A STUDY OF INVERTASE DEVELOPMENT IN EXCISED CARROT TISSUE

JERRY DEXTER GREGORY

To the Graduate Council:

I am submitting herewith a thesis written by Jerry Dexter Gregory entitled "A Study of Invertase Development in Excised Carrot Tissue." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biology.

Benj. P. Stone
Major Professor

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A STUDY OF INVERTASE DEVELOPMENT IN EXCISED CARROT TISSUE

An Abstract

Presented to

the Graduate Council of

Austin Peay State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Jerry Dexter Gregory

July, 1970

ABSTRACT

This study was undertaken to determine the inhibitory effect of ethionine on invertase development in carrot root tissue. Attempts were made to reverse the inhibition of ethionine by the addition of methionine and adenosine triphosphate (ATP). Labeled precursors were used to determine the inhibitory effect of ethionine, 5-fluorouracil, and actinomycin D upon synthesis and methylation of ribonucleic acid (RNA). Other inhibitors such as 6-methylpurine and cycloheximide were employed to observe their effect on the development of invertase. Additional studies were undertaken to determine the relationship of gibberellic acid (GA_3) stimulation of invertase and synthesis and methylation of RNA in carrot tissue.

Invertase activity increased four fold over a period of forty-eight hours in washed excised carrot root tissue. The addition of ethionine 0.01 molar (M.) to the carrot tissue resulted in a severe inhibition of invertase development (approximately 75 percent) and methylation of RNA (80 percent) with a lesser inhibition of RNA synthesis (55 percent). Methionine 0.01 M. and 0.005 M. reversed the inhibition of ethionine on invertase development. Adenosine triphosphate, 0.01 millimolar to 15 millimolar (mM.), did not

significantly reverse ethionine inhibition of invertase development. Cycloheximide and 6-methylpurine 10 micrograms per milliliter and 20 micrograms per milliliter (μg./ml.) severely inhibited the development of invertase. Actinomycin D (50 μg./ml.) severely inhibited invertase development and RNA synthesis (approximately 80 percent) with a lesser inhibition on RNA methylation (60 percent). The addition of 5-fluorouracil (100 μg./ml.) significantly inhibited synthesis of RNA (50 percent) but only slightly inhibited invertase development and methylation of RNA (approximately 20 percent). Gibberellic acid (10 μg./ml.) stimulated invertase development (25 to 60 percent) but exhibited no significant effect on synthesis and methylation of RNA. These results indicate that protein and RNA synthesis as well as methylation of RNA are essential for invertase development in excised carrot tissue.

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ACKNOWLEDGEMENTS

I wish to gratefully acknowledge the assistance and continual encouragement of my major professor, Dr. Benjamin P. Stone. Further acknowledgement is extended to Dr. Haskell Phillips and Dr. Floyd Ford for the reading of this manuscript and assistance throughout this investigation.

I also wish to thank my wife Janice for her contined encouragement and criticism which contributed in many ways toward the completion of this thesis.

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INTRODUCTION

Factors which regulate enzyme activity in higher plants have received increasing attention in recent years. Most of this research has involved the application of chemical growth regulators.

The objective of this research was to determine the inhibitory effect of ethionine on invertase development in carrot root tissue. Attempts were made to reverse the inhibition of ethionine by the addition of methionine and adenosine triphosphate. Other inhibitors such as 6-methylpurine and cycloheximide were employed to observe their effect on the development of invertase.

Labeled precursors were used to determine the inhibitory effect of ethionine, 5-fluorouracil, and actinomycin D upon synthesis and methylation of ribonucleic acid. Additional studies were undertaken to determine the relationship of gibberellic acid stimulation of invertase and synthesis methylation of RNA in carrot tissue.

CHAPTER II

LITERATURE REVIEW

When thin slices are excised from intact storage tissue (roots and tubers) of plants, a rapid and dramatic change in the metabolic activity of the tissue occurs. The most obvious measure of this metabolic change is a time-dependent increase in respiration, which differs quantitatively from the basal respiration of the intact tissue (ApRees and Beevers, 1960; and Adams, 1970). Invertase is one of a number of enzymes absent or at very low concentrations in mature storage tissue of sugar beet and artichoke but rapidly appears in washed aerated discs after a period of twenty-four hours (Bacon et al., 1965; and Edelman and Hall, 1965). Excised carrot and potato tissue also exhibits an increase in invertase level as well as excised sugar cane tissue, rapidly growing epicotyls of lentil seedlings, and Avena stem segments (Vaughan and MacDonald, 1967; Glasziou et al., 1966; Seitz and Lang, 1968; and Kaufman et al., 1968).

Rapid growth and respiration of plant cells and the action of plant growth hormones on these processes depend upon synthesis of protein and RNA (Key, 1964; and Nooden and Thimann, 1966). Among the proteins which are synthesized during cell growth are enzymes,

which play an important function in growth. One such enzyme is invertase. Click and Hackett (1963), using potato slices, showed respiration increase to be dependent on protein and RNA synthesis. Protein synthesis has been shown to be necessary for growth and invertase activity in Avena stem segments (Kaufman et al., 1968). Leaver and Edelman (1965) found that during aging of carrot tissue slices, RNA synthesis increased by fifty percent during the first twenty-four hours. (Cherry ,1969), using sugar beet, concluded that protein synthesis and deoxyribonucleic acid (DNA) directed RNA synthesis were necessary for the production of invertase. Glasziou et al. (1965) indicated that glucose regulated the rate of invertase by controlling the destruction of messenger RNA (m-RNA) required for production of invertase. Gayler and Glasziou (1969) found RNA synthesis to be rate-limiting for invertase synthesis in sugar cane tissue.

Recently it was observed in washed carrot (Leaver and Key, 1967) and sugar beet discs (Cherry, 1969) that in vivo polyribosome formation occurred within a short period of time and preceded any measurable increase in invertase activity.

In recent years factors which regulate enzyme activity in higher plants have received increasing attention. Gibberellic acid

has been shown to enhance the synthesis of α-amylase and ribonuclease in isolated aleurone layers of barley (Chrispeels and Varner, 1967). It has been postulated that GA_3 controls the synthesis of α-amylase and other heat-stable proteins in aleurone cells by causing the production of specific messenger RNA's (Wakel, et al., 1964). Edelman and Hall (1964 and 1965) indicated that the development of invertase in artichoke is stimulated by GA_3.

Gibberellic acid was shown to promote or inhibit the production of invertase in sugar beet discs depending on the stage of washing (Cherry, 1969). Gayler and Glasziou (1969) found GA_3 increased the enzyne-forming-capacity for invertase by stablization of m-RNA necessary for invertase synthesis. Other investigators, Kaufman et al. (1968) and Seitz and Lang (1968), found GA_3 stimulated invertase development of Avena stem segments and lentil epicotyls, respectively.

