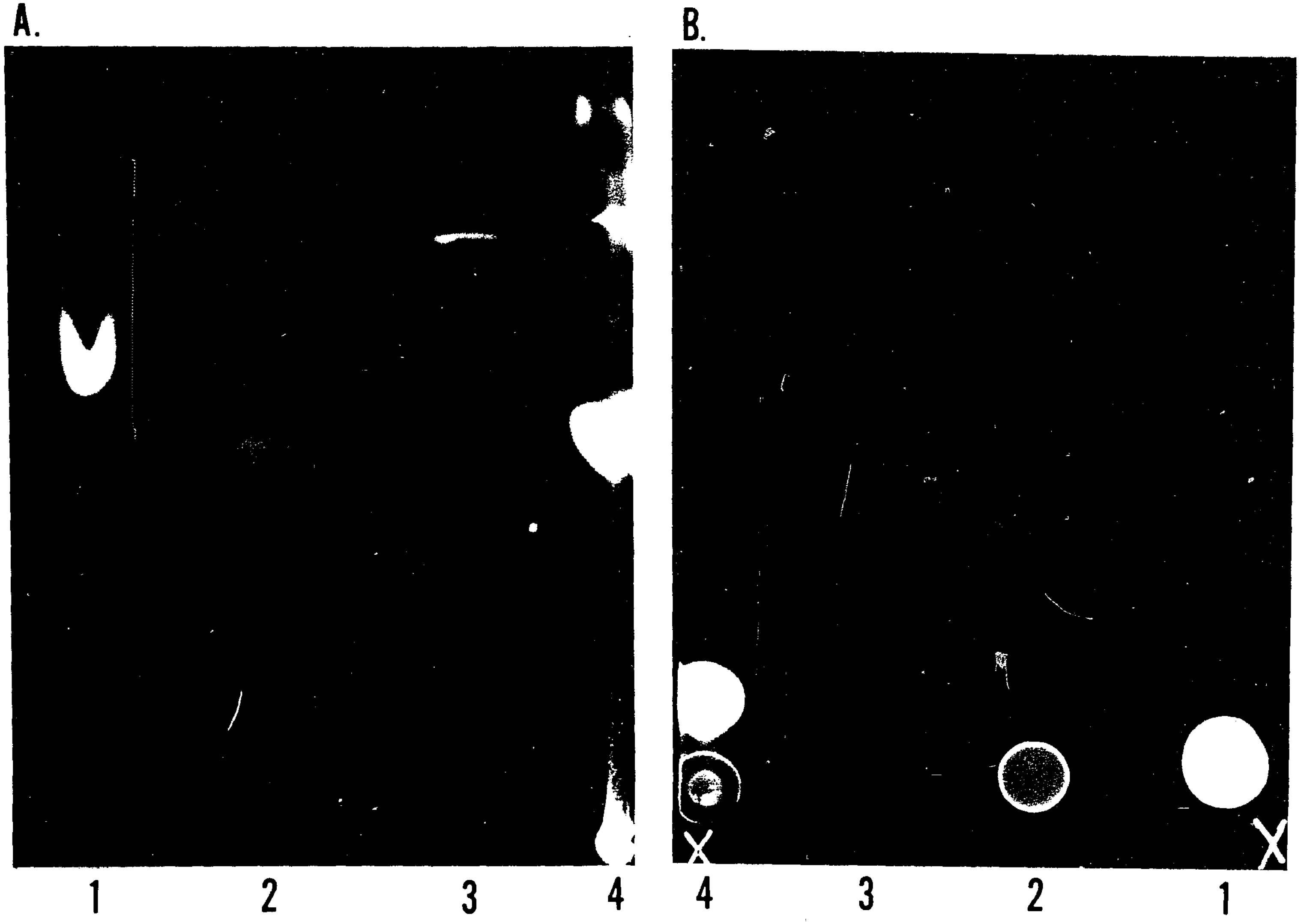

A.
1
2
3
4
B.
4
3
2
1
X
X

TABLE 6

Enzymatic conversion of peak II material to AIR.

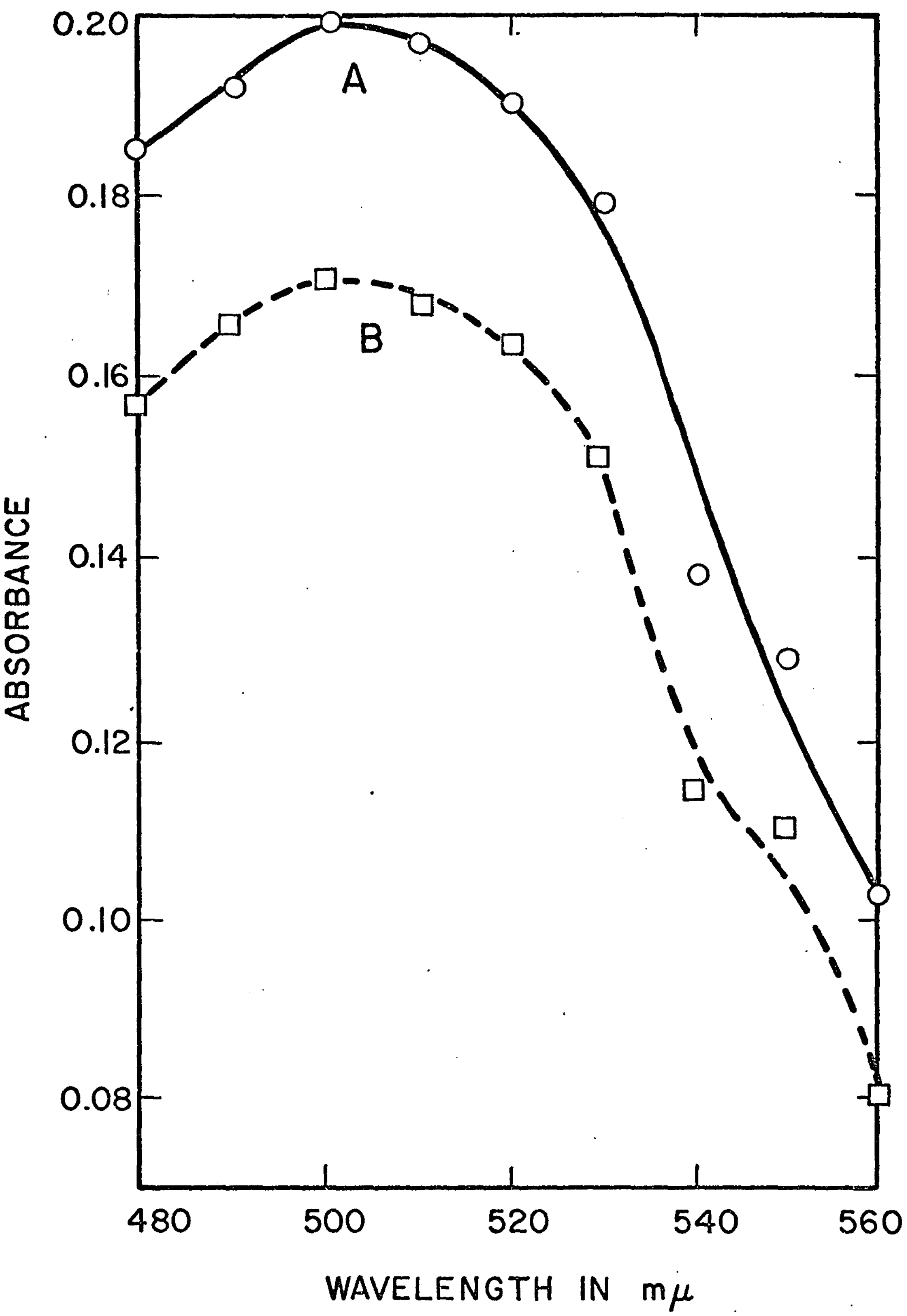
Reaction mixture	O.D.$_{500\ m\mu}$ (after acetylation)
zero-time, complete	0.162
no substrate (peak II)	0.157
no ATP	0.173
no glutamine	0.197
60 minute, complete	0.192

Each reaction vessel contained the following: 2.5 µM potassium ATP, 6.0 µM $MgCl_2$, 10.0 µM L-glutamine, 10.0 µM potassium PGA, 18.6 mg crude dialyzed ad_2 extract, 10.0 µM potassium phosphate buffer, pH 7.4, containing 0.5 µM EDTA and 0.5 µM L-glutamine. Omissions of compounds from the control reaction mixtures were made as indicated in the table. The zero-time and 60 minute vessels contained complete reaction mixtures, however, 0.2 ml of 1.33 M potassium phosphate buffer, pH 1.4, containing 20% TCA was added to the zero-time vessel before the addition of enzyme. All vessels were incubated 60 minutes at 30°. AIR was assayed as described in Materials and Methods.

crude enzyme system was low as seen by the small increase in Bratton-Marshall positive material observed in the 60 minute reaction mixture. Flaks and Lukens (1963) report that in Neurospora negligible enzymatic activity of the purine pathway enzymes can be demonstrated without a preliminary fractionation of the crude extract. Since no fractionation of the yeast enzymes was attempted, this may explain the low activity observed. No glutamine dependence of the reaction could be demonstrated, since the procedure followed (cf. Flaks and Lukens, 1963) calls for the addition of L-glutamine to the buffer in which the enzymes are prepared. Nevertheless, as shown in Fig 34, the absorption spectrum of the non-acetylatable Bratton-Marshall positive material formed in the reaction is that of AIR and represents an increase in Bratton-Marshall positive material over the control reaction mixtures. Although the ad_2 enzymes are dialyzed, some AIR does apparently remain tightly bound to the enzyme and accounts for the Bratton-Marshall chromophore observed in the zero-hour sample (Fig 34).

