I. CHEMOTHERAPY OF HIGH TEMPERATURE INHIBITION OF PLANT GROWTH

II. STUDIES ON THE RELATIONSHIP BETWEEN TOBACCO MOSAIC VIRUS INFECTION AND THE DNA METABOLISM OF TOBACCO LEAVES

III. THE CONSERVATION OF MICROSOMAL RNA IN ESCHERICHIA COLI

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ABSTRACT

I. Chemotherapy of High Temperature Inhibition of Plant Growth

The effects of biochemical supplements on the growth of five subterranean clover varieties grown under supra-optimal temperature conditions were studied. Some evidence of a chemotherapeutic effect of the high temperature growth inhibition was obtained.

II. Studies on the Relationship between Tobacco Mosaic Virus Infection and the DNA Metabolism of Tobacco Leaves

A comparison of the DNA metabolisms of uninfected and TMV infected excised tobacco leaves, using P^{32}-orthophosphate incorporation into the DNA as a measure of its metabolism, indicated that the DNA metabolism is not affected by TMV infection. This result was corroborated by the results of studies on the effect of 5-fluorouracil, a specific inhibitor of DNA synthesis, on the multiplication of TMV in tobacco-leaf discs. Although partial inhibition of TMV multiplication was observed, the absence of inhibition reversal by thymidine, indicated that the mechanism of TMV inhibition probably did not involve a specific block of DNA synthesis. Finally unsuccessful attempts were made to see if intact host DNA was necessary for TMV infection by treating tobacco-leaf discs with DNAase.

III. The Conservation of Microsomal RNA in Escherichia coli

A uniformly C^{13} N^{15} labeled exponentially growing culture of E. coli was transferred to light isotope substrates, and the metabolic fate of the pre-transfer synthesized heavy isotope microsomal RNA molecules followed by means of equilibrium sedimentation analysis of the RNA molecules in a density gradient. The results demonstrated complete conservation of the heavy isotopes by the pre-transfer RNA molecules remaining intact after transfer.

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SECTION I

CHEMOTHERAPY OF HIGH TEMPERATURE INHIBITION OF PLANT GROWTH

INTRODUCTION

The measure of a plant's adaptation is the efficiency with which it converts light energy into fixed chemical energy. The underlying mechanism of this conversion is a complex of biochemical reactions, each possessing genetically controlled reaction norms whose specific characteristics have developed in response to natural selection. A plant grown in an environment which is marginal for one or more of its component metabolic processes shows, a relatively inefficient fixing of light energy.

Most variables of the environment have been brought largely under control, but temperature variation is still a major limiting factor of energy fixation. Because of the day-to-day and season-to-season variation in temperature, precise adaptation of a genotype is improbable. Hence, even in the region where a plant is said to be "fully adapted," marginal conditions must arise from time to time for some of its component processes.

Although the higher plant has always been considered an autotrophic organism, there exists the possibility of relieving temperature-induced biochemical stresses by the application of appropriate organic nutrients (1). By the use of such nutrients, it may be possible to improve yields of crops in their present areas of cultivation, and perhaps also to extend these areas into marginal regions. This concept of the chemical

cure of climatic lesions may even be extended to the replacement of specific temperature effects in plant differentiation, such as in floral induction.

In the work reported here, attention will be confined to the chemotherapy of growth inhibition by unfavorably high temperatures. However, work on the substitution of chemical treatments for the specific temperature requirements of floral induction will be discussed in the literature review.

LITERATURE REVIEW

The first section of the review is devoted to studies made with biochemical temperature sensitive mutants found in microorganisms, especially those occurring in Neurospora.

The second section of the review deals with published and unpublished reports of successful chemotherapy of temperature effects on the growth of higher plants, and with the cases of chemically directed differentiation involved in flower formation, which are normally dependent upon specific temperature stimuli.

Temperature Sensitive Mutants in Microorganisms

In the course of study of biochemical mutants in Neurospora crassa and Escherichia coli, a number of mutants were found which displayed a prototrophic phenotype at low temperatures, but needed specific biochemical supplements for normal growth at higher temperatures. These mutants were called temperature-sensitive mutants.

The first such mutant was reported in 1946 (2). It showed normal growth at temperatures up to 28°C, but did not grow at all above this temperature. Growth at high temperatures was partially restored by the addition of adenine to the medium. Complete restoration of normal growth was dependent upon the simultaneous addition of adenine, histidine, and methionine. The latter two substances were completely ineffective when supplied singly or together in the absence of

adenine. This case is particularly interesting in that it shows that even a single lesion may have a complex requirement for a complete cure.

A riboflavin-requiring temperature sensitive mutant (3), gave an unusual temperature riboflavin dose growth response interaction. The growth response to a limiting dose of riboflavin decreased with increasing temperature to 30°C, then increased to a maximum response at 37°C. Furthermore, this mutant proved to be only partially blocked for riboflavin synthesis at high temperatures; it could synthesize its own riboflavin when supplemented with riboflavin.

A temperature sensitive uracil-requiring mutant has been isolated (4). Two other uracil requiring mutants occur at the same locus, but these are complete blocks with respect to uracil requirement.

In E. coli, a pantothenate-requiring temperature sensitive mutant was isolated. The enzyme catalyzing the condensation of β-alanine with pantoic acid to form pantothenic acid in this mutant was more heat labile than the normal enzyme.

The interpretation of the temperature sensitivity of the adenine and the uracil auxotrophs in Neurospora would be consonant with the production of a heat labile enzyme. The case of the riboflavin temperature sensitive auxotroph seems to be more complex. One interpretation could be that Neurospora possesses two genes for the production of riboflavin, one which is active over one temperature range, and the other

which is operative over another temperature range. The switch from one template to the other is under the control of a temperature modulated switch gene. The mutant is visualized as a defect in this switch control gene.

<u>Chemotherapy of the effects of unfavorable temperatures on growth and survival in plants</u>

The partial removal of low temperature growth inhibition in Cosmos by thiamine, reported in 1943 (6), is the first recorded instance of a successful chemical cure of a climatic lesion. Here thiamine elicited a significant growth response only in plants grown at temperatures below the optimum for Cosmos. At temperatures where the dry weight accumulation of the plants was 5% of comparable plants grown under optimal temperature conditions, the application of thiamine stimulated a 40% increase in dry weight yield. At temperatures where the dry weight yield was one half maximum, thiamine stimulated a 20% increase.

The maximum possible response to the chemical alleviation of a specific low temperature induced deficiency is limited by the overall slowing down of the metabolism by low temperatures. Thus the thiamine treatment may well have effected a complete cure at the half maximum temperature.

The removal of the high temperature inhibition of extension growth in excised pea stem sections by adenine has been studied (7). Again a temperature-adenine growth response

interaction was obtained. The adenine stimulated growth increment at the optimal temperature (30°C) was half the increment obtained at 35°C. These results indicate that high temperature growth inhibition in whole pea plants may be in part due to an adenine deficiency. However, further studies on pea growth (8) in the temperature controlled facilities of Earhart Laboratory gave very little, if any, indication that adenine is effective in relieving high temperature growth inhibition. In this experiment, the day temperature was kept constant and the night temperature was varied from low to high levels. Perhaps adenine limitation is a lesion of high day temperatures.

In another experiment with the same pea variety (9), a different system of temperature treatments, wherein the night temperature was 6°C lower than the day temperature, was unable to elicit any growth response to adenine at any temperature level. Despite these negative results with whole plant studies, the adenine effect may have some significance, in that the total adenine level of a high temperature tolerant pea strain increases with increasing growing temperature, whilst the total adenine level of a high temperature sensitive strain decreases with increasing growing temperature (10). Of course, these changes in adenine level may merely reflect changes in RNA status associated with meristematic activity, which are the result, and not the cause of the primary lesion. In the whole plant studies, other high temperature induced limitations could be occurring, which would preclude the detection of an

adenine effect unless these other limitations were also removed.

Adenosine and guanosine were found to be effective in preventing high temperature death in Lemna minor cultured at 29°C (11). This result has unfortunately not been repeated.

A genotype dependent auxin limitation in two corn varieties was shown to be affected by temperature (8). At 20°C day/14°C night, the "silkless" variety gave a 50% elongation response to applied indole acetic acid, whereas the "normal" variety gave no response. However at 26°C day/20°C night, the "silkless" variety showed no response to the applied auxin, whilst the "normal" variety gave a 16% elongation response.

Gibberellin applied to Alaska peas growing at high temperatures has a striking effect in delaying the high temperature accelerated senescence which accompanies the onset of maturity (9).

A very clear cut case of effective chemotherapy of high temperature growth and development inhibition has been reported (12). In this case, a spontaneous mutant of Arabidopsis thalliana, which shows arrested growth and abnormal development above 27°C, showed a restoration of normal growth and development when supplemented with 20 µg of choline per plant. This case is completely analogous to the cases of temperature sensitive mutants in Neurospora.

Temperature mimetic chemicals in floral induction

The cell elongation hormone, gibberellin, has recently been found to be involved in the temperature response mechanism for floral induction. Biennial long day plants, such as Hyoscyamus niger, require a period of low temperature exposure in order to initiate floral differentiation. The application of gibberellin to Hyoscyamus grown in warm temperatures under short day conditions, results in bolting, but no flowering (13). If the experiment is conducted under long day conditions, flowering occurs. Thus gibberellin replaces the need for a low temperature exposure normally essential for induction of stem elongation. Other biennials, such as Daucus, Samolus, and Crepis species (14) also respond to gibberellin application by flowering during warm temperature and long days.

Vernalization factors have been extracted from spring rye and vernalized winter rye (10). These extracts, when applied to unvernalized winter rye, replace its vernalization requirement for flowering. Cytidine and guanosine, although not identified with natural vernalization factors, show vernalization activity when applied to cold-requiring pea varieties.

These results constitute a start in the understanding of the chemical basis of temperature mediated differentiation processes in plants, and should eventually provide a fruitful field in the chemical control of differentiation--an aspect of the overall problem of the chemical cure of climatic lesions.

PROBLEM OUTLINE

At this early stage of the problem, the seeking of evidence concerning the chemical basis of unfavorable temperature damage to plants must necessarily be empirical in approach.

It was decided to study high temperature lesions rather than low temperature lesions, in order to avoid the complication of the overall depression of metabolic rate by low temperatures and the consequent reduction of the potential response range.

Trifolium subterraneum was chosen as the assay material. It has the advantage of being an obligate inbreeder, and genetic variation is thus reduced to a minimum. It is also a small plant with hirsute leaflets ideally suited to retaining spray droplets.

The temperature control facilities are restricted to those provided by the Earhart Plant Research Laboratory (15).

The chemicals for the spraying program were chosen to cover a wide range of metabolically important substances. The chemicals were applied as mixtures. Ideally, one should first apply a "complete" mixture; then, if a cure is obtained, the chemical or chemicals responsible for the cure can be assayed for by application of the whole range of "complete minus one ingredient" mixtures. In the experiments reported here, the mixtures used were casein hydrolysate (as a complete amino acid source), water soluble vitamins, and nucleosides.

Gibberellin was applied in some instances. Partial mixtures of amino acids and of the water soluble vitamins were used in cases where responses to the complete mixtures were obtained.

MATERIALS AND METHODS

Description of plant material

Trifolium subterraneum L. consists of numerous self-pollinating varieties which show a wide morphological and physiological diversity. The natural habitat of the species is the Mediterranean region, the Iberian peninsula, France and the British Isles. Many varieties from these areas were introduced to Australia and flourished there in the Mediterranean climate of the southern part of the continent (16).

Classification of the Australian varieties was made by naming the varieties after the locality where they were first recognized. Varietal classification of T. subterraneum collected in Europe and Africa was made by naming the varieties after the country of origin and adding a collecting number.

Physiological studies (17) show that all varieties are winter annuals, whose flowering is accelerated by long days, vernalization, and low night temperatures. The physiological requirement for long days and low temperature varies enormously among the varieties, ranging from a mild acceleration of flowering time to an absolute requirement for flowering. The inbreeding habit of the species ensures both homozygosity and genetic uniformity within any variety which has been recently purified by single plant pedigreeing. All material used in the experiments reported here is derived by single plant pedigreeing no more than one generation prior to the generation

actually grown.

Five varieties, Wenigup, Mt. Barker, Clare, Dwalganup, and Portugal 19464, were studied. They are all prostrate in habit, with a compressed central axis. The experiments were terminated before the development of lateral runners. The varieties' physiological traits, as far as they are known, are listed below:

Dwalganup: Early flowering, with no cold requirement, and no long day requirement for flowering (18)

Clare : Early flowering, with no cold requirement, and no long day requirement for flowering (17)

Mt.Barker: Mid-season to late flowering, with a pronounced cold requirement for floral initiation, and accelerated flowering under long days (18)

Wenigup : Late flowering, with a slight cold requirement, and a pronounced long day requirement for flowering (17).

Portugal 19464 : The physiological requirements for flowering have not been determined. It was chosen for this study because it developed leaf damage when grown in non-air conditioned glasshouse in summer. The leaf damage was presumed to be a high temperature effect; it consisted of a progressive chlorosis commencing at the leaflet margin, followed by progressive necrosis.

The varieties Wenigup, Mt. Barker and Portugal 19464 were used in the majority of the high temperature studies. Dwalganup and Clare were studied in one experiment only.

Temperature control facilities

All the experiments were conducted in the Earhart Plant Research Laboratory. Three types of temperature controlled facilities were used (15).

1. Air conditioned greenhouses: These have a diurnal temperature control pattern. From 0800 hours to 1600 hours, a constant temperature is maintained and is designated the day temperature. At 1600 hours the temperature is lowered to a new constant temperature, which is maintained until 0800 hours and is designated the night temperature. Natural daylight supplies the light energy for photosynthesis; the photoperiod is maintained at 16 hours by artificial lighting. Three greenhouses were used: 30°C day/24°C night,* 26°C day/20°C night, and 23°C day/17°C night.

2. Artificial light rooms: These rooms run at a constant temperature. In all experiments performed in these rooms, the plants were grown under long day conditions (16 hours light, 8 hours dark). Three constant temperature levels were used, 26°C, 23°C, and 20°C.

3. Modified artificial light room: This room was fitted

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* This was changed to 23°C night after April 3, 1958.

with two adjustable thermostats with time controlled actuation. This arrangement permitted a two level temperature pattern on a 24 hour cycle, wherein both the temperatures of each level and their period could be set at will. All experiments here were under long day conditions.

Growing methods

Seed selection and preparation: Seed to be used in the experiments were hand-graded for uniformity, their testas pricked to permit imbibition and germinated.

Germination.--In general, germination at room temperature was fairly irregular. To cope with this irregularity two methods were used.

In one method, a large excess of seeds was sown on wet filter paper in petri dishes and allowed to imbibe for two to four hours at room temperature. Imbibed seeds were then sown in plastic cups containing the potting mixture. Two seeds were sown per cup, and about a 50% excess of cups was sown above the number needed for the experiment. By means of a combined selection of plants and cups, a uniform batch of germinated seedlings could be obtained for the experiment.

In the other method, the imbibed seeds were germinated at 4°C for periods varying from one to seven days prior to transplantation to the plastic cups. This cold treatment both accelerated germination and removed the irregularity in germination time. The specific germination technique used

for each experiment is described in the Experimental Details section of Materials and Methods.

Growing conditions.--Germinated seeds were transplanted into plastic cups containing a 1:1 mixture of gravel and vermiculite. One plant per cup was used in all experiments. For some experiments the plants were grown under the experimental temperature conditions from the time of transplantation. In other experiments the plants were grown for variable periods at optimal temperatures before being transferred to the experimental temperature conditions. The latter course was adopted in order to select for uniformity at the seedling stage, prior to the random allocation of the plants to the various treatment groups. The plants were fed Hoagland's nutrient solution every morning and watered every afternoon with de-ionized water. It should be noted that the temperature of these solutions was not adjusted to the temperature of the growth chambers. Thus in many instances the temperature of the watering solution was as much as 10°C below the air temperature of the high temperature growth chambers.

Application of chemicals

All chemicals used were made up as aqueous solutions with de-ionized water and were 0.01% with respect to Tween 20 (polyoxyethylene sorbitan monolaurate) as a wetting agent. The solutions were applied as a fine mist, generated by a compressed air sprayer. The plants were sprayed once every

two days during the treatment period. They were transferred from the greenhouses or artificial light rooms to a spraying room for their spray treatment, and were absent from their experimental growing conditions for about twenty minutes. The unsprayed control plants were also removed from their experimental growing conditions for an equivalent period.