Cycloheximide, and antibotic produced by Streptomyces griseus, inhibits protein and nucleic acid synthesis in chlorella. The inhibition of nucleic acid synthesis is not immediate, however, and inhibition of RNA synthesis is more delayed than is DNA (Morris, 1966). Siegel and Sisler (1963) observed that the primary effect of cycloheximide on yeast cells was to inhibit protein synthesis by preventing the transfer of amino acids from the transfer RNA-amino acid complex to the polypeptide chain. deKloet (1966), also working with yeast, indicated

that cycloheximide caused the release of the control that amino acids have over RNA synthesis which causes a gradual decrease in RNA formation.

Cycloheximide inhibits the development of invertase in sugar beets (Cherry, 1969) lentil epicotyls (Seitz and Lang, 1968), Avena stem segments (Kaufman et al., 1968), and sugar cane (Gayler and Glasziou, 1969). Chrispeels and Varner (1967) observed the inhibtion of α-amylase on exposure of the aleurone layer to cycloheximide. In rat tissue, cycloheximide decreases the incorporation of amino acids into protein (Korner, 1966).

Another widely used inhibitor, actinomycin D, was shown by Leaver and Key (1967) to inhibit transition of monoribosomes to polyribosomes during the aging of carrot tissue. Chrispeels and Varner (1967) observed that actinomycin D prevented the synthesis of α-amylase formation if added immediately to the aleurone layer, but inhibited α-amylase formation to a much lesser extent if added during mid-course of α-amylase synthesis. Cherry (1969) suggested that actinomycin D inhibits DNA directed RNA synthesis thus preventing invertase development in sugar beet tissue.

Growth and invertase development are inhibited by actinomycin D in Avena stem segments (Kaufman et al., 1968), lentil epicotyls (Seitz and Lang, 1968) and sugar cane (Glasziou et al., 1966).

Cavalieri and Nemchin (1964) using calf thymus tissue and Escherichia coli, suggested that, actinomycin D inhibits RNA polymerase by competing with it for the binding sites on the DNA.

A purine analogue, 6-methylpurine, is another widely used nucleic acid inhibitor. Gayler and Glasziou (1969) observed that 6-methylpurine blocked RNA synthesis in sugar cane tissue. Soybean hypocotyls exposed to 6-methylpurine resulted in an inhibition RNA and protein synthesis, (Key, 1966) as well as α-amylase formation in aleurone layers of barley (Chrispeels and Varner, 1967).

A pyrimidine analogue, 5-fluorouracil, was demonstrated by Key (1966) to selectively inhibit ribosomal (r-RNA) and t-RNA synthesis without effecting the synthesis of r-RNA. Mayo et al. (1968), working with yeast cells, indicated that 5-fluorouracil selectively inhibits the formation of 28-S-RNA with no effect on the formation of 18-S-RNA. deKloet (1968) found that yeast cells exposed to 5-fluorouracil accumulated an abnormal high molecular weight RNA with a more DNA-like base composition. Incubation of Escherichia coli with 5-fluorouracil prevented the formation of normal 50-S ribosome particles. The r-RNA produced in the presence of 5-fluorouracil had sedimention characteristics similar to the normal 23-S and 16-S-RNA, however, the amount of the component corresponding to 23-S-RNA was much lower than normal (Sell and Crudup, 1966).

Ethionine is a classical example of an antimetabolite and has been widely used as a competitive inhibitor of methionine metabolism. Methionine adenosyltransferase catalyzes the synthesis of S-adenosylmethionine from L-methionine and adenosine triphosphate. Ethionine, an analogue of methionine, is also activated by this enzyme to form S-adenosylethionine (Stekol, 1964). S-adenosyl-L-methionine was shown by Fleissner and Borek (1962) to serve as the methyl donor for the methylation of certain nucleotides in various ribonucleic and deoxyribonucleic acids at the polynucleotide level. The exact function of these methylated bases remains uncertain, although recent findings have correlated amino acid activation to the degree of methylation of bacterial transfer RNA (Shugart et al., 1968).

Peterkofsky (1964) and Peterkofsky et al. (1966) working with Escherichia coli and yeast cells found that the leucyl-t-RNA synthetase from E. coli successfully attached leucine to t-RNA regardless of whether or not the transfer RNA (t-RNA) was methylated. However, they found the yeast enzyme non-functional if the t-RNA was not methylated. Mehtylated bases were demonstrated by Isaksson and Phillips (1968) to exist in both r-RNA and t-RNA of yeast cells.

Ortwerth and Novelli (1969) observed that when rats were injected with L-ethionine-1-^{14}C, the t-RNA contained a much greater

quantity of the ethionine label than r-RNA contained, indicating that t-RNA contained many more methylated bases than r-RNA. Moore and Smith (1969) demonstrated S-adenosylethionine to be an inhibitor of both bacterial and rat t-RNA methylation. Cox and Smith (1969) also showed ethionine to be a competitive inhibitor of methionine activation to S-adenosylmethionine.

Schrank (1956) observed that elongation of Avena coleoptile sections were inhibited by ethionine and that this inhibition was reversed by the addition of methionine. Cleland (1960) showed ethionine to be an inhibitor of auxin-induced elongation of Avena coleoptiles and that this inhibition could be reversed by methionine. Working with rats, Celander and George (1963) found that ethionine depressed the rate of growth and this depression could be reversed by the addition of methionine to the rats' diet.

Norris (1964) suggested that ethionine may exert its inhibitory effect on protein synthesis necessary for elongation of the oat coleoptile by interferring with ATP metabolism. Ethionine decreases the hepatic ATP concentration of rats. (Smith and Salmon, 1965 and Shull et al., 1966) It was postulated from this data that ethionine traps adenosine as S-adenosylethionine thus decreasing the concentration of ATP in the cell. A significant decrease in the cellular

concentration of ATP would inhibit the formation of S-adenosylmethionine from methionine and ATP, thus possibly preventing the methylation of RNA.

CHAPTER III

MATERIALS AND METHODS

Preparation and Washing of Tissue

Carrots were purchased locally in large batches, washed in alconox, rinsed with distilled water and stored for further use at 2-4°C. A number 6 cork-borer was employed to obtain rods 1 centimeter (cm.) in diameter from the outer phloem parenchyma region of the carrot root. These rods were sliced with a plexiglass-razor blade into discs approximately 1 millimeter (mm.) thick. The discs were packed in ice until washing (no longer than twenty-four hours).

