Doctoral Thesis

Investigations on the metabolism of Fusarium lycopersici Sacc. with the aid of radioactive carbon

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Publication Date:
1956

Permanent Link:
https://doi.org/10.3929/ethz-a-000099025

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THESIS
PRESENTED TO
THE
SWISS FEDERAL INSTITUTE OF TECHNOLOGY
ZURICH
FOR THE DEGREE OF
DOCTOR OF NATURAL SCIENCES
BY
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Accepted on the Recommendation of
Prof. Dr. E. GÄUMANN and Dr. H. KERN

1956

Druck von A. W. Hayn's Erben, Berlin SO 36
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*With 18 Figures*  

**Contents:** A. Introduction. — B. Materials and Methods: I. Materials. II. Mycological and Pathological Techniques. III. Biochemical Techniques. IV. Isotope Techniques; a) Radioautography and Radiodchromatography; b) Preparation of samples for radioactive assay. — C. Metabolism of *Fusarium lycopersici* in vitro: I. Effect of substrate on toxin production. II. Chemical changes in artificial cultures during fusaric acid production. — D. Studies on the metabolism of *Fusarium lycopersici* in vivo: I. The secretion of radioactive metabolic products into the host. II. The distribution of radioactive metabolic products in the host tissues. III. Chemical substances secreted by the pathogen in the host. — E. The role and fate of C\textsuperscript{14}-marked fusaric acid in the tomato plants: I. Decarboxylation of C\textsuperscript{14}-marked fusaric acid in plants under different conditions; a) Effect of pH of C\textsuperscript{14}-marked fusaric acid on C\textsuperscript{14}O\textsubscript{2} evolution; b) Effect of continuous light and continuous darkness on the breakdown of fusaric acid. II. The intermediate products of metabolism of fusaric acid. III. Quantitative breakdown of fusaric acid in plants. — F. Discussion. — Summary. — Zusammenfassung. — Literature cited.

**A. Introduction**

*Fusarium lycopersici* Sacc., causing a wilt disease of tomato plants, is known to produce three wilting toxins in artificial cultures. These toxins bear no chemical relationship with one another. Thus, *lycomarasmin* (empirical formula C\textsubscript{9}H\textsubscript{13}O\textsubscript{7}N\textsubscript{3}) is a dipeptide with a molecular weight of 277.3 (CLAUSON-KAAS, PLATTNER and GÄUMANN, 1944; PLATTNER and CLAUSON-KAAS, 1944, 1945; PLATTNER, CLAUSON-KAAS, BOLLER and NAGER, 1948; Phytopath. Z., Bd. 25, Heft 4 22.
Fusaric acid, the second toxin, is 5n-butylpyridine-2-carboxylic acid (empirical formula \( \text{C}_{10}\text{H}_{13}\text{O}_{2}\text{N} \)) with a molecular weight of 179. It was first isolated by Yabuta, Kambe and Hayashi (1934) from the culture filtrates of *Fusarium heterosporum* Nees. In contradistinction to lycomarasmin, *f usar ic a c id* is produced by many *Fusarium* species and is not specific to *F. lycopersici* alone (Gäumann, Naef-Roth and Kobel, 1952). The third toxin, *vasinfuscari n* (Gäumann, Stoll and Kern, 1953) has not yet been completely purified and like fusaric acid is not specific to *F. lycopersici*, but is also produced by *Gibberella fujikuroi* (Saw.) Woll. (Stoll, 1954). Most probably it is a protein of an enzymatic nature. The pectic enzymes, demonstrated in the cultures of *F. lycopersici* by American authors (Davis, 1953; Gothoskar and Scheffer, 1953; Gothoskar, Walker and Stahmann, 1953; Winstead and Walker, 1954), may or may not be similar to vasinfuscarin.