Formulation of spray solutions

The sprays used can be classified into four general types--amino acids, water soluble vitamins, ribonucleosides, and gibberellic acid. The formulation of the various spray solutions used are shown in Table 1-I. The vitamin solution "Complete Vitamin Solution minus Ascorbic acid" showed precipitation on standing; thus, the concentrations of vitamins in Vitamin Solutions I, II, and III were reduced.

Plant measurements

Whole plant dry weights were used to determine the effects of temperature on growth and the response to the applied chemicals. Plants were harvested, washed free of rooting medium, and placed in kraft bags for drying. Drying was effected in a forced draught drying oven at 70°C for 24 hours. In some instances, the number of expanded leaves per plant was recorded at harvest.

In the case of the variety Portugal 19464, a lesion index was computed for each plant at harvest. The lesion index was derived in the following manner: each expanded leaf on a

TABLE 1 - I

Formulations of Spray Solutions

Type	Ingredients	Concentration
AMINO ACID	Casein Hydrolysate (Vitamin Free) enzymatically digested.	0.025% and 0.1%
	Casein Hydrolysate (Vitamin Free) HCL digested.	0.025%
	Amino Acid Solution I L. glycine L. alanine L. valine L. isoleucine L. leucine L. serine L. threonine	10^{-4} M (each)
	Amino Acid Solution II L. asparagine L. glutamine L. lysine L. arginine L. cysteine L. cystine L. methionine	10^{-4} M (each)
	Amino Acid Solution III L. histidine L. proline L. hydroxyproline L. tryptophan L. phenylalanine L. tyrosine	10^{-4} M (each)
RIBO-NUCLEOSIDES	Adenosine Guanosine Uridine Cytidine	5×10^{-4} M (each)

TABLE 1 - I (continued)

Type	Ingredients	Concentration
WATER SOLUBLE VITAMINS	Vitamin Solution I	
	Ascorbic acid	500 mgs/liter
	Choline HCl	10 " "
	Inositol	200 " "
	Vitamin Solution II	
	Pyridoxine HCl	10 " "
	Nicotinic acid	50 " "
	Riboflavin	10 " "
	Thiamin HCl	10 " "
	Vitamin Solution III	
	Biotin	1 " "
	Calcium pantothenate	10 " "
	Folic acid	10 " "
	p-Aminobenzoic acid	50 " "
	Complete Vitamin Solution A (Solutions I, II and III combined)	as above
	Complete Vitamin Solution B *minus* Ascorbic acid	
	Choline·HCl	10 " "
	Inositol	500 " "
	Pyridoxine·HCl	100 " "
	Nicotinic acid	500 " "
	Riboflavin	50 " "
	Thiamin HCl	100 " "
	Biotin	5 " "
	Calcium pantothenate	800 " "
	Folic acid	100 " "
	p-Aminobenzoic acid	50 " "
	Ascorbic Acid Solution	1,000 " "
GIBBERELLIC ACID	Gibberellic acid	200 " "

plant was given a grade ranging from 0 through 5 for leaf injury, where the grade 5 indicates total necrosis. These grades were summed over the whole plant and the sum divided by the total number of expanded leaves for that plant. This mean value constitutes the lesion index.

Analysis of results

The dry weight data and the lesion index data were treated by the analysis of variance technique for spray treatment effects on each variety within each temperature condition. Where the analysis of variance indicated significant spray treatment effects, the means of each spray treatment within each experiment were tabulated,and their 95% and 99% fiducial semi-intervals calculated from the error variance estimate derived from the appropriate analysis of variance. Twice the value of these 95% and 99% semi-intervals were used as a guide to the significance of treatment effects.

In using these values as a guide to the statistical significance of any difference between a chemical spray treatment and an unsprayed or water-sprayed control it should be realized that in an experiment involving n such comparisons, the probability that any 1 of the differences will exceed the 5% or 1% confidence value is approximately $1 - (0.95)^n$ or $1 - (0.99)^n$ respectively.

Presentation of results

For simplicity of presentation, the data for each variety have been collected and are presented for each variety in turn. As some of the experiments were run with more than one variety, a complete outline of the experimental procedure involved in each experiment is given below. In the course of reporting the results for each variety, reference will be made by experiment number to these experimental details.

Experimental details

Experiment 1.--Seeds of the varieties Wenigup and Mt. Barker were scarified, imbibed at room temperature, and then sown directly into the growing cups on 1/19/57. The cups were immediately transferred to the experimental temperature conditions, viz. the 30°C/23°C, the 26°C/20°C and the 23°C/17°C greenhouses. On 1/27/57, the seedlings were randomly allocated to four treatment groups of ten seedlings each, in each of the greenhouses. The four treatments were: 1) unsprayed control, 2) 0.025% casein hydrolysate (vitamin free) enzymatically digested, 3) complete vitamin solution minus ascorbic acid, and 4) 0.1% ascorbic acid. Spraying commenced 1/28/57, and was repeated every two days until 2/20/57. The plants were harvested on 2/22/57, 34 days after sowing. Leaf counts and dry weights were recorded.

Experiment 2.--Imbibed seeds of Mt. Barker were sown into growing cups on 3/1/57 and transferred immediately to the

23°C/17°C greenhouse, where they germinated and grew until 3/15/57. The seedlings were selected for uniformity and randomly allocated to three treatment groups of nine plants each and then transferred to the 30°C/23°C greenhouse. The three treatments were: 1) unsprayed control, 2) water and 3) 0.1% casein hydrolysate (vitamin free)-enzymatically digested. Spraying commenced 3/15/57 and was repeated every two days until 4/9/57. The plants were harvested on 4/11/57, 41 days after sowing. Dry weights were recorded.

Experiment 3.--Imbibed seeds of Wenigup were sown on dampened filter paper in petri dishes on 3/15/57, and cold treated for seven days at 4°C. The germinated seeds were then transplanted into growing cups, and randomly allocated to three treatment groups of nine plants each for both the 30°C/23°C and the 26°C/20°C greenhouses. The three treatments were the same as in Experiment 2. Spraying commenced 3/29/57 and was repeated every two days until 4/17/57. Plants were visually inspected for high temperature growth inhibition and growth response to the treatments.

Experiment 4.--Imbibed seeds of the varieties Wenigup and Portugal 19464 were sown into growing cups on 8/23/57, and transferred to the modified artificial light room (see Temperature Control Facilities), where the plants were grown at 23°C under 16 hours light per day until 9/14/57. Then the temperature was raised to 35°C for 3 hours at the eighth hour of the light period. This temperature regime was maintained until the

plants were harvested on 9/26/57. Prior to raising the temperature, the plants were randomly allocated to eight treatment groups of 5 plants each for Portugal 19464 and 9 plants each for Wenigup. One treatment group for each variety was transferred to an adjacent 23°C artificial light room when the temperature was raised. This group constituted an unsprayed normal temperature control. The other seven groups remained in the high temperature room. The seven treatments were: 1) unsprayed control, 2) water, 3) 0.025% casein hydrolysate (vitamin free) enzymatically digested, 4) ribonucleosides, 5) complete vitamin solution B plus ascorbic acid, 6) gibberellic acid, and 7) combination of all ingredients at the same concentration as in the individual spray solutions (see Table 1-I). Spraying commenced 9/14/57 and was repeated every two days until 9/24/57. At harvest, dry weights were recorded for Wenigup, and leaf numbers, lesion indices, and dry weights were recorded for Portugal 19464.

Experiment 5.--Imbibed seeds of Wenigup, Portugal 19464, Clare, and Dwalganup were cold treated for 24 hours at 4°C on 10/4/57 and sown into growing cups in the modified artificial light room running at 20°C with a 16 hour light period per day. On 10/15/57 the plants were randomly allocated to six treatment groups and the temperature raised to 23°C, with a three hour period at 35°C commencing at the eighth hour of the light period. The six treatments were: 1) unsprayed control, 2) water, 3) 0.025% casein hydrolysate (vitamin free)

enzymatically digested, 4) amino acid solution I, 5) amino acid solution II, and 6) amino acid solution III (see Table 1-I). Spraying commenced 10/15/57. As the variety Portugal 19464 was not showing any signs of developing leaf injury, the 23°C temperature was raised to 25°C on 11/4/57. Spraying ceased on 11/12/57, and the plants were harvested two days later, 39 days after sowing. Dry weights were recorded for all varieties, and lesion indices were recorded for the variety Portugal 19464.

Experiment 6.--Imbibed seeds of Portugal 19464 were cold treated at 4°C for 24 hours on 2/19/58 and then sown into growing cups in each of three constant temperature artificial light rooms, viz. 26°C, 23°C and 20°C under 16 hours light per day. Within each room the plants were allocated at random to four treatment groups of nine plants each. The four treatments were: 1) water, 2) 0.025% casein hydrolysate (vitamin free) enzymatically digested, 3) as in 2 but minus Tween 20, 4) 0.025% casein hydrolysate (vitamin free) HCl digested. Spraying commenced 3/3/58 and was repeated every two days until 4/4/58, and the plants were harvested three days later--47 days after sowing. During this experiment the plants suffered from fumigation damage. Dry weights and lesion indices were recorded. In the case of the latter, the high temperature damage could be distinguished from fumigation damage except in the 20°C room where fumigation damage was most severe.

Experiment 7.--Imbibed seeds of Wenigup were cold treated at 4°C for two days and then transferred to the 23°C/17°C greenhouse on 4/11/58. In this case the seeds were sown in vermiculite-gravel mixture in seedling trays. On 4/27/58 the seedlings were selected for uniformity and transplanted to growing cups. The plants were randomly allocated to six treatment groups of nine plants each for each of two temperature conditions. The two temperature conditions were 30°C/23°C and 23°C/17°C respectively, where the higher temperature for each condition was obtained from 0800 hours-1600 hours in the appropriate greenhouse, and the lower temperature for each condition was obtained from 1600 hours-0800 hours in the appropriate dark room. Thus, unlike the other experiments reported, this one was conducted under short day conditions. The six treatments were: 1) water, 2) 0.025% casein hydrolysate (vitamin free) enzymatically digested, 3) vitamin solution I, 4) vitamin solution II, 5) vitamin solution III, and 6) complete vitamin solution A plus 0.025% casein hydrolysate. Spraying commenced 5/2/58 and was repeated every two days. The plants were harvested on 5/14/58--35 days after sowing. Dry weights and leaf numbers were recorded.

Experiment 8.--A batch of seeds from each of the two varieties Mt. Barker and Wenigup was germinated at 4°C for 6 days, commencing 2/27/59. A second batch of seeds was imbibed and sown directly into plastic cups in the 30°C/23°C and the 26°C/20°C greenhouses on 3/4/59. The cold-treated

seeds were sown out into the two greenhouses on 3/6/59. In each greenhouse, both cold-treated and non-cold-treated plants were selected for uniformity and randomly allocated to 3 treatment groups on 3/12/59. The three spray treatments were 1) water, 2) 0.025% enzyme digested vitamin free casein hydrolysate, and 3) complete vitamin solution A. Spraying commenced on 3/12/59 and was repeated every 2 days. The plants were harvested on 3/28/59, dried, and weighed.

RESULTS

I. Studies with the Mount Barker variety

Experiment 1.--In this experiment, the seeds were sown directly into the experimental temperature conditions without any cold treatment.

The analyses of variance for treatment effects on whole plant dry weight for each of the three temperature conditions are shown in Table 2-I. The treatment group means for dry weight, and leaf number per plant are shown in Table 3-I. The treatment effects are very highly significant in the 30°C/24°C greenhouse, are significant at the 1% level in the 26°C/20°C greenhouse, and are not significant in the 23°C/17°C greenhouse.

Unsprayed plants grown at 30°C/24°C produced 11.5% as much dry matter, and had fewer and smaller leaves than plants grown at 26°C/20°C, which was the optimum temperature in this experiment. All the spray treatments gave large increases in yield, leaf size, and leaf numbers for the plants grown at 30°C/24°C. No positive spray responses were obtained in either of the other two temperature conditions.

The casein hydrolysate spray (0.025% solution) gave the largest increase in dry matter yield (350% increase over the unsprayed control). The vitamin mixture spray gave a 310% yield increase, and the Vitamin C spray gave a 195% increase. These results strongly suggest that the applied chemicals had

TABLE 2 - I

Analyses of Variance of Dry Weight Yields
of Mt. Barker (Experiment 1)

Growth temperature	Source of variation	Degrees of freedom	Mean sums of squares	F
30°C day,	Between treatments	3	12,982	55***
23°C night	Error	35	236	
26°C day,	Between treatments	3	7,768	6.3**
20°C night	Error	35	1,238	
23°C day,	Between treatments	3	1,960	2.8*
17°C night	Error	32	713	

*Not significant.

**Significant at 1% level of confidence.

***Very highly significant.

TABLE 3 - I

Mean Dry Weight (in mg) and Leaf Number of Mt. Barker (Experiment 1) under Various Spray Treatments

Growth temperature	Spray treatment	Number of plants	Mean dry weight (mg)	Mean leaf number
30°C day, 23°C night	Unsprayed control	9	24	4.1
	Casein hydrolysate	10	107	7.4
	Vitamin mixture	10	99	6.8
	Vitamin C	10	71	5.8
	95% double fiducial interval = 20 mg (n = 10)			
	99% " " " = 26 mg (n = 10)			
26°C day, 20°C night	Unsprayed control	10	205	8.4
	Casein hydrolysate	10	188	7.9
	Vitamin mixture	9	218	9.1
	Vitamin C	10	153	6.9
	95% double fiducial interval = 46 mg (n = 9 and 10)			
	99% " " " = 62 mg (n = 9 and 10)			
23°C day, 17°C night	Unsprayed control	9	114	5.5
	Casein hydrolysate	9	129	6.0
	Vitamin mixture	9	146	6.1
	Vitamin C	9	117	5.9
	No significant difference.			

a therapeutic effect in relieving high temperature stress. There is however the possibility that water itself may have some effect.

Experiment 2.--This experiment was designed to test the repeatability of the casein hydrolysate effect obtained in the 30°C/24°C greenhouse in experiment 1. The plants were germinated and grown in the 23°C/17°C greenhouse for 14 days prior to their transfer to the 30°C/24°C greenhouse. The results are shown in Table 4-I. No spray effects were obtained.

Experiment 8.--This experiment was designed to investigate the effect of germination temperature on the plant's subsequent temperature sensitivity, and thus on their response patterns to the spray treatments which were used in experiment 1. Completely unexpected results were obtained. No high temperature inhibition was observed in the 30°C/23°C greenhouse--in fact, the dry weight yields for all spray seed treatment subgroups were higher in the 30°C/23°C greenhouse. This same result was also observed for Wenigup grown in the same experiment. No spray effects were observed in either of the temperature conditions, whether the seeds were cold treated or not cold treated during germination (see Table 5-I). The only known differences between the conditions of experiment 1 and this experiment were the time of year (January-February versus March), and the 1°C lower night temperature in experiment 8.

TABLE 4 - I

Mean Dry Weight (in mg) of Mt. Barker (Experiment 2)

under Various Spray Treatments

Growth temperature	Spray treatment	Number of plants	Mean dry weight (mg)
30°C day, 23°C night	Unsprayed control	7	127
	Water	8	109
	Casein hydrolysate	9	94

Analysis of Variance

Source of variation	Degrees of freedom	Mean sums of squares	F
Between treatments	2	2195	3.1*
Error	21	703	

*Not significant.

TABLE 5 - I

Mean Dry Weight (in mg) of Mt. Barker (Experiment 8) in Two Greenhouses, with and without Cold Treatment

30°C/23°C Greenhouse

Germination condition	Spray treatment: Water	Spray treatment: Casein hydrolysate	Spray treatment: Vitamin mixture
Cold-treated	187	191	187
Non cold-treated	211	200	200

26°C/20°C Greenhouse

Germination condition	Spray treatment: Water	Spray treatment: Casein hydrolysate	Spray treatment: Vitamin mixture
Cold-treated	133	144	105
Non cold-treated	167	173	165

II. Studies with the Wenigup variety

Experiment 1.--The seeds were sown directly into the three experimental temperature conditions without any cold treatment.

The analyses of variance for treatment effects on whole plant dry weight for each of the three temperature conditions are shown in Table 6-I. The treatment group means for dry weight and leaf number per plant are shown in Table 7-I. The treatment effects are very highly significant in the 30°C/24°C greenhouse, just significant at the 5% level in the 26°C/20°C greenhouse, and not significant in the 23°C/17°C greenhouse.