The carrot discs were rinsed in distilled water, blotted on absorbent paper, and transferred to 50 mm. diameter petri dishes (fifteen discs per dish) containing 1 ml. of 0.01 M. phosphate buffer (PO_4), pH 6.5 with 50 µg./ml. chloramphenicol for the desired elapsed time. All experimental treatments were prepared in 0.01 M. PO_4 buffer, pH 6.5, chloramphenicol 50 µg./ml. Chloramphenicol inhibits bacterial growth without effecting the metabolic activity of tissue during washing (Leaver and Edelman, 1965). All treatments were replicated. The solutions were removed every 12 hours and replaced with fresh solutions.

Estimation of Invertase Activity

The carrot discs were aerated twenty-four and forty-eight hours, washed with cold distilled water, blotted on absorbent paper and dropped into a beaker cooled in ice which contained enough ethyl acetate to cover the discs (Bacon et al., 1965). The beakers were occasionally shaken by hand, during a ten minute period. The ethyl acetate was then decanted and the discs washed twice more with cold ethyl acetate for the same length of time. The ethyl acetate-treated discs were washed with cold distilled water, blotted on absorbent paper, transferred to 50 ml. flasks, and stoppered with plastic caps. Invertase activity of the treated tissue was assayed by incubating the discs in a 20 ml. solution of 0.16 M. sucrose and 0.045 M. sodium acetate, pH 5 (Cherry, 1969). The discs were gently shaken in a water bath at 30°C. for two hours.

The enzyme reaction was stopped after two hours by pipetting 5 ml. of cold five percent (w/v) sodium carbonate ($Na_2 CO_3$). Samples were shaken and kept cold until assayed. The amount of reducing sugar was determined according to Nelson (1944) on an alequot (0.1 ml.) of each Na_2CO_3 stopped samples. Nelson's alkaline copper reagent consists of 12.5 ml. of Nelson's reagent A (see appendix) mixed with 0.5 ml. of Nelson's reagent B (see appendix).

A 0.1 ml. volume of each Na_2CO_3 stopped sample was added to 1 ml. of Nelson's alkaline copper reagent and then diluted to 2 ml. with distilled water. Replicates of all samples were run. The tubes were then shaken, stoppered, and placed in a boiling water bath for twenty minutes. The tubes were shaken occasionally by hand during the heating. After twenty minutes the tubes were placed into a cold water bath and cooled to approximately 25°C. Then 1 ml. of arsenomolybdate (see appendix) was added to each sample. The tubes were shaken to enhance the dissolving of the copper oxide and diluted with distilled water to 10 ml. The tubes were shaken to facilitate thorough mixing of the solutions.

The optical density of each sample was read on the Gilford spectrophotometer at 540 nanometers (nm.). A blank sample (no sugar), treated in the same manner as the experimental samples, was used to set the absorbance to zero. During each assay three glucose standards (100, 50, and 10 µg./ml.) were included, thus enabling the amount of reducing sugar in the unknown samples to be calculated. The number of µg. hexose/milligram (mg.) fresh weight/hour in the fifteen discs was calculated.

Incorporation of Radioactive Precursors into Carrot Tissue

Synthesis and methylation of carrot RNA was studied by the incorporation of ^{3}H-uridine (specific activity 21.7 curies per millimole and ^{14}C-methionine (specific activity 0.25 microcuries per 4.1

milligrams) into isolated RNA. Carrot discs were prepared as previously described and packed in ice. The carrot tissue (25 grams per treatment) was washed in sterile distilled water, blotted on sterile absorbent paper, and placed into sterile 250 ml. flasks containing 35 ml. of filter sterilized 0.01 M. PO_4 buffer, pH 6.5, chloramphenicol 50 µg./ml. or other experimental treatments as described in the results. To each flask was then added 50 microcuries (µci.) ^{3}H-uridine and 20 µci. ^{14}C-methionine. The tissue was vacuum infiltrated for ten minutes.

The flasks were incubated in a 25°C. water bath and shaken vigorously for six hours after which solutions were decanted. The discs were washed three times with 150 ml. sterile distilled water and blotted on sterile absorbent paper.

Extraction and Characterization of RNA

Carrot discs were homogenized with a Virtis homogenizer in a solution (Cherry et al., 1965) containing 30 ml. of cold 0.01 M. Tris, pH 7.6; 0.06 M. potassium chloride; 0.01 M. magnesium chloride; 1.5 ml. bentonite (38 mg./ml.); 4.5 ml. of 11 percent Dupanol; and 33 ml. of cold 0.01 M. Tris washed phenol. The homogenizing flask was packed in ice during the homogenizing period. The discs were homogenized for one minute medium speed, thirty seconds low speed, one minute high speed, and again, thirty seconds at low speed. The homogenate transferred to 50 ml. polypropylene centrifuge tubes and

centrifuged in an International Refrigerated Centrifuge (Model B-20) at 20,000 x g. for ten minutes.

The aqueous layer, containing the radioactively labeled RNA, was carefully removed with a large syringe and needle. To this solution (kept on ice) was added 0.5 ml. bentonite and an equal volume of cold Tris washed phenol. The RNA mixture was stirred for ten minutes, centrifuged for ten minutes at 20,000 x g., and again the aqueous layer was removed. The phenol treatment was repeated and the mixture centrifuged at 20,000 x g. for ten minutes. Aqueous phases of each treatment were made 0.2 M. with potassium acetate after which two volumes of cold ninety-five percent ethanol were added to precipitate the RNA. All RNA solutions were stored 6-12 hours in the freezer. The RNA precipitant was collected by centrifuging the ethanol-potassium acetate-mixture for twenty minutes at 30,000 x g. The supernate was decanted and the precipitate dissolved into 2 ml. of ten percent 3-E buffer (see appendix). Once the radioactively labeled RNA precipitant was dissolved, 25 microliters (µl.) were removed and diluted to 1.5 ml. with ten percent 3-E buffer.

A ratio for estimation of radioactive labeled RNA present in the 25 µl. was obtained by reading the absorbancy at 260 and 280 nm. with a Gilford 240 spectrophotometer. The 280 nm. reading was divided by the 260 nm. absorbance and if the ratio was greater than 0.6, further

phenol extractions were necessary for removal of protein bound to the radioactively labeled RNA. The ug. RNA was determined by subtracting the absorbancy at 260 nm. from the 280 nm. reading and multiplying by the extinction coefficient of 50 (Cherry, 1962).

Specific activity (CPM/ug. RNA) was determined as follows: A 0.1 ml. volume of Carrier-RNA (1 mg. per ml.) was added to each 25 µl. sample of radioactively labeled RNA. The RNA was precipitated by adding two volumes of cold ten percent trichloroacetic acid (TCA) and stored in a cold room for a minimum of thirty minutes. The precipitated RNA was collected on a Glass Fibre/Type A filter and dried with an infra-red lamp. The dried filter was placed into scintillation vials containing 10-15 ml. of scintillation solution (see appendix) and the ^{3}H-uridine and ^{14}C-methionine activity determined (Wang and Willis, 1965). The counts per minute (CPM) minus the background for each sample divided by the µg. RNA yields the specific activity. Double label equations were used to estimate the disintegrations per minute of ^{3}H-uridine and ^{14}C-methionine incorporated into the isolated RNA.

