NUCLEIC ACID METABOLISM IN YEAST: (1) STUDIES ON TRANSFORMATION OF THE ADENINE LOCUS BY CELL EXTRACTS; (2) STUDIES ON ADENOSINE DIPHOSPHATE SULFURYLASE

Thesis for the Degree of M. S.

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ABSTRACT

NUCLEIC ACID METABOLISM IN YEAST: (1) STUDIES ON TRANSFORMATION OF THE ADENINE LOCUS BY CELL EXTRACTS; (2) STUDIES ON ADENOSINE DIPHOSPHATE SULFURYLASE.

By Stuart William Bradford

I. Experiments were performed which were designed to determine if extracts of a yeast mutant could be used to alter the hereditary characteristics of the prototroph yeast. Cell free extracts of an adenine independent, red Saccharomyces were used to treat the adenine dependent, red prototroph cells. Such a treatment resulted in an increase in the number of reversions to white, adenine independent cells. The results, although preliminary, suggest that the mutants at the adenine locus in Saccharomyces provide a system which can be conveniently utilized in further studies concerned with establishing the existence of yeast transformations. The alteration of heretable characteristics of bacteria by essentially pure deoxyribonucleic acid (1) is well known and the mechanisms involved have been studied. Transformations have not, however, been conclusively demonstrated in other organisms. Yeasts are genetically among the most thoroughly investigated microorganisms and studies on the feasibility of yeast transformations are, consequently, of particular interest.

II. The phosphorolysis of adenosine-5'-phosphosulfate (APS) by inorganic phosphate to form adenosine diphosphate and sulfate, which is catalyzed by the enzyme ADP sulfurylase, has been reported (2). Measurement of this reaction in the reverse direction was investigated by substitution of molybdate ion, an ion which has been shown to act as an analog for sulfate in reactions catalyzed by adenosine triphosphate sulfurylase (3), for sulfate. The back reaction, which is in the direction of APS formation, could serve as an alternative to the adenosine triphosphate

sulfurylase catalyzed reaction, as a means of APS synthesis. With molybdate ion the reaction could not be demonstrated to proceed in the direction of APS synthesis, indicating that either the reaction has such a high equilibrium constant as to be effectively irreversible or that in the ADP sulfurylase reaction molybdate ion cannot substitute for sulfate.

A divalent zinc activated inorganic pyrophosphatase was observed in yeast extracts. This enzyme may be identical to the zinc activated yeast pyrophosphatase recently reported on (4). Earlier reports on yeast pyrophosphatase had implicated only magnesium ion as an effective activator.

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STUART WILLIAM BRADFORD

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TABLE OF CONTENTS

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PART I: STUDIES ON TRANSFORMATION OF THE ADENINE LOCUS BY CELL EXTRACTS

1. INTRODUCTION AND REVIEW OF LITERATURE

Genetic transformation by cell free extracts has only been demonstrated to occur in bacteria; nevertheless, it seems reasonable to believe that it may be possible to extend it to other microorganisms. In this thesis the mutants at the adenine locus in the yeast genus Saccharomyces were utilized to study the possibility of transformation of yeasts. Extension of the transformation phenomenon to include yeasts would provide a genetic operation of great value. Yeasts possess a number of characteristics which are advantageous for fundamental genetic studies. Many genera grow equally well as haploids or diploids, single spores can be isolated and hybrids rapidly produced, and finally tetrad analysis can be performed.

A. Genetic Transformation in Bacteria

Bacterial transformations may be simply represented as:

$$DNA^{1}\text{-}X + \text{bacterium-}Y \longrightarrow \text{bacterium-}X$$

where bacterium-X possesses heritable characteristics not possessed by Y, and DNA-X is DNA extracted from cells of bacteria-X. In general, transformations are reciprocal and

$$DNA\text{-}Y + \text{bacterium-}X \longrightarrow \text{bacterium-}Y$$

may occur. This phenomenon, whose dependency on DNA was discovered by Avery, MacLeod, and McCarty for Diplococcus pneumoniae in 1944 (1), has since been extended to a number of other species of bacteria. Transformations have been reported in Neisseria meingitidis (2), Hemophilus influenzae (3),

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[1]The following abbreviations are used: RNA, polyribonucleic acid; DNA, polydeoxyribonucleic acid; Tris, tris (hydroxylmethyl) aminomethane; TCA, trichloroacetic acid.

Xanthomonas phaseoli (4), Salmonella typhemurim (5), Rhizobium meliloti (6), and Bacillus subtilis (7). The reported transformations encompass a variety of characteristics: antigenic specificity, drug resistance, cellular and colonial morphology, and the ability to utilize particular nutrients. In addition, transformations between bacteria regarded as separate species have been reported (8,9). Although the work of Avery and his collaborators was performed with highly purified DNA, in work by subsequent experimenters transformations have often been performed with cruder preparations (4,5,7) but in all cases the ability to initiate transformation was reported to be DNase sensitive.

Studies on transformation have been pursued most thoroughly in Hemophilus influenzae and Pneumococcus. In Pneumococcus transformations do not occur unless bovine serum albumin and calcium ion are present (1). The exact role that these two factors play is unknown, but it is very likely a membrane permeability effect.

In H. influenzae transformations can take place in simple saline solution (10). In other species the conditions which might be necessary have not been established. For example, in Xanthomonas phaseoli, in which transformations were effected with a crude extract, removal of protein from the active nucleic acid preparation increased the rate of transformation 10--100 fold, and addition of 0.01% commercial yeast extract increased the rate as much as 10,000 times (4)!

The stability of H. influenzae transforming DNA under a variety of physical and chemical conditions has been investigated (11). No loss of activity was reported to occur after heating for one hour at 81° in citrate buffer or from treatment of the DNA with solutions of pH 5.0 to 10.5, but about 80% of the activity was lost in a solution of pH 3.5 or in a solution of pH 11.0. DNA solutions in distilled water were quickly

inactivated and highly purified preparations lost activity when vacuum dried. Litt (12) has shown in Pneumococcus that mechanical breakage of DNA molecules diminishes the number of transformations obtained. Instability on freezing and thawing has been reported for a crude preparation of transforming DNA (4). Marmur and Doty (13), working with Diplococcus pneumoniae, have concluded that the double helix of DNA is necessary for transforming ability. In heated solutions of DNA the strands of the helix separate and transforming activity is lost, while upon slow cooling the strands reunite and regain the ability to transform. Hybrid helices, which are reconstituted between transforming and non-transforming DNA, also possess transforming activity.

It is now well established that a single DNA molecule is sufficient to effect a transformation, and, therefore, there is a linear relationship between the concentration of transforming DNA to which the cells are exposed and the number of transformed cells obtained (14). Further studies have demonstrated that a single DNA molecule may transform more than one characteristic of a cell. This is illustrated by the mannitol utilization (M) and streptomycin resistance (S) mutations in Pneumococcus (15). DNA extracted from the double mutant (presumably MS - DNA), transforms a much higher proportion of wild type cells into MS-cells than are transformed by a mixture of DNA obtained from the single mutants (M-DNA plus S-DNA).

The mechanisms involved in the genetic conversion of cells by DNA have received considerable study and it is now known that transformation involves a series of steps. First, the bacterium must be physiologically competent to undergo transformation. This receptive state, although occurring at random in normal growing cultures, may be made to appear cyclically by brief exposure of the culture to a low temperature (16).

Fox and Hotchkiss (17) have shown that the development of competence consists in the appearance of sites with which the transforming DNA combines reversibly. Populations of competent cells were frozen in 10% glycerol. Under these conditions the cells retained their competence and could be thawed, treated with DNA under a variety of conditions and the reaction terminated by DNase. At low concentration of DNA the response obtained was linear, but at higher concentration DNA became saturating and the number of transformed cells obtained did not increase. Such a relationship can be treated according to conventional enzyme kinetics:

$$B_{(s)} + DNA \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B_{(s)} + \text{DNA complex} \xrightarrow{k_3} B_{(tr)}$$

where $B_{(s)}$ represents bacterial sites, and $B_{(tr)}$ represents transformed bacteria. Hotchkiss' and Fox's calculations indicate between 33 and 75 sites per cell. The actual nature of the sites is unknown; however, several observations suggest that enzymatic processes are involved: their formation is stimulated by an apparent protein synthesizing system (17); the absorption of DNA onto the sites is a temperature dependent reaction (18), and they react with some DNA more readily than others, thus exhibiting a specificity (19). In addition, Schaeffer (9) has shown that the sites must be on the surface of the bacterium.

Following adsorption onto a site, the transforming DNA is retained in a DNase resistant form, presumably representing penetration into the cell. The use of P^{32} labeled DNA has established that the number of transformed cells obtained is directly related to the amount of DNA which is permanently bound by the cells (19). Non-transforming DNA can also be taken up and Lerman and Tolmach (18) have shown in Pneumococcus that E. coli DNA will accumulate to the same extent as the genetically active DNA extracted from Pneumococcus itself. However, when several species of

bacteria which have not been transformed were exposed to Pneumococcus DNA, no uptake of DNA occurred. In view of this, the authors suggested that measurement of the incorporation of labeled DNA into microorganisms might prove to be valuable in screening for those organisms in which transformation is feasible.