Plants grown in the 30°C/24°C greenhouse were 88.5% lower in dry matter yield, and had 55% fewer leaves than plants grown at 26°C/20°C (the optimal temperature for growth in this experiment).

All the spray treatments had large positive effects on the growth of plants in the highest temperature condition. No positive responses were observed in either of the two lower temperature conditions.

The vitamin mixture spray gave the largest increase in dry matter yield (438% over the unsprayed control). The casein hydrolysate sprayed plants were 306% higher in yield than the unsprayed controls, and the vitamin C sprayed plants were 197% higher in yield. The yield increases were expressed in larger leaves, petioles, rooting systems and leaf numbers.

Thus, as in the case of Mount Barker grown in the same

TABLE 6 - I

Analyses of Variance of Dry Weight Yields of Wenigup (Experiment 1)

Growth temperature	Source of variation	Degrees of freedom	Mean sums of squares	F
30°C day,	Between treatments	3	94,067	27.5***
23°C night	Error	33	3,417	
26°C day,	Between treatments	3	29,687	3.7**
20°C night	Error	31	8,024	
23°C day,	Between treatments	3	3,529	0.7*
17°C night	Error	32	4,967	

*Not significant.

**Significant at 5% level of confidence.

***Very highly significant.

TABLE 7 - I

Mean Dry Weight (in mg) and Leaf Number of Wenigup (Experiment 1) under Various Spray Treatments

Growth temperature	Spray treatment	Number of plants	Mean dry weight (mg)	Mean leaf number
30°C day, 23°C night	Unsprayed control	9	56	4.3
	Casein hydrolysate	9	227	7.4
	Vitamin mixture	9	297	7.8
	Vitamin C	10	166	6.7
	95% double fiducial interval = 79 mg (n = 9)			
	99% " " " = 106 mg (n = 9)			
26°C day, 20°C night	Unsprayed control	9	489	9.5
	Casein hydrolysate	8	455	9.1
	Vitamin mixture	9	557	10.5
	Vitamin C	9	422	8.4
	95% double fiducial interval = 122 mg (n = 9)			
	99% " " " = 126 mg (n = 8 and 9)			
23°C day, 17°C night	Unsprayed control	9	267	5.8
	Casein hydrolysate	9	270	6.4
	Vitamin mixture	9	289	6.4
	Vitamin C	9	241	5.9
	No significant difference.			

experiment, large responses to the applied chemicals were obtained in the high temperature condition.

Experiment 3.--This experiment was designed to further investigate the casein hydrolysate effect obtained in the 30°C/24°C greenhouse, reported in experiment 1. In contrast to experiment 1, the imbibed seeds were germinated at 4°C for 7 days prior to sowing them out into the 30°C/24°C greenhouse. The plants showed much more vigorous growth in this experiment than in experiment 1. No growth differences were observed among the 3 spray treatments (unsprayed, water, and casein hydrolysate) after eighteen days of treatment. The experiment was discontinued and the plants discarded. The reason for the absence of high temperature growth inhibition could possibly be attributed either to a difference in solar irradiation or to the cold treatment during germination.

Experiment 4.--This experiment was conducted in an artificial light room to remove the radiation variable possibly responsible for the non-repeatability of high temperature lesion induction in the previously conducted greenhouse experiments. The plants were germinated at 23°C and grown at this temperature until they were 22 days old. One group of plants was then transferred to an adjacent 23°C artificial light room, and the other 7 groups were subjected to the high temperature treatment as described in Materials and Methods.

The analysis of variance for spray treatments in the high temperature condition (Table 8-I) shows a significant effect of

TABLE 8 - I

Analysis of Variance of Dry Weight Yields

of Wenigup (Experiment 4)

Growth temperature	Source of variation	Degrees of freedom	Mean sums of squares	F
23°C (3 hours at 35°C)	Between treatments	6	84,635	6.97*
	Error	56	12,145	

*Significant at 1% level of confidence.

treatments. The mean dry weights for the treatment groups in the high temperature condition and the mean dry weight for the unsprayed group in the 23°C room are shown in Table 9-I. The unsprayed high temperature group was 34% inhibited in dry matter yield relative to the group of plants grown continuously at 23°C. At the high temperature, the water sprayed group is not significantly larger than the unsprayed group. Both the vitamin mixture spray and the combined spray groups gave 36% greater yields than the water sprayed group. The casein hydrolysate sprayed plants gave 16.5% higher yields than the water sprayed plants. The ribonucleoside spray and the gibberellin spray were ineffective. Although the yield increases obtained with the vitamin mixture, combined, and casein hydrolysate sprays cannot be considered individually statistically significant, these results, when combined with those from the high temperature condition in experiment 1, are highly suggestive of real chemotherapeutic effects.

Experiment 5.--The conditions of this experiment were designed for investigations with the variety Portugal 19464; however, as space was available, the opportunity was taken to study Wenigup further. The seeds were cold treated for 24 hours during germination and grown at 20°C for 10 days in the artificial light room prior to the high temperature treatment. The experiment was designed to do a preliminary crude analysis of the casein hydrolysate effect suggested by the results of experiments 1 and 4. Twenty amino acids were divided into

TABLE 9 - I

Mean Dry Weight (in mg) of Wenigup (Experiment 4) under Various Spray Treatments

Growth temperature	Spray treatment	Number of plants	Mean dry weight (mg)
23°C (3 hours at 35°C)	1 Unsprayed control	9	375
	2 Water	9	465
	3 Casein hydrolysate	9	540
	4 Ribonucleosides	9	446
	5 Vitamin mixture	9	632
	6 Gibberellic acid	9	521
	Combined (3,4,5,6)	9	633
23°C	Unsprayed control	9	569

95% double fiducial interval = 148 mg
99% " " " = 196 mg

three spray mixtures as shown in Table 1-I. These three amino acid mixtures, 0.025% casein hydrolysate, and water, constituted the 5 sprays assayed in this experiment. In addition, one group of plants was left unsprayed. The analysis of variance for dry weight yields (Table 10-I) demonstrated significant spray treatment effects at the 1% level of confidence. The mean dry weights for each treatment group are shown in Table 11-I. Again the casein hydrolysate elicited a positive growth response (22% greater yields than the water sprayed group). In addition, the amino acid mixture II sprayed group was 18.5% higher in yield than the water sprayed group. However neither of these positive responses is statistically significant. The difference between the water sprayed plants and the unsprayed plants is unusually large, but this is unlikely to be a real effect, as such differences were not obtained in any other experiment in which positive chemical spray effects were obtained.

Experiment 7.--In this experiment, the positive vitamin mixture effects obtained in experiments 1 and 5 were further investigated. The vitamins used in the mixture were divided into three spray mixtures, as shown in Table 1-I. These three mixtures, casein hydrolysate, a combined mixture of all the vitamins plus casein hydrolysate, and water constituted the 6 spray treatments used. The imbibed seeds were cold treated for 48 hours and grown for 16 days at 23°C for 8 hours in the greenhouse, and 16 hours at 17°C in the darkroom. Half the

TABLE 10 - I

Analysis of Variance of Dry Weight Yields
of Wenigup (Experiment 5)

Growth temperature	Source of variation	Degrees of freedom	Mean sums of squares	F
23°C (3 hours at 35°C)	Between treatments	5	44,407	7.6*
	Error	29	5,848	

*Significant at 1% level of confidence.

TABLE 11 - I

Mean Dry Weight (in mg) of Wenigup (Experiment 5)
under Various Spray Treatments

Growth temperature	Spray treatment	Number of plants	Mean dry weight (mg)
23°C (3 hours at 35°C) 20 days,	Unsprayed control	6	307
25°C (3 hours at 35°C) 8 days	Water	6	447
	Casein hydrolysate	5	546
	Amino acid mix I	6	480
	Amino acid mix II	6	539
	Amino acid mix III	6	433

95% double fiducial interval = 128 mg (n = 6)
" " " " = 134 mg (n = 5,6)
99% " " " = 172 mg (n = 6)
" " " " = 180 mg (n = 5,6)

plants were then transferred to the 30°C day/23°C night, on the same short day schedule.

The analyses of variance of dry weight yields for the spray treatments are shown in Table 12-I. Significant treatment effects were observed in the 30°C/23°C condition only. The mean dry weight yields for spray treatments at each temperature are shown in Table 13-I. There are no significant positive responses to the applied chemicals. However, the 28% higher yields obtained with the combined vitamin and casein hydrolysate spray, and the 15.5% increase obtained with the casein hydrolysate spray, are in accord with the results of experiments 1, 4 and 5. None of the vitamin submixtures gave responses meriting attention.

Experiment 8.--The variability experienced in generation of the high temperature lesion could possibly be due to variation in temperature conditions experienced by the germinating seeds. For instance, in experiments 1 and 4, in which the seeds were not subjected to a cold treatment, high temperature growth inhibition and positive chemotherapeutic responses were obtained. Plants grown in experiments 3, 5, and 7, in which the seeds were cold treated to various extents in order to improve germination, either exhibited no growth inhibition by high temperatures, as in experiments 3 and 7, or exhibited too small an inhibition to permit the detection of statistically significant chemotherapeutic effects. Experiment 8 was designed to test the effect of germination temperature on the

TABLE 12 - I

Analyses of Variance of Dry Weight Yields of Wenigup (Experiment 7)

Growth temperature	Source of variation	Degrees of freedom	Mean sums of squares	F
30°C day,	Between treatments	5	17,962	2.6*
23°C night	Error	48	6,733	
23°C day,	Between treatments	5	415	1.06**
17°C night	Error	48	439	

*Significant at 5% level of confidence.

**Not significant.

TABLE 13 - I

Mean Dry Weight (in mg) of Wenigup (Experiment 7) under Various Spray Treatments at Two Temperatures

Spray treatment	Number of plants	Growth temperature	
		30°C day, 23°C night	23°C day, 17°C night
Water	9	294	251
Vitamin solution I	9	327	218
Vitamin solution II	9	259	263
Vitamin solution III	9	271	225
Complete vitamin solution (I+II+III+cas. hyd.)	9	377	227
Casein hydrolysate	9	340	203

95% double fiducial interval = 112 mg
(for the 30°C/23°C growth only).

subsequent temperature responses of the plants. The experiment was conducted in the 30°C/23°C and 26°C/20°C greenhouses, one group of seeds being cold treated at 4°C for 6 days prior to planting out, and the other group being sown directly in the two greenhouses.

The mean dry weights for each spray treatment group for each germination treatment in each greenhouse are shown in Table 14-I. Analyses of variance for spray treatments within seed treatment groups at each temperature condition revealed no spray effects. Furthermore, the yield for each seed treatment spray subgroup was higher in the 30°C/23°C greenhouse than in the 26°C/20°C greenhouse. This completely unexpected result is unexplainable and renders the experiment uninformative.

TABLE 14 - I

Mean Dry Weight (in mg) of Wenigup (Experiment 8)

in Two Greenhouses, with and

without Cold Treatment

30°C/23°C Greenhouse

Germination condition	Spray treatment: Water	Spray treatment: Casein hydrolysate	Spray treatment: Vitamin mixture
Cold-treated	328	331	370
Non cold-treated	420	398	387

26°C/20°C Greenhouse

Germination condition	Spray treatment: Water	Spray treatment: Casein hydrolysate	Spray treatment: Vitamin mixture
Cold-treated	278	237	243
Non cold-treated	328	302	314

III. Studies with Portugal 19464

Experiment 4.--Since the optimal temperature and high temperature sensitivity of this variety were unknown, some preliminary temperature studies were made in the artificial light rooms to aid in the subsequent choice of a suitable high temperature condition for assay of the chemotherapeutic properties of applied organic supplements. Six constant temperature conditions were tested: 14°C, 17°C, 20°C, 23°C, 26°C, and 35°C. All these artificial light rooms were operated on a 16 hour photoperiod. Leaf injury occurred to a similar extent in all the temperature conditions from 20°C and higher except at 35°C where all the plants died. The optimal growth temperature for dry weight yield was 17°C. For experiment 4, a temperature regime of 23°C with a three hour period at 35°C (in the middle of the photoperiod) was chosen as the high temperature treatment. Unfortunately, space was available to run an unsprayed control group only at 23°C. The seeds were germinated at 23°C and grown at this temperature for 22 days prior to subjecting the plants to the high temperature, and to the spray treatments.

The analyses of variance for treatment effects on both dry weight yield and lesion index were significant at the 1% level (Table 15-I).

Plants grown in the high temperature condition gave 43% less dry matter yield than the unsprayed control plants grown

TABLE 15 - I

Analysis of Variance of Dry Weight Yields
of Portugal 19464 (Experiment 4)

Growth temperature	Source of variation	Degrees of freedom	Mean sums of squares	F
23°C (3 hours at 35°C)	Between treatments	6	33,618	4.4*
	Error	28	7,639	

Analysis of Variance of Lesion Index

Growth temperature	Source of variation	Degrees of freedom	Mean sums of squares	F
	Between treatments	6	3,333	11.9*
	Error	28	2,279	

*Significant at 1% level of confidence.

at 23°C; the high temperature grown plants also showed greater leaf injury than the 23°C controls (Table 16-I). Examination of the specific spray treatment effects listed in Table 16-I shows that the casein hydrolysate spray may have been the only one to exert a real chemotherapeutic effect. The casein hydrolysate sprayed plants were 120% heavier than the water sprayed plants at the same temperature condition. They even exceeded the unsprayed 23°C control plants by 25.5%. In addition to the increase in yield, the casein hydrolysate sprayed plants showed 78% less leaf injury than the water sprayed plants. However, the combined spray treatment failed to stimulate a significant increase in yield, despite the presence of casein hydrolysate, and in fact, this group of plants showed the highest degree of leaf injury in the whole experiment. Perhaps the other components in the combination spray interfered with the casein hydrolysate effect.

Experiment 5.--The results of the previous experiment strongly suggest that casein hydrolysate may have a real effect in relieving high temperature growth inhibition and leaf injury. Experiment 5 was designed to investigate further the casein hydrolysate effect under conditions similar to those of experiment 4. In addition an attempt was made to narrow down the range of possible amino acids responsible for the casein hydrolysate effect by assaying 3 different amino acid mixtures, described in Table 1-I. In this experiment, owing to the irregular germination of Portugal 19464 in experiment 4,

TABLE 16 - I

Mean Dry Weight (in mg), Lesion Index, and Leaves per Plant of Portugal 19464 (Experiment 4) under Various Spray Treatments

Growth temperature	Spray treatment	Number of plants	Mean dry weight (mg)*	Mean lesion index	Mean leaf number
23°C (3 hours at 35°C)	1. Unsprayed control	5	219	2.10	9.4
	2. Water	5	213	1.88	8.8
	3. Casein hydrolysate	5	460	0.48	14.6
	4. Ribonucleosides	5	295	1.69	10.2
	5. Vitamin mixture	5	307	1.72	10.6
	6. Gibberellic acid	5	311	1.02	10.0
	7. Combined spray (3,4,5,6)	5	276	2.30	9.4
23°C	8. Unsprayed control	5	366	1.44	11.6

*95% double fiducial interval = 160 mg
99% " " " = 216 mg

the imbibed seeds were cold treated at 4°C prior to planting. The plants were grown for 10 days at 20°C before being subjected to the experimental temperature regime. After 20 days growth at the high temperature, no signs of leaf injury were observed, so the 23°C period of the temperature regime was increased to 25°C for a further 10 days. The plants were then harvested and scored.

The degree of leaf injury in this experiment was only half that of experiment 4. In addition, the spray treatments had no significant effect on the degree of leaf injury (Table 17-I). Despite the absence of treatment effects on leaf injury, significant spray treatment effects were again observed for dry weight yield (Table 17-I).

As in experiment 4, the casein hydrolysate sprayed plants gave the highest yield (Table 18-I). They were 24% larger than the water sprayed group. Even though this response is not statistically significant for this experiment alone, when it is considered in conjunction with the results of experiment 4, there is a considerable chance that the effect is real. The smaller positive yield responses obtained with amino acid mixtures I and II also lend confidence to this conclusion.