The chemicals were purchased as follows:
D-L ethionine and chloramphenicol from Sigma Chemical Company, St. Louis, Missouri; L-methionine from Aceto Chemical Company, Flushing, New York; Gibberellic Acid (GA_3) from Eastman Organic Chemicals, Rochester, New York; 5-Fluorouracil from Hoffman-La Roche Incorporated, Nutley, New Jersey; cycloheximide, 6-methylpurine

and adenosine triphosphate from Nutritional Biochemical Corporation, Cleveland, Ohio; Uridine-5-^{3}H from International Chemical and Nuclear Corporation-Chemical and Radioisotope Division, Irvine California; L-methionine-methyl-^{14}C, New England Nuclear, Boston, Massachusetts, and actionomycin D, a gift from the Merch and Company, Rahway, New Jersey.

CHAPTER IV

RESULTS

Effect of Washing on the Development of Invertase

Cherry (1969) and Bacon et al. (1965) reported that invertase development in washed sugar beet tissue increases over a period of days. Carrot tissue was found to exhibit a similar response (Figure 1). A 2.4 and 4 fold increase in invertase development was observed after twenty-four and forty-eight hours respectively.

Effect of Ethionine, Gibberellic Acid, and Methionine on the Development of Invertase

Ethionine, the ethyl analogue of methionine, was shown by Schrank (1956) and Cleland (1960) to inhibit elogation of Avena coleoptiles. In agreement with the work previously mentioned, ethionine was found to inhibit invertase development in carrot tissue (Figure 2). Ethionine 0.075 M., 0.025 M., and 0.0125 M. exhibited approximately the same inhibition of invertase development in both the twenty-four and forty-eight hour washed tissue. Ethionine 0.01 M. was the threshold concentration of invertase inhibition.

It was desirable to determine at what time ethionine exerted its greatest inhibition on the development of invertase. Table I illustrates the effect of ethionine at various time intervals on the development of

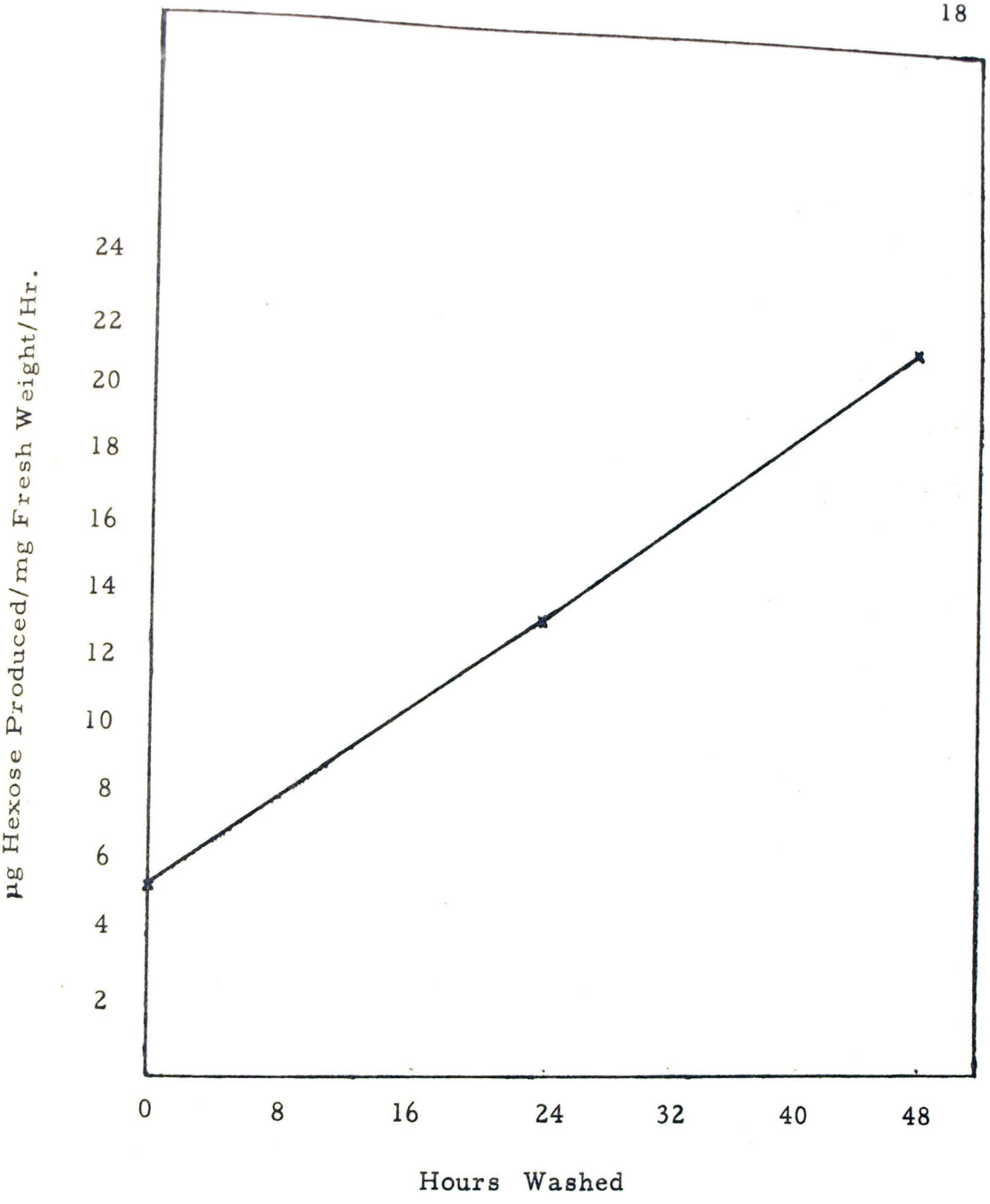

Figure 1. Effect of washing on the development of invertase. Tissue was washed 24 and 48 hours in 0.01M PO_4 buffer, pH 6.5 with chloramphenicol 50 µg/ml. Unwashed tissue was packed in ice until invertase assay.