The actual amount of DNA taken up by receptive cells can be inhibited by addition of foreign DNA - non-transforming DNA from the same species, or even DNA from other species; but the degree of inhibition varies with the particular DNA employed. DNA from more closely related species inhibits more, while that from completely unrelated organisms, such as the DNA from calf thymus glands, is much less effective (20). The number of DNA molecules incorporated for each cell transformed, in the case of streptomycin resistance in Hemophilus, was found to be 120 (19). This, in view of the fact that one DNA molecule is believed to be sufficient to effect a transformation, strongly suggests that for Hemophilus one out of every 120 DNA molecules carries the gene for streptomycin resistance.

The occurrence of reciprocal transformations almost certainly establishes that transforming DNA is not merely carried along, but, on the contrary, becomes eventually incorporated into the "chromosomal" material of the recipient bacterium. This incorporation must logically be preceded by a pairing of the exogenous DNA with some complementary site on a chromosomal structure of the bacterium. The necessity for such specific pairing or synapsis is indicated by Schaeffer's (9) studies on transformation to streptomycin resistance performed between separate species of bacteria. Such interspecific transformations occur with a much lower frequency than those within a species, although there is no reduction in uptake of the transforming DNA. This situation may be interpreted as follows: in interspecific transformations structural differences around the streptomycin

region act as obstacles to pairing and thereby reduce the frequency of transformations, while in intraspecific transformations the donor DNA is structurally similar to the DNA of the recipient bacteria and pairing may occur more easily.

The final step in the series of events leading to a transformed cell is the incorporation (or copying) of the genetic factors, brought within the cell by the DNA molecule, into a chromosomal structure. Hotchkiss (21) has described a complex single locus which he designates as abd, conveying a high level of streptomycin resistance. A molecule of transforming DNA carrying the abd complement may transform a recipient cell to any one of the different characteristic low levels of resistance corresponding to the a, b, or c sites on the complex locus or less frequently to the characteristic higher levels corresponding to the ab, bd, or abd parts of the complex locus. However, if a is incorporated into the recipient cell then the bd sites are not given phenotypic expressions; similarly, if ab is incorporated into the recipient cell then d is not expressed. Therefore only a portion of DNA molecule is incorporated, or copied, by a cell undergoing transformation and the genetic markers on the remaining portion of the molecule appear to exert no influence. The incorporation of the transforming DNA apparently does not occur until after several cellular divisions, because transformed cells give rise, for the first few divisions, to both normal cells and transformed cells.

A few accounts have appeared of the altering of heritable characteristics in bacteria by exposure to suitable RNA preparations. Kramer and Straub (22) were able to induce the formation of penicillinase in strains of Bacillus cerus lacking the enzyme by extracts from a penicillinase producing strain. The time required for the formation of this induced enzyme was considerably reduced by treatment with RNase, suggesting that the active

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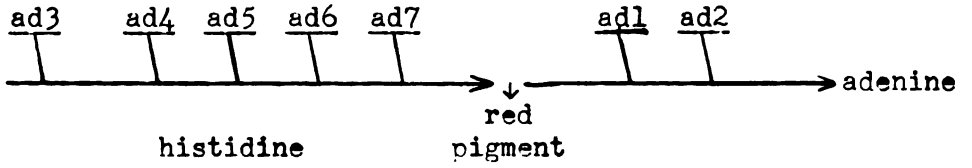
moieties were low molecular weight poly- or oligonucleotides. In a similar manner Reiner and Goodman (23) found that formation of the induced enzyme glucokinase in E. coli could be accelerated by the dialyzable fragments of RNase treated RNA from a strain of E. coli producing glucokinase. These RNA mediated "transformations" appear to bear little relationship to the DNA transformations, since the activity of the RNA is not lost upon depolymerization.

B. The Adenine Dependent Loci in Saccharomyces

Roman has studied mutations to adenine dependence in Saccharomyces (24) and describes recessive mutations at seven loci. At two of the loci the mutants form a red pigment when supplied with adenine, but the mutants at the remaining five loci, although adenine requiring, remain normal colored. These seven loci are shown below in relationship to the formation of red pigment and adenine (the mutant ad3 also requires histidine for growth):

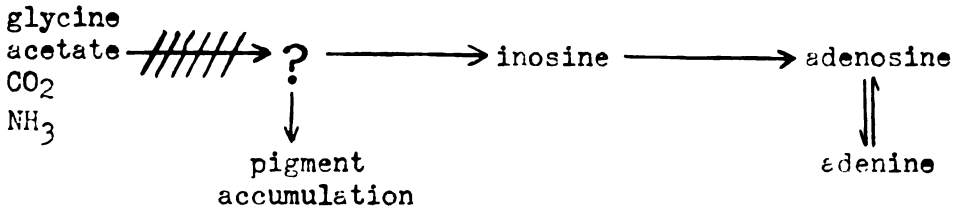


ad5 and ad7 are tightly linked, but at the remaining loci the genes exhibit regular Mendelian inheritance and segregate independently. ad3, ad4, ad5 and ad6 are leaky mutants; i.e., they allow some small amount of growth in the absence of adenine, and form pink colonies.

Evidence has been provided by Roman for the occurrence of crossing over during mitotic division in the diploid strains of the ad mutants. When two haploid strains, containing mutation of different origin but of the same locus, are crossed to provide diploids, reversions to adenine independence occurs with a frequency of .01% to .0001%. For example,

mutant strain ad2-1 crossed with mutant strain ad2-2 gave a small number of adenine independent reversions. If ad2-1 and ad2-2 represent different defective parts in the same locus then crossing over between these two points would restore the normal adenine independent gene. Analysis of the revertant diploid showed that crossing-over had occurred, although in a few cases non-reciprocal recombination was demonstrated. By use of this technique the various ad loci were found to each contain from 4 to 29 separable alleles. However, these represent minima and additional alleles will probably be disclosed as more mutants are studied.

Similar adenine requiring mutants occur in Neurospora and have been utilized to formulate some of the metabolic pathways involved in adenine synthesis:



in which the diagonal lines represent different mutants. The red pigment is a mixture of water soluble polymers related in structure to amino imidzacle carboxamide. This pigment or some of its precursors are slightly inhibitory to growth and cultures of the red mutant have been observed to become white but still adenine requiring owing to selection for a second mutant preventing the formation of the pigment (25). There is some question whether adenine dependent mutants always represent a defect in biosynthetic pathway leading to the production of adenine. Abram (26) has described a red adenine requiring mutant in Saccharomyces, which is capable of synthesizing adenine at a rate only slightly less than that exhibited in the wild type.

Red strains of Saccharomyces are known to be unstable and, as would be expected, the instability is most pronounced in the haploids (27).

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On solid medium haploid colonies produce white papillae, while in liquid medium the white variants rapidly outgrow their red progenitors. This selection is very much more rapid in shaken cultures than in standing cultures, with the exception of red respiratory mutants of the petite type in which the selection pressure is the same under both conditions. The cause for the selection remains unknown: very little selection occurs under anaerobic conditions and very little red pigment is produced under these conditions. Therefore, it is likely that either the pigment itself or some precursor has a deleterious effect on the growth of the red strains thus allowing selection for a mutant which inhibits pigment production (28). Srb (29) has also noted that there is a strong selection for the white prototroph when red strains are grown at a pH below 5.

C. Deoxyribonucleic Acid in Yeasts

In yeasts the amount of DNA is greatly exceeded by the amount of RNA. RNA composes about 95% of their total nucleic acid and consequently the extraction and isolation of yeast DNA poses special problems. Chargaff has given the only report of isolation of yeast DNA. He extracted with 1 N NaCl for 72 hours, and removed RNA by a combination of precipitation with calcium ion and dialysis with RNase (30).

A general review of methods for isolation of DNA appears in the volume edited by Chargaff and Davidson (31). In general, the isolation methods involve extraction with 1 N NaCl or with detergents. Extraction with cold 20% alcohol (32) and with the aid of cetyl trimethylammonium bromide (Cetavlon) have also been reported (33). Removal of protein is usually accomplished by shaking with a mixture of chloroform and octonal, but dialysis with chymotrypsin may be used and neither of these methods appears to degrade DNA (34). The removal of contaminating RNA is often

difficult, and although RNase can be used, it leaves an enzyme resistant polynucleotide core. Adsorption of DNA on charcoal (35) or precipitation of DNA by cetyl-trimethylammonium bromide has proved effective in some cases.