When applied to healthy tomato cuttings in particular concentrations, the toxins produce approximately the same symptoms as are present in infected plants. Thus, vasinfuscarin causes the typical browning of the vascular bundles, which is a very striking symptom of naturally infected tomato plants. Lycomarasmin acts specifically on the areas between the midribs of the leaves and causes necrosis there, which again is a typical symptom of the wilt disease of tomato (Gäumann, 1951 a). Depending upon the pH, fusaric acid causes either injury to the stems and leaves (at pH 4.3) or to the leaves alone (at pH 6.0 or above, see Gäumann, Naef-Roth and Kobel, 1952). The symptoms on the stems take the form of depression of the areas adjoining the vascular bundles and an ultimate collapse of the stem. The leaf symptoms generally appear as water-soaked spots all over the leaves (see Linskens, 1955).

Various indirect evidences (Gäumann, 1951 a) are available to show that all the above named toxins are also produced by the pathogen in vivo during its pathogenic phase of life. However, these toxins have never been isolated from the naturally infected plants. The reasons are very obvious. Even if we do not reckon with the technical difficulties in the isolation of the minute quantities of toxins elaborated by the pathogen in vivo, we have yet to take into consideration the possibility that these may be metabolised by the host cells with their consequent transformation into unknown substances. We refer here to the general experience of authors who have worked with wilting toxins. Lycomarasmin, for instance, perhaps chelates the iron ions in the tomato plants and thereby changes into a lycomarasmin-iron-complex (Gäumann and Naef-Roth, 1954, 1955; Deuel, 1954). Fusaric acid, according to the experience of the Japanese authors (Tamari and Kaji, 1952, 1953 a, b; Deuel, 1954), perhaps forms in the plants water insoluble chelate complexes with metal ions. Yet another toxin, patulin, when introduced in the tomato plants, combines with the thiol groups of biological importance in the cells and cannot be recovered back (Miescher, 1950). We deduce from these observations of a number of authors in this field that it
might not always be possible to isolate the toxins from the diseased plants in the original state in which they were produced by the pathogen. Before we attempt to isolate the toxins from the diseased plants, therefore, we must know the metabolic fate of the toxins in question, once they have been secreted by the pathogen. Only after acquiring such knowledge can we attempt to isolate either the toxins themselves or their degradation products. Success in such isolation would provide a direct evidence for the toxin theory of the wilt diseases.

Apart from the attempt to isolate the toxins from the diseased plants, it is important to follow the metabolism of the pathogen in vivo and correlate such studies with the possible mode of toxin formation in infected plants.

There are, however, many technical difficulties in undertaking investigations of the above nature. The difficulties have been solved to some extent, in the following studies by using C\textsuperscript{14} as a tagging material for both mycelia and fusaric acid (Wheeler, 1952, 1953; Hoffmann and Zuckerman, 1954; Kern and Sanwal, 1954).

B. Materials and Methods

I. Materials

The strains of *Fusarium lycopersici* used in the following investigations originated from the laboratories of Dr. T. Fontaine of U.S. Department of Agriculture in Beltsville, Md. (Kern, 1952). Strain R-5-6 built on malt-agar medium large amounts of aerial mycelia and was very strongly pathogenic to tomato plants. Strain 257, on the contrary, built very little, if at all, aerial mycelium and was practically non-pathogenic.

Throughout the following investigations Tuckswood variety of tomato plants were used, unless otherwise stated. They were cultivated in green houses throughout the year with supplement of fluorescent lights during the winter months.

2-C\textsuperscript{14}-glycine was purchased from the Harwell Isotope Center, England. When obtained it had a specific activity of 14.9 \mu C per mg.

C\textsuperscript{14}-fusaric acid, marked in the carboxyl group was synthesized in the Organic Chemistry laboratories of the Swiss Federal Institute of Technology by Dr. E. Hardegger and Mr. E. Nikles. The original material was very active (14.7 mC/mM) and was diluted by the addition of inactive fusaric acid. To obtain homogeneous material, the mixture of radioactive and non-radioactive fusaric acid was crystallized from petroleum ether.