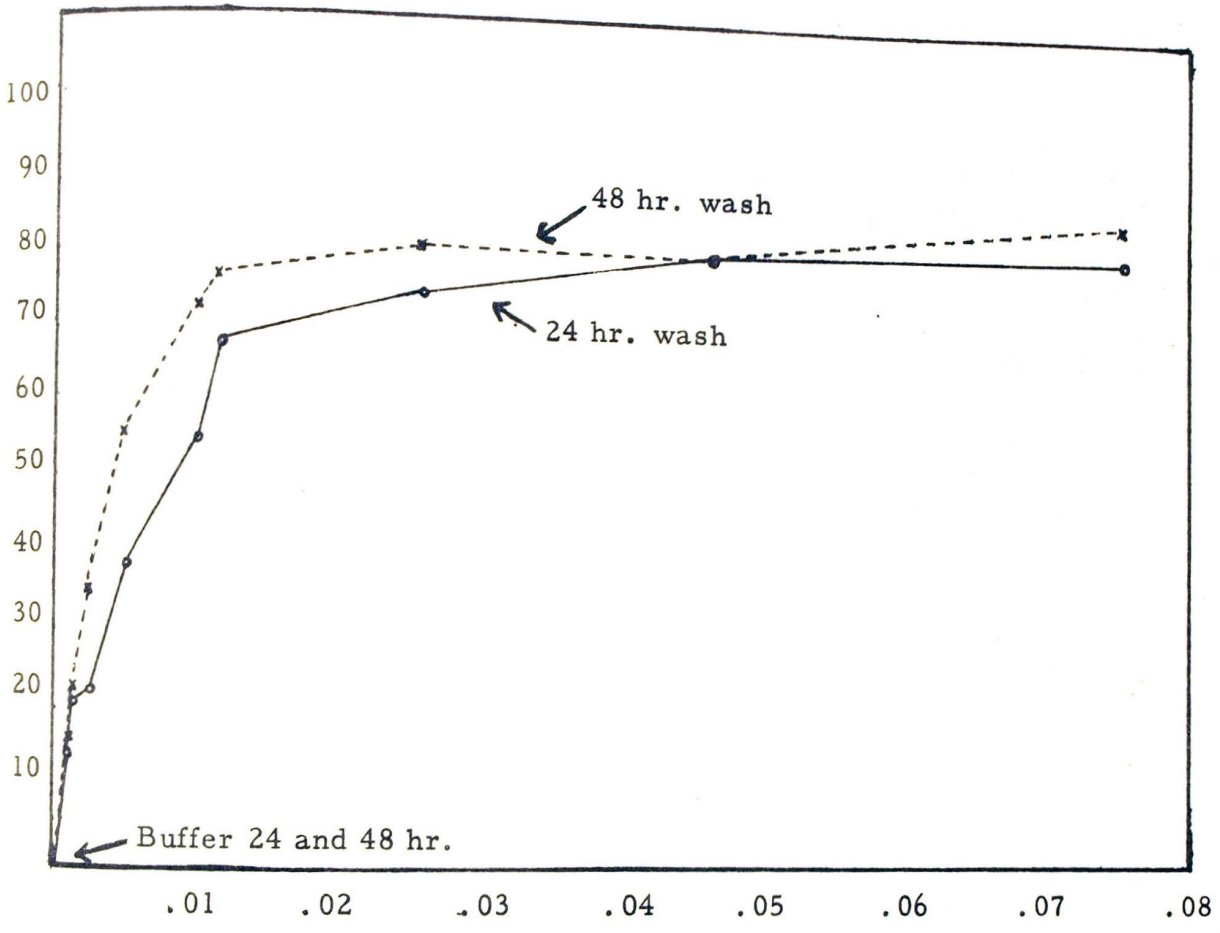

Figure 2. Effect of Ethionine on the development of invertase. Control tissue was washed 24 and 48 hours in 0.01M PO_4 buffer, pH 6.5 with chloramphenicol 50 µg/ml. and subsequent invertase activity in buffer washed tissue was 31.4 and 29 respectively. Unwashed tissue after 24 and 48 hours was 6.3 and 2.6 respectively.

TABLE I

EFFECT OF ETHIONINE AT VARIOUS TIME INTERVALS ON THE DEVELOPMENT OF INVERTASE

Treatment Duration in Ethionine	µg Hexose Produced/mg Fresh Weight/Hr Percent of Control 24 Hour Wash*
0-24 hours	33%
1-24 hours	31%
3-24 hours	40%
6-24 hours	58%
8-24 hours	58%
10-24 hours	69%
12-24 hours	76%
15-24 hours	95%

*Control tissue was washed 24 hours in 0.01m PO_4 buffer, pH 6.5 with chloramphenicol 50µg/ml. and subsequent invertase activity in the buffer washed tissue was 20.3. Unwashed tissue 4.4. Ethionine concentration 0.01M.

invertase in carrot tissue. If the carrot tissue was allowed to age for fifteen hours without exposure to ethionine no significant inhibition was observed. A twenty-four and thirty-one percent inhibition of invertase development was observed in carrot tissue exposed to ethionine after ten and twelve hours respectively. The data presented in Table I indicated that the critical period of ethionine inhibition was between 0 and 8 hours.

Gibberellic acid (GA_3) was shown by Edelman and Hall (1964 and 1965) and Cherry (1969) to stimulate invertase development in excised storage tissue. Gibberellic acid was demonstrated to have a similar stimulatory effect on invertase development in carrot tissue (Table II). Gibberellic acid (10 µg./ml.) exerted a sixty-two percent increase in invertase development.

Schrank (1956) and Cleland (1960) observed that ethionine inhibited Avena coleoptile elongation and that the inhibition was reversible by the addition of methionine. Similar effects were observed with the development of invertase in two different experiments conducted with carrot tissue (Table III). Also illustrated in Table III is the stimulatory effect of GA_3 on the development of invertase. When ethionine was present in the GA_3 treated tissue, the GA_3 stimulation was diminished to the level of ethionine alone. The GA_3 stimulation was reestablished when the tissue treated with ethionine was subjected

TABLE II

EFFECT OF GIBBERELLIC ACID ON THE DEVELOPMENT OF INVERTASE

Treatment	µg Hexose Produced/mg Fresh Weight/Hr. Percent of Control
	48 Hour Wash*
GA 0.01 µg/ml	103%
GA 0.1 µg/ml	128%
GA 1 µg/ml	128%
GA 5 µg/ml	144%
GA 10 µg/ml	162%
GA 25 µg/ml	135%
GA 50 µg/ml	135%
GA 75 µg/ml	156%
GA 100 µg/ml	157%

* Control tissue was washed 48 hours in 0.01M PO_4 buffer, pH 6.5 with chloramphenicol 50 µg/ml and subsequent invertase activity in buffer washed tissue was 26.9. Unwashed tissue 2.1.