Experiment 6.--Three groups of imbibed seed were cold treated at 4°C for 24 hours and sown in each of the following constant temperature artificial light rooms--26°C, 23°C, and 20°C. Two types of casein hydrolysate were investigated:

TABLE 17 - I

Analysis of Variance of Dry Weight Yields

of Portugal 19464 (Experiment 5)

Growth temperature	Source of variation	Degrees of freedom	Mean Sums of squares	F
23°C (3 hours at 35°C) 20 days,	Between treatments	5	18,589	3.46*
25°C (3 hours at 35°C) 8 days	Error	48	5,377	

Analysis of Variance of Lesion Index

Growth temperature	Source of variation	Degrees of freedom	Mean Sums of squares	F
	Between treatments	5	0.2251	1.7**
	Error	48	0.1273	

*Significant at 1% level of confidence

**Not significant.

TABLE 18 - I

Mean Dry Weight (in mg) of Portugal 19464 (Experiment 5) under Various Spray Treatments

Growth temperature	Spray treatment	Number of plants	Mean dry weight (mg)*	Lesion index
23°C (3 hours at 35°C) 20 days,	Unsprayed control	9	202	0.99
25°C (3 hours at 35°C) 8 days	Water	9	267	0.54
	Casein hydrolysate	9	331	0.72
	Amino acid mix I	9	282	0.80
	Amino acid mix II	9	309	0.82
	Amino acid mix III	9	250	0.62

*95% double fiducial interval = 98 mg
99% " " " = 131 mg

1) acid-digested and 2) enzymatically digested. The latter hydrolysate was made up into 2 spray solutions--one containing Tween 20 and the other without it. No significant spray response was obtained for either dry weight yield or leaf injury in any of the temperature conditions (Tables 19-I and 20-I). Yet obvious growth inhibition was obtained at 26°C and 23°C, where the mean dry weight yields for the water sprayed groups were respectively 279 mg and 267 mg, compared to 594 mg for the 20°C group.

TABLE 19 - I

Analyses of Variance of Dry Weight Yields of Portugal 19464 (Experiment 6)

Growth temperature	Source of variation	Degrees of freedom	Mean sums of squares	F
26°C	Between treatments	3	312	1.1*
	Error	27	273	
23°C	Between treatments	3	1599	2.63*
	Error	27	609	
20°C	Between treatments	3	3001	1.2*
	Error	28	2444	

*Not significant.

TABLE 20 - I

Analyses of Variance of Lesion Index of

Portugal 19464 (Experiment 6)

Growth temperature	Source of variation	Degrees of freedom	Mean sums of squares	F
26°C	Between treatments	3	0.9543	2.28*
	Error	27	0.4190	
23°C	Between treatments	3	0.4190	
	Error	26	0.5798	1*

*Not significant.

IV. Studies with the Clare variety

Experiment 5.--This variety was studied concurrently with Portugal 19464 in an experiment in which the effects of casein hydrolysate and of the three amino acid mixtures (Table 1-I) were investigated. The imbibed seeds were cold treated for 24 hours prior to sowing. Results are shown in Table 21-I.

Spray treatments had a significant effect on dry weight yield, the casein hydrolysate spray causing the largest positive effect (33% larger yield than the water sprayed group). The yield increases observed for the amino acid sprays were 20% for mixture I, 11.5% for mixture II, and 8% for mixture III. Even though these responses are not individually significant, considered together as a non-specified amino acid effect, they are highly significant.

TABLE 21 - I

Mean Dry Weight (in mg) of Clare (Experiment 5) under Various Spray Treatments

Growth temperature	Spray treatment	Number of plants	Mean dry weight (mg)*
23°C	Unsprayed control	9	330
	Water	8	396
	Casein hydrolysate	8	526
	Amino acid mix I	8	474
	Amino acid mix II	9	442
	Amino acid mix III	9	427

*95% double fiducial interval = 122 mg (n = 8)
99% " " " = 164 mg (n = 8)

Analysis of Variance of Dry Weight Yields of Clare (Experiment 5)

Source of variation	Degrees of freedom	Mean sums of squares	F
Between treatments	5	37,993	5.1**
Error	45	7,384	

**Significant at 1% level of confidence.

V. Studies with the Dwalganup variety

Experiment 5.--The experimental conditions for this variety were identical to those for Portugal 19464 and Clare studied in the same experiment.

The analysis of variance for spray treatment effect on dry weight yields indicated a significant effect at the 1% level (Table 22-I). Only the amino acid mixture I gave an appreciable increase in yield (61% higher than the water sprayed plants). This response is significant at the 5% level. However, little confidence can be placed in such an isolated result, and further investigation is needed to test the reality of this effect.

TABLE 22 - I

Mean Dry Weight (in mg) of Dwalganup (Experiment 5)

under Various Spray Treatments

Growth temperature	Spray treatment	Number of plants	Mean dry weight (mg)*
23°C	Unsprayed control	8	270
	Water	7	253
	Casein hydrolysate	8	294
	Amino acid mix I	7	371
	Amino acid mix II	8	298
	Amino acid mix III	8	252

*95% double fiducial interval = 86 mg (n = 7)
99% " " " = 115 mg (n = 7)

Analysis of Variance of Dry Weight Yields

of Dwalganup (Experiment 5)

Source of variation	Degrees of freedom	Mean sums of squares	F
Between treatments	5	14,201	4.4**
Error	40	3,173	

**Significant at 1% level of confidence

DISCUSSION AND CONCLUSIONS

In all, 14 investigations were made on the effect of organic supplements on the alleviation of high temperature growth inhibition. Of the 9 experiments which possessed control groups of plants grown under optimal temperature conditions, 5 experiments showed growth inhibition in the high temperature. Significant spray effects were observed in the high temperature condition in 4 of these 5 experiments. In the 5 experiments which lacked an optimal temperature control, 4 gave significant spray effects.

The results are discussed in turn for each variety below.

Mt. Barker.--High temperature growth inhibition was obtained only in experiment 1. The spray supplement effects were very large, and were restricted to the plants grown in the 30°C/24°C greenhouse. The temperature dependence of the supplement effects suggests that at least one metabolic reaction is particularly high temperature sensitive. The fact that each of the 3 types of supplement (casein hydrolysate, vitamin mixture, and vitamin C) caused larger yields than the control does not imply that more than one reaction was limiting growth. The supplements could have overlapping functions, alleviating a particular metabolic block induced by the high temperature treatment.

Wenigup variety.--Large, highly significant chemotherapeutic responses were obtained in experiment 1. In contrast to the results with Mt. Barker, the vitamin mixture spray was more effective than the casein hydrolysate spray. The vitamin mixture spray was again more effective in experiment 4, and the combined vitamin mixture and casein hydrolysate spray was more effective than the casein hydrolysate spray alone in experiment 7. This consistent superiority of the vitamin mixture spray is evidence for the reality of its chemotherapeutic properties. In experiments 1, 4, 5, and 7, casein hydrolysate gave positive yield increases in the high temperature condition, indicating that one or more amino acids are effective chemotherapeutic agents for high temperature inhibition. This indication is confirmed by the results of experiment 5, in which amino acid mixture II caused a yield increase.

Portugal 19464.--Again a chemotherapeutic effect may be attributed to amino acids. In experiment 4, casein hydrolysate is 3 times more effective than the vitamin mixture. Whether this is a real superiority or not, cannot be judged from this single experiment. If the responses to casein hydrolysate and the three amino acid mixtures are considered a general amino acid effect, the data of experiment 5 indicate a chemotherapeutic effect for amino acids.

Dwalganup 19464.--No explanation can be offered for the anomalous results obtained with this variety in experiment 5. Here a significant yield increase was observed in the plants

sprayed with amino acid mixture I, whereas no increase was observed for the casein hydrolysate sprayed group.

Clare.--The casein hydrolysate spray and each of the three amino acid mixtures gave small positive growth responses, which, considered together as a generalized amino acid effect, is highly significant.

Generation of the high temperature lesion

Considerable difficulty was encountered in the attempt to obtain repeatable high temperature inhibition of growth or leaf damage. This was especially true for the experiments conducted in the greenhouses. In particular, the striking high temperature effect obtained in experiment 1, for the varieties Mt. Barker and Wenigup, was not repeatable in experiments 2, 3, and 8. In experiments 1 and 8, in which both varieties were being studied simultaneously, both varieties responded similarly within each experiment. This suggested that the source of variability in obtaining a high temperature lesion may have been some environmental factor which varied between experiments.

The night temperature was 1°C lower in experiment 8, and this may in part account for the different temperature response between the 2 experiments. In experiments 2 and 3 the night temperature was the same as in experiment 1; yet no high temperature lesion was apparent in these 2 experiments. However, the seed used in experiments 2 and 3 were cold treated at 4°C

during germination. Perhaps the cold treatment affects the temperature response pattern in subsequent growth. Vernalization is known to increase the high temperature tolerance of peas (10). This possibility was not borne out by the results of experiment 8, in which the effect of cold treatment was specifically investigated. However, experiment 8 may not have been a valid test, owing to the confounding influence of the 1°C difference in night temperature.

Other factors may be responsible for the non-repeatability of the high temperature inhibition. For instance, plant temperature is a function of light intensity as well as the ambient air temperature, and differences in light intensity between experiments could be responsible for the variation in growth response under conditions of controlled ambient temperature.

In the artificial light rooms, where the variation in light intensity between experiments is much less than in the naturally lighted greenhouses, the repeatability was still poor (see experiments 4 and 5 for the variety Portugal 19464). Here again the variation in germination conditions and the pre-experimental growth temperatures may be responsible for the variability in temperature response.

Conclusions

The overall impression gathered from the foregoing results indicates that high temperature induced derangements of

plant metabolism are amenable to some degree of repair by the exogenous supply of biochemical supplements.

At this stage, no information is available on the specific nature of the high temperature lesion. However, the results suggest that the relative effectiveness of the amino acid supplements on the one hand, and the vitamin mixture supplement on the other, varies with the genotype. This genotype-supplement interaction may indicate specific differences in the metabolic site of the high temperature lesion for different genotypes. This possibility is also suggested by the genotype-amino acid mixture interactions observed in experiment 5.

The results justify further investigation of the problem of the chemical cure of climatic lesions. However a much better system is required, both in terms of more precise environmental control and a larger degree of flexibility in the temperature control facilities.

Some theoretical considerations

To say that unfavorable temperature effects on plant metabolism may be relieved by specific metabolites, implies that the temperature effect is analogous to the effect of a biochemical mutation. It is possible that this type of temperature effect may occur in some cases, but there is no a priori reason to expect it, since temperature influences all reactions.

However, if Blackman's model (19) for metabolic rate

control by a single rate-limiting reaction is correct, the temperature effect would be restricted to a rate-limiting reaction whose specific nature is a function of the temperature. But the validity of this model is questionable in relation to the effect of temperature on the relative availability of endogenous metabolites. The model was developed from studies made on the influence of temperature on the rate of utilization of CO_2 and light in photosynthesis (20). Thus the study was concerned with the interaction between exogenously supplied requirements and the overall effect of temperature on plant metabolism.

An extension of the Blackman theory by Crozier (reviewed in (21)) may be more applicable, for it was derived from studies on the effect of temperature on the rate of various physiological processes when other exogenous variables were held constant. The kinetics obtained were interpreted as functions of changes in the nature of the rate-limiting reaction.

Arrhenius plots of the log of the reaction rate against the reciprocal of the absolute temperature, for the majority of the physiological processes studied, were linear with sharp changes in slope. It was assumed that a single specific reaction is limiting over a temperature range which shows constant slope. Each change in slope then indicates a transition to a different rate-limiting reaction with a different activation energy.

The assumptions of the interpretation seem oversimplified. If each of a sequence of many limiting reactions may have similar activation energies, then the entire sequence over the temperature range would also give an Arrhenius plot of constant slope. Furthermore fitting a line of constant slope to the experimental points and assuming their deviations to be due to experimental errors is a doubtful practice. These deviations may be due to the operation of a sequence of rate-limiting reactions of varying activation energies. Thus, there is no a priori reason to expect a specific reaction to be rate limiting over temperature ranges which give a linear Arrhenius plot of constant slope.

Even though the concept of the temperature-dependent, rate-limiting reaction may be correct, the effective range of temperature over which any one reaction may be rate limiting could be so small as to render the concept practically useless. The more extreme the temperature conditions, the greater the accumulation of potential rate-limiting reactions, all, or most of which, would have to be relieved by specific supplements in order to obtain a reasonable restoration of growth rate.

Evolutionary considerations would lead one to surmise that natural selection would tend to develop some system of regulation for the reaction rate potentials of all the metabolic steps, in order to maximize metabolic efficiency. If this were the case, it would be unlikely that any one reaction

would be especially limiting over the range of temperature normally encountered by the plant in its natural environment. However, for plants which are the recent products of hybridization, and unusual selection pressures, this metabolic balance may be disturbed. In this case, one, or a few of the reaction steps, may be especially sensitive to temperature changes, and thus be amenable to effective chemotherapy.

At this stage, our knowledge of the integration and regulation of metabolism is insufficient to make any firm predictions about the feasibility of chemotherapy for temperature lesions. Instead, one may look to experimental chemotherapy as a technique for providing information on the organization of plant metabolism under varying temperature conditions.

SECTION I

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SECTION II

STUDIES ON THE RELATIONSHIP BETWEEN TOBACCO MOSAIC VIRUS INFECTION AND THE DNA METABOLISM OF TOBACCO LEAVES

INTRODUCTION

Current hypotheses concerning the perpetuation and control of biological specificity envisage a transfer of information from DNA through RNA to protein, by a sequence of template mechanisms (1). The specificity transfer and replication processes are seen as identical. DNA is usually considered the only molecule in the hierarchy capable of self replication, and the specificity transfer is thought to be unidirectional.

To this hypothesis, the RNA viruses present a paradox. Either viral RNA is capable of self-replication, or the specificity transfer is not unidirectional. The latter possibility implies that the RNA should produce a specific DNA, which would in turn act as a template to replicate viral RNA. The experiments reported here attempt to determine if DNA is involved in TMV infection of tobacco leaves.

Three experimental approaches to the problem were used. In all three, young, fully expanded leaves were chosen as the host material, since at this stage of leaf development, cell division has ceased (2), and host DNA synthesis should therefore be minimal.

The first experiment compared the DNA metabolisms of TMV infected and healthy excised leaves, using P^{32}-orthophosphate incorporation as the measure of metabolic activity. The second experiment investigated the effect on virus production of a specific inhibitor of DNA synthesis, 5-fluoro-uracil (3). The third experiment attempted to test the possible influence of deoxyribonuclease (DNAase) on TMV multiplication.

PART 1

P^{32}-ORTHOPHOSPHATE INCORPORATION INTO DNA OF HEALTHY AND TMV-INFECTED TOBACCO LEAVES

The effect of TMV infection on the metabolism of DNA has been investigated by the incorporation of radioactive phosphorus, supplied as orthophosphate to infected and uninfected excised leaves at the time of inoculation. The DNA metabolism in infected and uninfected leaves has been compared by measuring the specific activity of the isolated DNA nucleotides.

MATERIALS AND METHODS

A modified version of the Schmidt-Tannhauser procedure (4) was used to isolate the DNA free of other phosphorus-containing components. The isolated DNA was then subjected to successive enzymatic hydrolysis by DNAase and phosphodiesterase, to release the nucleotides (5). Finally, the DNA nucleotides were separated from the other constituents of the enzyme digest by electrophoresis (6), and their specific activity determined.

Details of the methods used are given below.

Four leaves (approximately 15 cm long at the midvein), of matched area and similar physiological age were selected from a group of 3 month old Nicotiana tabacum var. Samsun plants. Two leaves were inoculated at zero time with the U 1 strain of TMV (10^{-2} mg virus per ml in 0.06 M phosphate buffer, pH 7.0), using 400 mesh carborundum as an abrasive. The

other two leaves served as uninoculated controls. The leaves were then excised from the plants, and a 5 mm section of their petioles cut off (under water, to ensure the absence of air blocks in the xylem vessels). One hour after inoculation, each leaf was transferred to a separate vial containing 0.38 mc of P^{32} as neutralized orthophosphate, in 10 ml of 0.001 M phosphate buffer. The specific activity of the phosphorus in the solution was 0.17 mc per µmole. The leaves were incubated under fluorescent lights (450 foot-candles) at room temperature (26°C). The liquid level in the vials was periodically replenished with distilled water. Leaves transpired 1-2 ml of water per hour.

At times 25.5 hours and 149.5 hours, one TMV-inoculated leaf and one uninoculated leaf were removed from the vials and killed by a 2 minute immersion in boiling 80% ethyl alcohol.

Isolation of DNA

The alcohol-killed leaves were further extracted with cold, 80% ethyl alcohol until all traces of leaf pigment were removed. The leaves were then dried in a 70°C oven, and subsequently homogenized with sea sand and 0.3 M KOH in a mortar and pestle. Each homogenate was made up to 30 ml with 0.3 M KOH and incubated at 30°C for 21 hours.