II. Mycological and Pathological techniques

The inoculum for general experiments discussed in Section C (I) was obtained by growing the fungus on rice cultures (20 gms. rice and 40 ml. water; sterilized in Autoclave) for 5 days. At the end of this period the
fungus built large quantities of conidia. For the inoculation of liquid cultures, the rice cultures were gently shaken with 100 ml. sterile distilled water to obtain a spore suspension. 1 ml. of this spore suspension was used to inoculate 100 ml. of liquid substrate.

For studying the metabolism of fusaric acid in vitro, discussed in Section II, the inoculum had to be much more uniform. To this end, single conidial cultures were made on solid malt-agar slants and one of those conidial cultures served as the mother culture for all subsequent sub-cultures. Whenever needed, sub-cultures were made on solidified Richard’s medium (subsequently abbreviated in the following work as Ris medium) in petri dishes. After 5 days growth at 27 °C in darkness, uniform discs of agar with mycelium were cut out from the periphery of the petri dishes by means of a flamed cork-borer and these discs served as the source of inocula for the liquid cultures in Erlenmeyer flasks.

Unless otherwise stated, Erlenmeyer flasks of 500 ml. capacity were used throughout. Each flask contained 100 ml. of Ris medium (modified after Luz, 1934: 50 gms. glucose, 10 gms. ammonium nitrate, 5 gms. monopotassium phosphate, 2.5 gms. magnesium sulphate and 0.02 gms. ferric chloride, distilled water to make up 1000 ml.). The medium had a pH of 4.0—4.2. It was sterilized in an Autoclave at a pressure of 1 atm. for 20 minutes.

All cultures were incubated in dark at a temperature of 27 °C.

Dry weight of the mycelium was taken as a standard of the amount of growth of the mycelium. For dry weight determinations, the contents of 10 Erlenmeyer flasks were filtered through a weighed filter paper. The mycelium was washed with 50 ml. distilled, luke-warm water 4 times and was dried along with the filter paper for 12 hours at 60 °C and further 4 hours at 103 °C and weighed.

The method of inoculation of the tomato plants was the same as proposed by Wellman (1938). They were inoculated at the four leaf stage by uprooting them from the soil and dipping the washed roots in a mycelial and conidial suspension and again planting in soil. Controls were treated in a similar way (Kern, 1952).

Tomato wilting tests were the same as proposed by Gäumann, Naef-Roth and Miescher (1950) and Gäumann, Naef-Roth, Reusser and Ammann (1952). The tomato plants were cut at the four leaf stage by means of a sharp razor and immediately placed in water. They were allowed to take up the required dose of the toxin by means of transpiration pull under similar conditions of temperature, humidity and light. After uptake of the test solutions, the plants were placed in tap water and symptoms checked after 48 hours (Linskens, 1955).

The Ustilago spore germination tests were made according to the method proposed by Kobel (1951) and Kern (1952) and the results were expressed as Ustilago units per ml. of the test solutions (U/ml.), i.e., the dilution of the original solution at which only 50 % of the control smut spores germinate.
Investigations on the metabolism of Fusarium lycopersici Sacc.

III. Biochemical techniques

a) Glucose determination

Glucose was estimated as reducing sugar by the method of Schaffer and Hartmann (1920) by iodometric titration. 2 ml. of clear culture filtrate was added to a mixture of 25 ml. of copper sulphate (CuSO₄·5H₂O; 69.28 gms. dissolved in 1 liter distilled water) and 25 ml. of Rochelle salt solution (346 gms. Rochelle salt with 100 gms. of sodium hydroxide dissolved in 1 liter distilled water) in a flask. Distilled water was added to this solution to make up the total volume to 100 ml. The flask, with its contents, was heated quickly to boiling and after allowing it to boil for 2—3 minutes, rapidly cooled under the tap. To this was added 50 ml. of potassium iodate-iodide solution (5.4 gms. KIO₃ and 60 gms. KI with a small amount of alkali, distilled water to make up 1 liter) followed by approximately 15 ml. of 5 N H₂SO₄. Immediately afterwards, 20 ml. of a saturated solution of potassium iodate was added to dissolve completely the cuprous iodide formed. This solution was titrated with 0.1 N sodium thiosulphate, using towards the end some drops of a starch solution as an indicator. A blank also was run side by side in boiling the Fehling’s solution, using distilled water instead of the culture filtrate. The former titration value was subtracted from the latter blank and the milligram copper equivalent was calculated out (1 ml. 0.1 N Na₂S₂O₃ corresponds to 6.36 mg. copper). From this value, the glucose present in the culture filtrate was calculated out by reference to the Munson-Walker tables (Methods of analysis of the Association of official Agricultural chemists, 1950).