A yeast DNase has been described by Chargaff and Zamenhof (36). The enzyme is released only gradually from yeast extracts and its appearance is controlled by the following sequence of events: upon standing a proteinase is released from the extract which digests an inhibitor of the enzyme resulting in the release of active DNase.

2. MATERIALS AND METHODS

A. Yeast Strains and Culture Methods

A red, adenine requiring, diploid strain of Saccharomyces was supplied by Dr. Herschel L. Roman (Roman #C92-0-106). The white, adenine independent wild type was obtained as a back mutant from this strain. In this study the two strains are designated as ad(+) and ad(-), respectively. The ad(+) and ad(-) mutants may be considered to differ in only one gene since adenine dependence and red color result from a recessive mutation at a single locus (24).

Types of media. The following types of media were employed:

SC: A synthetic medium containing the following ingredients per liter of solution:

ammonium sulfate	1 g	thiamine-HCL	4000 μg
glucose	20 g	$FeCl_3 \cdot 6\ H_2O$	500 μg
histidine-HCL	10 mg	$ZnSO_4 \cdot 7H_2O$	700 μg
methionine	10 mg	H_3BO_3	100 μg
tryptophane	10 mg	$CuSO_4 \cdot 5H_2O$	100 μg
uracil	10 mg	KI	100 μg
adenine sulfate	10 mg	KH_2PO_4	875 mg
biotin	20 μg	$KHPO_4$	125 mg
inositol	20 mg	$MgSO_4 \cdot 7H_2O$	500 mg
calcium pantothenate	4000 μg	NaCl	100 mg
pyridoxine-HCL	4000 μg	$CaCl_2 \cdot 2H_2O$	100 mg

15 grams of agar were added to prepare solid media.

WAD: SC minus adenine.

C-30: A complex medium containing 2.5% glucose, 0.3% bactopeptone, 0.2% bacto-malt extract and the same concentrations of all the minerals used in SC medium with the exception that no NH_4SO_4 was added. 1.5% agar was added to prepare solid medium.

In all cases the media were autoclaved for 20 to 30 minutes, the exact time depending on the volume being sterilized. The pH before autoclaving of the SC medium was 6.0 and the pH for the C-30 medium was 5.8.

Maintenance of cultures. Cultures were maintained by weekly subculture on C-30 slants. Red ad(+) cells became visibly pigmented sooner on C-30 medium than on WAD medium but after 4 or 5 days both were equally dark red. White mutant colonies appeared frequently on ad(+) slants and in 2 or 3 weeks, upon solid medium; or in 3 or 4 days, in the case of populations transferred daily in liquid medium, completely outgrow their red progenitors. Therefore ad(+) slants were always inoculated with red colonies appearing from single cell isolates of the ad(+) strain.

B. Preparation of ad(-) Acetone Powder

The ad(-) cells were grown in C-30 medium in shaken Erlenmeyer flasks at 30°C. The cells were harvested by centrifugation, washed with pH 7.5 M/10 sodium citrate followed by distilled water. They were then dispersed in a few ml of distilled water, mixed well with 150 ml of -20° acetone in a high speed mixer and dried in a sterile tube under vacuum. From 600 ml of medium containing 3×10^{10} cells, 510 mg of very fine white powder was obtained. This powder, although free of bacterial and fungal contamination, still contained an average of 10 viable yeast cells per mg of acetone powder. Since neither a second cold acetone treatment nor exposure of the powder to the fumes of propylene oxide in an evacuated

desiccator served to decrease this number, the powder was completely sterilized before each experiment by addition of 0.5 ml of cold 95% ethanol for each mg of acetone powder contained in a sterile tube; after 30 minutes at 1° this mixture was diluted with sufficient WAD medium to bring the concentration of powder to 1 mg per ml.

C. Method of Determining Activity of the Acetone Powder

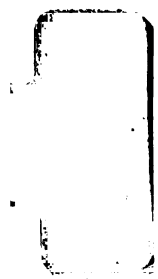
The ad(+) cultures from a sufficient number of 6 to 8 day old C-30 slants were suspended in WAD medium, cell counts made with a Neubauer hemocytometer, and the cell suspensions diluted to the desired density with additional WAD medium. 20 ml of WAD medium containing 1 mg per ml of ethanol sterilized acetone powder was mixed with 20 ml of cell suspension in a 250 ml erlenmeyer flask (or half these quantities in a 125 ml flask) and shaken at 30° for 8 hours. At the same time 20 ml of cell suspension was shaken with 20 ml of WAD medium containing an amount of alcohol equal to that found in the acetone powder suspension. At the end of the period of shaking, aliquots were withdrawn and pipetted into 41° WAD agar in the amount of 1 ml for every 19 ml of medium. The cells were mixed thoroughly throughout the warm agar by means of an enclosed magnetic stirrer and the agar poured in the amount of approximately 20 ml per petri plate. Although the amount of medium poured and thus the number of cells per plate was not controlled, a measured total volume of agar was poured in each experiment; and, therefore, the total number of cells plated was always known.

For studying the effect of anaerobic conditions on colony growth appropriate numbers of cells were spread on the surface of WC agar by tilting and rotating the plates and after 4 hours incubation were covered with a 1-2 mm layer of 41°C WC agar.

D. Extraction and Determination of Yeast Nucleic Acids.

Twenty-five grams of acetone powder of baker's yeast was stirred for 20 minutes at 5°-10°C with a high speed mixer at 10,000 r.p.m. in 100 ml of water containing the following: 0.1 M NaCl, 0.01 M pH 8.0 Tris-HCl, 0.5 mM EDTA, 1 gram sodium dodecyl sulfate, and 50 grams 200μ glass beads. The resulting mixture was decanted, centrifuged at 10,000 x g for 10 minutes, the supernatant precipitated with 2 volumes of cold 100% ethanol and centrifuged at 22,000 x g for 15 minutes. The alcohol precipitate was taken up in 50 ml 1 M NaCl containing .01 M pH 8.0 Tris-HCl, and .05 M EDTA, and freed of protein by stirring in the high speed mixer with an equal volume of 9:1 chloroform-octonal, centrifuging at 2500 r.p.m., and removing the upper aqueous layer. This process was repeated with 0.5 volume portions of chloroform-octonal mixture until no more proteinaseous scum formed at the aqueous-organic interface; generally 4 additional times were sufficient. All the chloroform-octonal mixture used in the deproteinization was combined and re-extracted twice with 50 ml of 1 M NaCl. All the NaCl extracts were combined, precipitated with 2 volumes cold 100% ethanol and centrifuged at 22,000 x g for 15 minutes. This residue could be dissolved in 1 M NaCl, giving a solution in which DNA composed about 12% of the nucleic acid present, or dissolved in 15 ml of 10% $CaCl_2$ and centrifuged at 22,000 x g for 50 minutes in which case 33% of the nucleic acid in the supernatant fluid was DNA.

Determination of nucleic acids. Samples were centrifuged at 5000 r.p.m., extracted twice with 2-1/2 volumes of 10% TCA and twice with 2-1/2 volumes 1:1 ethanol-ethyl ether. The residue was hydrolyzed by heating in a water bath at 90° for 15 min. centrifuged at 5000 r.p.m. and the supernatant fluid saved for the determination of RNA and DNA. RNA was determined by the method of Dische (37) involving reaction with



concentrated H_2SO_4 and cysteine. DNA was determined by the diphenylamine method of Dische (38).

3. RESULTS

A. Yeast Transformation Studies

The effects of shaking a suspension containing 1 x 10^6 ad(+) cells per ml with and without the addition of acetone powder from ad(-) cells are shown in Table 1. The level of acetone powder employed, 1 mg per ml, corresponds to 2 x 10^6 living ad(-) cells.

The diploid strain of yeast used in these studies is quite stable and ad(+) slants containing as many as 75 x 10^6 cells may, upon plating, give no adenine independent colonies. However, on occasional slants, sectors of white revertant cells do occur. In preparation of suspensions of red cells, slants which have visible growth of white cells are discarded, but slants which may contain sub-visible white colonies cannot be eliminated. In consequence, the number of white colonies obtained upon plating the suspension represents the number of white cells and their progeny originally present on the slants used plus whatever number of cells (and their progeny) which revert to adenine independence during the period of shaking. Two hundred eighty-five more colonies appear on plates obtained from the population shaken with acetone powder, Table 1. In all 15 pairs of plates, the plates which represent cells treated with the acetone powder preparation have a larger number of colonies. If all the additional colonies were the result of reversions occurring within a short time following the mixing of the acetone powder with the cell suspensions, then a reversion or transformation rate of 2.5 to 5.0 cells/10^6 cells originally present could account for the additional colonies. If, on the other hand, the reversions occurred with equal frequency throughout the period of shaking, then the approximate rate per

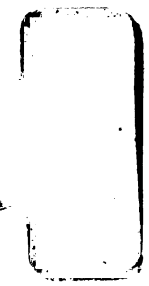


TABLE 1

Number of Reversions of Adenine Requiring, Red Cells to White Adenine Independent Cells Following Treatment with Ad(-) Acetone Powder

Ad(-) Acetone Powder	No. of White Colonies Appearing — Plate Number															Total No. of White Colonies	Average No. Per Plate
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
-	44	59	52	57	41	71	51	59	53	32	49	39	56	49	46	758	50.5
+	60	79	84	59	77	81	87	62	54	59	63	65	74	67	72	1043	69.5

Conditions as described under methods of determining activity of the powder. 15×10^6 cells shaken with and without acetone powder: each plate innoculated with approximately 1×10^6 cells.

generation time can be simply arrived at. With a generation time of 2 hours, the reversions occurring at times 0-2 hours would multiply to give 8 cells at the end of the shaking time, those at 2-4 hours would give 4, 4-6 hours would give 2, and 6-8 hours would give 1. The total number of reversions or transformations obtained is equal to the above numbers summed times the mutation rate per generation time (X). Therefore, 15X must equal 285 and X must equal approximately 20. Since 15×10^6 cells were used a frequency of $1\text{-}1/3 \times 10^{-6}$ cells reverted or transformed per generation time of 2 hours could account for the additional colonies obtained from plates of the acetone treated cells.