TABLE III

EFFECT OF GIBBERELLIC ACID, ETHIONINE, AND METHIONINE ON THE DEVELOPMENT OF INVERTASE

Treatment	µg Hexose Produced/mg Fresh Weight/Hr. Percent of Control	
	48 Hour Wash*	
	Experiment 1	Experiment 2
GA 10 µg ml.	139%	135%
Ethionine 0.01M	22%	22%
Methionine 0.01M	85%	93%
GA 10 µg ml +Eth. 0.01M	29%	19%
Eth. 0.01M+Meth. 0.01M	90%	93%
Eth. 0.01M+Meth. 0.005M	84%	95%
GA 10 µg/ml +Eth. 0.01M+ Meth. 0.01M	125%	139%

* Control tissue was washed 48 hours in 0.01M PO_4 buffer, pH 6.5 chloramphenicol 50 µg/ml. and subsequent invertase activity in buffer washed tissue in experiment 1 and 2 was 26 and 36 respectively. Unwashed tissue in experiment 1 and 2 was 5.1 and 8.7 respectively.

to methionine. Methionine had no significant effect on the development of invertase (Table III).

Effect of Ethionine and Adenosine Triphosphate on the Development of Invertase

Norris (1964) reported ethionine to be an inhibitor of Avena coleoptile elongation and that the inhibition could be reversed (85-90 percent of control) by the addition of ATP. He also reported ATP to exhibit a stimulatory effect on Avena coleoptile elongation. No significant reversal of ethionine inhibition of invertase development was observed by the addition of ATP to carrot tissue during aeration (Table IV). Adenosine triphosphate exhibited no significant effect on invertase development.

Effect of Cycloheximide, 5-Fluorouracil, 6-Methylpurine, and Actinomycin D on the Development of Invertase

A number of chemicals have been reported to interfere with protein and nucleic acid synthesis resulting in inhibition of plant growth. It was desirable to determine whether or not these inhibitors would interfere with invertase development in washed carrot tissue. Cycloheximide was shown to inhibit invertase development in sugar beet (Cherry, 1969) and sugar cane (Gayler and Glasziou, 1969). It was found that cycloheximide had a similar effect on the development of invertase in carrot tissue (Table V). Cycloheximide at 10 and 20 µg./ml. was shown to inhibit invertase development 54 and 91 percent

TABLE IV

EFFECT OF ETHIONINE AND ADENOSINE TRIPHOSPHATE ON THE DEVELOPMENT OF INVERTASE

	µg Hexose Produced/mg Fresh Weight/Hr. Percent of Control	
	48 Hour Wash*	
	Experiment 1	Experiment 2
Ethionine 0.01M	20%	23%
Eth. 0.01M+ATP 15mM	---	18%
Eth. 0.01M+ATP 10mM	36%	---
Eth. 0.01M+ATP 1mM	34%	40%
Eth. 0.01M+ATP 0.1mM	33%	36%
Eth. 0.01M+ATP0.1mM	19%	25%
ATP 15 mM	---	92%
ATP 10 mM	85%	---
ATP 1 mM	80%	95%
ATP 0.1mM	81%	97%
ATP 0.01mM	94%	93%

* Control tissue was washed 48 hours in 0.01M PO_4 buffer, pH 6.5 with chloramphenicol 50 µg/ml. and subsequent invertase activity in experiment 1 and 2 was 17.4 and 23.9 respectively. Unwashed tissue in experiment 1 and 2 was 4.7 and 5.7 respectively.

TABLE V

EFFECT OF CYCLOHEXIMIDE, 5-FLUOROURACIL, AND 6-METHYLPURINE ON THE DEVELOPMENT OF INVERTASE

Treatment	µg Hexose Produced/mg Fresh Weight/Hr. Percent of Control	
	24 Hour Wash*	48 Hour Wash*
Cycloheximide 20µg/ml.	29%	9%
Cycloheximide 10 µg/ml.	35%	46%
5-Fluorouracil 250 µg/ml.	70%	85%
5-Fluorouracil 100 µg/ml.	80%	81%
5-Fluorouracil 50 µg/ml.	88%	76%
6-Methylpurine 20 µg/ml.	20%	38%
6-Methylpurine 10 µg/ml.	43%	53%

* Control tissue was washed 24 and 48 hours in 0.01M PO_4 buffer, pH 6.5 with chloramphenicol 50µg/ml. and subsequent invertase activity in buffer washed tissue was 27.9 and 25.9 respectively. Unwashed tissue after 24 and 48 hours was 8.42 and 3.9 respectively.

respectively, in tissue washed for forty-eight hours. Tissue washed for twenty-four hours in cycloheximide exhibited approximately the same inhibition for both concentrations of cycloheximide.

An analogue of uracil, 5-fluorouracil, was reported by Key (1966) to have no effect on growth and protein synthesis of soybean hypocotyl, but selectively inhibited r-RNA and t-RNA synthesis without effecting the synthesis of m-RNA. Contrary to the work of Key, the data in Table V indicated that 5-fluorouracil was a slight inhibitor of invertase development in carrot tissue. Carrot tissue washed in 5-fluorouracil (250 µg./ml.) for 24 hours exhibited the greatest inhibitory effect on invertase development (30 percent inhibition).

Another widely used inhibitor of nucleic acid synthesis is 6-methylpurine. Gayler and Glasziou (1969) reported 6-methylpurine to inhibit invertase development in sugar cane. In agreement with the work of Gayler and Glasziou (1969) 6-methylpurine inhibited invertase development in carrot tissue (Table V). The concentration at which 6-methylpurine exhibited maximum inhibition of invertase development in both the twenty-four and forty-eight hour washed tissue was 20 µg./ml.

Cherry, (1969) indicated that sugar beet tissue washed with actinomycin D for only the first six or twelve hours severely inhibited the development of invertase. However, tissue exposed to actinomycin D at six-hour intervals after twelve hours progressively inhibited

TABLE VI

EFFECT OF ACTINOMYCIN D ON THE DEVELOPMENT OF INVERTASE

Treatment	µg Hexose Produced/mg Fresh Weight/Hr. Percent of Control	
	24 Hour Wash*	48 Hour Wash*
Actinomycin D 1 µg/ml	100%	113%
Actinomycin D 5 µg/ml	82%	90%
Actinomycin D 10 µg/ml	74%	72%
Actinomycin D 25 µg/ml	45%	38%
Actinomycin D 50 µg/ml	20%	18.5%
Actinomycin D 75 µg/ml	5%	18%

* Control tissue was washed 24 and 48 hours in 0.01M PO_4 buffer, pH 6.5 with chloramphenicol 50 µg/ml. Subsequent invertase activity in buffer washed tissue was 25.4 and 29.6 respectively. Unwashed tissue after 24 and 48 hours was 5.18 and 5.75 respectively.

invertase development to a lesser extent. Actinomycin D inhibited the development of invertase in carrot tissue (Table VI). Actinomycin D (75 μg./ml.) exhibited the greatest inhibitory effect on invertase development in both the twenty-four (95 percent inhibition) and forty-eight hour (82 percent inhibition) washed tissue. At 1 μg./ml., actinomycin D slightly stimulated invertase development in forty-eight hour washed tissue.