The alkaline digest was centrifuged at 5000 rpm in the Servall SS-1 for 20 min to remove leaf debris. Three centrifuged washings of the pellet were combined with the original

supernatant. Perchloric acid was added to this solution to bring it to pH 8. After an hour in the cold, the potassium perchlorate precipitate was centrifuged out.

To precipitate the DNA, the supernatant was brought to pH 4 with acetic acid, made 0.001 M with respect to magnesium chloride, and ethyl alcohol added to 50%. After refrigerated cooling for two hours, a white, flocculent precipitate had formed, which was centrifuged down at 10,000 rpm for 30 min. The pellet was resuspended, washed, and centrifuged five times with the acid alcohol solution. The final pellet was washed once with 1:1 acetone-ether, once with ether, and then dried.

Enzymatic hydrolysis of the DNA

Some of the dried DNA preparation was dissolved in 0.5 ml of 0.5 M glycine buffer, pH 7.2, plus 0.2 ml of 0.3 M magnesium chloride. To this solution was added 0.3 ml of 1 mg per ml crystalline pancreatic DNAase (Worthington Chemical Corp.). The four samples were incubated under hexane at 37°C for 22 hours. Then 1 ml of 0.5 M glycine buffer, pH 9.2, and 0.1 ml of snake venom phosphodiesterase solution* were added to each. Preparations from the 25.5 hour leaves were

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*Phosphodiesterase was prepared by an unpublished method of Sinsheimer, using acetone fractionation (see (5)). Agkistrodon Piscovorus and Crotalus Adamanteus venoms were used as source material. The acetone fractions were assayed for 5'-nucleotidase activity and phosphodiesterase activity. The best fraction (least nucleotidase activity) of each preparation was used.

incubated 12 hours at 37°C; the preparations from the 149.5 hour leaves were incubated 7 hours. After the reaction had been stopped with 0.1N HCl, the enzyme digests were evaporated to dryness under vacuum.

Electrophoretic separation of the DNA nucleotides

The Spinco Durrum Model R Series D paper electrophoresis apparatus was used to separate the DNA nucleotides from other materials in the enzyme digest. An aliquot of each digest was applied 3 cm from the cathode end of separate 30 cm long Whatman no. 3 filter paper strips. In addition, an aliquot of P^{32}-orthophosphate solution was applied to a separate strip. The samples were electrophoresed in 0.05 M formate buffer, pH 3.5 for 10-11.5 hours at 8 volts per cm. At pH 3.5, all nucleotides possess a net negative charge, and the differences among their electrophoretic mobilities are maximal (6). After electrophoresis, the strips were dried and inspected for ultraviolet absorbing areas, which indicated the location of nucleotide material. The strip was then scanned for radioactivity, in a Nuclear-Chicago Actigraph fitted with a Model D47 Micromil gas-flow counter.

Determination of specific activity of nucleotides

An equivalent section of each electrophoresis strip containing radioactive and UV-absorbing material was eluted with pH 8.5 ammonia water and made up to 3 ml. The UV absorption spectrum of each eluate was read in a Spinco Model DU Spectro-

photometer against an equivalent eluate obtained from a blank electrophoresis strip. An aliquot of each eluate was plated out in an aluminum planchet and assayed for radioactivity in a Nuclear-Chicago Model D47 Micromil gas-flow counter, equipped with a Model 181 scalar and automatic sample changer. The counting efficiency was 30%; the thin samples required no correction for self-absorption. The specific activity of the UV absorbing substance was expressed in counts per minute per optical density unit at 265 mμ.

RESULTS

The 25.5 hour sample

Similar patterns of UV absorbing material were observed on the electrophoretograms of the DNA nucleotides from the uninfected and infected leaf. The UV absorbing material was located in a single broad band extending three to ten cm from the origin toward the anode. The absence of discrete bands of UV absorbing material possibly indicated the incomplete degradation of the DNA to mononucleotides.

Ninety percent of the radioactivity on the electrophoretograms was associated with this UV absorbing material. A small, discrete peak of radioactivity remained at the origin. Another small peak of radioactivity occurred 13 to 17 cm from the origin. No UV absorbing material could be detected in this region of the electrophoretogram, and it was assumed that this peak was free phosphate, hydrolyzed from the nucleotides by

a trace of 5'-nucleotidase in the phosphodiesterase preparation. On the control electrophoretogram of P^{32}-orthophosphate, the radioactivity had advanced the full length of the strip (30 cm). Salt effects might have delayed the orthophosphate migration in the sample electrophoretograms.

The ultraviolet spectra of the two 3 ml eluates from a 4 cm section of each electrophoretogram, commencing 6 cm from the origin, are shown in Fig. 1-II. The spectra do not precisely correspond to any of the nucleotide spectra reported in (7). This is not surprising, since the eluate probably contains a mixture of oligonucleotides. However, the spectra are sufficiently similar to one another to justify the calculation of the specific activity of the DNA-nucleotide phosphorus, on the basis of optical density measurements at 265 mμ.

The radioactivity of the 0.5 ml aliquots from the two eluates (corrected for 30% counting efficiency and background) were 140 ± 7 cpm for the uninfected leaf DNA nucleotides, and 143 ± 7 cpm for the virus-infected leaf DNA nucleotides. The specific activities were 226 ± 10 cpm and 212 ± 10 cpm per $O.D._{265}$, respectively. Thus, there seems to be no difference between the rates of P^{32} incorporation into DNA of TMV-infected and uninfected tobacco leaves, for the period up to 25.5 hours after inoculation.

A very approximate minimal estimate of the extent of DNA metabolism can be derived from the previous data. The specific activity of the supplied orthophosphate was

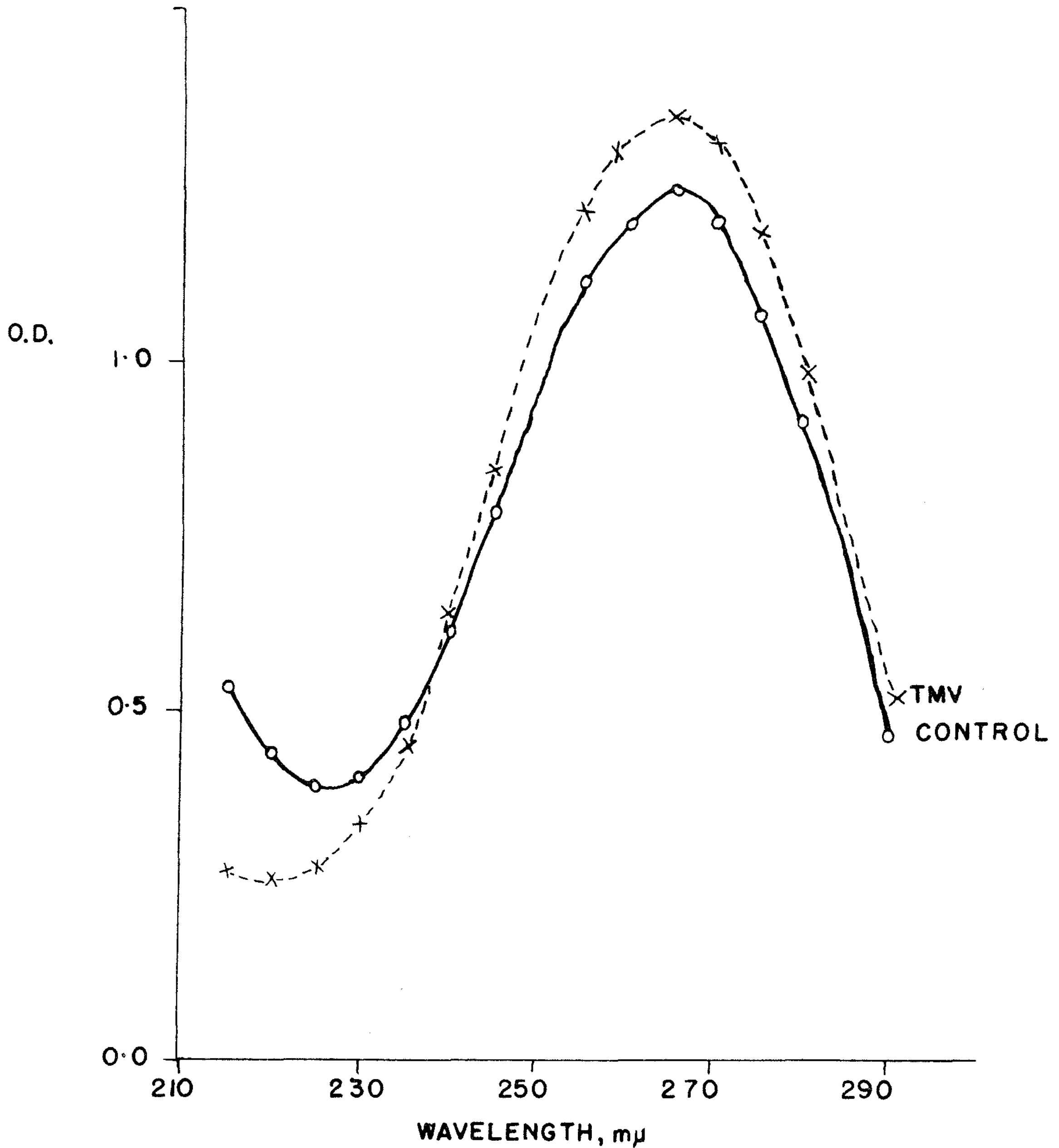

Fig. 1-II. Absorption spectra of 3 ml eluates (pH 8.5) from electrophoretograms of the hydrolyzed DNA preparations from TMV infected and uninfected control tobacco leaves. Leaves harvested for extraction 25.5 hours after infection.

3.8×10^8 cpm per µmole P. Assuming a molar extinction coefficient of 10,000 for the nucleotide material in the eluate, the mean specific activity of the P in the leaf nucleotides was 5,300 cpm per µmole P (corrected for P^{32} decay). Thus, a minimum of 0.0014% increase or turnover of leaf DNA must have occurred in 24.5 hours.

The 149.5 hour sample

During this long period of incubation, considerable difficulty was encountered in maintaining the turgor of the leaves. The xylem became periodically blocked. Sections of the petiole were excised to relieve the block. The leaves showed visible degeneration after the third day. It is unlikely that the results obtained from these leaves will be very meaningful, because of this degeneration and the possibility of bacterial contamination.

No UV absorbing material could be observed on the dried electrophoretograms of either the virus infected or the uninfected leaf DNA nucleotide preparations. The radioactivity distributions on the paper strips were similar for the two leaf preparations. Apart from a small peak of radioactivity at the origin, the radioactivity was distributed fairly uniformly from 3 cm to 17 cm from the origin in the direction of the anode. The radioactivity peak in a parallel P^{32}-orthophosphate electrophoretogram were located between 20 and 24 cm from the origin.

The spectra of the 3 ml eluates obtained from the section of each electrophoretogram 8 to 17 cm from the origin are shown in Fig. 2-II. The concentration of the UV absorbing material was very low. At this level of concentration, errors due to differences in the cuvettes and sensitivity of the spectrophotometer approach the magnitude of the reading. Hence, the specific activities calculated from these data can only give an approximate indication of the status of the leaf DNA metabolism.

The radioactivity of the 0.2 ml aliquots from the two eluates (corrected for 30% counting efficiency and background) were 5,900 ± 300 cpm for the uninfected leaf and 4,500 ± 230 cpm for the TMV infected leaf DNA nucleotides. The specific activities were 118,000 ± 5,900 cpm and 88,000 ± 4,400 cpm per $O.D._{265}$, respectively. Owing to the low accuracy involved in the determination of the UV spectra, little confidence can be placed in these figures. However, there is no indication of increased DNA metabolism in the TMV infected leaf.

An approximate minimal estimate of the extent of the DNA metabolism in the uninfected leaf during the 148.5 hour incubation in the presence of P^{32} can be derived from the previous data. Correcting for P^{32} decay, the specific activity of the P incorporated into DNA nucleotides was 4×10^6 cpm per µmole P. This incorporation is equivalent to a minimum of 1% increase or turnover of the leaf DNA. The possibility that this figure includes a contribution from the DNA metabolism of microorganisms growing in the degenerating leaf tissue cannot be ignored.

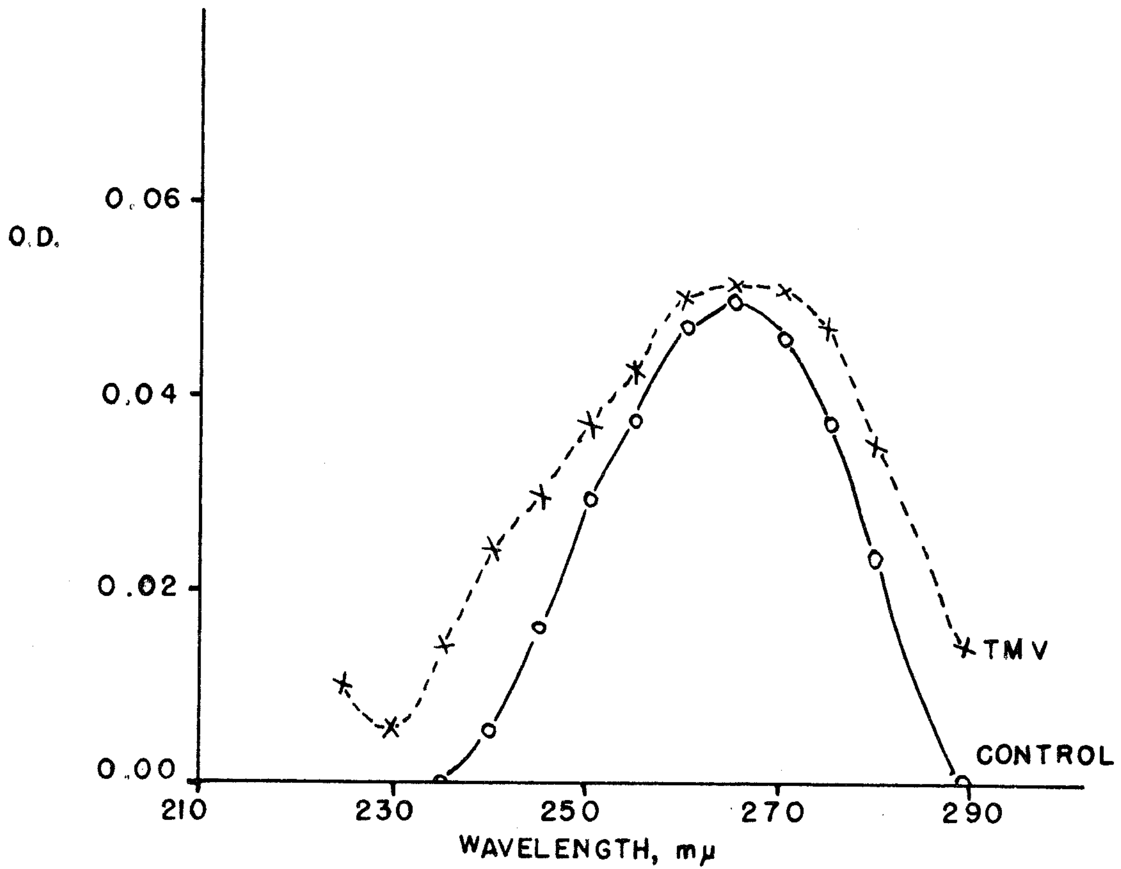

Fig. 2-II. Absorption spectra of 3 ml eluates (pH 8.5) from electrophoretograms of the hydrolyzed DNA preparations from TMV infected and uninfected control tobacco leaves. Leaves harvested for extraction 149.5 hours after infection.

PART 2

THE INFLUENCE OF 5-FLUOROURACIL ON TOBACCO-MOSAIC VIRUS PRODUCTION IN TOBACCO-LEAF DISCS

The influence of 5-fluorouracil on Tobacco-Mosaic Virus production in tobacco-leaf discs

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The influence of 5-fluorouracil on Tobacco-Mosaic Virus production in tobacco-leaf discs

In harmony with the concept of template replication as involved in the formation of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein, one could envisage that the multiplication of an RNA-virus in host tissue is basically a template process. Among the various possibilities is that the virus may act as an inducer of new template activity in the host. In particular, the RNA-virus, upon entry into the plant, might evoke the synthesis of a new DNA template by the host. This possibility has been subjected to a preliminary test by use of the metabolite antagonist, 5-fluorouracil (5-FU). 5-FU inhibits the formation of thymidine in Ehrlich-ascites-tumor cells of mice[1,2] and in human-tumor transplants[3] presumably by suppression of the methylation of deoxyuridine to thymidine[1,2,4]. Growth inhibition by 5-FU is reversed by thymidine in the case of *Lactobacillus leuchmannii*[1,5]. This is not true, however, for the case of *Escherichia coli*, strain K-12, in which 5-FU-induced inhibition of growth of the organism is not reversed by thymidine although it is reversible by uracil[4]. Thus, although 5-FU can clearly act as an inhibitor of DNA synthesis, it is not evident that 5-FU always exerts its inhibition in this way.