The question arises as to whether the white colonies shown in Table 1 represent, in actuality, ad(-) cells since the production of red pigment by adenine dependent mutants is inhibited by growth under anaerobic conditions. Furthermore, the addition of yeast acetone powder to the adenine deficient medium might promote the growth of ad(-) cells. To answer the first of these two questions, suitable dilutions of ad(-) cells were grown on the surface of SC agar or between layers of SC agar. It was shown, Table 2, that under these conditions the colonies growing under an agar layer accumulate red pigment more slowly and never obtain so deep a pigmentation as those grown aerobically. Nevertheless, the ad(-) colonies do exhibit a light pink color after 10 days which is easily distinguishable from the white colonies formed by ad(-) cells. All the white colonies listed in Table 1 remained completely white for at least 3 weeks after inoculation.

In regard to the second consideration, it was found that yeast acetone powder does not serve to increase the rate of multiplication of ad(+) cells, when they are grown in adenine deficient medium. Table 3 shows that even when ad(-) acetone powder is supplied at the rate of 1 mg -

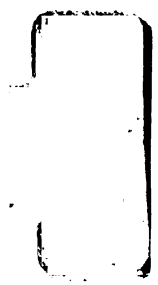


TABLE 2

Effect of Aeration on Development of Red Color in Ad(-) Colonies and upon the Number of Colonies Formed

Growth conditions	Average No. of colonies/ plate	Color after 5 days	Color after 10 days
Aerobic	192	Pink	Red
Anaerobic	188	White	Light Pink

Conditions as described under methods of determining activity of acetone powder. Each an average of 4 plates.

TABLE 3

Effect of Ad(+) Acetone Powder on Growth of Ad(-) Cells

Ad(-) cells shaken in:	Average No. of colonies/plate	
	0 hours	8 hours
WAD media	83	49
WAD media with 1 mg/ml Ad(+) acetone pwd.	86	55

Plated on SC media. Each an average of 5 plates.

which represents the yield from about 2 x 10^6 ad(-) cells - for approximately every 100 ad(+) cells, there is no stimulatory effect on the growth of these cells.

A considerable decrease in viable cell number occurs in populations of ad(+) cells following 8 hours of shaking in an adenineless medium, Tables 1 and 3. Although the zero time cell counts, shown in Table 1, were made with a hemocytometer and therefore did not distinguish between living and dead cells, separate counts made both by dilution and colony counting and by use of the hemocytometer established that all or practically all cells on fresh ad(+) slants are living and viable.

B. Nucleic acid Extraction

Because of the preponderance of RNA present in yeast (Table 4) nucleic acid extractions were performed mainly with the view of increasing DNA yield. For this reason one molar NaCl was employed, without regard to the fact that RNA nucleoproteins are insoluble in this concentration of NaCl. Preliminary experiments demonstrated that extractions of whole cells or of acetone powder for 24 hours with molar NaCl resulted in a very low yield - in the latter case only 4% of the total DNA was extracted. Grinding the acetone powder increased the yield several fold, while with the procedure finally adopted, which utilized grinding in the presence of sodium dodecyl sulfate, as much as 42% of the total DNA was extracted (Table 4). However, this high yield occurred only from acetone powder #1, whereas subsequent extractions from acetone powder #2 gave a maximum of only 16% DNA. Although the basis for this difference was not established, a possible explanation is suggested by the fact that powder #1 was made from yeast that was frozen and thawed before treatments with cold acetone, while #2 represents fresh cells processed directly with acetone.

TABLE 4

Extraction of Nucleic Acids from Baker's Yeast

Fraction	DNA (mg)	RNA (mg)	DNA % In Total Nucleic Acids	Recovery % Total RNA	Recovery % Total DNA
Total Nucleic Acids	231	7120	3.12	---	---
I. Acetone powder No. 1, Na-dodecyl extract	99	1420	6.5	10.2	42.9
II. Acetone powder No. 2, Na-dodecyl extract	37	515	6.7	17.0	7.23
1. Deproteinized supernatant	10.2	54	16.9		
2. First extraction with $HCCl_3$-Octanol	4.8	46	9.4		
3. Second extraction with $HCCl_3$-Octanol	9.6	113	8.5		
4. Total protein-free nucleic acids	24.6	213	10.3	10.6	3.0
5. $CaCl_2$ supernatant	9.9	24	29.2		
6. $CaCl_2$ precipitate	10.3	234	2.4		

4. DISCUSSION

The well established studies on the genetic role of DNA in bacterial transformation suggested that it would be worthwhile to seek a similar system in yeasts. However, in view of the occasional reports of transformation-like phenomena mediated by RNA, it seemed advisable to investigate first the possible hereditary effects of crude, mixed nucleic acid extracts on yeasts. A system has been described in this thesis, utilizing an adenine mutant of Saccharomyces, in which extracts having possible transforming activity might be assayed relatively easily. Also, a procedure is outlined for obtaining DNA enriched nucleic acid extracts from yeasts.

The preliminary results which have been reported here indicate that yeast cells can be modified by exposure to cell-free extracts. This transformation of adenine negative to adenine positive cells by unfractionated acetone powders of the latter suggests that genetic transformation may have occurred.

In experiments of this sort it is often possible to explain the differential effect as being a matter of selection. Such an explanation is particularly in order in the experiments reported on, where a control of ad(+) cells treated with ad(+) acetone powder was not provided. (It proved difficult to grow sufficient quantities of ad(+) to produce an acetone powder, due to back mutation to, and selection for, the white prototroph.) Thus the yeast acetone powder may be suggested to have had a growth promoting effect on the ad(-) cells which were already present, or which arose by mutation from ad(+) cells during the shaking period. It was demonstrated that the growth of ad(+) cells was not stimulated, in adenineless media, by high concentrations of ad(-) acetone powder. In adenineless media viable ad(+) cells gradually decrease in number, and the

addition of ad(-) acetone powder in a ratio of powder/living cells 10,000 times as high as that used in the transformation experiments does not affect this decrease.

An effect of a non-specific factor on alteration of an inherited characteristic in yeast has been reported (39). Galactose-positive cells were converted to galactose-negative cells by extracts not only from galactose positive cells, but from extracts of baker's yeast and even from galactose negative cells.

Ashida, Minagawa, and co-workers have studied extensively an alteration in copper ion sensitivity of a Saccharomyces ellipsoideus strain, induced by a specific but not reproduced factor (40,41). If this yeast is plated on a medium containing Cu^{+2}, only 60% of the cells plated form colonies; but resistant strains can be selected in which 100% of the cells plated give colonies. RNA extracted from resistant strains can convey resistance to the sensitive strains, while the corresponding extract from sensitive cells does not. However, the effective RNA does not seem to be reproduced in cells which are treated with a RNA extract (42).

During the time that this thesis was being written a paper appeared by Oppenoarth (43), in which he reported his experiments on yeast transformation. A crude nucleic acid extract from a yeast strain able to ferment maltose, sucrose, and other dissacharides was added to cultures of a strain not able to ferment dissacharides. The non-fermentors were left to grow in the presence of the yeast extract for several days, then transferred to fermentation tubes. The cells were now found to possess the ability to ferment one or more dissacharides. Some of the transformed yeast retained the acquired ability following sub-culturing 20 times, while other could no longer ferment after being re-inoculated only a few times. On the basis of experiments with added RNase, DNase, or proteinases, the author believed

that the permanent transformation resulted from ingestion of DNA, whereas the temporary or pseudo-transformations were RNA induced. The latter case resembles the specific RNA induced Cu^{+2} resistance in Saccharomyces.