Effect of Gibberellic Acid, Ethionine, and Actinomycin D on the Development of Invertase

The stimulatory effect of GA_3 (Tables II and III) and the inhibitory effect of actinomycin D (Table VI) and ethionine (Figure 1 and Table I) on the development of invertase has already been illustrated. Similar results are presented in Table VII. With the addition of actinomycin D to the GA_3 treated tissue, the level of invertase reached that of actinomycin D alone. The combination of ethionine and actinomycin D resulted in a invertase level similar to that of actinomycin D alone. Assuming that ethionine inhibited methylation of RNA and actinomycin D inhibits synthesis of RNA, no definite conclusions concerning the effects of synthesis and methylation of RNA on invertase development may be drawn from the data illustrated in Table VII.

TABLE VII

EFFECT OF ETHIONINE, GIBBERELLIC ACID, AND ACTINOMYCIN D ON THE DEVELOPMENT OF INVERTASE

Treatment	µg Hexose Produced/mg Fresh Weight/Hr. Percent of Control
	24 Hour Wash *
Actinomycin D 75 µg/ml.	17%
Actinomycin D 25 µg/ml.	22%
Gibberellic Acid 10 µg/ml.	128%
Ethionine 0.01M	32%
GA_3 10 µg/ml +Act. D 75 µg/ml.	13%
Act. D 25 µg/ml. +Eth. 0.01M	18%

* Control tissue was washed 24 hours in 0.01M PO_4 buffer, pH 6.5 with chloramphenicol 50 µg/ml. and subsequent invertase activity in the buffer washed tissue was 26.1. Unwashed tissue 7.8.

Effect of Gibberellic Acid, Ethionine, Actinomycin D, and 5-Fluorouracil on Synthesis and Methylation of Carrot Tissue Nucleic Acids

Gibberellic acid had no significant effect on synthesis (^{3}H-uridine incorporation) and methylation (^{14}C-methionine incorporation) of carrot tissue ribonucleic acid (Table VIII). Ethionine severely inhibited methylation (81 percent) with a less severe effect on synthesis (54 percent) of carrot tissue RNA. The addition of GA_3 to the ethionine treated tissue exhibited no significant change in synthesis or methylation of RNA (Table VIII).

Actinomycin D (50 µg./ml.) severely inhibited synthesis (78 percent) with a less severe effect on methylation (59 percent) of carrot tissue RNA (Table IX). Invertase activity was inhibited eighty percent by actinomycin D, 50 µg./ml. (Table VI). At a concentration of 10 µg./ml. actinomycin D inhibited synthesis (41 percent) and methylation (30 percent) of carrot tissue RNA. This was approximately fifty percent of the inhibition observed at 50 µg./ml. The inhibition of invertase activity at 10 µg./ml. was only twenty-five percent of that observed at 50 µg./ml. (Table VI).

At a concentration (100 µg./ml.) 5-fluorouracil was shown to inhibit synthesis (50 percent) and methylation (25 percent) of carrot RNA. At the same concentration, 5-fluorouracil slightly inhibited invertase development (Table V). As indicated by the $^{3}H/^{14}C$ ratio (Table IX),

the RNA extracted from the tissue treated with 5-fluorouracil and actinomycin D contained more of the ^{14}C label per μg. RNA than the control tissue. This suggests the possibility that performed RNA may be methylated in the early stage of tissue aeration.

TABLE VIII

INCORPORATION OF ^{3}H-URIDINE AND ^{14}C-METHIONINE INTO CARROT TISSUE NUCLEIC ACID

Treatment	Disintegration Rate/Minute/µg Nucleic Acid		
	H ^{3}-Uridine	^{14}C-Methionine	$^{3}H/^{14}C$ Ratio
Control (buffer)*	1,058,320	3312	320
Gibberellic Acid 10 µg/ml	945,120	2832	334
Ethionine 0.01M	489,280	640	764
Eth. 0.01M+GA_3 10 µg/ml	554,960	800	694

* Control tissue was washed in 0.01M PO_4 buffer, pH 6.5 with chloramphenicol 50 µg/ml. Twenty-five grams of carrot discs were washed for 6 hours in 35 ml. of the prescribed treatment. Included was 50 µci uridine-5-^{3}H and 20µci L-methionine-methyl-^{14}C.

TABLE IX

INCORPORATION OF ^{3}H-URIDINE AND ^{14}C-METHIONINE INTO CARROT TISSUE NUCLEIC ACID

Treatment	Disintegration Rate/Minute/µg Nucleic Acid		
	^{3}H-Uridine	^{14}C-Methionine	$^{3}H/^{14}C$ Ratio
Control (buffer)*	983,760	4928	200
5-Fluorouracil 100 µg/ml.	493,920	3672	135
Actinomycin D 50 µg/ml.	220,960	2008	110
Actinomycin D 10 µg/ml.	583,440	3424	170

* Control tissue was washed in 0.01M PO_4 buffer, pH 6.5 with chloramphenicol 50 µg/ml. Twenty-five grams of carrot discs were washed for 6 hours in 35 ml. of the prescribed treatment. Included was 50 µci uridine-5-^{3}H and 20 µci L-methionine-methyl-^{14}C.

CHAPTER V

DISCUSSION

The present study shows that invertase development of washed excised carrot tissue increases four fold over a period of forty-eight hours. Similar results occur in sugar beet (Bacon et al., 1965) and carrot (Vaughan and MacDonald, 1967). In agreement with Cherry (1969), Edelman and Hall (1964 and 1965), and Gayler and Glasziou (1969), gibberellic acid GA_3 was shown to increase invertase development. However, in the present study, GA_3 had no significant effect on synthesis or methylation of carrot tissue RNA. This data suggests that GA_3 stimulation is not due to the increase in synthesis or methylation of RNA. However, it may be possible that GA_3 accelerates the synthesis of a specific m-RNA which is necessary for invertase development without a quantitative change in the RNA concentration. At the present no definite explanation for GA_3 stimulation of invertase can be offered.

For the synthesis of protein to occur, information from DNA is programmed on m-RNA and the information carried to the ribosome, at which point the m-RNA and ribosome combine to form a m-RNA-ribosomal complex. The amino acids necessary for the synthesis of

the programmed protein are activated by a specific adaptor molecule called t-RNA. The t-RNAs and amino acids are linked by a covalent bond to form amino acyl-S-RNAs, These complexes move to specific sites on the m-RNA-ribosomal complex where the orderly peptide bond formation of amino acids take place with a discharge of the t-RNA moieties (Conn and Stumpf, 1967). S-adenosylmethionine serves as the methyl donor for the methylation of certain nucleotides in various ribonucleic and deoxyrobonucleic acids. (Fleissner and Borek, 1962) The exact function of these methylated bases remains uncertain but recent findings have correlated amino acid activation to the degree of methylation (Shugart et al., 1968; Peterkofsky, 1964; and Peterkofsky et al., 1965).