Further identification of peak II as FGAR, was carried out as follows: C^{14}-glycine-labeled FGAR (peak II) was isolated on a Dowex-1-formate column. The FGAR was subjected to the deformylation procedure described above, rechromatographed, and the material eluted in fractions 4-7 was collected and lyophilized. The lyophilized residue was dissolved in water and assayed for the presence of GAR by the procedure of Nierlich and Magasanik (1961). The AICAR formed as a result of the degradation of IMP was assayed by the Bratton-Marshall procedure described by these authors. While the zero-hour control contained no Bratton-Marshall positive material, the Bratton-Marshall chromophore

Fig. 34. Absorption spectrum of non-acetylatable Bratton-Marshall positive material formed in the enzymatic conversion of peak II material to AIR.
(A) 60 minute complete reaction vessel (B) zero minute complete reaction vessel.

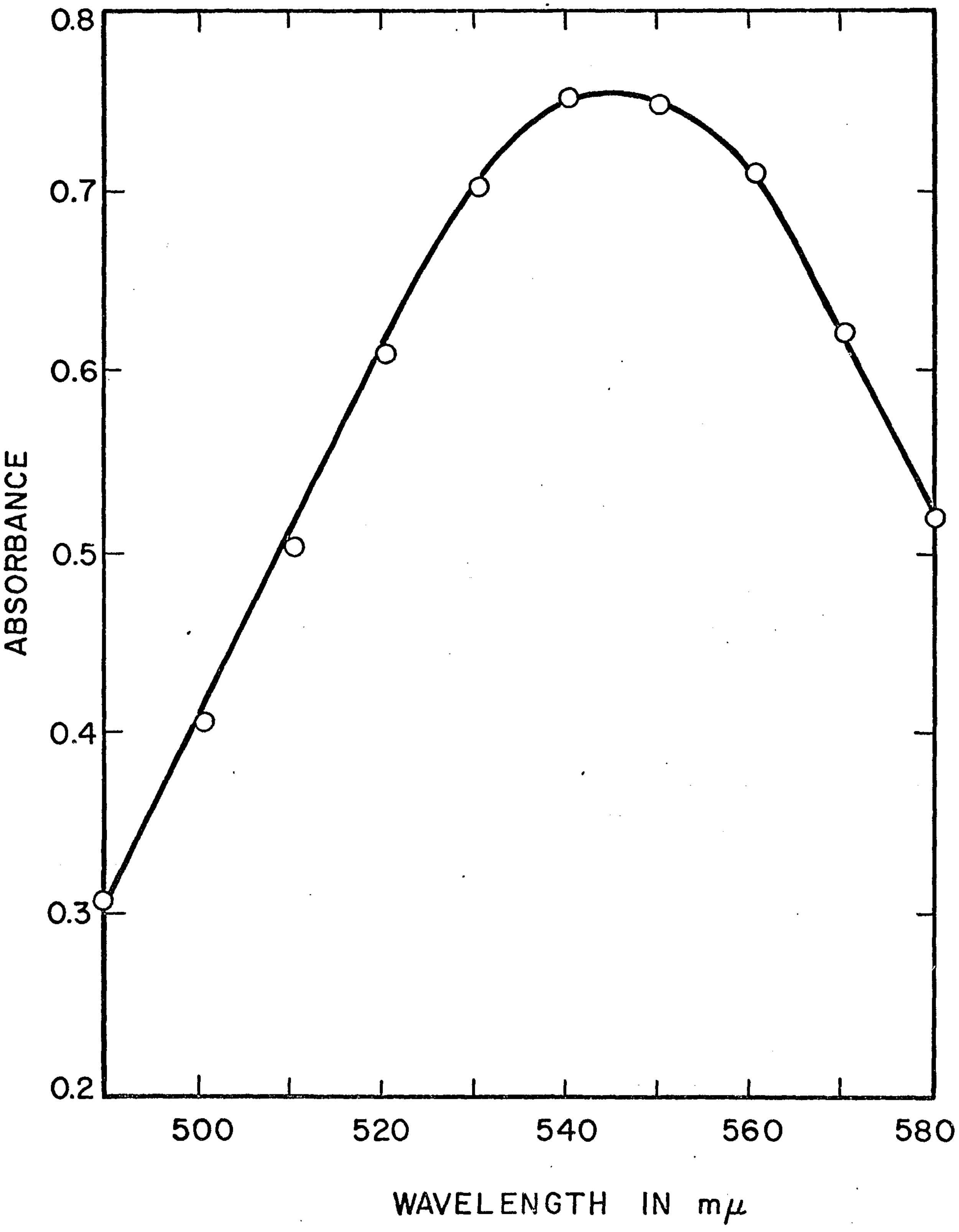

0.20
0.18
0.16
0.14
0.12
0.10
0.08
ABSORBANCE
A
B
480
500
520
540
560
WAVELENGTH IN mμ

found in the supernatant of the 45 minute reaction mixture, has the absorption spectrum of that of AICAR (Fig 35).

In summary, peak II, which can be labeled both by C^{14}-glycine and C^{14}-formate, is subject to feedback inhibition by adenine. This intermediate is ninhydrin negative, contains sugar and phosphate and can be converted to AIR by yeast enzymes. In addition, the intermediate can be deformylated to a ninhydrin positive material which has the chromatographic properties of GAR. One may conclude therefore that the material isolated as peak II is FGAR.

The demonstration of FGAR as the intermediate of purine biosynthesis which accumulates in the ad_6 mutant, confirms the above proposal that this mutant is blocked at the conversion of FGAR to FGAM (step V, Fig 1). Since ad_7 also accumulates FGAR, but was shown to be blocked at a step later than ad_6, the ad_7 mutant lacks the ability to convert FGAM to AIR (step VI, Fig 1).

Fig. 35. Absorption spectrum of the Bratton-Marshall chromophore formed in the GAR assay : deformylated peak II material. Peak II material was isolated on a Dowex-1X8-formate column, deformylated, rechromatographed and the material eluted in fractions 4-7 was collected and lyophilized. The lyophilized residue was dissolved in water and assayed for the presence of GAR by the procedure of Nierlich and Magasanik.



0.8
0.7
0.6
0.5
0.4
0.3
0.2
ABSORBANCE
500
520
540
560
580
WAVELENGTH IN mμ

CHAPTER IV

Functional Blocks of AD_4 and AD_5

Since characterization of the accumulated C^{14}-glycine-labeled intermediates was used successfully to localize the functional blocks of mutants ad_6 and ad_7 (Chapt III), a similar approach was used to study the mutants ad_4 and ad_5. The mutants were grown in SC medium containing C^{14}-glycine, ethanol-deproteinized extracts were made and these were compared by TLC in the PAW solvent (Fig 36). In contrast to the results obtained with extracts of ad_6 and ad_7, although some heavily labeled materials are present, the extracts of ad_4 and ad_5 contained no identifiable characteristic spots.

An attempt was made to determine whether any of the C^{14}-glycine-labeled materials observed in these extracts represented purine intermediates, using the criterion of feedback inhibition. Some suggestion was found of feedback inhibition of the accumulation of labeled material in the ad_4 extract (Fig 37). The extract of ad_5, however, could not be resolved into a discrete pattern. Thus, no determination of feedback inhibition could be made in this mutant.

Further evidence that the labeled materials which accumulate in ad_4 represent adenine pathway intermediates is supplied by the observation that the introduction of an ad_8 marker into an ad_4 strain will cause an alteration in the TLC-pattern of the latter (Fig 38). On the other hand, a similar procedure with ad_5 gives no marked reduction in the accumulation of C^{14}-glycine-labeled material by the ad_5 mutant (Fig 39). The above procedure suggests that some of the C^{14}-glycine-

Fig. 36. TLC-autoradiograms of extracts of C^{14}-glycine-labeled ad_4 and ad_5. The chromatograms were developed in the PAW solvent system.

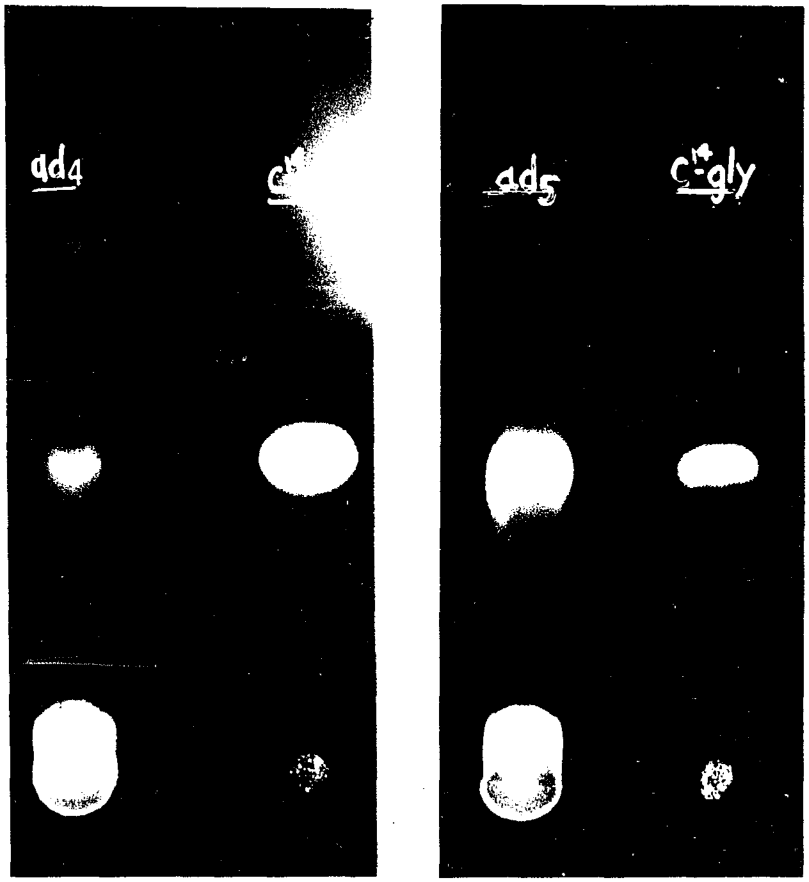

ad4
C
ad5
C^{14}-gly

Fig. 37. Feedback inhibition by adenine of the accumulation of C^{14}-glycine-labeled material in ad_4. (1) represents the C^{14}-glycine standard (2) an extract of cells incubated in the absence of adenine and (3) an extract of cells incubated in the presence of adenine.