The influence of 5-FU on multiplication of tobacco-mosaic virus (TMV) has been studied in excised tobacco-leaf discs. Half-expanded leaves of *Nicotiana tabacum* var. Samsun were inoculated, while still attached to the plant, with the common strain of TMV (U-2). In one case, Expt. 2, leaves were inoculated after excision. Following inoculation, leaf discs 12 mm in diameter were punched from the leaves, washed in distilled water, and floated on the treatment solution in covered petri dishes. The solutions were made up in half-strength Hoagland's solution and adjusted to pH 6.5. The discs were then incubated at 26° in continuous light and harvested as indicated below. At the end of the incubation period the discs were washed and frozen until use. The virus was then extracted by the method of SCHNEIDER[6] and assayed spectrophotometrically.

The data of Table I show that virus production is inhibited by 5-FU at concentrations of 10^{-2} and 10^{-3} *M* but not at 10^{-4} *M*. This inhibition is not reversed by thymidine supplied at concentrations equal to or greater (10x) than that of the 5-FU. Uracil also is ineffective in reversal of the 5-FU-induced inhibition of virus multiplication (Table II). It would appear, therefore, that the inhibition of virus production in tobacco leaves by 5-FU is not to be attributed to effects of 5-FU on the DNA metabolism of the host, but to other and more general inhibitory effects of the substance. Thus, 5-FU at a concentration of 10^{-2} *M* is clearly unfavorable for tobacco discs since it induces a marked chlorosis of the tissue, beginning at about the third day of incubation.

That the biological properties of the virus are affected directly by 5-FU is also clear. The specific infectivity of virus produced in the presence of 5-FU has been compared with that of normal virus produced in the absence of the inhibitor. Specific infectivity was assayed in all cases by local lesion counts on the primary leaves of pinto beans. The specific infectivity of virus produced in the presence of 10^{-2} *M* 5-FU but not in the presence of 10^{-3} *M* 5-FU is significantly lower than that of normal virus.

TABLE I

EFFECT OF 5-FU ON TMV PRODUCTION BY LEAF DISCS INCUBATED AT 26°, CONTINUOUS ILLUMINATION

The discs were incubated in ½-strength Hoagland's solution with or without 5-FU. The values given are the means of two measurements of the absorbance at 260 mμ of aqueous aliquots of TMV. Differences > 0.14 are significant at 5 % level.

Experiment No.	*1*	*2*
Time incubation commenced	*12 h after incoulation*	*6 h prior to inoculation*
Days incubated in treatment solutions	7.5	7
A. *Virus inoculated*		
I. No 5-FU	0.450	0.510
II. 10^{-2} *M* 5-FU	0.045	0.150
III. 10^{-3} *M* 5-FU	0.205	0.300
IV. 10^{-4} *M* 5-FU	0.370	0.450
B. *Healthy leaf discs*		
No 5-FU	0.005	0.020

TABLE II

EFFECT OF THYMIDINE AND URACIL ON THE 5-FU INHIBITION OF TMV PRODUCTION

Leaves inoculated on plant 7 h before harvesting discs and floating them on treatment solutions. Results expressed as absorbance at 260 mμ of aqueous virus aliquots. The values are the means of two measurements. Differences > 0.30 are significant at 5% level. All solutions made up in ½-strength Hoagland's solution. Incubated for 7 days

	10^{-3} M 5-FU	*No 5-FU*
Basal solution	0.440	0.880
10^{-2} *M* uracil	0.455	0.795
10^{-3} *M* uracil	0.520	0.825
10^{-2} *M* thymidine	0.520	1.495
10^{-3} thymidine	0.525	0.840

The high concentration of 5-FU needed to affect inhibition of TMV multiplication in leaf discs, coupled with the obvious 5-FU-induced derangement of host tissue metabolism, indicates that the mechanism of inhibition may lie in some general and unfavorable disturbance of the host tissue. The fact that this inhibition is not reversed by either uracil or thymidine appears to rule out the possibility that the mechanism involves a block in the formation of thymidine. It may be tentatively concluded that the mechanism of 5-FU inhibition of TMV multiplication is not directly concerned with DNA synthesis.

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Part 2

In the original copy of this thesis the pages indicated above contain the text of a published article:

"The Influence of 5-Fluorouracil on Tobacco-Mosaic Virus Production in Tobacco-Leaf Discs"

by C. I. Davern and James Bonner

Biochimica et Biophysica Acta, vol. 29, pp. 205-206 (1958)

PART 3

INVESTIGATIONS OF THE EFFECT OF DNAase ON THE MULTIPLICATION OF TMV IN TOBACCO LEAF DISCS

The possibility that DNA is involved as a template in the multiplication of TMV could be tested by specifically destroying the DNA in the host tissue prior to infection. DNAase, if it could penetrate into the cell nucleus, should be a suitable destructive agent with the desired specificity.

The permeability of intact tobacco leaf discs to DNAase, and the possible influence of this enzyme on TMV multiplication, were investigated.

a. Influence of DNAase on the DNA of Intact Tobacco Leaf Discs

The Feulgen staining technique (8) was used to assay for the penetration of active DNAase into the nuclei of intact tobacco leaf discs.

MATERIALS AND METHODS

Discs 1 cm in diameter were punched out of fully expanded leaves of three-month old plants, Nicotiana tobacum, var. Samsun. One group of leaf discs was cytolyzed in ether for two minutes. Cytolyzed and intact leaf discs were then divided into two treatment groups. One group of leaf discs was vacuum infiltrated with a buffered DNAase solution (1 mg per ml DNAase in 0.01 M phosphate buffer, pH 7, 0.01 M magnesium

chloride). The other group of leaf discs was vacuum infiltrated with the buffer only. After vacuum infiltration, the leaf discs were incubated for 16 hours in their respective treatment solutions. All tests were performed in duplicate. At the end of the incubation period, the discs were fixed in Carnoy's fluid and stained by the Feulgen procedure. Subsequently, the leaf discs were macerated, mounted in glycerol, and inspected microscopically, at 440x.

RESULTS

Cytological observations were restricted to the epidermal guard cells, since their nuclei were the least obscured by other cellular components. The nuclei of the DNAase infiltrated, ether-cytolyzed leaf discs were Feulgen negative; all other treatments gave Feulgen positive nuclei.

Thus, active DNAase cannot penetrate into intact cells of tobacco leaf discs.

b. Influence of DNAase Treatment on TMV Development

Although intact leaf tissue is impermeable to DNAase, DNAase may be able to penetrate injured cells by the same paths taken by invading TMV. Therefore the effect of DNAase application before and after inoculation with TMV was studied.

Experiment 1 - DNAase treatment prior to TMV inoculation.--Twenty leaf discs were vacuum infiltrated with the DNAase solution. Another twenty were infiltrated with buffer only.

The discs were then placed on damp filter paper to allow them to lose the solution from their intercellular spaces. Eighteen hours after infiltration they were inoculated with TMV, strain U1 (0.1 mg per ml), using 600 mesh Celite as an abrasive. The discs were incubated on half-strength Hoagland's solution in Petri dishes for 146 hours, illuminated by 400 fc fluorescent light. The discs were then harvested, washed with distilled water, and frozen.

Virus was isolated from the leaf discs by the procedure of Schneider (9). The total yield of purified TMV was suspended in 10 ml of distilled water, and titer assayed by UV absorption at 260 mμ.

Experiment 2 - DNAase treatment after TMV inoculation.--Freshly excised tobacco leaves, with their petioles immersed in half-strength Hoagland's solution, were inoculated with TMV as above. Discs were punched from these leaves 15.5 hours later and divided into two groups of 20 discs each. One group was vacuum infiltrated with DNAase, and the other with buffer only, 17.5 hours after inoculation. Leaf discs were incubated under fluorescent light, as before, for 161.5 hours. TMV was then isolated from the leaf discs and assayed, as in experiment 1.

RESULTS

The yields of virus from the DNAase and buffer infiltrated leaf discs are shown in Table 1-II. There was no

TABLE 1 - II

Effect of DNAase treatment on the Yield of TMV from 20 Leaf Discs 1 cm in Diameter. Yield Expressed as OD_{260} of an Aqueous Suspension of Total Extracted Virus

	Treatment	Incubation time	Yield of TMV (OD_{260}) Replication 1	Replication 2
Exp. 1	18 hrs. before inoculation:			
	DNAase (mg/ml)	146 hours	0.50	0.69
	BUFFER	" "	0.61	0.58
Exp. 2	17.5 hrs. after inoculation:			
	DNAase (mg/ml)	161.5 hours	0.41	0.42
	BUFFER	" "	0.43	0.46

significant effect of the DNAase on the development of TMV in the leaf discs.

DISCUSSION AND CONCLUSIONS

The only safe conclusion which can be drawn from these data is that DNAase probably did not penetrate into the leaf cells. To test the hypothesis that DNAase can penetrate into the cell by the route taken by infecting TMV, the DNAase should have been applied simultaneously with the virus.

Even with an ideal experimental design, a negative result would be meaningless. Even a positive result would be difficult to interpret, because the destruction of the host DNA may interfere with TMV replication through indirect effects on the cell metabolism.

GENERAL DISCUSSION

Further studies on the inhibition of TMV development by 5-fluorouracil (10) have demonstrated that 5-fluorouracil is extensively incorporated into the TMV RNA. This may in part explain the inhibition. However, it is strange that uracil does not alleviate the inhibition. Since thymidine was also ineffective, it is unlikely that blocking of DNA synthesis is the cause of the observed inhibition of virus multiplication.

This conclusion is consistent with the results of the P^{32}-orthophosphate experiment, in which no increase in the DNA metabolism was observed in TMV-infected leaves.

There is evidence that DNA may be implicated in normal RNA synthesis. For example, the inability of enucleated Acetabularia (11) and enucleated amoeba (12) to synthesize RNA, despite their large cytoplasmic RNA concentrations, indicates that the nucleus is essential for RNA synthesis, and that cytoplasmic RNA is incapable of self-replication. These results, together with others (13,14,15,16,17) which suggest that RNA is synthesized in the nucleus and later moves to the cytoplasm, have been considered as evidence for the implication of the nucleus as the sole site of RNA synthesis in the cell.

Apparent DNA independent self replication is not unique to the RNA viruses. Chloroplasts (18), mitochondria (19), and possibly other cellular organelles possess this property,

as demonstrated by the phenomenon of cytoplasmic inheritance.

Thus the concept of the DNA- RNA-protein sequence of specificity transfer is not adequate to explain the replication of RNA viruses or the occurrence of cytoplasmic inheritance. Since chloroplasts (20) and mitochondria contain RNA (21), it is tempting to suggest that some RNA may share with DNA the properties of genetic specificity and self replication. Again, the absence of demonstrable cytoplasmic RNA synthesis in enucleated cells would tend to negate this hypothesis; however, the postulated role of the nucleus could involve the production of non-genetic agents essential for cytoplasmic RNA synthesis.

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SECTION III

THE CONSERVATION OF MICROSOMAL RNA IN ESCHERICHIA COLI

INTRODUCTION

Recent radioactive tracer studies on the metabolic status of various macromolecular fractions in exponentially growing cultures of micro-organisms have demonstrated repeatedly that these fractions conserve their incorporated tracer (1,2,3,4). These findings cast doubt on the Schoenheimer concept of "The dynamic state of cellular constituents" (5), in which the cell is considered to be in a steady state such that its functional integrity is conserved while its macromolecular components are subjected to a continuing metabolic breakdown and synthesis. While casting doubt, these tracer studies do not disprove the dynamic state concept, for metabolic turnover coupled with an efficient mechanism for salvaging breakdown products for use in resynthesis would lead to the observed result of conservation of label. However such a scheme would lead to the redistribution of the label among the molecules of the macromolecular fraction.

The existence of such a redistribution of label can now be experimentally detected by the use of heavy isotope labeling in conjunction with a subsequent analysis of the density distribution of the macromolecules by the method of equilibrium density-gradient sedimentation (6). This method has been used to study the distribution of the heavy nitrogen isotope N^{15} among the molecules of DNA after the transfer of a uniformly N^{15} labeled, exponentially growing E. coli population to a

N^{14} medium (7). In this experiment it was found that N^{15} was totally conserved within a molecular sub-unit of the DNA.

This thesis reports the results of a similar heavy to light isotope transfer experiment, designed to investigate the metabolic stability of high molecular weight (microsomal) RNA in an exponentially growing E. coli B culture.

In a constant centrifugal field, the concentration distribution of a single macromolecular species of uniform density, at equilibrium in a constant density gradient, is Gaussian. The distribution is centered about that point in the gradient where the density of the solution, and the buoyant density of the macromolecule are equal (6). Because it is labeled with heavy isotope, the RNA synthesized up to the time of transfer will band at a higher density in the gradient than RNA synthesized from light isotope precursors. Thus the population of pre-transfer RNA molecules, if conserved after the transfer of the bacterial culture to light isotope substrates, will be distinguishable from the population of RNA molecules synthesized during the post-transfer period by virtue of their higher density.

In the previously mentioned DNA transfer experiment, the density difference imparted by the substitution of N^{15} for N^{14} in the DNA molecule gave sufficient resolution of the DNA density species in the density gradient. However, since the standard deviation of the Gaussian concentration distribution for a single macromolecular species is inversely proportional to the

square root of the molecular weight, and the molecular weight of E. coli microsomal RNA (6×10^5)(8) is only one twelfth that of E. coli DNA, a greater density difference between the labeled and unlabeled molecules was necessary for adequate band separation, in the density gradient. Thus both C^{13}, and N^{15} heavy isotopes were used to label the RNA in the transfer experiment.

In addition to containing high molecular weight microsomal RNA, E. coli contains a low molecular weight soluble RNA (20% of the total RNA). Because its molecular weight is only 3×10^4 (9), its band width would be approximately four times broader than the microsomal RNA band and thus should not interfere with the density gradient analysis of the microsomal RNA. Preliminary experiments comparing the microsomal RNA bands formed in the density gradient of whole cell lysates and of purified microsome lysates confirmed this expectation.

E. coli microsomal RNA has a buoyant density of 2.04 gm cm^{-3} (8). Cesium formate was chosen as the salt to form the density gradient since it forms solutions of high density and low UV absorption.

In addition to analyzing the microsomal RNA, the distribution of C^{13} N^{15} label among the molecules of DNA was also observed in the same transfer experiment.

MATERIALS AND METHODS

Preparation of C^{13} N^{15} medium

The C^{13} and N^{15} E. coli growth medium in the transfer experiment was derived from acid-hydrolyzed algae (Ankistrodesmus Sp.). The algae were cultured in a closed system, using $C^{13}O_2$ as sole carbon source and $N^{15}H_4Cl$ as sole nitrogen source. (See details below).

1. Preparation of C^{13} N^{15} algae

The nutrient solution for the algae was prepared from the following stock solutions (all concentrations are expressed in gm per liter of solution)

Stock solution 1 : KCl, 59.6 gm; $CaCl_2 \cdot 6H_2O$, 2.2 gm; $MgSO_4 \cdot 7H_2O$, 24.7 gm.

Stock solution 2 : $Na_2HPO_4 \cdot 2H_2O$, 35.6 gm; $NaH_2PO_4 \cdot 2H_2O$, 31.2 gm.

Stock solution 3 : $FeSO_4 \cdot 7H_2O$, 10 gm; $MnCl_2 \cdot 4H_2O$, 0.2 gm; $ZnSO_4 \cdot 7H_2O$, 0.1 gm; ethylene-diamine-tetra-acetate, 1.0 gm.

A liter of nutrient solution at pH 6.5, was made up under sterile conditions, containing 10 ml of stock solution 1, 10 ml of stock solution 2, and 1 ml of stock solution 3. To this solution was added 438 mg of $N^{15}H_4Cl$ of 97% isotopic purity (Isomet Corp.). The nutrient solution was then transferred to a 12-liter 3-necked flask and inoculated with 3.5×10^6 algae in 1 ml of water.