b) Determination of total nitrogen

Total nitrogen in the culture filtrates was determined by the method of Pepkowitz and Shive (1942). 1 ml. of clear culture filtrate was filled into a thick-walled boiling tube and evaporated to dryness under vacuum. Then 1—2 ml. of Ranker’s solution (32 gms. salicylic acid dissolved in 1000 ml. concentrated sulphuric acid) was added and allowed to stand in the cold for 20—30 mts. To this were added 3 drops of sodium thiosulphate solution (50 gms. of sodium thiosulphate monohydrate per 100 ml. of distilled water) and 0.5 ml. of selenium oxychloride-sulphuric acid solution (12 gms. of selenium oxychloride per liter of conc. H₂SO₄). The contents of the test-tube were vigorously boiled for 15 minutes and then cooled, first at room temperature and then under cold tapwater. 2 drops of a 35% perchloric acid (made from 70% acid with distilled water) solution were added right in the middle of the tube by means of a pipette and the tube heated for 15 minutes in the digestion rack over a low flame so that the temperature remained below the boiling point. The test tube at the end of this operation was cooled again, the contents diluted with cold distilled water to a volume of 30 ml. and nitrogen estimated in 10 ml. lots of this solution by the method discussed under the determination of ammonia. A blank
c) Determination of ammonia

Ammonia was determined by the method proposed by Raynand (1948). To 1 ml. of clear culture filtrate was added approximately 20 ml. of 95% ethyl alcohol along with a drop of 1% alcoholic solution of Thymolphthalein. The whole solution was neutralized by 0.01 N NaOH till the colour turned blue, after which a little more alkali was added to get an alkaline reaction. When the original culture filtrate or solution (as in Kjeldahl nitrogen determinations) was very strongly acidic, neutralization was begun with conc. NaOH and ended up with 0.01 N NaOH solution. The alkalinised solution was placed in a steam distillation apparatus, the original container rinsed three times with 3—5 ml. quantities of 95% alcohol and the rinsings combined with the original solution. The receiving flask contained, before the distillation was started, 2 ml. of 95% alcohol with 2 drops of 1% alcoholic sodium alizarine sulphonate. Steam was now turned on and distillation continued till all the alcohol was collected in the distillate. The pink coloured distillate was then titrated to neutrality by means of 0.02 N H₂SO₄, delivered from a microburette. Milligrams nitrogen present in the distillate were calculated in multiplying by 0.28 the number of ml. of 0.02 N H₂SO₄ used.

d) Determination of nitrate nitrogen

Nitrate was determined by a volumetric method described by Treadwell (1947). 2 ml. of clear filtrate were taken, made acidic by adding a little 2 N H₂SO₄ and diluted with distilled water to 70—80 ml. This solution was heated to boiling and to it was added 15 ml. of a solution of Nitron reagent (10 gms. Nitron dissolved in 5 ml. of acetic acid and 95 ml. distilled water). The container was immediately placed in ice-cold water and left there for 2 hours. During this time the nitrate salt of Nitron crystallizes out of the solution because of its non-solubility. The crystals were separated by means of a glass Gooch crucible (Gooch G 3), washed three times with 3—5 ml. distilled water and allowed to dry in an oven for 2 hours at 110° C. From the weight of the Nitron nitrate, so obtained, the milligrams of nitrate nitrogen were calculated.