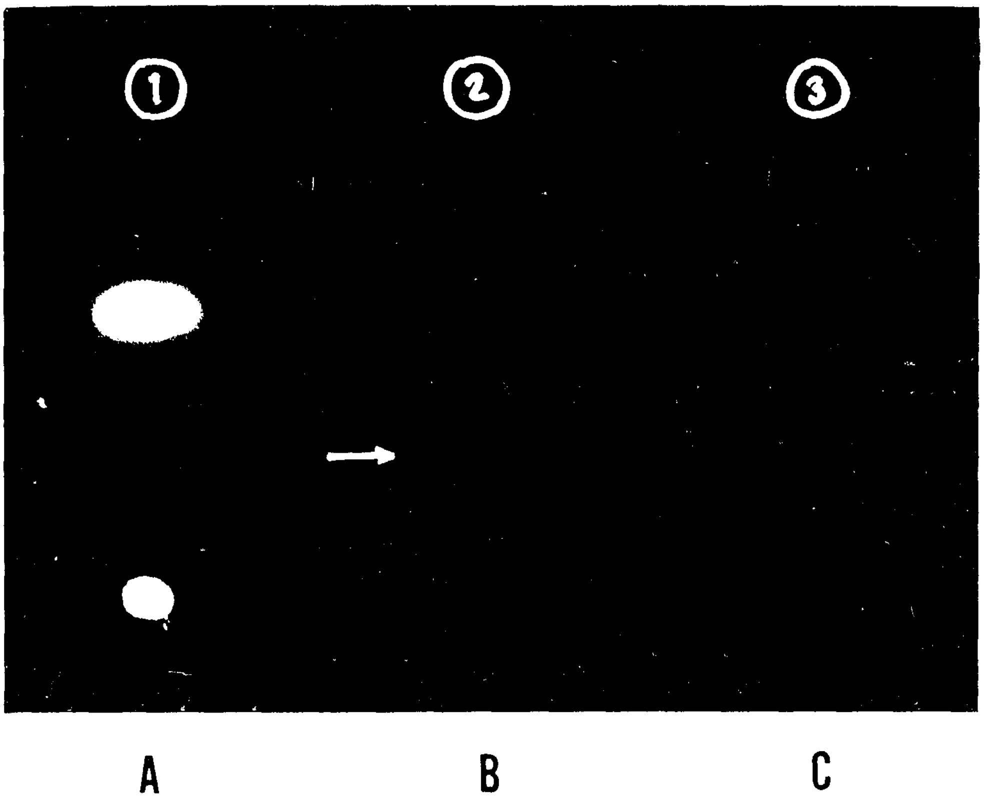

①
②
③
A
B
C

Fig. 38. TLC-comparison of extracts from C^{14}-gycine-labeled ad_4, ad_8 and ad_4ad_8.

Solvent: PAW.

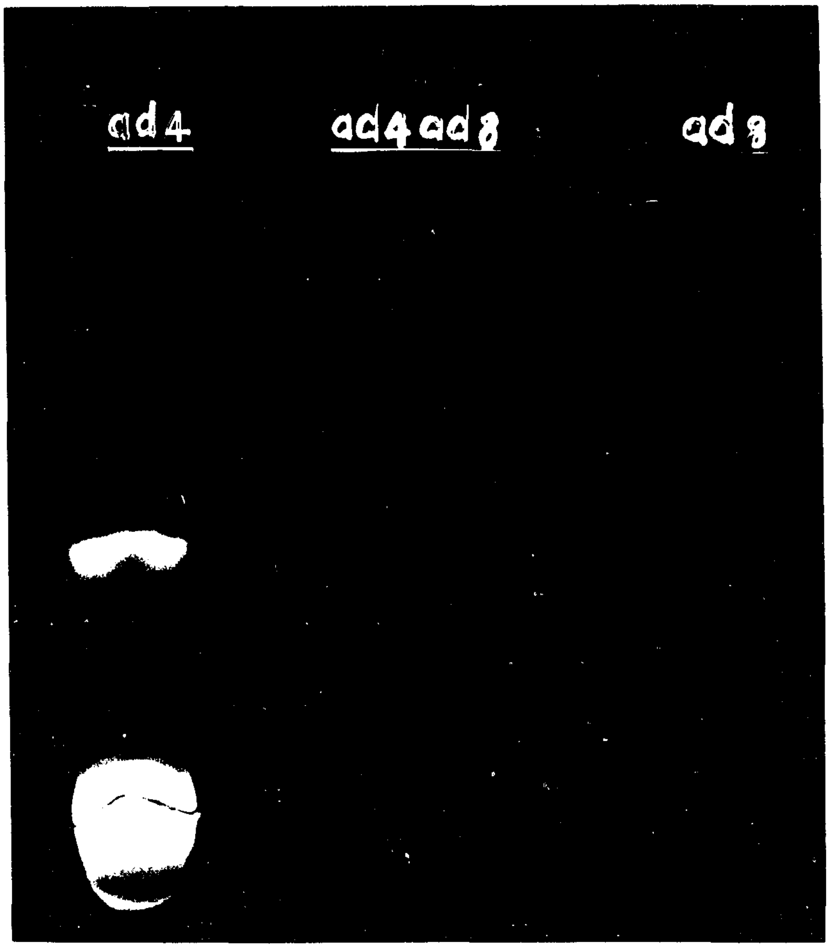
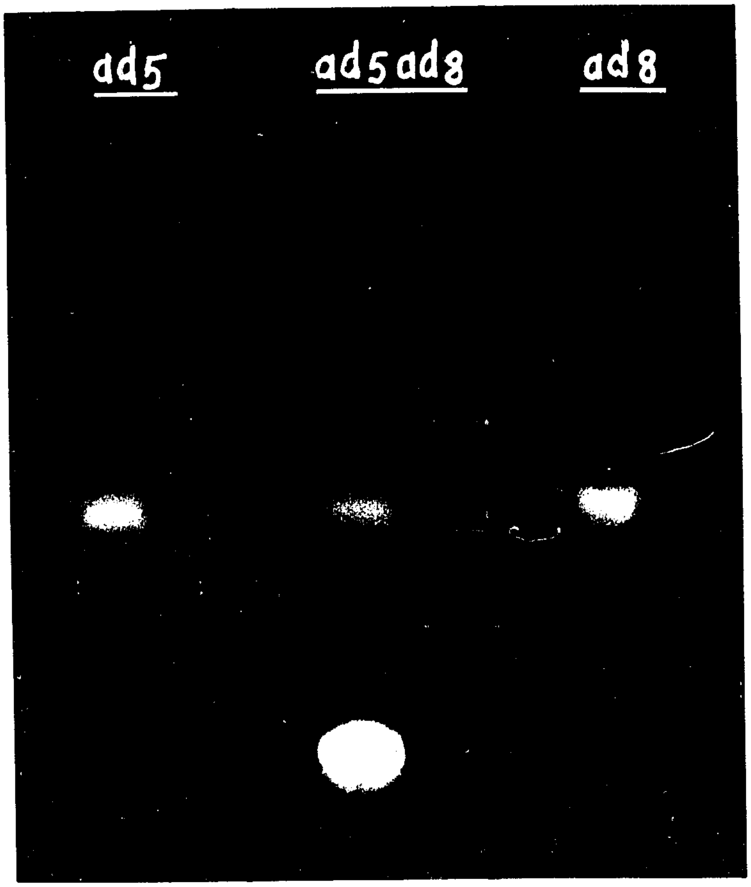

ad4
ad4 ad8
ad8

Fig. 39. TLC-comparison of extracts from C^{14}-glycine-labeled ad_5, ad_8 and ad_5ad_8.
Solvent: PAW.