The culture flask was fitted with a mercury manometer and connected to a vacuum pump. The third neck was connected to a CO_2 generator. A diagram of the complete apparatus is shown in Fig. 1-III. 5.82 gm of $BaC^{13}O_3$ of 67% isotopic purity (Isomet Corp.), was placed in the 200 ml three-necked flask of the CO_2 generator, and 60 ml of 22 N phosphoric acid was added to the dropping reservoir. The flask was connected on one side with the culture flask, and on the other with the CO_2 scrubber.

After the culture flask and CO_2 generator were evacuated to 1 cm Hg, they were returned to atmospheric pressure with CO_2-free air drawn through the scrubber. This flushing cycle was repeated twice. Then the system was again evacuated to 1 cm Hg, and closed off from the outside atmosphere. The $C^{13}O_2$ was generated over a period of about 10 min by adding the phosphoric acid to the $BaC^{13}O_3$. When the evolution of $C^{13}O_2$ had ceased, the stopcock connecting the CO_2 scrubber to the generator was opened, and CO_2-free air was allowed to sweep through the generator into the culture flask. When the pressure had reached 4 cm Hg below atmospheric pressure, the culture flask was isolated from the CO_2 generator. The $C^{13}O_2$ concentration in the culture flask was approximately 6%.

The culture, magnetically stirred, was incubated for 7 days at room temperature (about 26°C), under continuous illumination by 2 General Electric "Power Groove" fluorescent tubes one inch from the side of the flask. Fans were used to dissipate the heat generated by the lights and magnetic stirrer.

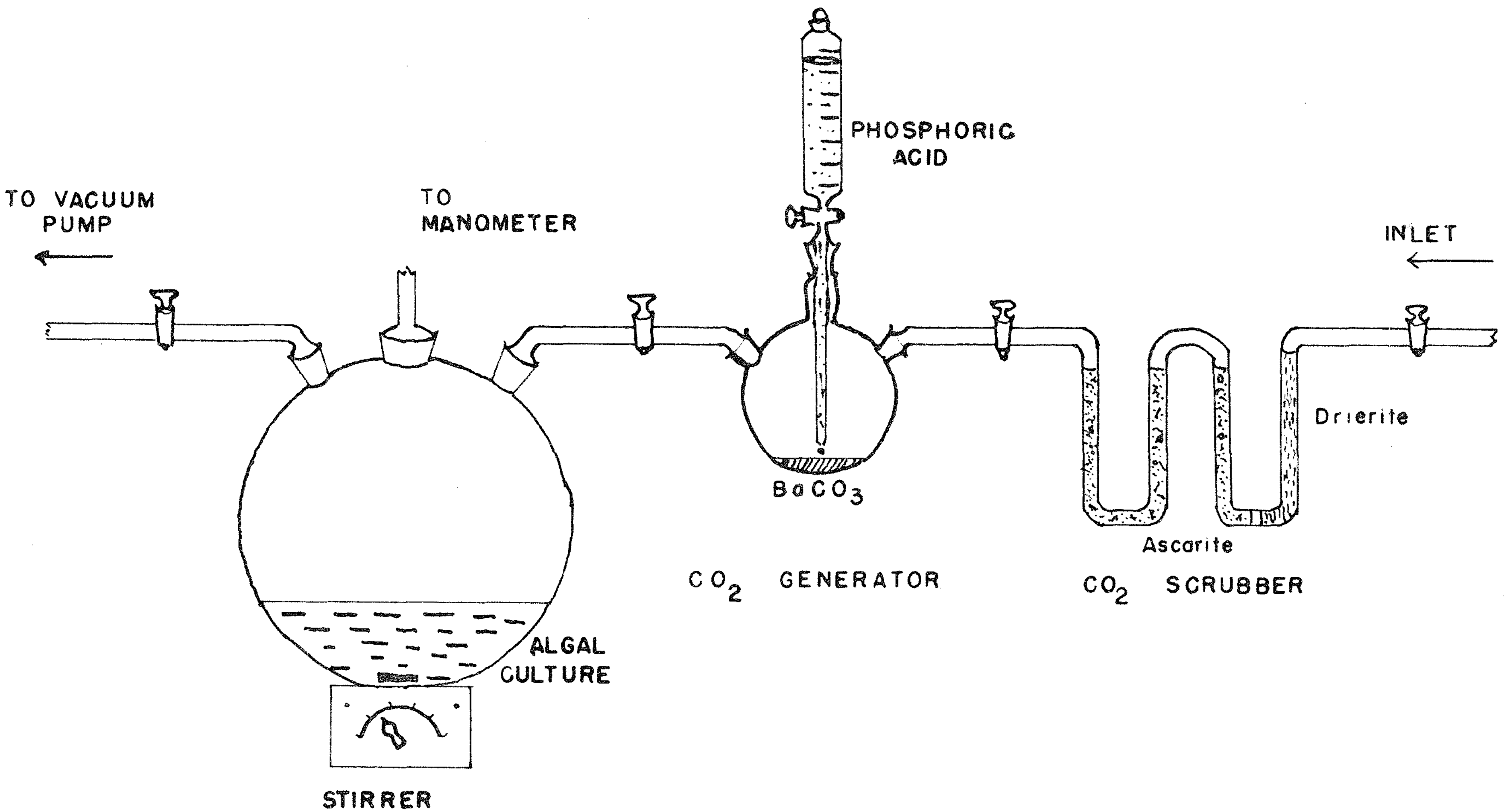

Fig. 1-III. Diagram of apparatus used to culture C^{13} N^{15} Ankistrodesmus sp.

From the start of the sixth day of incubation, small aliquots of the culture atmosphere were assayed for the presence of CO_2 by an infrared analyzer (Beckman), until less than 0.01% CO_2 could be detected. The culture flask was then removed from the rest of the system, and the algae harvested by centrifugation in the cold. The pellet of algae was frozen, lyophilized, and stored at 0°C. The culture yielded 414 mg of lyophilized algae.

2. Hydrolysis of algae

The lyophilized algae were taken up in 5 ml of N HCl and refluxed in a boiling water bath for 2 hours. The resultant digest was filtered through sintered glass, and the residue washed with three 2 ml aliquots of N HCl. The washed residue was resuspended in 9 ml of 6 N HCl and refluxed for 25.5 hours in a boiling water bath. This acid hydrolysate was filtered through sintered glass, and the residue washed with three 5 ml aliquots of hot distilled water. The filtrate and washes were combined and evaporated on a flash evaporator to a gum-like residue. This residue was resuspended in 20 ml of distilled water and re-evaporated. The residue was again resuspended in 20 ml of distilled water, and decolorized with about 0.1 gm acid-washed Norit A for one hour. The decolorized solution was filtered through sintered glass, and the residue washed with four 5 ml aliquots of distilled water. The filtrate and washes were combined and vacuum-evaporated to a gum.

The gum was transferred to a tared weighing bottle with three 1 ml aliquots of distilled water and placed in a desiccator

over solid KOH, and dried under vacuum for 24 hours to give 71 mg of dry material. This was then dissolved in 0.71 ml of distilled water. The solution was autoclaved and stored in the refrigerator until use; it is called the algal hydrolysate.

Preparation of cesium formate

Cesium chloride (Maywood Chemical Works) was converted to cesium formate by passing it through an anion-exchange resin in the formate form.

250 gm damp weight of analytical grade Dowex 2-X 8, 200-400 mesh anion exchange resin, in the chloride form (Biorad Laboratories), was converted to the hydroxide form by elution with three liters of 2N KOH. The resin was washed free of excess KOH with 5 liters of doubly distilled water. It was then converted to the formate form by elution with 2 liters of 2 N formic acid.

After the excess formate was washed through the column with 3 liters of doubly distilled water, 60 gm of CsCl in a 2 N solution was added to the column, and the column was eluted with one bed volume of doubly distilled water (about 500 ml). The effluent was collected and treated with 0.5 gm acid washed Norit A to remove the UV absorbing material shed by the resin. The cesium formate solution was then filtered through sintered glass and evaporated in a flash evaporator to approximately 30 ml. The solution was titrated to pH 7.1 with concentrated formic acid. The resultant solution had a density of 2.32 gm per cm^3 and an optical density of 0.09 at 265 mμ.

Design of the transfer experiments

Two transfer experiments were performed. In the first, a total bacterial lysate was analyzed in the density gradient for the distribution of microsomal RNA. In the second, the microsomal particles were first isolated and then degraded with sodium dodecyl sulfate to yield the RNA subsequently analyzed in the density gradient.

Experiment 1.--Escherichia coli B, adapted to algal hydrolysate growth medium, was cultured at 37°C with mild aeration, in M9-salts medium (10), made with $N^{15}H_4Cl$.

In addition, the culture medium contained the C^{13} N^{15} algal hydrolysate as a sole carbon source.

Bacteria, uniformly labeled with C^{13} and N^{15} were prepared by growing cells for 11 generations (to a titer of 3.1×10^8 per ml) in 21 ml of the above medium, containing 100 μg of $N^{15}H_4Cl$ per ml and 0.2 ml of C^{13} N^{15} algal hydrolysate. The growth of the bacteria was followed by colony assays and direct cell counts (Fig. 2-III). After harvesting 9.4 ml of the culture to obtain the zero time sample, an abrupt change to C^{12} N^{14} medium was accomplished by adding to the remaining 10 ml of culture 10 ml of M9-salts medium, containing 2 mg per ml of NH_4Cl, 6 mg per ml of casamino acids, and 10 μg of each of the four ribosides, adenosine, guanosine, cytidine, and uridine.

Further 10 ml harvests were made at 33, 66, 100, and 133 minutes after zero time. At 33, 66, and 100 minutes, the volume

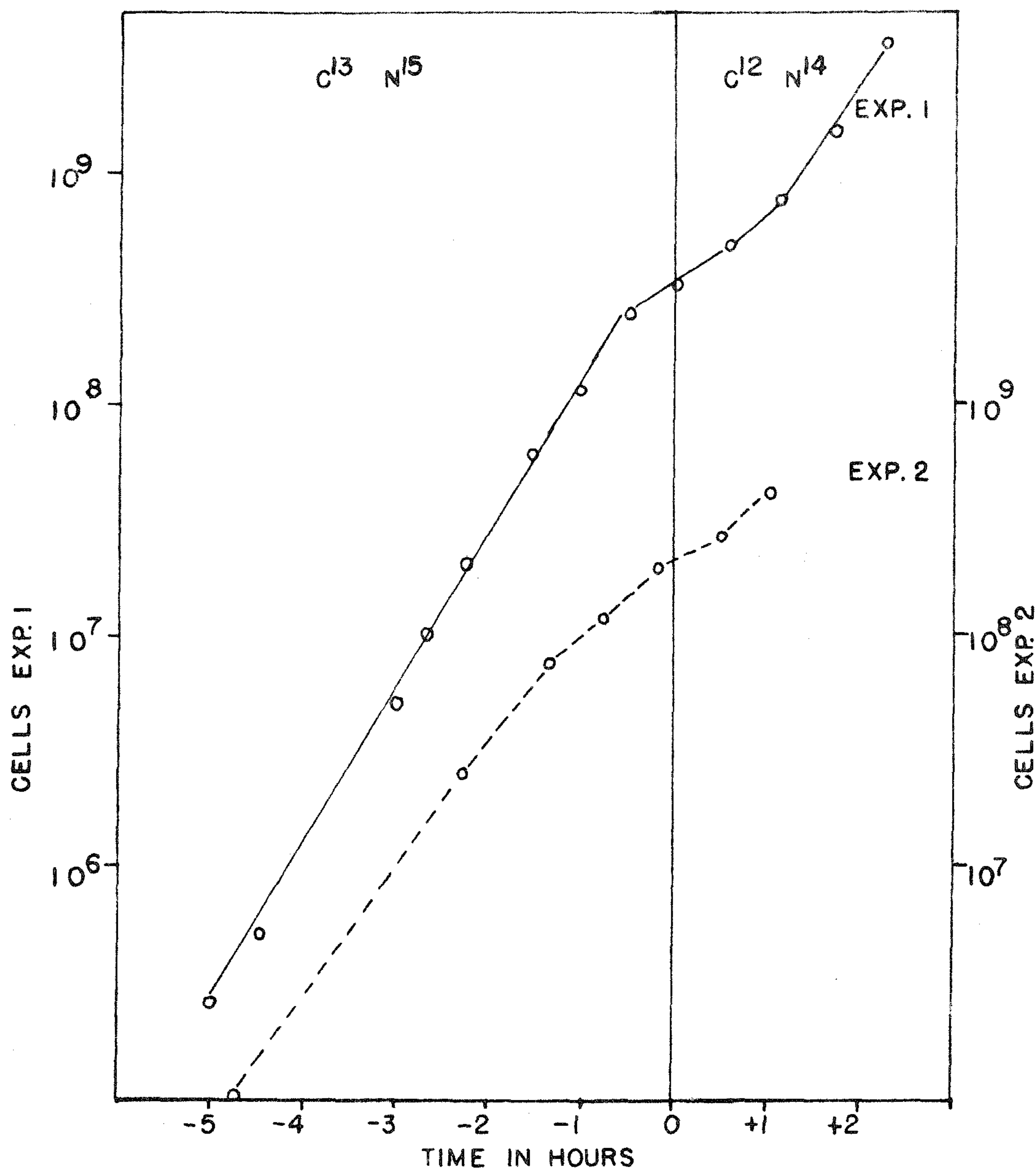

Fig. 2-III. Growth of bacterial populations first in C^{13} N^{15} and then in C^{12} N^{14} medium. The values on the ordinates give the actual titers of the cultures up to the time of addition of C^{12} N^{15}. Thereafter, during the period when samples were being withdrawn for density-gradient centrifugation, the actual titer was kept between 1 and 2.5 x 10^8 by additions of fresh medium. The titers shown during this later period have been corrected for the withdrawals and additions.

of the culture was restored to 20 ml with casamino M9-salts medium. After removing 0.1 ml of the sample for counting the cell population each sample was immediately chilled and centrifuged in the cold for 5 minutes at 2000 rpm in the International centrifuge. Bacterial pellets were then resuspended in 0.4 ml of a cold solution, 0.015 M in NaCl and 0.01 M in ethylene-diamine tetra-acetate (EDTA). After lysis of the cells by the addition of 0.1 ml of 10% sodium dodecyl sulfate, the lysates were frozen and stored.

Experiment 2.--E. coli B were grown for 8 generations (to 2.1×10^8 per ml) in 91 ml of the C^{13} N^{15} algal hydrolysate $N^{15}H_4Cl$ M9-salts medium. The growth of the bacteria was followed by colony assays and microscopic cell counts (Fig. 2-III). At zero time, 30 ml of culture were harvested, and 60 ml of pre-warmed casamino acids nucleosides M9-salts medium added to the remaining 60 ml of growing cells. Thirty min after zero time, a 60 ml harvest was replaced with 60 ml of pre-warmed casamino acids M9-salts medium. Sixty min after zero time, the entire 120 ml of culture was harvested.

The harvests were immediately processed in the cold. Bacteria were pelleted by centrifugation 10 min at 3000 rpm in the International centrifuge. The bacteria were resuspended in 30 ml of 0.001 M magnesium acetate 0.01 M tris, pH 7.4 and again pelleted by a 10 min centrifugation. The pellet was suspended in 5 ml of the tris magnesium acetate buffer, containing 2.5 μg of DNAase per ml.

The bacterial suspension was placed in a chilled, stainless steel Nossal shaker (11) canister with 5 gm of Balatini beads (grade 14). It was then shaken, to break open the cells, for 2 one minute periods, with ice water cooling of the canister between the shaking periods.

After withdrawing the disrupted cell suspension from the Nossal canister, the glass beads were washed with two 2 ml aliquots of buffer. The disrupted cell suspension and the combined washes were centrifuged separately at 6000 rpm in the Servall SS-1 for 15 minutes. The optical density of the supernatant of the disrupted cell suspension was read at 260 mμ to provide an estimate of the RNA content. This supernatant and the wash supernatants were then placed in separate polyethylene vials and frozen.

Both supernatants of each sample were thawed 6 hours later, and 1.5 ml of the disrupted bacterial cell supernatant combined with the 4 ml of wash supernatant. The volumes were made up to 11 ml with buffer in lusteroid centrifuge tubes and centrifuged in the Spinco Model L for a total of 10 hours at 40,000 rpm. (The run was interrupted after 4 hours by a machine failure, with the resultant partial loss of the 30 minute sample). The supernatants were discarded, and the pellets dispersed, each in 0.5 ml of 2% sodium dodecyl sulfate, 0.015 M NaCl, 0.01 M EDTA. These microsomal lysates were frozen.