e) Determination of amino-nitrogen

A method proposed by Pope and Stevens (1939) was adopted (see also, Schroeder, Kay and Mills, 1950). 10 ml. of the clear culture filtrate were pipetted in a 50 ml. volumetric flask, 2 drops of 1% alcoholic Thymolphthalein solution followed by N NaOH were added till the colour turned blue. To this solution were added approximately 30 ml. of copper phosphate suspension (made by mixing 1 vol. of 0.16 M solution of copper chloride with 2 vols. of borate buffer, made by dissolving 28.6 gms. sodium borate
Investigations on the metabolism of *Fusarium lycopersici* Sacc.

with 50 ml. N HCl in 700 ml. water and 2 vols. of a solution containing 64.5 gms. dihydrogen phosphate plus 7.2 gms. sodium hydroxide per liter, the volume made up to 50 ml. by adding distilled water, mixed well and the whole solution filtered through a Whatman filter paper no. 5. The copper content of the filtrate was determined iodometrically. To 10 ml. of the filtrate was added about 1 gm. of potassium iodide dissolved in water and acidified with about 0.5 ml. acetic acid. The free iodine was titrated by 0.01 N sodium thiosulphate, using a microburette for the purpose and adding a starch solution (*Pope and Stevens, 1939*) as an indicator towards the end of the titration. Milligram amino-nitrogen present in 1 ml. culture filtrate was calculated as follows (see *Schaffer und Hartmann, 1920—21*):

\[
\frac{t \times 0.28}{2} = \text{mg. amino-N in 1 ml. of culture filtrate (where } t = \text{the amount of 0.01N } \text{Na}_2\text{S}_2\text{O}_3 \text{ used for titration).}
\]

**f) Determination of purine nitrogen**

The method of *Krüger and Schmid (1905)* for determination of purine bases was used (see also, *Hitchings and Fiske, 1941*). 30 ml. of deproteinized culture filtrate was neutralized to phenolphthalein and heated in a boiling water bath. The purine bases were precipitated by adding 2 ml. of a 40 % solution of sodium bisulphite and 2 ml. of a 10 % solution of copper sulphate. The mixture was set to boiling and continued for three minutes. The precipitate was centrifuged and washed successively with 1 % acetic acid and 10 ml. portions of hot water. The precipitate was handled in the same way as in the determination of total Kjeldahl nitrogen discussed in part b).

**g) Fusaric acid nitrogen**

This part of the analytical work presented the most difficulties. In order to determine the nitrogen of fusaric acid we had to obtain it in pure form. We tried to extract the fusaric acid from the culture filtrates from model experimental cultures, where a known quantity of fusaric acid was added to the culture filtrate immediately after sterilization and extracted with the method used by *Platten, Keller and Boller (1954)*. The culture filtrate was mixed with Norit (Norit superactive coal 0.5 %) twice and well mixed in a vibrator. By applying suction, the coal with the adsorbed fusaric acid was separated from the culture fluid in a Buchner funnel. The fusaric acid was eluted first by methanol and lastly by methanol-ammonia. All the methanol extracts were combined and reduced to a very small volume (calculated to contain about 1 mg. fusaric acid/ml.). A known quantity of this solution was applied as a band, about 3 cms. long on the starting line of a Whatman paper no. 1 and the chromatogram run with secondary butanol, formic acid and water mixture (75:15:10). After drying the chromatogram the fusaric acid band was cut out, eluted and quantity of fusaric acid determined by ultra-violet absorption in a Beckmann spectro-
photometer (for further details see Zähner, 1954). Using such a procedure it was found that absorption on active coal does not remove all the fusaric acid from the culture filtrates. In very favourable cases a recovery of about 55% is obtained.

In view of the above we were obliged to take recourse to other methods of extraction. After a good deal of experimentation, the following method was adopted, which gave an error of round 10%:

300 ml. of a clear culture filtrate was adjusted to a pH of 4.0 and extracted 5 times with 80 ml. of ethyl acetate. All the ethyl acetate was combined and completely dried under vacuum at room temperature. The solid material was taken up in 1 ml. of 80% ethanol and 0.05 ml. of this solution was applied as a 5 cms. long band on Whatman no. 1 chromatographic paper. The chromatogram was run with the elsewhere mentioned butanol-formic acid and water mixture. After drying, the position of fusaric acid was located under ultra-violet light. The band was cut and eluated with about 5 ml. of ethanol in a Wasitzki eluation apparatus (Morton, 1938). The eluate was dried in a thick-walled boiling tube and handled in the same way as described in section b).

h) pH determination

All pH measurements were done with a glass electrode.