ad5
ad5ad8
ad8

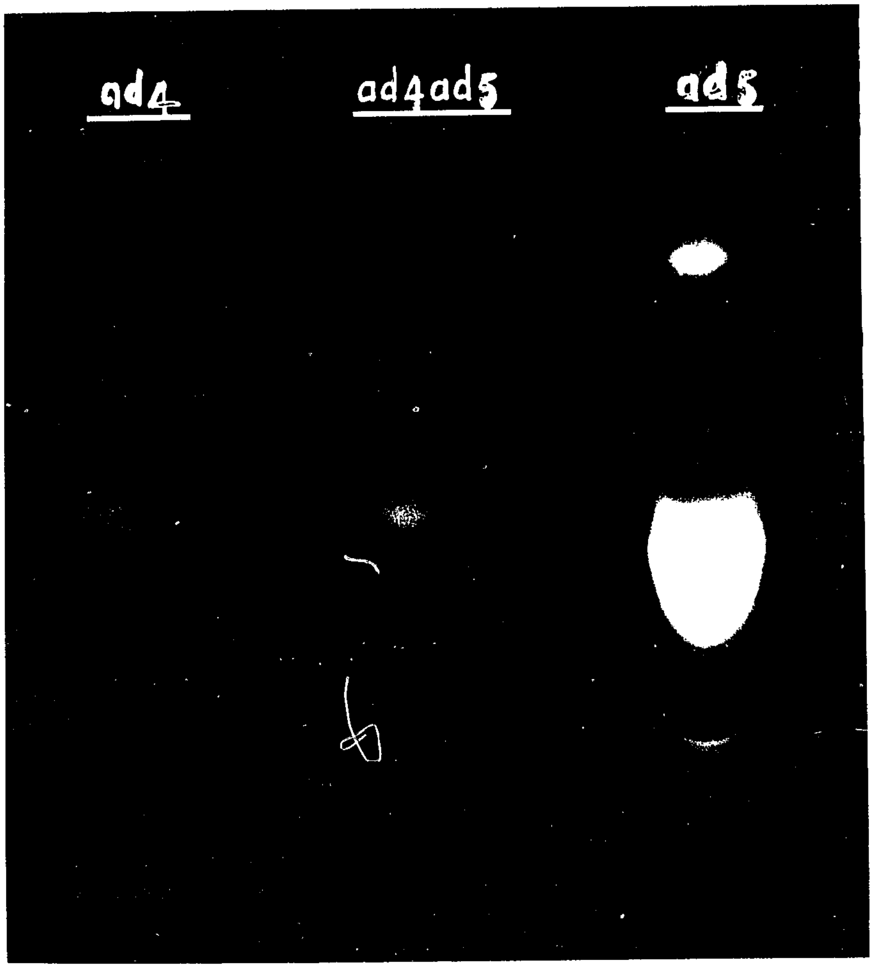
labeled products in the ad_4 extract are purine pathway intermediates, and the double mutant studies place the ad_8 mutational block earlier in the pathway than that of ad_4.

Attempts to order ad_4 and ad_5 in an ad_4ad_5 double mutant led to ambiguous results (Fig 40).

Evidence presented in Chapt III, showed that both the ad_4 and ad_5 mutants are blocked before ad_6 and ad_7, and therefore occur at, or before, the steps at which formylated intermediates are synthesized (i.e. before step IV, Fig 1). Nevertheless, a test for the presence of FGAR in the ad_4 and ad_5 mutants was made by chromatographing extracts of each, using the ion-exchange procedure described in Chapt III. No radioactivity was eluted in the FGAR region in either case, nor from extracts of the wild-type cells (Figs 22, 23 and 26). This is consistent with the preceding data which indicated that these mutants are blocked at, or before, step IV.

Based on their ability to incorporate C^{14}-glycine and on the absence of FGAR, the mutants ad_4 and ad_5 were thought to be blocked after step III in the pathway but before the incorporation of formate. In the pigeon liver system (Fig 1), there is only one step after the incorporation of glycine (step III) but before the incorporation of formate (step IV), and a mutant blocked at this step would be expected to accumulate GAR. Although there are at least two reasons for an inability to carry out the formylation of GAR, i.e. the lack of cofactor (N^5N^{10}methenyl THFA) or a lack of GAR transformylase activity, either deficiency should result in the accumulation of GAR. Accumulation of this intermediate was therefore investigated in both ad_4 and ad_5.

Fig. 40. TLC-comparison of extracts from C^{14}-glycine-labeled ad_4, ad_5 and ad_4ad_5.
Solvent: BAW


ad4
ad4ad5
ad5

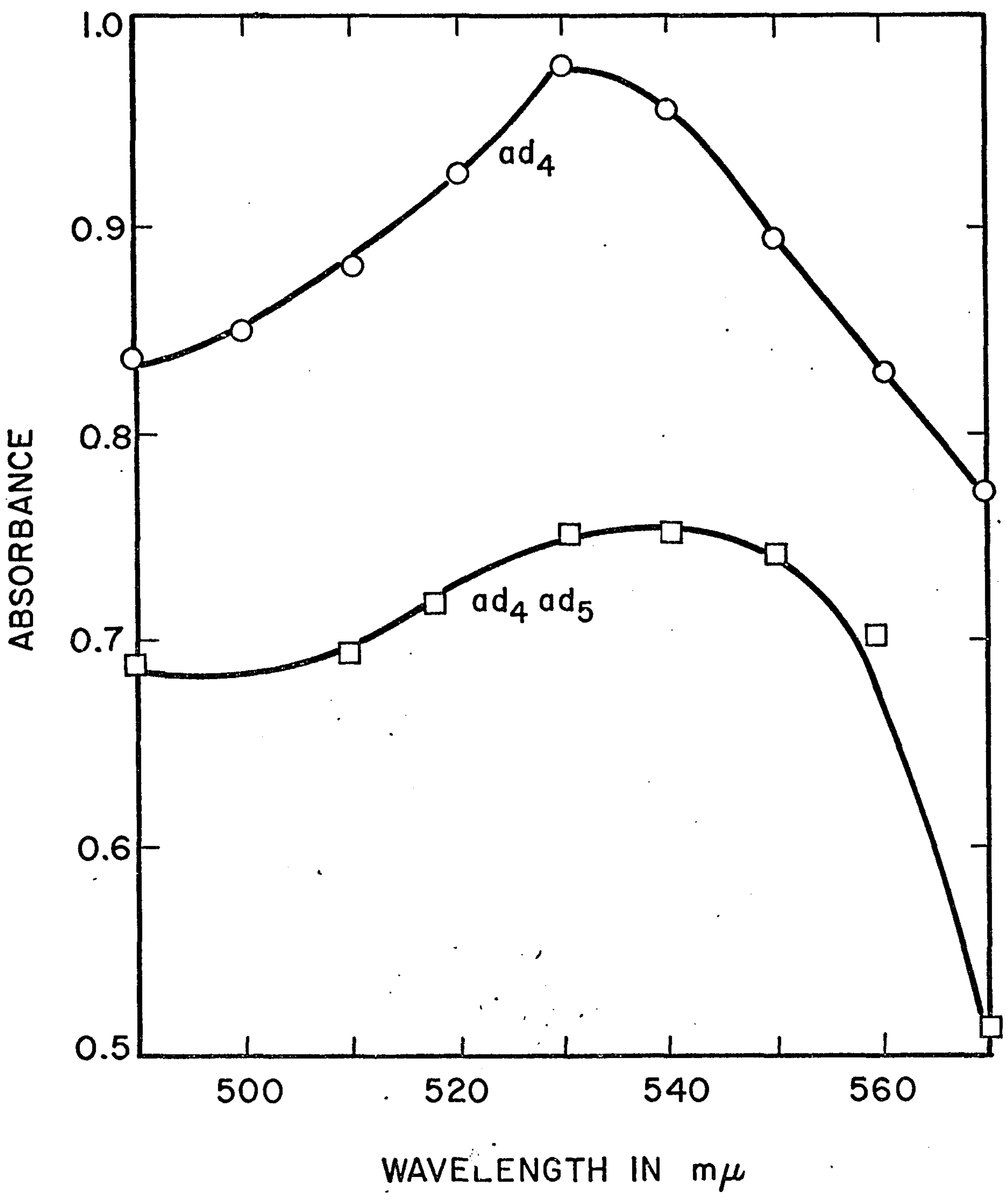
Results of attempts to detect GAR by ion-exchange chromatography, showed that ad_4 and ad_5 have a C^{14}-glycine-labeled peak in the GAR region (Figs 22 and 23). However, this peak was present also in all of the other adenine mutants tested (ad_6, ad_7, ad_8 and ad_9) as well as in the wild type (Figs 19, 20, 24, 25 and 26).

Although there is some indication that small amounts of GAR are eluted in this region using an ad_4 extract (Mazlen, 1968), no feedback inhibition of the accumulation of C^{14}-glycine-labeled material could be demonstrated in either ad_4 or ad_5. Since the wildtype, which should accumulate the purine pathway intermediate, also has a C^{14}-peak in this region, one may conclude that at least part of the material elated in the GAR region is not an intermediate of the purine pathway. It is possible that the presence of this C^{14}-glycine-labeled compound of unknown identity masks any inhibiting effect that adenine may have.

Although no demonstration of GAR was achieved by the chromatographic procedures, the presence of GAR was assayed in crude extracts of ad_4, ad_5 and the double mutant ad_4ad_5, by the modified procedure of Nierlich and Magasanik (1961). Extracts of mutants ad_8 and ad_9, which are not expected to accumulate GAR (see Chapt V) were used as controls. The results are presented in Table 7. Confirmation of the formation of AICAR, expected from the reaction when GAR is present, was obtained by examination of absorption spectra of the Bratton-Marshall reaction products (Fig 41). These results show that ad_4 does indeed accumulate GAR, and suggests that this mutant is blocked at step IV. No GAR accumulates in ad_5, ad_8 or ad_9. The fact that ad_5 does not accumulate GAR suggests that it is blocked before ad_4. Surprisingly, however,

Fig. 41. Absorption spectra of Bratton-Marshall chromophores formed in the GAR assays: crude extracts of ad_4 and ad_4ad_5.

GAR was assayed by the procedure of Nierlich and Magasanik. The curves represent the difference between the 45-minute reaction vessel and the zero-time control.



1.0
0.9
0.8
0.7
0.6
0.5
ABSORBANCE
ad$_4$
ad$_4$ ad$_5$
500
520
540
560
WAVELENGTH IN mμ

TABLE 7

Enzymatic determination of GAR in crude extracts of various adenine mutants.