The Saccharomyces mutants at the adenine loci can be conveniently utilized as a means of assaying yeast extracts for genetic transforming activity. The adenine requiring yeast cells, which are red, may be mixed with extracts from the white, adenine independent cells, and plated on adenineless media. Transformed cells will give rise to colonies on the adenineless plates and, further, the colonies which represent transformations will appear as white and not red.

Genetically the adenine mutants have been rather thoroughly investigated, and crossover frequencies between loci and within loci measured. In addition, the metabolic pathways involved in red pigment formation and adenine biosynthesis have to some extent been established. In contrast, in the copper resistant mutants of yeast (40), neither the genetics nor the physiological processes involved are clear. In the dissacharide utilizing mutants of yeast (43), quantitative analysis for transformations are difficult because fermentation measurements are required.

Extraction of nucleic acids from yeast cells proved difficult and detergent extraction of acetone powders yielded less than 10% of the total nucleic acid present. Similar low yields were reported by Oppenoarth (43). However, he was later able to increase considerably the amount of nucleic acid obtained by using the gut juice of Helix pomatia to produce yeast protoplasts. From the protoplasts DNA and RNA could be extracted in high yields.

PART II. STUDIES ON ADENOSINE DIPHOSPHATE SULFURYLASE

1. INTRODUCTION AND REVIEW OF LITERATURE

Mononucleotide acid anhydrides are usually biosynthesized by the pyrophosphorolytic cleavage of a nucleoside triphosphate. Thus the biosynthesis of the mixed acid anhydride adenosine—5'-phosphosulfate, APS[2], has been shown to be linked to the cleavage of pyrophosphate from ATP. In addition, however, Robbins and Lipmann (44) have reported the phosphorolysis of APS with the resultant formation of ADP - in the reverse reaction phosphate would be cleaved from ADP and APS synthesized. Synthesis of APS by this reaction mechanism therefore would represent an unusual mode of formation of nucleoside acid anhydride. The reaction is of further, more general interest in that the energy used to drive the reaction in the direction of APS synthesis would have to be derived not from the phosphate bond energy of ATP but from that of ADP.

It was the purpose of this study to measure the ADP sulfurylase reaction in the direction of APS synthesis. The enzymatic cleavage of P_i from ADP in the present of MoO_4^{-2} was utilized as a means of assay for the reaction in this direction. Molybdate ion has been demonstrated to substitute for sulfate ion in reactions catalyzed by ATP sulfurylase (45).

A. The Formation of Acid Adenylates

Among the mononucleotide acid anhydrides those of AMP (the acid adenylates) participate most frequently in biochemical reactions. In addition to the phosphoric or pyrophosphoric acid adenylates (ADP, ATP),

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[2]The following abbreviations are used: APS, adenosine-5'-phosphosulfate; PAPS, 3'-phosphoadenosine-5'phosphosulfate; PP, pyrophosphate; P_i, orthophosphate; PPase, inorganic pyrophosphatase; ATP, adenosine triphosphate; AMP, adenosine monophosphate; Tris, tris (hydroxylmethyl) aminomethane; EDTA, ethylenediaminetetraacetate.

within the last ten years a large variety of mixed acid adenylates, functioning as important metabolic intermediates, have been described (46). In the formation of the mixed acid adenylates inorganic pyrophosphate is split out from ATP and the reaction sequence is:

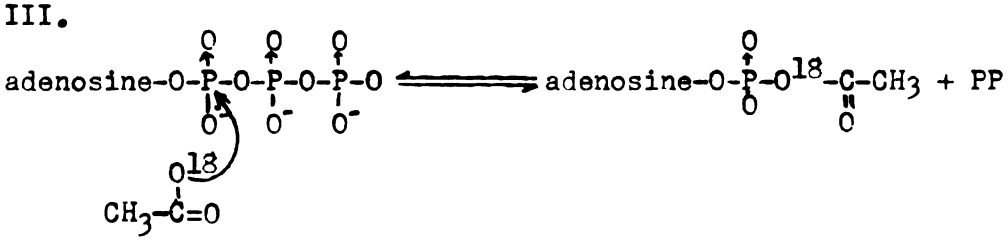
I. ATP + anion $\rightleftharpoons$ acid adenylate + PP

Such a pyrophosphorolytic reaction was demonstrated by Berg (47) as occurring in the activation of acetate. In the presence of enzyme he found that acetate was required for labeled PP exchange and that synthetic adenyl acetate could be pyrophosphorylized to form ATP. Thus:

II. ATP + acetate $\rightleftharpoons$ adenyl acetate + PP

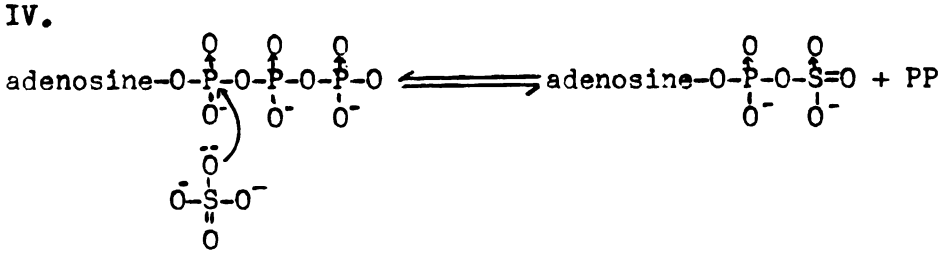
Study of reaction II with O^{18} has established that the oxygen of the acetate is transferred to the phosphate of the adenylate and has permitted the formulation of the reaction as follows:

III.



in which the oxygen of the acetate makes an attack on the first phosphate of ATP (48). If the same reaction mechanism is applied to the sulfate activating-pyrophosphorylase reaction, then the formation of APS would occur as shown in IV.

IV.



In this reaction the anionic attack is believed to involve an enzyme-magnesium-sulfate complex (49).

(1.) Sulfuric acid adenylates: A sulfuric adenylic acid anhydride was first described in 1955 by Hilz and Lipmann (50). The mixed anhydride was tentatively identified as APS and postulated to serve as the sulfate donor (active sulfate) for the esterification of sulfate with phenol. In subsequent work, however, Robbins and Lipmann (51) established that active sulfate was not APS but adenosine-3'-phosphate-5'-phosphosulfate, PAPS. The metabolic relation between APS and PAPS was established by the elaboration of the reaction sequence involved in their formation. Wilson and Bandurski (52) and Robbins and Lipmann (53) showed that the reaction

$$\text{V.}\quad \text{ATP} + SO_4^{-2} \rightleftharpoons \text{APS} + \text{PP}$$

is followed by a second reaction

$$\text{VI.}\quad \text{APS} + \text{ATP} \rightleftharpoons \text{PAPS} + \text{ADP}$$

which results in the synthesis of active sulfate, PAPS. The first reaction is catalyzed by ATP sulfurylase and the second reaction by APS kinase.

During the course of separation of the ATP sulfurylase and APS kinase enzyme, Robbins and Lipmann (44,53) obtained a third enzyme which catalyzed the reaction

$$\text{VII.}\quad \text{APS} + P_i \rightleftharpoons \text{ADP} + SO_4^{-2}$$

The enzyme, ADP sulfurylase, was purified threefold from yeast extract and freed of ATP sulfurylase. Activity determinations were made by incubation with synthetically prepared APS (54) and measuring P_i disappearance, with checks on ADP formation by electrophoresis or chromatography. While Robbins and Lipmann have not investigated ADP sulfurylase extensively, they have

reported it to be unstable and to require no added cation activator.

(2.) Biosynthetic reactions involving ADP: At the present time only two classes of enzyme catalyzed reactions are known in which a synthetic or endergonic reaction is coupled to cleavage of phosphate from ADP (or other dinucleotides). They are the synthesis of polyribonucleic acid and the phosphate transferring reactions catalyzed by nucleoside monophosphate kinases. The formation of APS from ADP may serve as a third example of an ADP coupled reaction; however, as yet, this reaction has not been conclusively demonstrated. In RNA synthesis, the enzyme polynucleotide phosphorylase discovered by Ochoa (55) catalyzed the reaction:

VIII. $${}_{n}x\text{-}P\text{-}P \rightleftharpoons (x\text{-}P)_{n} + n^{P}i$$

in which x represents a nucleoside. In this reaction the cleavage of a phosphate is followed by the formation of an ester bond.

The nucleoside monophosphate kinases can be considered to catalyze the transfer of phosphate from a nucleoside triphosphate to a nucleoside monophosphate; or, in the reverse reaction, to catalyze the attack of one nucleoside diphosphate on another to yield one molecule of nucleoside monophosphate and one molecule of nucleoside triphosphate (56):

Nucleoside-A triphosphate + Nucleoside-B monophosphate $\rightleftharpoons$

Nucleoside-A diphosphate + Nucleoside-B diphosphate.

The earliest known nucleoside monophosphate kinase reaction is that catalyzed by the ATP-AMP kinase, adenylate kinase (originally termed myokinase)(57):

IX. $$2\ ADP \rightleftharpoons AMP + ATP$$

in which only adenine nucleoside phosphates are involved.