Density gradient centrifugation

The density gradient formed by a cesium formate solution (density 2.072 gm cm^{-3}) at 52,640 rpm and 20°C reaches equilibrium in less than 11 hours, and has a value of 0.087 gm cm^{-4}.

Since the buoyant density of E. coli microsomal RNA is 2.04 gm cm^{-3}, the buoyant density of the 67.1% C^{13}, 97.0% N^{15} RNA would be expected to be in the vicinity of 2.09 gm cm^{-3} (see later). Thus the density of the cesium formate-lysate solution for each sample time was adjusted to a value approximately intermediate between these two densities. In making the adjustments, the density was measured by reading the refractive index of small aliquots in the Abbe refractometer (Zeiss), using the following relationship for cesium formate at 25°C:

$$\text{Density} = 12.8755 \text{ ref. index} - 16.209$$

The compositions of the cesium formate-lysate solutions for density gradient analysis are shown in Table 1-III for both experiments. Since leaks occurred during most of centrifugations, the density of the cesium formate-lysate solutions was redetermined after the runs.

For each centrifugation, 0.7 ml of the cesium formate-lysate solution was added to a 12 mm, 4°, Kel-F centrifuge cell, fitted with a -1° wedge window. Solutions were centrifuged at 52,640 rpm at 20°C in the Spinco Model E ultracentrifuge for 53-61 hours.

TABLE 1 - III

Composition and Density of Samples for Density Gradient Centrifugation of RNA

Experiment #	Sample time min	ml Lysate	ml Cs formate (density 2.32 gm cm^{-3}	ml H_2O	ml in cell	Density gm cm^{-3}
1	0	0.04	0.80	0.150	0.7	2.080
	33	0.08	0.80	0.113	0.7	2.072
	66	0.19	0.80		0.7*	2.077
	100	0.19	0.80		0.7*	2.077
	133	0.19	0.80		0.7*	2.075
	0 133	0.08 0.08	0.80	0.045	0.7	2.087
2	0	0.09	0.93	0.115	0.7*	2.085
	30	0.14	0.95	0.045	0.7*	2.084
	60	0.18	0.94	0.025	0.6*	2.083

*Cell leaked partially during centrifugation

Ultraviolet absorption photographs of the RNA in the density gradient were taken during the course of each centrifugation and scanned with a double beam automatic recording microdensitometer (Joyce Loebl).

In addition to analyzing the microsomal RNA by density-gradient centrifugation, aliquots of the bacterial lysates from Experiment 1 were analyzed for the density distribution of DNA, in a CsCl density gradient. Samples for centrifugation were prepared as shown in Table 2-III. 0.7 ml of each sample was centrifuged at 44,770 rpm in the Spinco Model E ultracentrifuge at 25°C for at least 25 hours. Ultraviolet absorption photographs were taken and scanned.

RESULTS

During the density-gradient centrifugation, the first clear separation of the C^{13} N^{15} RNA and the C^{12} N^{14} RNA into discrete bands occurred after about 22 hours. Thereafter, the rate of increase in separation between the RNA bands decreased exponentially with time of centrifugation. All comparisons of the RNA distribution in the density gradient for the various samples were made from enlarged microdensitometer tracings of UV absorption photographs taken between the 53rd and 61st hour of centrifugation. Over this 8 hour period, the separation between the bands increased by 2%.

Experiment 1.--The microdensitometer tracings of the microsomal RNA distributions for the zero time sample, the 4 post-

TABLE 2 - III

Composition and Density of Samples for Density Gradient Centrifugation of DNA (Experiment 1)

Sample time min	ml Lysate	ml CsCl Density = 1.852 gm cm^{-3}	ml H_2O	ml in cell	Density gm cm^{-3}
0	0.09	0.80	0.060	0.7	1.726
33	0.05	0.80	0.094	0.7	1.731
66	0.15	0.80		0.7	1.728
100	0.15	0.80		0.7	1.724

transfer samples, and a sample containing aliquots of the zero time and fourth post-transfer samples are shown in Fig. 3-III. In addition, the results of the density gradient centrifugation of the DNA from the zero time and the first three post-transfer samples are shown in the same figure.

The RNA from each of the post-transfer samples was distributed in 2 discrete bands, one corresponding to the C^{13} N^{15} band in the zero time sample, and the other to C^{12} N^{14} RNA.

A comparison of the microdensitometer tracings from the 33 min sample and the combined 0 min and 133 min sample gave no evidence for the existence of microsomal RNA of density intermediate between the heavy and light isotope bands in the 33 min sample.

The relative proportion of the C^{13} N^{15} microsomal RNA decreased with time of culture after transfer. The actual distance between the modes of the RNA bands in each post-transfer sample are given in Table 3-III. These distances were corrected for variations among the samples in the density gradient over the region of RNA band formation (the density gradient varies directly with distance along the radius of rotation). In addition, by using a plot of seperation against centrifuge time obtained from the 66 min sample, the distances were corrected to a centrifugation time of 60 hours. (See last column of Table 3-III).

Taking the base composition of E. coli microsomal RNA to be 1 : 0.69 : 0.79 : 0.63 for guanine, cytosine, adenine, and

See Reverse

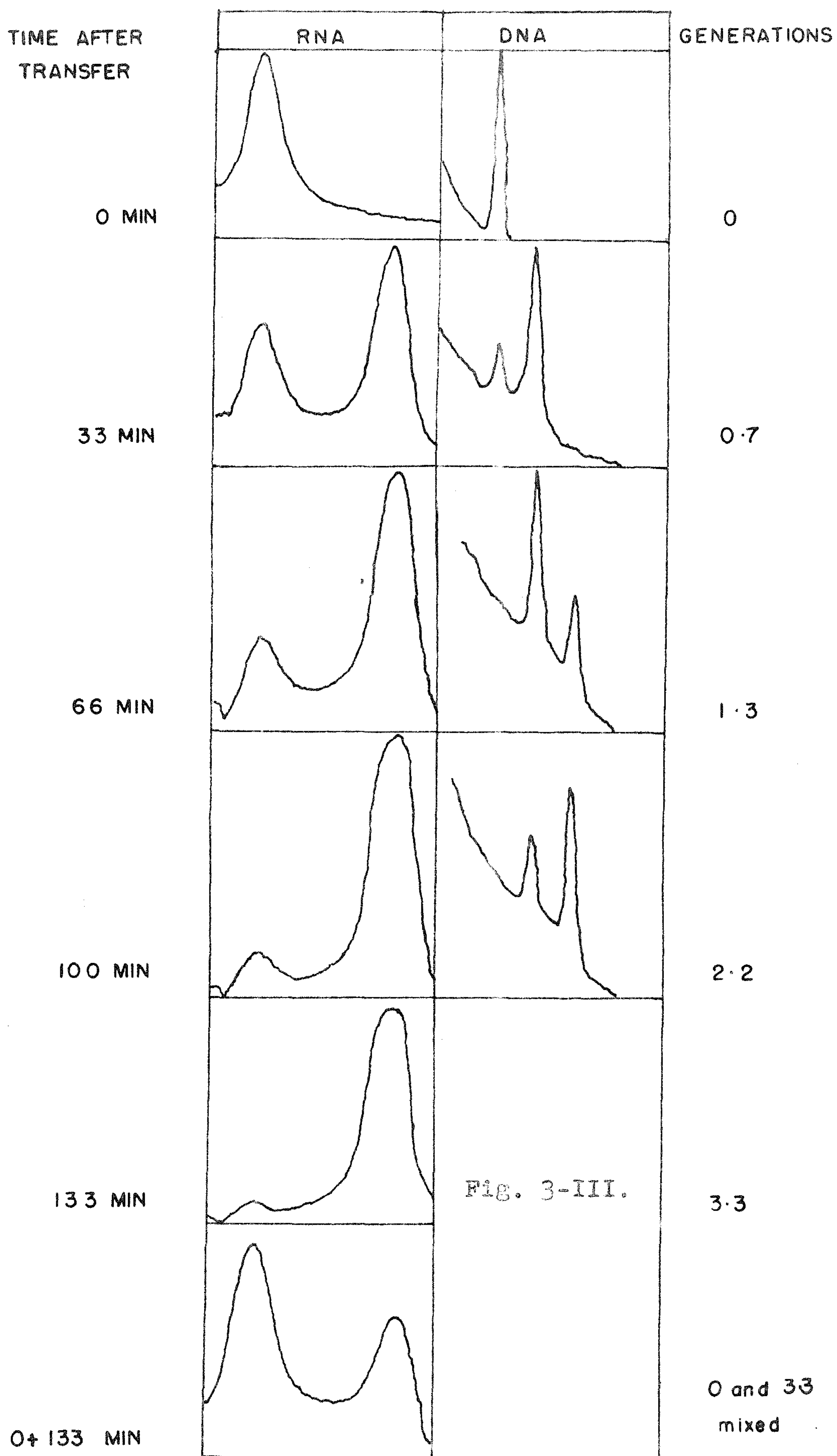

Fig. 3-III.

TABLE 3 - III

Distance Separating Modes of RNA Bands in the Density Gradient at Equilibrium

Experiment #	Sample (time after transfer in min)	Time centrifuged at 52,640 rpm (hours)	Distance between band modes in cm*	Distance corrected for centrifugation time and gradient (cm)
1	33	53	0.542	0.573
	66	58	0.563	0.585
	99	54	0.542	0.568
	133	58	0.563	0.585
	0 + 133	61	0.592	0.590
2	30	60	0.534	0.553
	60	60	0.556	0.583

* Measurement error 3%.

uracil (12), the expected density difference between C^{12} N^{14} and 67.1% C^{13} 97.0% N^{15} RNA (as the cesium salt) may be calculated by assuming that the extra neutrons contributed by the heavy isotopes increase the mass without changing the volume of the molecule. This expected density difference was 0.0458 gm cm^{-3}. The mean density difference between the two density species of RNA in all the post transfer samples of experiment 1 was calculated from their separation in the density gradient corrected to a constant gradient and centrifuge time of 60 hours. Using the density gradient value of 0.087 gm cm^{-4} at radius 6.5 cm, the mean density difference was calculated to be 0.0505 gm cm^{-3}. This value exceeds the expected density difference by 9.3%.

This calculation ignores the additional density gradient caused by compression. A value for this gradient was estimated. Assuming a compressibility equal to that of water (41.6×10^{-6} cc per atm at 500 atm), a 1 cm column of cesium formate (density 2 gm cm^{-3}), 6.5 cm from the center of a rotor at 52,640 rpm, would have a gradient due to compression of about 0.03 gm cm^{-4}. Because the addition of salts usually lowers the compressibility of water, this value is probably a maximum. Thus, the density difference calculated from the observed separation may exceed by as much as 46% that calculated from isotopic substitution.

Unfortunately, the leaks which occurred during the centrifugation of the second, third, and fourth post-transfer

samples, and the variable recovery of microsomal RNA in the lysates prohibit quantitative estimates of the absolute amount of C^{13} N^{15} RNA remaining in the bacterial population after its transfer to the C^{12} N^{14} medium.

In the density gradient analysis of the DNA from the zero time and post-transfer samples, three density species of DNA were observed (Fig. 3-III). In the zero time sample only one density species, viz. fully labeled DNA, was present. By 33 min after transfer from the C^{13} N^{15} to the C^{12} N^{14} medium, the fully labeled DNA had decreased in amount and a new density species of DNA had appeared. The latter species was intermediate in density between fully labeled and unlabeled DNA. In the two subsequent samples, fully labeled DNA was no longer detectable, and the half-labeled DNA appeared to be conserved. In addition new unlabeled DNA had made its appearance and was increasing in amount.

<u>Experiment 2</u>.--The results of experiment 2 confirmed those of experiment 1. The microdensitometer tracings for the RNA distributions in the density gradients for the time zero and the 2 post-transfer samples are shown in Fig. 4-III. The distances between the modes of the two RNA bands appearing in the two post-transfer samples are listed in Table 3-III. The RNA distribution in the C^{12} N^{14} band of the second post-transfer sample was non-Gaussian; and exhibited a sharp increase in UV absorption on the meniscus side of the band center.

At various times during each centrifugation, the cell

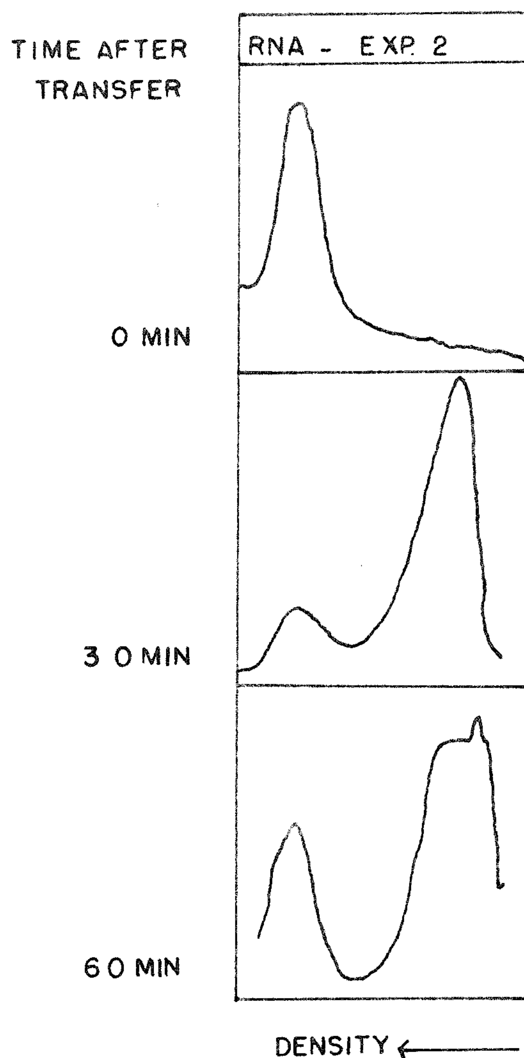

Fig. 4-III. Microdensitometer tracings of UV absorption photographs from experiment 2, showing microsomal RNA bands resulting from density-gradient centrifugation in cesium formate of lysates of purified microsomes prepared from bacteria sampled at various times after the addition of an excess of C^{12} N^{15} substrates to a growing C^{13} N^{15} labeled culture. The photographs were taken after 60 hours of centrifugation at 52,640 rpm.

leaked approximately 20% of its liquid volume and then stabilized. Thus, again, as in experiment 1, quantitative estimates of the absolute amount of C^{13} N^{15} RNA remaining in the bacterial population after transfer to the C^{12} N^{14} medium are prohibited.

DISCUSSION AND CONCLUSIONS

In both experiments 1 and 2, within the limits of measurement errors, the density of the heavy isotope pre-transfer microsomal RNA was constant for at least 133 min after transfer in the first experiment and for at least 60 min after transfer in the second experiment. Thus, it may be concluded that microsomal RNA molecules do not participate in exchange reactions, either with precursors or among themselves in exponentially growing E. coli.

The inability to detect RNA of intermediate densities demonstrates that the precursor pool for microsomal RNA is either very small and/or accessible to rapid exchange with externally supplied precursors. In addition the absence of intermediates indicates that the time involved for the complete synthesis of an individual microsomal RNA molecule must be short relative to the generation time. Even though no density species corresponding to a duplex entity composed of one "heavy" parental sub-unit and one "light" progeny sub-unit could be observed, this result does not rule out the possibility that a transient duplex structure is formed at the time of synthesis of a new RNA molecule.

The results of the DNA transfer experiment with C^{13} and N^{15}, are in complete agreement to those of the N^{15} transfer experiment in E. coli (6). Thus the carbon, like the nitrogen, of the DNA molecule is divided equally between two sub-units at

replication, and these sub-units are conserved through subsequent replications.

The reason for the discrepancy between the expected density difference and the observed density difference for the heavy and light isotope RNA is unknown. If the RNA preferentially bound a lighter cation than Cs^+, such as Na^+, the expected density difference would be 20% larger, and thus more nearly comparable to the density difference calculated from the separation of the two RNA density species in the density gradient.

The lack of complementarity in the base ratios of microsomal RNA (12), coupled with its likely single-strandedness (based on studies of TMV RNA (13,14)), would argue against it having a replication system like the one suggested for DNA (15). However, base-pair complementarity is not a necessary concomitant of a template replication system characterized by stringent base-pairing restrictions if one of the complementary strands is a preferred template.

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