	Mutant:				
	ad_4	ad_5	ad_8	ad_9	ad_4ad_5
ΔO.D. at 540 mμ[a]	0.906	0.018	0.065	(-).038	0.764
ΔO.D./gram[b]	0.248	0.003	0.016	(-).013	0.168

GAR was assayed by the procedure of Nierlich and Magasanik.

a. ΔO.D. represents the difference in the optical density between the 45 minute and zero time samples.

b. ΔO.D./gram is the optical density of each sample standardized to one gram (wet weight) of cells.

GAR was present in the extract of the double mutant ad_4ad_5. The basis for the accumulation of GAR by the double mutant is not clear. One possible explanation for this is that the ad_5 marker in the double mutant is leaky. This would allow the accumulation of some GAR. It should be noted that the amount of GAR in the double mutant is approximately half that found in ad_4 (Table 7). This result might be expected if ad_5 were only partially blocked. Since the ad_5 parent used to construct this particular ad_4ad_5 double mutant was not tested, no definite conclusion can be drawn with respect to the location of the block in ad_5.

CHAPTER V

Functional Blocks of AD_8 and AD_9

The approaches used in characterizing the accumulating products of the mutants discussed in the preceding chapters were applied as well to the study of the adenine mutants ad_8 and ad_9. Unlike the outer adenine mutants tested however, ad_8 and ad_9 incorporate only small amounts of C^{14}-glycine into compounds which can be resolved by TLC (Fig 42). This behavior is consistant with the tentative assignment of ad_8 and ad_9 to steps in the pathway preceding that at which glycine is incorporated (i.e. step III, Fig 1).

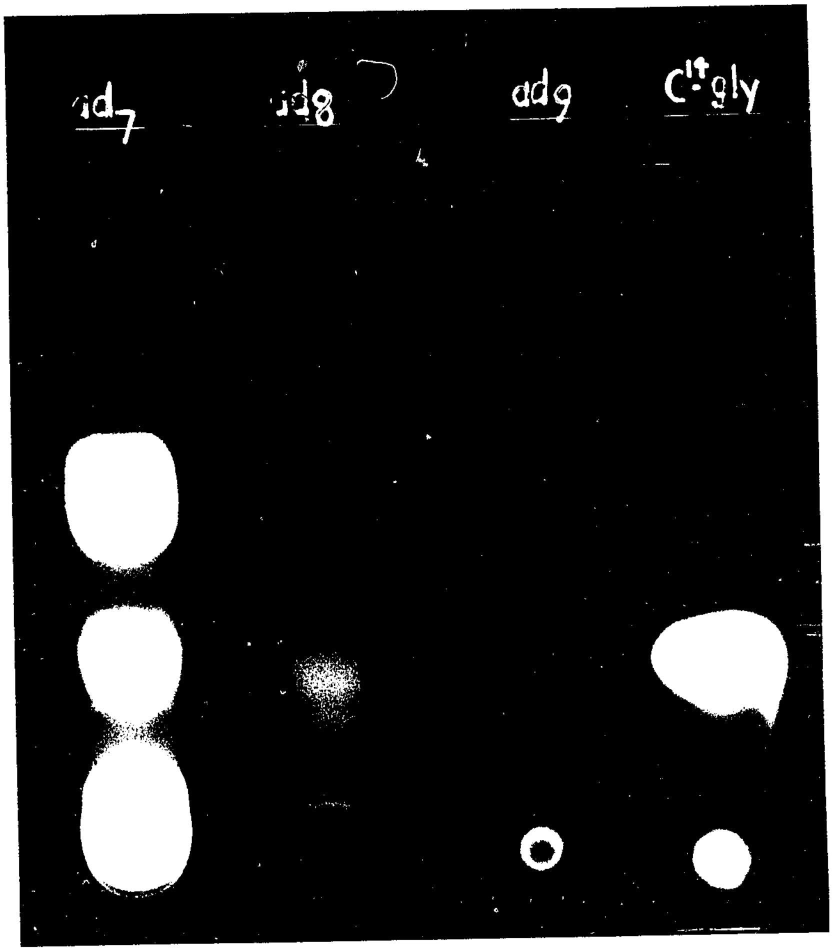
The lack of significant incorporation of C^{14}-glycine by ad_8 and ad_9 precluded their testing for feedback inhibition.

Additional evidence that ad_8 is blocked at a relatively early step in the pathway is supplied by the fact that the introduction of an ad_8 marker into the ad_4, ad_6 or an ad_7 strain, results in a reduction in the accumulation of C^{14}-glycine-labeled intermediates characteristic of the latter strains (Figs 12, 13 and 38). The position of the ad_8 mutation with respect to ad_5 and ad_9 however, could not be ascertained.

Studies of double mutants carrying an ad_9 marker are complicated by the "leaky" nature of this mutant. In liquid SC medium, no growth of this mutant is observed until approximately 50 hours, at which time the culture enters log phase (Fig 43). Stationary phase is not reached until approximately 100 hours. Extracts from stationary phase cultures give characteristic ad_9 TLC patterns, i.e., there is little or no accumulation of C^{14}-glycine-labeled material. However, it should

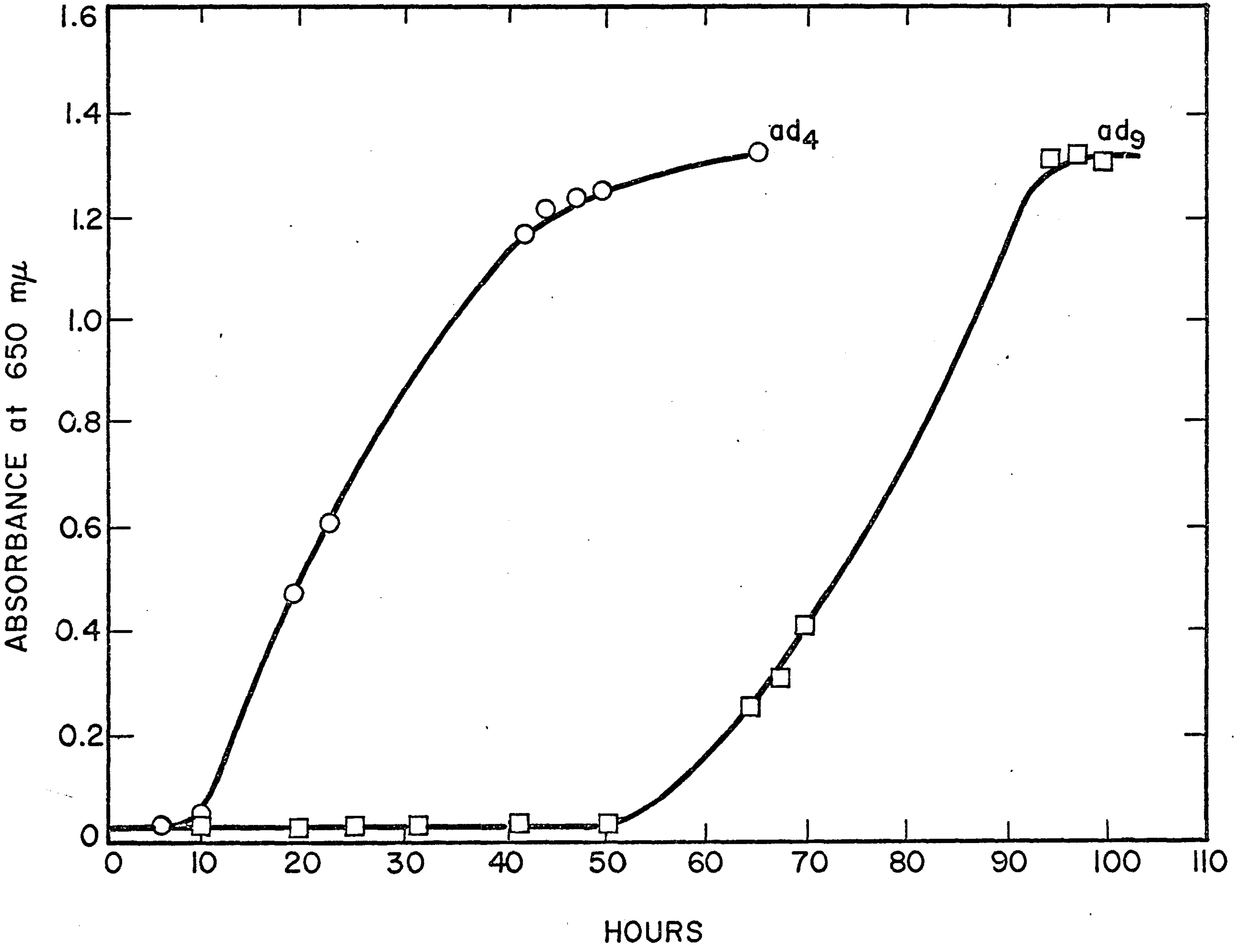
Fig. 42. TLC-comparison of extracts from C^{14}-glycine-labeled ad_7, ad_8 and ad_9.

Solvent: PAW.



ad7
ad8
ad9
C14 gly

Fig. 43. Growth curves of ad_4 and ad_9. The cells were grown in SC medium with shaking at 30°C.


ABSORBANCE at 650 mμ
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
80
90
100
110
HOURS
ad4
ad9

be noted that at this time the culture contains large numbers of adenine independent revertants, and after 72 hours on adenine-less agar plates, the entire population appears to lose its adenine requirement. Because of this behavior, the loss of the effect of the ad_9 marker from a double mutant can not be strictly controlled, and in most cases the double mutant pattern obtained from chromatographs appeared similar to the pattern of the other adenine marker. In the ad_6ad_9 double mutant, however, (Fig 14), the complete loss of the characteristic ad_6 spots was observed. Although the basis of the adenine requirement in ad_9 is not well understood, since this mutant incorporates glycine poorly it is considered to be blocked before step III (Fig 1).