(3.) Sulfurylase reactions with molybdate: Work by Wilson and Bandurski (45) has established that other group VI oxyanions, in addition

to sulfate, can serve as substrates for ATP sulfurylase. The substitution of MoO_4^{-2} for SO_4^{-2} was found to lead to a much greater PP release from ATP, and consequently could serve as a convenient means of assay for ATP sulfurylase.

Assuming that MoO_4^{-2} would also serve as a substrate for ADP sulfurylase, the reaction

$$\text{X.} \qquad ADP \xrightarrow{MoO_4^{-2}} AMP + P_i$$

in which the anionic attack on ADP is made by molybdate, would be expected to occur. In this thesis, MoO_4^{-2} was used and P_i release measured to assay for ADP sulfurylase activity.

B. Inorganic Pyrophosphatase

Yeast pyrophosphorylase hydrolyzes the acid anhydride bond in pyrophosphate and liberates two molecules of P_i for every molecule of pyrophosphate hydrolyzed:

$$\text{XI.} \qquad PP \rightleftharpoons 2\ P_i$$

The enzyme is specific for inorganic PP and will not hydrolyze other PP linkages. Bailey and Webb (58) prepared highly purified yeast PPase and found the presence of Mg^{+2} essential for activity, while Ca^{+2}, Mn^{+2}, and Zn^{+2} were inhibitory in the presence of Mg^{+2}. Divalent magnesium was required as an activator in the yeast PPase preparations of Heppel and Hilmoe (59). Kunitz (60) has crystallized yeast PPase and found in addition to Mg^{+2} activation a slight activation by Co^{+2} and Mn^{+2}. In potatoes, Nagawa, et al. (61) report the occurrence of an acid and an alkali PPase (pH optima of 5 and 8.5). The acid PPase is not activated by Mg^{+2} but not inhibited by MoO_4^{-2}. In either case no activation is obtained by Co^{+3}, Mn^{+2}, or Zn^{+2}.

2. MATERIAL AND METHODS

Pyrophosphatase: Yeast PPase, prepared by the Heppel and Hilmoe method, was supplied by Dr. Lloyd Wilson.

Preparation of adenosine phosphosulfate. APS was synthesized by the method of Baddiley, et al. (54), 400 mg of dry pyridine - SO_3 complex (prepared by Mr. Craig Squires) was slowly added with stirring to 200 mg AMP and 350 mg $NaHCO_3$ contained in 5 ml of water. The resulting reaction mixture was stirred for 10-20 minutes at 42°C.

The yield of APS was estimated by paper electrophoresis of a small quantity of the reaction mixture at 1-2° in M/10 pH 5.0 acetate buffer. The nucleotide spots located by their uv absorption, were cut out, eluted with water for 1/2 hour at 50-60°, and the concentration of nucleotide determined from optical density readings at 260 mμ (62). Yields of APS were obtained corresponding to about 8.5% of the AMP supplied.

Isolation of APS. Previous attempts in this laboratory to isolate APS by means of the formate column described by Baddiley were unsuccessful. The description by Brunngraber (63) of the isolation of PAPS by NaCl elution from a Dowex-1, chloride column, led to several unsuccessful attempts to isolate APS on ion exchange columns of this type. A new method for separation of nucleotides was suggested by the reported use of ion exchange resins in determinations of stability constants of cation-nucleotide complexes (64). Approximately 2 grams of 200-400 mesh, 10% crossed linked, chloride, Dowex-1 resin was formed into a 7.5 x 1 cm column. From 10 to 100 μmoles of nucleotide in 20 ml of water were put on the column and eluted with a solution containing .005 mole $MnCl_2$, .05 mole of NaCl, and .01 mole pH 8.2 tris-HCl per liter. The strength of the cation nucleotide complex depends on the length of the phosphate chain in the nucleotide, and dinucleotides

form Mn-complexes which are less strongly absorbed to the exchange resin than those of mononucleotides. The Dowex column described was capable of separating ADP, which was eluted first, from admixed AMP. However, it was ineffective in separating APS from AMP because of the rapid hydrolysis of APS, apparently catalyzed by Mn ion. Small quantities of APS could be separated by use of a continuous flow electrophoresis apparatus built by Mr. Robert Hamilton. Separation was accomplished by applying APS reaction mixture by means of a wick onto a hanging curtain of Whatman #3 paper. M/20 pH 5 acetate was employed as a solvent and a field strength of approximately 14.3 v/cm applied for 24 hours, while the apparatus was kept in a 1° cold room. The upper edges of the curtain were serrated to provide a slower flow of solvent. The APS fraction was adsorbed on charcoal and eluted according to the method given by Baddiley (54). Alcohol was removed from the eluate with a flash evaporator at 50° and the remaining solution freeze-dried in a lypophil apparatus. A mixture of 4.2 μM APS and 4.6 μM AMP was obtained.

Determination of Orthophosphate. The method of Fiske and Subbarrow (65) was used to determine inorganic phosphate.

Estimation of protein. Samples were diluted with M/2 KCl and shaken with an equal volume of 5% trichloroacetic acid. The resulting turbidity was measured in a Klett-Summerson colorimeter with a #54 filter and compared with crystallized bovine serum albumin as a standard.

Sulfurylase assay. Sulfurylase activity was determined by measuring molybdate stimulated release of P_i. The reaction mixture contained 50 μmoles tris-HCl buffer, pH 7.5; 4 μmoles Na_2MoO_4, 0.3 μmoles EDTA; 2 μmoles of either $MgCl_2$ or other cation as specified, 2.0 μmoles ADP

or ATP, and enzyme in a total volume of 0.50 ml. Parallel tubes were also run without addition of molybdate. Incubation was for 60 minutes at 37°C.

Preparation of enzyme. Twelve grams of baker's yeast were suspended in 100 ml of .01 M pH 7.5 tris-HCl containing .001 M EDTA by grinding in a porcelain mortar. This suspension was then stirred at 14,000 r.p.m. in a high speed mixer for 10 minutes with 35 grams of 200 μ glass beads. The extract was centrifuged, frozen, thawed, filtered through silk and centrifuged again at 22,000 x g for 10 minutes. The supernatant liquid was dialyzed for 20 hours at 1° against two one liter portions of .005 M tris-HCl, .0001 M EDTA. A small precipitate was removed by centrifuging for 10 minutes at 22,000 x g, and the supernatant liquid saved (stage II).

Heat precipitation. Dialyzed extract was heated to 49-50° while being stirred, kept at this temperature for 2 minutes, then cooled in an ice bath. The suspension was frozen, thawed, and centrifuged at 22,000 x g for 10 minutes (stage III). Loss of activity occurred quickly at temperatures above 50°, and only 15% of the activity present after heating at 49° was obtained following heating to 53°.

Acid precipitation. Portions of the heated supernatant fraction were dialyzed against acid buffer solutions. Dialysis was done against 250 volumes of 0.2 M acetate, 0.0005 M EDTA buffer of the appropriate pH at 1°. After 3 hours the suspension was removed from the dialysis bag, and centrifuged at 22,000 x g for 10 minutes. The precipitate was dissolved in a volume of M/10 pH 7.5 tris-HCl equal to the supernatant volume. The pH of the supernatant was recorded and found to range from 0.05 to 0.10 pH unit higher than the pH of the dialysis buffer employed.

Enzyme fractionation. Table 5 summarizes the enzyme fractionation. One unit of enzyme activity is defined as the amount of enzyme which produced one μmole of inorganic phosphate from ADP when incubated in the presence of MoO_4^{-2} at 37° for one hour. All the fractions obtained had greater activity with ATP than ADP. In addition pH precipitations were made at several other pH values at which the concentration of protein present was not measured. The activity of these fractions is given in Table 7.

3. RESULTS

A. Release of Phosphate from ADP

An enzymatic release of phosphate was observed when ADP was incubated with dialyzed yeast extract, Figure 1. The increment in P_i liberation obtained by addition of molybdate indicates the amount of sulfurylase activity present (45).

Electrophoresis of the incubation mixture demonstrated the presence of adenylate kinase in the enzyme preparation. Upon electrophoresis AMP, ADP, and ATP spots of approximately equal size were obtained. Furthermore, the incubation mixture was demonstrated to have PPase activity. Therefore, the relative contributions of ATP sulfurylase and possible ADP sulfurylase to the release of P_i could not be determined.