IV. Isotope techniques

a) Radioautography and Radiochromatography

In the following work, depending upon requirements, radioautography has been used either on a 'macro' or a 'micro' scale. In the 'macro' work, like in the radioautography of developed chromatograms (where only a rough image is required to locate the radioactive spots on chromatograms), No-Screen X-ray films manufactured by EASTMAN KODAK Co., U. S. A., have been used. The chromatograms, after drying, were stamped on the edges with radioactive ink (Sanwal, 1955) in order to mark its position on the X-ray photographic emulsion after development. The chromatogram in contact with the emulsion was covered with black paper and a lead weight of the same size as the film was put on top of it. This procedure ensured a close contact of the chromatogram with the emulsion and an uniform distribution of pressure on the whole film (since it is known that unequal pressure can give rise to artefacts). The films were exposed in a light-tight box at 0 °C for the required number of days. The time required to obtain a satisfactory development of the silver grains (in general, an optical density of \( D = 0.5 \) over the controls) depends on the activity of the radioactive spots and was always found out empirically (generally, one square centimeter of the emulsion must receive \( 10^6 \) disintegrations to get a satisfactory image, see Kamen, 1951; Sanwal, 1955). After exposure the emulsion was developed with KODAK rapid X-ray developer for 8 minutes and fixed in an
Investigations on the metabolism of *Fusarium lycopersici* Sacc. 341

acidic fixing bath. The position of the radioactive spots was determined by viewing the radioautograph through scattered light.

In the 'micro' work, as for instance, radioautography of thin tissue sections to locate the distribution of radioactive substances in the cells, two types of film emulsions were used. The 'stripping emulsion' (Pelc, 1947) was obtained by the courtesy of Prof. Dr. J. Eggert of the Department of Photography, Federal Institute of Technology, Zurich, to whom the writer is grateful for general advice on photography. This emulsion was manufactured by Kodak Co., England and consisted of a glass plate on which the dried emulsion was spread in a layer 4 μ thick with another supporting gelatine layer at the top.

The microscope slides to be used for mounting the tissue sections, containing the radioactive material, were 'subbed' before use, i.e., they were cleaned by soaking overnight in a solution containing the following constituents:

- Potassium dichromate: 100 gms.
- Sulphuric acid (conc.): 100 ml.
- Distilled water to: 1000 ml.

They were next washed in water and dipped in the following solution at 18 °C:

- Gelatine: 5 gms.
- Chromealum: 0.5 gm.
- Water to: 1000 ml.

After this treatment the slides were dried overnight at 30 °C. This treatment obviated the use of a sticking substance for holding the paraffin sections of the tissues. The paraffin ribbons were laid on top of the slides so treated, stretched by heating at 40 °C, dried and stored till required for use. For exposure of the 'stripping emulsion', pieces were cut out from the plate, sufficient to cover the entire area of the paraffin ribbons on the 'subbed' plates plus a margin about 8 mm. all round (Herz, 1951; Pelc, 1947) by means of a sharp knife and floated on the surface of distilled filtered water, with the emulsion surface facing downwards. The dried emulsion, on coming in contact with water stretched itself. The slide bearing the specimens was so placed under the emulsion layer that it formed an angle of 30 ° from the horizontal and one side touched the emulsion first. The whole slide, with the emulsion, was lifted clear of water and dried by means of a stream of cool air, placed in a light-tight box and exposed for the required length of time (maximum time of exposure 40 days).