The present study showed that ethionine severely inhibited the synthesis and methylation of carrot tissue RNA as well as the development of invertase. Considering the work of Shugart et al. (1968), Peterkofsky (1964) and Peterkofsky et al. (1965) the data obtained in this research suggested the possibility that ethionine inhibits methylation of t-RNA which is necessary for amino acid activation. The inhibition of amino acid activation results in a decrease in protein synthesis and a subsequent decrease in invertase development. It is possible that ethionine has a direct inhibitory effect on both synthesis and methylation

of RNA, which would result in a decrease in invertase development.

In agreement with Schrank (1956) and Cleland (1960) methionine was found to reverse the inhibition of ethionine. Methionine may be reversing the inhibition of ethionine by serving as an indirect methyl donor to the t-RNA, thus allowing amino acid activation and protein synthesis to occur at the normal rate necessary for invertase development. Contrary to the work of Norris (1964) ATP had no significant effect on the reversal of ethionine inhibition. Due to the fact that Norris (1964) was working with a different system (Avena coleoptiles), ethionine may have exerted its inhibitory effect on a different cellular mechanism. This could possibly explain why ATP did not reverse the ethionine inhibition of invertase development in carrot tissue. Further research may reveal the mechanism by which ethionine exerts its inhibition of invertase development in excised storage tissue.

Morris (1966) and Siegel and Sisler (1963) and deKloet (1966) found that cycloheximide inhibits protein synthesis. In the present study cycloheximide inhibited the development of invertase. If cycloheximide only inhibits protein synthesis, this data suggests that protein synthesis is necessary for development of invertase. However, unknown inhibitory effects of cycloheximide may exist and cannot be eliminated.

A selective inhibitor of r-RNA synthesis (deKloet, 1968; Mayo et al., 1968; and Sell and Crudup, 1966), 5-fluorouracil was shown to slightly inhibit invertase development and methylation of RNA, but inhibited synthesis of RNA by fifty percent. This data suggests that synthesis and methylation of the other RNA types are more essential for invertase development than r-RNA synthesis and methylation.

Actinomycin D and 6-methylpurine, widely used nucleic acid synthesis inhibitors, severely inhibited the development of invertase. Cherry (1969), Glasziour et al. (1965). Gayler and Glasziou (1969) and Key (1966) obtained similar results. Biochemical analysis of radioactively labeled precursors into RNA of carrot tissue revealed that actinomycin D (50 μg./ml.) exhibited approximately the same inhibition of RNA synthesis as invertase development but inhibited methylation of RNA to a lesser degree. This data suggests that actinomycin D primarily inhibits RNA synthesis with a secondary effect on methylation. Ethionine 0.01 M. inhibited methylation of RNA to approximately the same extent as actinomycin D (50 μg./ml.) inhibited synthesis of RNA. However, actinomycin D (50 μg./ml.) inhibited methylation of RNA to the same extent as ethionine 0.01 M. inhibited RNA synthesis. Actinomycin D (50 μg./ml.) and ethionine 0.01 M. inhibited invertase development to approximately the same extent (80 percent). This data suggests that DNA directed RNA synthesis and methylation of RNA are equally necessary for invertase development.

CHAPTER VI

SUMMARY

The objective of this research was to determine the inhibitory effect of ethionine on invertase development in carrot tissue. Attempts were made to reverse the inhibition of ethionine by the addition of methionine and adenosine triphosphate. Tritiated uridine and methionine-methyl-^{14}C were employed to determine the inhibitory effect of ethionine, 5-fluorouracil, and actinomycin D upon synthesis and methylation of ribonucleic acid (RNA). Other inhibitors such as 6-methylpurine and cycloheximide were used to observe their effect on invertase development. Gibberellic acid (GA_3) was employed to determine the interrelationship of GA_3 stimulation of invertase and synthesis and methylation of carrot tissue RNA.

A four fold increase in development of invertase in excised carrot tissue was observed after a forty-eight hour washing. Ethionine 0.01 M. severely inhibited invertase development (approximately 75 percent) and methylation of RNA (80 percent) with a lesser inhibition of RNA synthesis (55 percent). Methionine 0.01 M. and 0.005 M. reversed the inhibition of ethionine on the development of invertase. Adenosine triphosphate (0.01 mM. to 15 mM.) had no significant reversal effect on ethionine

inhibition of invertase development. Cycloheximide and 6-methylpurine (10 μg./ml. and 20 μg./ml.) significantly inhibited the development of invertase. Actinomycin D (50 μg./ml.) severely inhibited invertase development and RNA synthesis (approximately 80 percent) with a lesser inhibition in RNA methylation (60 percent). The addition 5-fluorouracil (100 μg./ml.) significantly inhibited synthesis of RNA (50 percent) but slightly inhibited invertase development and methylation of RNA (approximately 20 percent). This data may indicate that the synthesis and methylation of other RNA species are more essential for invertase development than the synthesis and methylation of r-RNA. Gibberellic acid (10 μg./ml.) stimulated invertase development (25 to 60 percent) but exhibited no significant effect on synthesis and methylation of RNA. These results indicate that protein and RNA synthesis as well as methylation of RNA are essential for invertase development in excised carrot tissue.

APPENDIX

PREPARATION OF CHEMICALS USED IN RESEARCH

Nelson's Reagent A: 12.5 g. anhydrous Na_2CO_3, 12.5 g. potassium tartrate, 10 g. $NaHCO_3$, and 100 g. anhydrous Na_2SO_4, diluted to 500 ml. with distilled water

Nelson's Reagent B: 7.5 g. $CuSO_4 \cdot 5H_2O$ in 50 ml. distilled water and add one drop of concentrated H_2SO_4

Arsenomolybdate: 25 g. $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 450 ml. distilled water and 21 ml. of concentrated H_2SO_4. Dissolve 3 g. of $Na_2HASO_4 \cdot 7H_2O$ and add to the above acid molybdate. Store in a brown bottle for twenty-four hours at 37°C. after which time it may be stored at room temperature.

3-E Buffer: 0.12 M. Tris, 0.06 M. Sodium acetate, 0.003 M. sodium EDTA per liter, pH 7.2 with glacial acetic acid

Scintillation Fluid: 4 g. 2, 5-diphenyloxazole and 50 mg. p-bis 2-(4 methyl-5-phenyloxasoly) benzene per liter

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