The effect of cations. The release of P_i from ADP through the action of ATP sulfurylase is dependent upon the presence of adenylate kinase and PPase in addition to the sulfurylase enzyme. The following series of reactions are involved:

XII. a. $2\ ADP \rightleftharpoons ATP + AMP$

b. $ATP \xrightarrow{MoO_4^{-2}} AMP + PP$

c. $PP \rightleftharpoons 2P_i$

TABLE 5

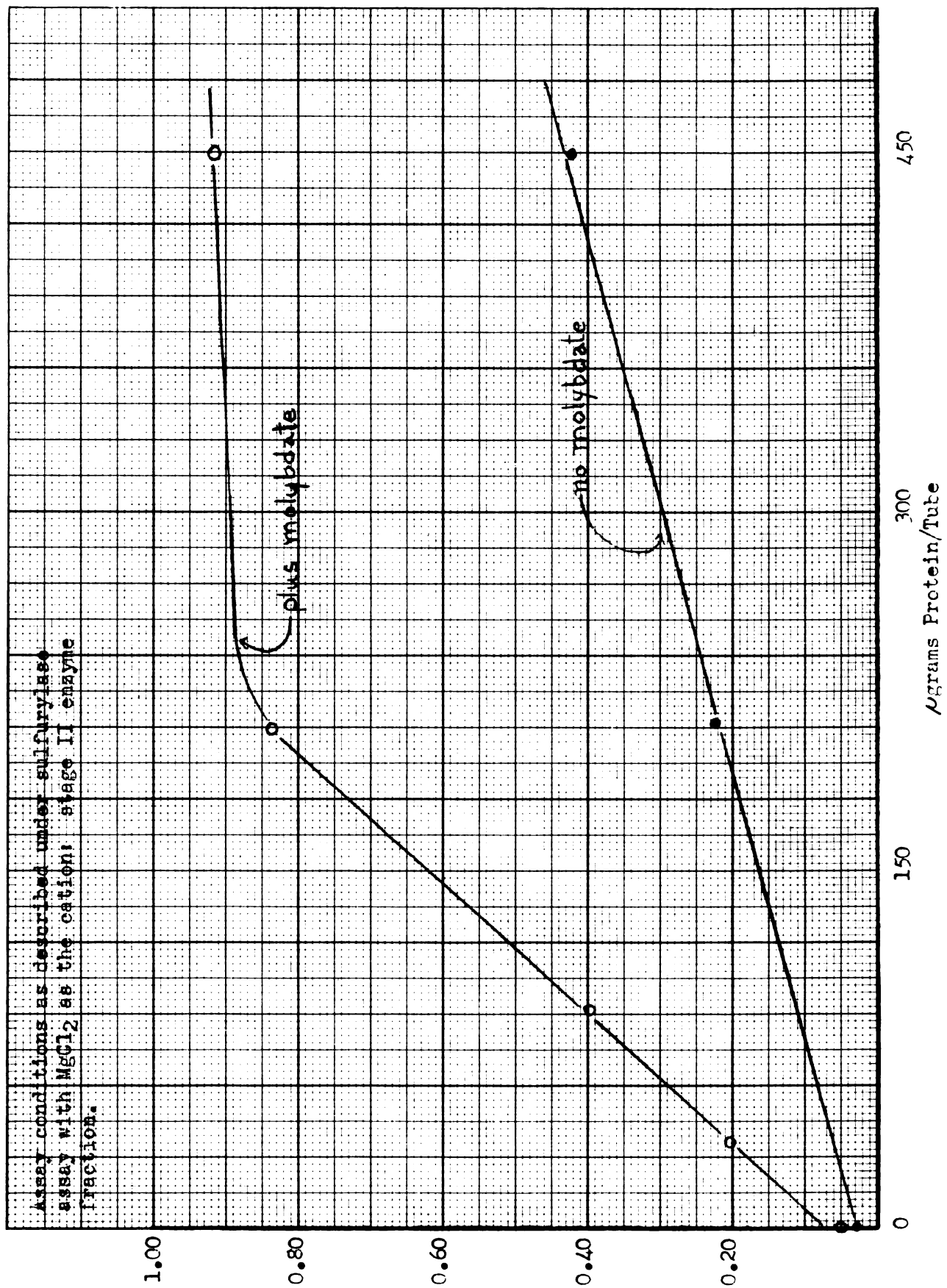
Purification of Sulfurylase Activity from Yeast

	Enzyme Fraction	Volume	Total Protein	Units	Specific Activity	Recovery
		ml	mg	μmoles P_i/hr.	units/mg protein	%
I.	Crude extract	56	55	4480	81.8	100
II.	Dialyzed extract	51	30.5	6630	217	148
III.	50° supernatant	50	13.2	3460	254	77
IV.	pH 5.5 supernatant	50	6.4	2620	407	58

Figure 1

Phosphate Liberation from ADP
As a Function of Enzyme Concentration

Figure 1


Phosphate Liberated μmoles/Tube
1.00
0.80
0.60
0.40
0.20
0
150
300
450
μgrams Protein/Tube
plus molybdate
no molybdate
Assay conditions as described under sulfurylase assay with MgCl2 as the cation: stage II enzyme fraction.

in which b. is probably a two step reaction involving an unstable AMP-MoO_2^{-4} mixed acid anhydride. The requirement of Mg^{+2} by PPase for high activity suggested elimination of magnesium ion from the incubation mixture might serve to prevent liberation of P_i through the ATP sulfurylase route. The reaction APS + P_i ⟶ ADP + SO_4^{-2} is reported to proceed without the addition of any cation (44). In measuring the reverse reaction with molybdate:

XIII. $\quad ADP + MoO_4^{-2} \longrightarrow AMP + P_i$

with the dialyzed yeast extract, used in the study reported on in this thesis, no appreciable release of P_i occurred in the absence of a cation. The effect of no added cation and of several different divalent cations on P_i liberation from ADP and ATP is shown in Table 6.

Substitution of Mn^{+2} for Mg^{+2} in the reaction mixture would be expected to reveal the presence of ADP sulfurylase. Also, the removal of any one of the enzymes shown in equations XIIa, XIIb, or XIIc from the enzyme fraction being assayed would prevent P_i release by the ATP sulfurylase route and permit the measurement of P_i liberated by ADP sulfurylase. Accordingly, the enzyme preparation was fractionated at different pH values and assayed in the presence of Mg^{+2} and Mn^{+2}, ADP and ATP. All the fractions show very similar ratios of ADP to ATP activity with either Mn^{+2} or Mg^{+2}, Table 7.

B. Divalent Zinc Activation of Phosphate Release from Pyrophosphate

Comparison of the PPase activity of stage IV enzyme and yeast PPase prepared by the Hippel and Hilmoe method in the presence of different activating cations disclosed an unexpected difference, Table 8. Both are highly activated by Mg^{+2} and slightly activated by Mn^{+2} - in agreement with the cation requirement of crystalline PPase (61). But PPase prepared by the Hippel and Hilmoe method shows only a slight activation by zinc, whereas

TABLE 6

Effect of Divalent Cations on Phosphate Liberation

Nucleotide and Cation Added		Micromoles Phosphate Released
ADP	Mg^{+2}	0.40
ADP	Mn^{+2}	0.21
ADP	Zn^{+2}	0.23
ADP	None	0.05
ATP	Mg^{+2}	0.71
ATP	Mn^{+2}	0.51
ATP	Zn^{+2}	0.55
ATP	None	0.07

Assay conditions as described for sulfurylase assay with nucleotides and cations as indicated: 0.003 ml (90 μg) dialyzed enzyme.

TABLE 7

Phosphate Release from ADP and ATP
with Mg^{+2} or Mn^{+2}

Enzyme Fraction	Micromoles Phosphate per Tube			
	ADP Mg/Mn	Ratio	ATP Mg/Mn	Ratio
Stage III				
50° supernatant	3.52/1.0	3.52	5.68/2.8	2.02
Stage IV				
pH 5.5 supernatant	2.48/0.98	2.53	5.84/3.02	1.93
pH 5.5 precipitate	0.86/0.20	4.30	1.02/0.64	1.59
pH 5.4 supernatant	0.92/0.36	2.56	1.20/0.76	1.58
pH 5.4 precipitate	2.40/0.48	5.00	6.04/2.32	2.60
pH 5.2 precipitate	2.72/0.46	5.91	6.20/2.3	2.70

Conditions as described for sulfurylase assay, Mg^{+2} or Mn^{+2}, and .05 ml of enzyme fraction indicated.

TABLE 8

Phosphate Liberation from Pyrophosphate
in the Presence of Divalent Cations

Cation	Enzyme	
	PPase	Stage IV Enzyme Fraction
	μmoles Pyrophosphate Liberated	
Mg^{+2}	2.02	2.04
Mn^{+2}	0.18	0.23
Zn^{+2}	0.30	1.92
None	0.06	0.07

Each tube contained in μmoles: tris-HCl, pH 7.5, 50; Na_2MoO_4, 5; EDTA, 0.3; cation, 2.0; and either 65 μg of stage IV enzyme fraction or PPase in a total volume of 0.5 ml. Incubation was at 37°C for 60 minutes.