The other kind of photographic emulsion used was the NTB (nuclear track plates) 'slide-coated' emulsion manufactured by Eastman Kodak Co., U.S.A. These plates carried an emulsion layer 10 μ thick coated directly on a microscope slide (1 inch × 3 inches). To expose these plates, the paraffin ribbons were first stretched at 40 °C over a hot plate and were immediately
float on filtered distilled water. The emulsion on the slides was wetted by
immersing it under water for 1 minute and the ribbons laid over the emulsion,
dried by means of a stream of cool air and exposed in light-tight boxes at
0 ° C for the required length of time (Evans, 1947).

After the desired exposure (found out empirically) the plates were
washed in xylol for about 15 minutes to remove the paraffin in order to
facilitate later the penetration of the processing material through the sections.
They were then dried for about 10 minutes (when it did not smell of xylol
any more) and developed with Kodak D-19 b developer for 20 minutes
without agitation. Next, they were rinsed in water and fixed in Kodak F 5
fixing bath about 40 minutes, after which they were washed thoroughly with
running water (1 hour). Staining of the sections, directly over the emulsion,
was done by haematoxylin with 4% iron-alum serving as a mordant. Stain¬
ing time had to be varied with different tissues and was largely determined
empirically. The developed and stained plates were next dehydrated through
various grades of alcohol, cleared in xylol and mounted in canada balsam
with a cover slip of convenient size on top.

Except staining, all operations were carried out in light from a Wratten
series 1 safelight (red). Whenever radioactivity was present in the tissues, the
silver grains directly over these areas were developed. Thus, by examining
slides prepared in the manner described above under a microscope, one could
determine exactly which tissues had accumulated radioactive substances.

Radiochromatography:

For want of any other suitable name we have called this technique radio-
chromatography (Lissitzky and Michel, 1952; Roche, Lissitzky and
Michel, 1954; Sanwal, 1955); it has been the most widely applied technique
for research with radioactive isotopes. The following procedure was adopted
by us:

A drop of mixture, containing radioactive substances to be separated
was applied on the starting line of Whatman no. 1 chromatographic paper.
The drops were delivered from a micropipette and it was rinsed after use
everytime, first with distilled water, then with alcohol and finally with
acetone and dried. Separation on the chromatogram was achieved by the
desired solvent mixture unidimensionally in an all-glass pyrex chromato-
graphic container by the descending method. The chromatogram was then
dried and strips, about 3 cm. wide (containing the radioactive spots), were
cut out in a longitudinal direction. This strip of paper was enclosed in a
small aluminium casket which had at one end a rectangular opening of
4 mm. × 20 mm. The opening was placed under a thin end-window Geiger-
Müller counter, the distance between the counter and the paper being 5 mm.
The paper strip was counted 4 mm. by 4 mm., beginning with the starting
point on the chromatogram to the solvent front by moving the chromatogram
after every measurement 4 mms. This movement was facilitated by a scale
fitted under the aluminium casket. Each portion was counted for 5 minutes.
The counts per minute were plotted against distance on the chromatogram
to obtain a radiochromatogram. The maxima on the radiochromatogram corresponded to the radioactive spots present on the paper chromatogram. By reference to the distance of such radioactive spots from the starting point and the solvent front, $R_f$ values could be easily calculated out.

b) Preparation of samples for radioactive assay

All the radioactive samples, unless otherwise stated were counted as barium carbonate. The combustion apparatus was constructed after Anderson, Delabarre and Bothnerby (1952) and is shown in figure 1. It consisted of a forked combustion tube at one end and a receiving flask on the other, with arrangements for evacuating the whole apparatus quickly (see legend to figure 1). All samples were oxidized using the wet combustion technique of Van Slyke et al (1940, 1951a and 1951b) using the following procedure:

In case of liquid samples to be oxidized, the liquid was placed in one arm of the forked combustion tube (previously weighed) and evaporated to dryness at room temperature under vacuum. The dried sample then was oxidized as written below. In case of tissue fractions or solid samples, they first were dried under vacuum over phosphorus pentoxide at room temperature, weighed and placed into one arm of the forked combustion tube. In no case (either of liquid samples or solid samples) the weight did exceed 15 milligrams. When measurements were to be made on a large amount of tissue, as for instance, whole plants, the plants were cut up in small individual parts, dried, weighed and then oxidized.