Double mutant studies indicated that ad_8 and ad_9 contain relatively early blocks. Furthermore, when tested on Dowex-1-formate columns, no FGAR could be detected (Fig 24 and 25). In addition, in contrast to the ad_4 mutant, the presence of GAR could not be demonstrated in extracts of ad_8 and ad_9 (see page 116, Chapt IV). This supports the view that these mutants are blocked early in the pathway (i.e. before step III).

CHAPTER VI

Functional Blocks of AD_1 and AD_2

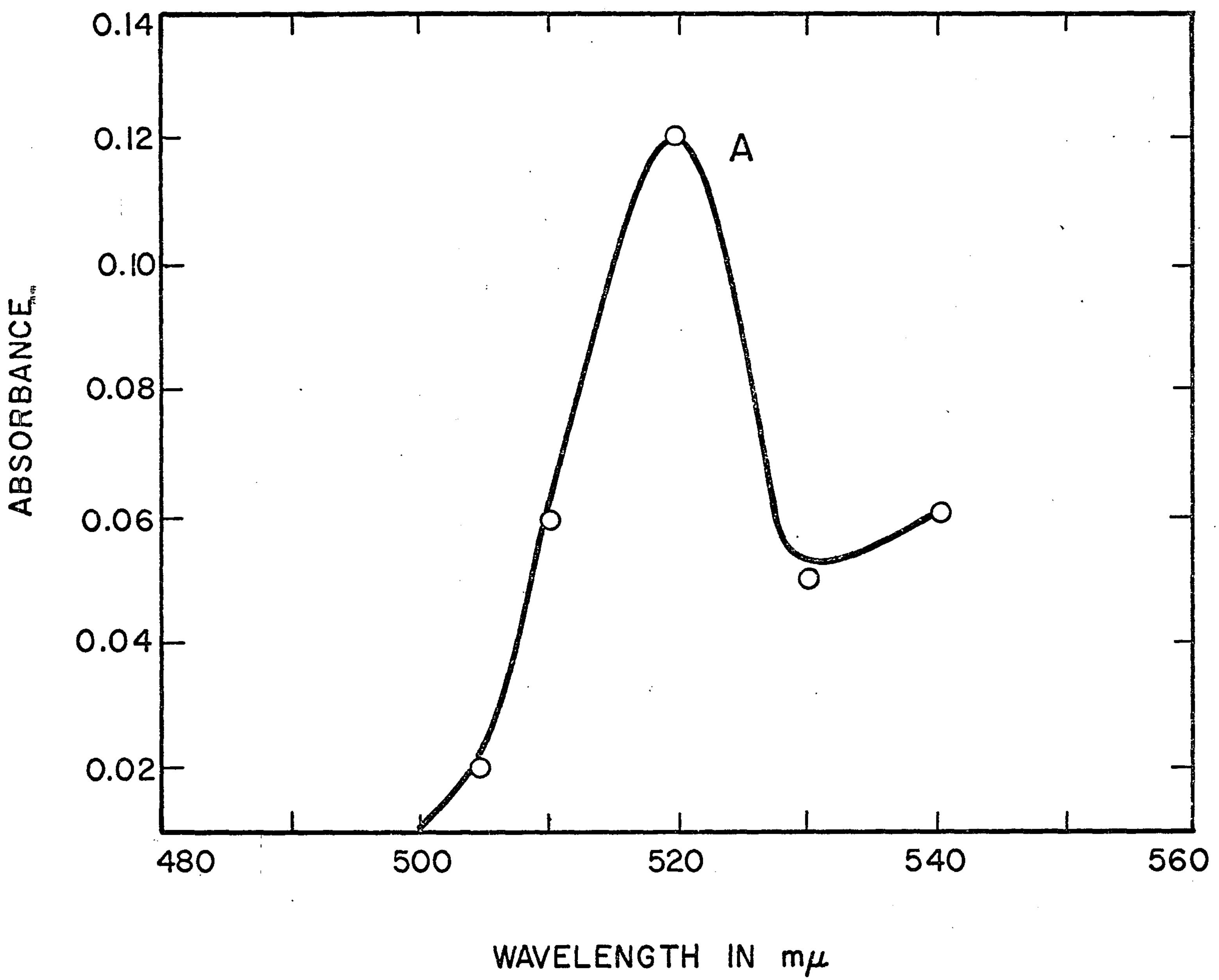
The assignment of the biosynthetic steps to which the adenine mutants ad_1 and ad_2 correspond, has not yet been confirmed. Reports to date have depended upon the identification of AIR and CAIR, which yield Bratton-Marshall chromophores with absorption maxima of 500 and 520 mμ respectively (Lukens and Buchanan, 1959), as the intermediates accumulated by these mutants.

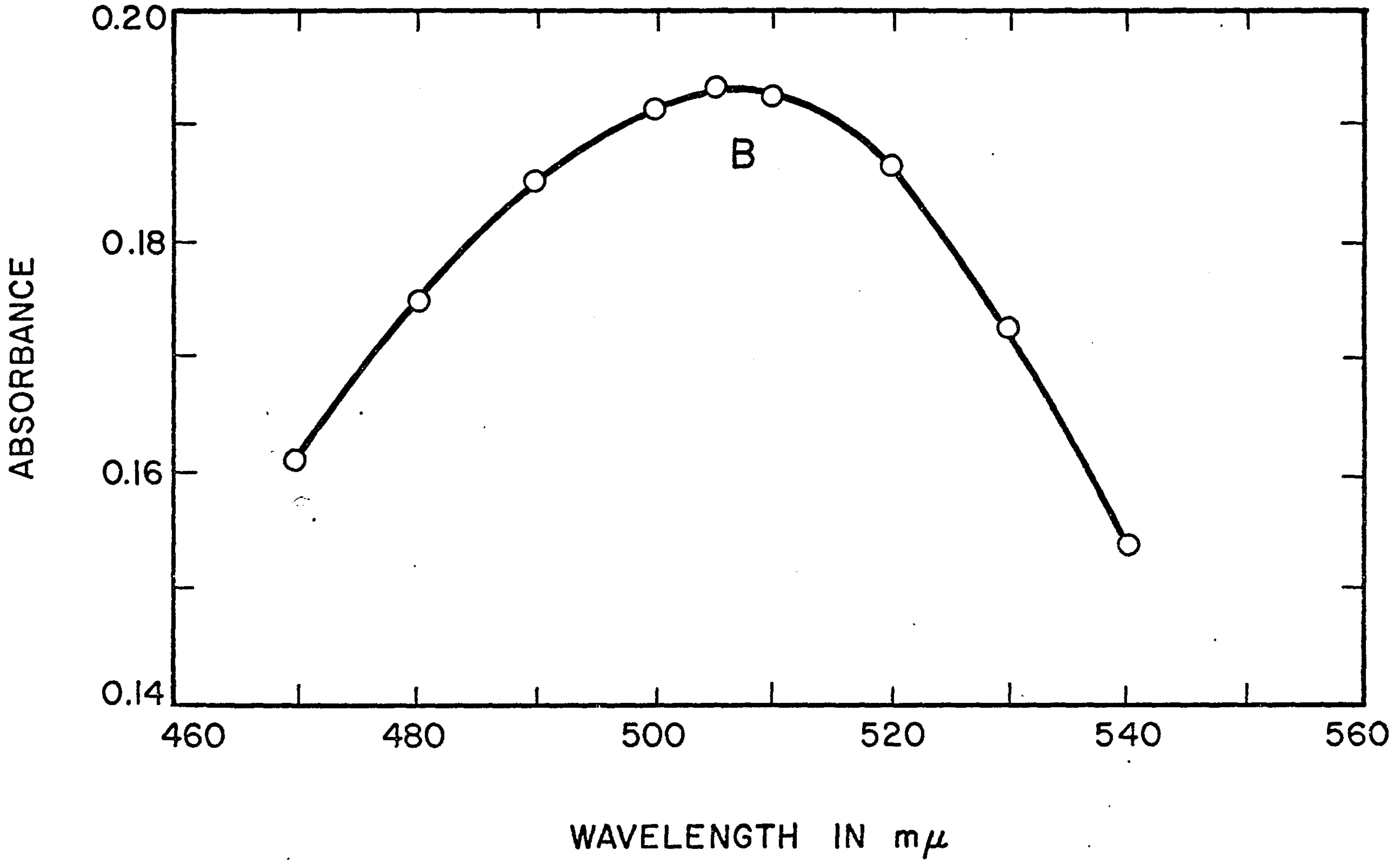
Considerable difficulty in the study of these two mutants arises from the fact that both may accumulate AIR as a result of a block either at step VIII or at step VII (Fig 1), and AIR may also be formed by the breakdown of CAIR, which is reported to be labile (Lukens and Buchanan, 1959). In addition, in the presence of oxygen AIR will polymerize to form a dark red pigment, which can interefere both with the spectrophotometric analysis and with the chromatographic resolution of components of cell extracts. Smirnov etal (1967), for example, report that the pigment in ad_1 has absorption maxima at 490 and 540 mμ and a Bratton-Marshall reaction product which gives an absorption maximum at 515 mμ. Further difficulty may result from a lack of strict control of the pH at which the Bratton-Marshall test is carried out. Lukens and Buchanan (1959) reported that the Bratton-Marshall chromophores of AIR and CAIR have absorption maxima at 500 and 520 mμ respectively at pH 1.4. At a more acid pH however, the absorption maximum of AIR may shift to wavelengths even larger than that of CAIR.