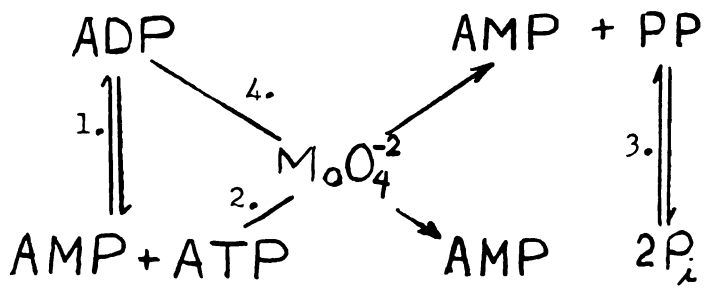
stage IV enzyme exhibits a high Zn^{+2} activation. The stage IV enzyme shows no phosphatase activity on ADP or ATP (in the absence of MoO_4^{-2}). Thus the presence of a yeast PPase is suggested, which may be differentiated from the previously reported pyrophosphatases on the basis of its activation by divalent zinc.

4. DISCUSSION

The reaction APS + P_i ⟶ ADP + SO_4^{-2} catalyzed by ADP sulfurylase is known to occur (44), although it has been only investigated briefly. The writer wished to study the ADP sulfurylase reaction in the opposite direction, ADP + SO_4^{-2} ⟶ APS + P_i , by use of the molybdate assay (45). Since MoO_4^{-2} has been shown to substitute for SO_4^{-2} in the reaction catalyzed by ATP sulfurylase, the occurrence of the reaction ADP $\xrightarrow{MoO_4^{-2}}$ AMP + P_i would be considered to demonstrate the presence of ADP sulfurylase. A MoO_4^{-2} dependent splitting of P_i from ADP was measured; however, a larger amount of P_i was released from ATP than from ADP with every enzyme fraction assayed.

ADP incubated with MoO_4^{-2} and yeast extract may undergo the reactions shown in Figure 2.

Figure 2. Reactions of ADP, ATP, and Molybdate

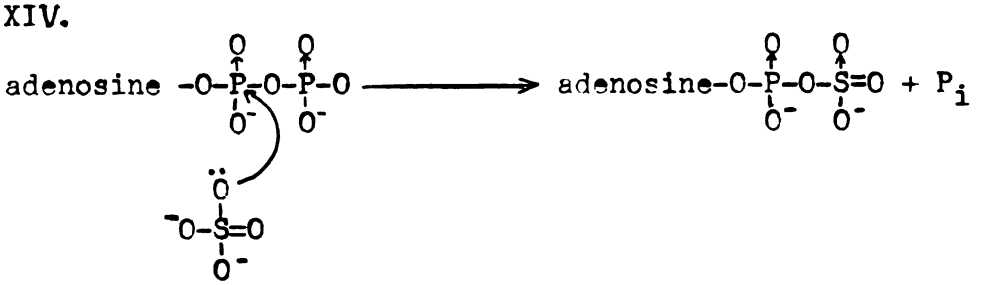


Reaction (1) is catalyzed by adenylate kinase, (2) by ATP sulfurylase, (3) by PPase, and (4) is that postulated for ADP sulfurylase.

Starting with ADP it is evident that P_i may be released by ADP sulfurylase; or phosphate may be released by means of an ATP sulfurylase route. The ratio of molybdate stimulated phosphate release from ATP and ADP was generally about 2:1 in all enzyme preparations. Fractionation of the enzyme preparation by dialysis against buffers of different pH values, in an attempt to separate ATP sulfurylase, PPase, or adenylate kinase from possible ADP sulfurylase, failed to change appreciably the ratio of P_i released from the two nucleotides. Substitution of Mn^{+2}, an ion which is a poor activator of PPase, for Mg^{+2} was regularly employed in an attempt to demonstrate ADP sulfurylase activity, but manganese decreased approximately proportionately the release of P_i from both ADP and ATP.

Thus, in yeast extracts, MoO_4^{-2} does not bring about the reaction $ADP + MoO_4^{-2} \longrightarrow AMP + P_i$ indicating either the failure of MoO_4^{-2} to substitute for SO_4^{-2} or the irreversibility of ADP sulfurylase. While in theory all reactions are reversible, many in fact are effectively irreversible because they do not proceed to any appreciable extent in one direction. The ADP sulfurylase reaction has previously been measured only in the direction of the phosphorolytic attack on APS (44,53,66). The reverse reaction, applying the reaction mechanism which has been postulated for the formation of nucleoside acid anhydrides, is depicted as:

XIV.



and this reaction would be unique in that no other enzyme catalyzed reaction has the formation of a <u>mixed</u> mononucleotide acid anhydride been demonstrated to be coupled with the cleavage of a high energy phosphate of a nucleoside diphosphate.

The writer was not successful in separating APS from ADP by means of ion exchange columns; but with Mn^{+2} used as an eluant ADP can be displaced from a cation exchange column ahead of admixed AMP. Under the same conditions a hydrolysis of APS occurs, probably catalyzed by the manganese ion. Continuous flow electrophoresis, while convenient, can be used only for separating small quantities of AMP and APS.

Yeast inorganic pyrophosphatase is reported to be chiefly activated by Mg^{+2}, with slight activation by Mn^{+2} and Co^{+2} (60). A pyrophosphatase activated by zinc ion has not been reported. The Zn^{+2} activated release of P_i from inorganic pyrophosphate, which was reported on in this thesis, with pH 5.5 supernatant enzyme is therefore of interest. This yeast enzyme fraction catalyzes only small amounts of P_i release from PP in the presence of Mn^{+2}, but catalyzes much greater amounts in the presence of Mg^{+2} or Zn^{+2}. Although a zinc activated PPase is thereby indicated, the extract has not been fractionated further with the purpose of separating Zn^{+2} dependent activity from Mg^{+2} dependent activity.

Very recently Schlesinger and Coon (67) also reported a yeast pyrophosphatase preparation which catalyzed the release of P_i from PP with either Zn ion or Mg ion as the activator. Furthermore, they found that the same preparation possessed nucleotidase activity; however, nucleotides were hydrolyzed at a very much slower rate then was inorganic pyrophosphate.

SUMMARY

I. The deoxyribonucleic acid (DNA) mediated genetic transformation of bacteria was discovered over 15 years ago. There have also been some reports of the apparent genetic alteration of bacteria and yeasts by ribonucleic acid. Transformations by DNA have not been demonstrated to occur in other organisms with the exception of the recent report of Oppenoarth's on yeast transformation (43). In the writer's experiments the genetic transformation of yeasts was attempted utilizing adenine mutants of Saccharomyces. The results of a single experiment are reported in which adenine requiring cells were treated with a crude extract of adenine independent cells. The treated cells, shaken for 8 hours with the crude extract, showed a greater number of cell reversions to adenine independence than those not so treated. These preliminary results suggest that for further experiments on yeast transformation, the mutants at the adenine loci in Saccharomyces may prove useful. The adenine dependent, red pigmented mutants may be treated with extracts from the white, adenine requiring prototrophs, plated on adenineless medium, and the colonies appearing, which represent reversions to adenine independence, counted after only a few days incubation. As a further check, the colonies which represent reversions or transformations must be white and not red.

A procedure for extraction of nucleic acids from yeasts is described in which an acetone powder in a sodium chloride - sodium dodecyl sulfate solution is stirred at a high speed with glass beads. Following removal of protein, the extract can be enriched in deoxyribonucleic acid by precipitating ribonucleic acid with a calcium chloride solution.

II. Adenosine diphosphate sulfurylase has been reported to occur in yeast and to catalyze the phosphorolysis of adenosine phosphosulfate (APS)

to adenosine diphosphate (ADP) and inorganic sulfate.

a.) $APS + P_i \longrightarrow ADP + SO_4^{-2}$

The present experiments were designed to test for the occurrence of the back reaction by making use of the ability of molybdate to serve as an analog of sulfate. Since the resultant phosphato-molybdate compound might be expected to be unstable the reaction expected was:

b.) $ADP + MoO_4^{-2} \longrightarrow AMP + P_i + MoO_4^{-2}$

Although a molybdate dependent enzymatic cleavage of P_i from ADP was obtained, a still larger release of P_i occurred whenever the enzyme fractions were incubated with ATP. In the presence of adenylate kinase, pyrophosphatase, and ATP sulfurylase, phosphate can be released from ADP. It was not possible to eliminate this means of P_i release from ADP by the substitution of manganese ion for magnesium ion nor could any of the three enzymes be removed from the enzyme preparation. Thus the ability of ADP sulfurylase, from baker's yeast, to catalyze reaction b could not be established, indicating either the failure of MoO_4^{-2} to substitute for sulfate or the irreversibility of ADP sulfurylase.

During the course of the experiments a divalent zinc ion activated enzymatic release of phosphate from inorganic pyrophosphate was observed. The presence of a yeast pyrophosphatase is therefore suggested which may be differentiated from previously reported pyrophosphatases on the basis of its activation by divalent zinc.

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