It has been reported by Dorfman (1964), that no imidazoles were detected in ad_1 and ad_2 when these mutants were grown anaerobically. In this laboratory, however, the same pattern of Pauly positive spots was obtained in chromatographed extracts from cells grown aerobically or anaerobically, in either YEP or SC medium. The only difference found was in the color of the initial extracts, all of which became an equivalent deep red color when stored under aerobic conditions. This dependence on oxygen for the polymerization of AIR has also been reported by Smirnov etal (1967).

It was considered desirable to reinvestigate the nature of the ad_1 and ad_2 mutational blocks, using conditions which avoid some of the above difficulties. Extracts of ad_1 and ad_2, which were to be analyzed by absorption spectra of the Bratton-Marshall reaction products, were therefore made from cells grown anaerobically, since less interfering pigment is formed under these conditions, and all Bratton-Marshall reactions were carried out at a pH of 1.4. The results are shown in Fig 44. The absorption maximum of the Bratton-Marshall chromophore of ad_2 extracts is close to the range of 500-502 mμ reported for AIR (Bernstein, 1961), whereas that of the ad_1 extract is identical to that reported for CAIR (520 mμ; Lukens and Buchanan, 1959). To ensure the complete removal of possible interfering pigment, the extract of ad_2 was subjected to gel filtration on a Sephadex G-25 column. The imidazoles eluted in fractions well separated from the pigment yielded a Bratton-Marshall reaction product which had an absorption maximum of 505 mμ, thus confirming that ad_2 accumulates AIR. These data indicate that ad_2 is blocked at the conversion of AIR to CAIR and that ad_1 is blocked at the conversion of CAIR to

Fig. 44. Absorption spectra of Bratton-Marshall chromophores in extracts of ad_1 and ad_2.
(A) is the absorption spectrum of the Bratton-Marshall chromophore in ad_1.
(B) is the absorption spectrum of the Bratton-Marshall chromophore in ad_2.


0.14
0.12
0.10
0.08
0.06
0.04
0.02
ABSORBANCE
A
480
500
520
540
560
WAVELENGTH IN mμ


0.20
0.18
0.16
0.14
ABSORBANCE
B
460
480
500
520
540
560
WAVELENGTH IN mμ

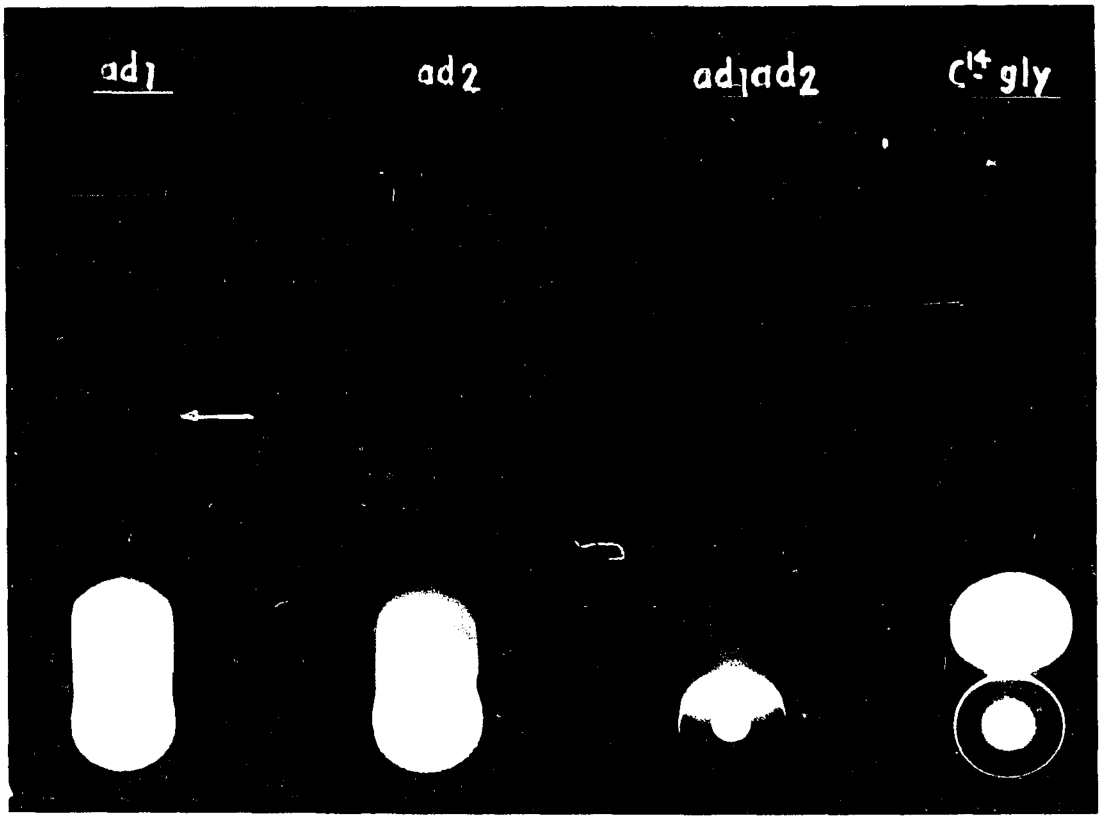
SAICAR. A similar conclusion was tentatively reached by Dorfman (1964), who found that acidification of an ad_1 extract caused the absorption maximum of the Bratton-Marshall derivative of this mutant to approach the absorption maximum characteristic of AIR, while that of the ad_2 derivative remained the same. This behavior of the material in the ad_1 extract is consistant with that expected of CAIR which is decarboxylated by acid to AIR (Lukens and Buchanan, 1959).

Further support for the idea that the block of ad_2 precedes that of ad_1 is obtained from the studies of the ad_1ad_2 double mutant. The introduction of an ad_2 marker into an ad_1 strain results in the elimination of a C^{14}-glycine-labeled, Pauly positive compound which is characteristic of the ad_1 strain (Fig 45). These and the foregoing data suggest that ad_2 is blocked at step VII, and ad_1 at step VIII.

Fig. 45. TLC-comparison of extracts of C^{14}-glycine-labeled ad_1, ad_2 and ad_1ad_2.

The arrow indicates the location of a C^{14}-glycine-labeled imidazole characteristic of ad_1.

Solvent: BAW.



ad1
ad2
ad1ad2
C14 gly

CHAPTER VII

DISCUSSION

The purine-requiring mutants discussed in the foregoing chapters were studied in order to permit their assignment to the respective steps in the pathway at which they are blocked. The purine pathway described for avian liver was used in this study as a point of reference, although there is no *a priori* reason for assuming that the pathway in *Saccharomyces* is exactly the same.

The possible approaches which might be applied to a study of this nature were limited by several factors. Since the yeast cells appear to be impermeable to exogenously supplied purine intermediates (Dorfman, 1964a) classical feeding experiments were not used. Furthermore, *in vitro* assays of the enzymes involved in the *de novo* synthesis of purines in *Saccharomyces* have been largely unsuccessful. Presumptive ordering of the mutants was made possible by using an approach which involved characterizing the purine precursors accumulating in the strains. It should be emphasized that the results of these studies lead to the localization of a functional block in the operation of the pathway, but can provide no evidence as to the nature of the defect. However it does provide a basis for the further examination of the proteins involved.

The adenine pathway in *Saccharomyces* has been the subject of numerous genetic analyses. For example, Roman (1956), Costello and Beven (1964) and Dorfman 1964a, 1964b) studied the ad_5 and ad_7 loci. Since these two loci are very closely linked and yield spontaneous mutations which fail to complement either ad_5 or ad_7 (Roman, 1956), the possibility was suggested that these mutants control the same enzymatic activity

(Costello and Bevan, 1964). The results of studies on meiotic recombination and complementation, however, led to the suggestion that ad_5 and ad_7 were genetically and functionally discrete and the observation that the spontaneously arising $\bar{ad}_5\bar{ad}_7$ double mutants were point mutations mapping in the ad_5 region (Dorfman, 1964b). It was deduced that the ad_5 and ad_7 cistrons were read as a unit and that these point mutations were frame-shift or nonsense mutations which simultaneously affected both cistrons. Since mutants containing a block affecting the same step in the pathway should accumulate the same intermediates, the absence in ad_5 of the formylated intermediates found in ad_7 supports the view that these loci are functionally discrete. The verification by biochemical methods of a model predicted from genetic studies supports the usefulness of such studies in attaining information on the functional relationship of genetic loci.

The location of the biosynthetic block of ad_5 could not be precisely determined. Based on the ability of ad_5 to accumulate heavily labeled materials derived from C^{14}-glycine and on the absence of FGAR, initially this mutant was tentatively placed at a step in the pathway after the incorporation of glycine but before that of formate. However, although the introduction of an ad_8 marker into an ad_4, ad_6 or ad_7 strain caused the alteration in the pattern of glycine incorporation of the latter strains a similar approach with ad_5 gave no marked reduction in the accumulation of C^{14}-glycine-labeled materials by the ad_5 mutant. Furthermore no data could be obtained on feedback inhibition of their accumulation. It therefore seems most likely that the materials derived from labeled glycine accumulated by the ad_5 mutant are not intermediates of adenine biosythesis.

Although no GAR was detected in extracts of ad_5, GAR was present in the extracts of both ad_4 and the double mutant ad_4ad_5. The basis for the accumulation of GAR by the double mutant has two possible explanations. If the ad_5 marker in the double mutant were leaky, even if the block were earlier than that in ad_4 some GAR might accumulate. This possibility is supported by the fact that the amount of GAR in the double mutant is approximately half that found in ad_4 (Table 7). It is also conceivable that the presence of GAR in the double mutant results from a block in ad_5 located after that of ad_4. If the pathways in pigeon liver and Saccharomyces are the same this explanation is unlikely, since there is no reported step in the pathway in pigeon liver which would correspond to such a mutant. On the other hand the possibility exists that the pathways are not identical and that there is an additional step in the pathway of adenine biosynthesis in Saccharomyces.

Since ad_8 and ad_9 accumulate only small amounts of materials derived from C^{14}-glycine these mutants were tentatively assigned to steps in the pathway preceding that at which glycine is incorporated. Additional evidence that ad_8 contains an early block is provided by the fact that the introduction of an ad_8 marker into an ad_4, ad_6 or ad_7 strain prevents the accumulation of characteristic intermediates in extracts of the latter strains.

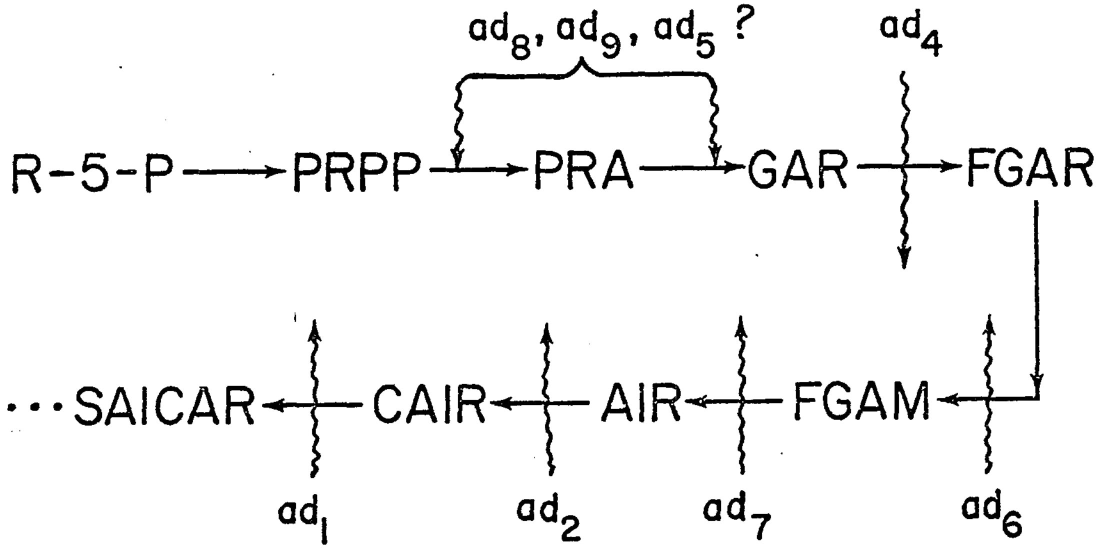
Studies of double mutants containing an ad_9 marker can not be precisely controlled. Ad_9 has a 50 hour lag phase when grown in liquid S.C. Stationary phase is not reached until approximately 100 hours, at which time the culture contains large numbers of adenine independent cells.

In addition the adenine requirement of ad_9 appears to be removed after 72 hours on adenine-less agar. Because of this behavior the effect of the ad_9 marker in the double mutant may be lost. It was possible, however, to demonstrate that the ad_9 marker prevented the accumulation of intermediates characteristic of ad_6 when ad_9 was present in an ad_6ad_9 double mutant. This is consistant with the evidence suggesting an early block in ad_9.

The localization of the blocks of ad_8, ad_9 and possibly that of ad_5 to steps before the formation of GAR, implies that one mutant should be unable to form PRPP, another should be unable to convert PRPP to PRA and the third should be blocked at the formation of GAR. Phophoribosylpyrophosphate, however, is required for the biosynthesis of histidine, tryptophan and uridine as well as adenine. Since these mutants do not have an absolute requirement for these nutrients it seems unlikely that they can not carry out the conversion of ribose-5-phosphate to PRPP. This leaves only two steps to which three mutants might be assigned, again suggesting the possibility of an additional step in the purine pathway of yeast. On the other hand, the peculiar growth characteristics of ad_9 suggests that the defective locus in this mutant may not directly control either of the two steps. If, however, ad_9 does represent a primary block, it's behavior might be explained by a faulty PRPP-amidotransferase which competes ineffectively for PRPP with enzymes of other pathways. The adenine requirement of ad_9 might be removed as stationary phase is approached, either as substrates of competing reactions become limiting, or alternatively, as accumulating end-products exert feedback inhibition on competing reactions. Phosphoribosylpyrophosphate thus would become available for purine biosynthesis and the requirement for adenine would be removed. Competition of this nature for PRPP has been cited by Rajalakshmi and Handschumacher (1968). The validity of this model might be determined by a test of sparing effects on the adenine requirement in response to end-products of known competing reactions.

The approaches used in this study were most successful in identifying mutational blocks after glycine incorporation. Comparisons of intermediates accumulating in single and double mutants enabled the assignment of the relative positions of the blocks in ad_1, ad_2, ad_4, ad_6 and ad_7. In addition, the presence of CAIR in extracts of ad_1 and AIR in extracts of ad_2 was demonstrated by spectrophotometric analyses. The expected presence of FGAR in extracts of ad_6 and ad_7 and GAR in extracts of ad_4, was subsequently confirmed by chromatographic and enzymatic procedures. A summary of these results appears in Fig 46.

Fig. 46. Summary of gene-enzyme relationships of eight non-allelic adenine-requiring mutants of *Saccharomyces cerevisiae*.


ad8, ad9, ad5 ?
ad4
R-5-P
PRPP
PRA
GAR
FGAR
···SAICAR
CAIR
AIR
FGAM
ad1
ad2
ad7
ad6

CHAPTER VIII

SUMMARY

The biosynthetic pathway of adenine in Saccharomyces cerevisiae has been studied with the aid of eight non-allelic adenine-requiring mutants arbitrarily numbered ad_1, ad_2, ad_4, ad_5, ad_6, ad_7, ad_8 and ad_9.

The presumptive ordering of these mutants along the pathway has been made by comparisons of substances derived from C^{14}-glycine which accumulate in extracts of the mutants, as detected by thin layer chromatography.

The introduction of an ad_4, ad_5, ad_8 or ad_9 marker into either an ad_6 or ad_7 strain resulted in the loss of the characteristic accumulations of the latter two mutants, indicating that ad_6 and ad_7 were blocked relatively late in the pathway with respect to the above mutants. Since the chromatographic pattern of the ad_6ad_7 double mutant resembled that of the ad_6 parent, it was concluded that ad_6 is blocked earlier than ad_7. Using a similar approach, ad_8 was referred to a step in the pathway earlier than ad_4. The exact location of the ad_5, ad_8 and ad_9 blocks with respect to one another, could not be determined by this method.

The ad_6 and ad_7 mutants do not accumulate any imidazoles not found in the wildtype. In addition, these strains are blocked relatively late with respect to all other mutants tested except ad_1 and ad_2. This suggested that ad_6 and ad_7 are blocked at steps immediately preceding ring closure and should therefore accumulate FGAR or FGAM. Extracts of the mutants were tested for the presence of these formylated intermediates and the presence of FGAR in both ad_6 and ad_7 confirmed by chromatographic and enzymatic studies. Since ad_7 was shown to be blocked at a step later than ad_6, the ad_7 block was placed at step VI (Fig 1) and the ad_6 block at step V.

Double mutant studies suggested that ad_4, ad_5, ad_8 and ad_9 were blocked at or before step IV of the pathway. Tests for the presence of formylated purine precursors in these mutants were negative, confirming the results obtained from double mutant studies.

Based on their ability to accumulate C^{14}-glycine-labeled materials and on the absence of FGAR, the mutants ad_4 and ad_5 were tentatively assigned to a step in the pathway after the incorporation of glycine but before the incorporation of formate, although there is only one reported step (step IV) which would correspond to such criteria (Buchanan, 1960). However, only ad_4 was found to accumulate GAR, confirming that this mutant is indeed blocked at step IV. The presence of GAR could not be demonstrated in extracts of ad_5. But, some GAR was found in extracts of the ad_4ad_5 double mutant. Because of these conflicting observations it was not possible to localize the ad_5 block.

Since crude extracts of ad_8 and ad_9 show comparatively little accumulation of C^{14}-glycine-labeled materials, these mutants were tentatively assigned to steps in the pathway preceding that at which glycine is incorporated (i.e. prior to step III, fig 1). Enzymatic assays confirmed the absence of GAR in extracts of these mutants. Although ad_8 and ad_9 are blocked early in the pathway, it is unlikely that these mutants can not carry out the conversion of ribose-5-phosphate to PRPP, since this compound is also required for the synthesis of histidine, tryptophan and uridine which are not required by these mutants for growth.

Of all the mutants studied only ad_1 and ad_2 accumulate Pauly positive compounds not found in the wildtype. Spectrophotometric analyses of Bratton-Marshall reaction products from extracts of anaerobically grown cells indicated that ad_2 was blocked at step VII and ad_1 at step VIII. Further evidence that ad_2 is blocked before ad_1 is provided by the observation that the introduction of an ad_2 marker into an ad_1 strain results in the elimination of a Pauly positive spot characteristic of the ad_1 mutant.

The data presented in this study lead to the assignment of the gene-enzyme relationships which are summarized in Fig 46.

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