Oxidation of the samples was done by placing in one arm of the forked combustion tube, along with the samples, the corresponding amount of Van Slyke-Folch solid reagent (2 parts by weight of KIO$_3$ and 1 part of K$_2$Cr$_2$O$_7$; Van Slyke, Plazin and Weisiger, 1951). The other arm of the combustion tube received a corresponding amount of liquid reagent (67 ml. of fuming sulphuric acid with a free SO$_3$ content of 20\%, 33 ml. of H$_3$PO$_4$ of sp. gr. 1.70—1.72 and 1 gm. of KIO$_3$, all three heated together to dissolve the KIO$_3$, cooled and stored in a clean Pyrex-
glass bottle.). The liquid reagent was always freshly made before use. In the receiving flask, on the other end of the apparatus, was placed 15 ml. 2 N carbonate free NaOH solution having a blank of about 3—4 mg. BaCO₃ per mole of NaOH.

The whole apparatus was quickly evacuated of air and the contents of the arm of the combustion tube containing Van Slyke-Folch reagent were tilted into the arm containing the solid reagent with the sample to be oxidized. The mixture was strongly heated for 5 minutes by an open flame applied directly under the tube. After removing the flame, the whole apparatus was left for 30 minutes with occasional shaking by hand to facilitate complete absorption of the liberated C¹⁴O₂ in NaOH solution. After all the C¹⁴O₂ had been absorbed, the receiving flask was removed and the contents rinsed into a clean dried centrifuge tube followed by an amount of 2 N ammonium nitrate solution equivalent to the alkali used, and immediately stoppered with an ascarite tube to protect the contents from atmospheric CO₂. The centrifuge tube was then immersed immediately in a boiling water bath and allowed to remain there for about 15 minutes, after which the ascarite tube was removed and 10—15 ml. of a solution of Ba(OH)₂ • BaCl₂ (for preparation see Anderson, Delabarre and Bothnerby, 1952) were added from a burette protected from atmospheric CO₂ to precipitate the BaC¹⁴O₃ from the solution. The centrifuge tube was again stoppered and placed in a water bath maintained at 90° C for 30 minutes. The crystalline BaCO₃ precipitate was centrifuged out, washed first with hot distilled water, then with alcohol and from this the samples for radioactive assay were made as follows:

The BaCO₃ precipitate was ground under absolute alcohol by means of a pyrex glass rod and the alcohol-barium carbonate slurry so obtained was transferred by means of an eye-dropper to small aluminium counting discs (area 3.47 sq. cms.). The alcohol was evaporated from the discs by drying the precipitate under an infra-red lamp and finally in an oven at 115° C for one minute (Yankwich, 1948). Such preparations, if not immediately counted were stored in a desiccator over dry NaOH pellets till further use.

It might be mentioned here that the BaCO₃ precipitate obtained by other procedures than those described above (see also Reid, Weil and Dunning, 1947; Calvin, Heidelberger, Reid, Tolbert and Yankwich, 1949) is ‘growing’ and has a tendency to stick on the glass walls of the dropper while transferring to counting discs. Besides this, the ‘growing’ precipitates never dry on the discs uniformly and this invariably results in serious counting errors due to the ununiform geometry of the sample (Kamen, 1951; Calvin et al, 1949).

**Counting of the samples and corrections applied:**

Counting was done by means of an end thin window Geiger-Müller counter. A “1000 Scaler” of Tracerlab, Boston, Mass. and a ELA 2 64-scaler
of Landis & Gyr A. G., Zug, Switzerland with a EQB 1 GM-tube having a window diameter of about 25 mm and thickness of about 2 mg/sq. cm, were used. The samples were counted always at a fixed distance from the window.

Samples having less than 100 counts per minute were counted for at least 30 minutes and those having greater activities were counted from 5 to 15 minutes depending upon the activity. Background counts were taken before and after every measurement for at least 5 minutes and were subtracted from the total counts of the samples in expressing the data.

The standard deviation of a series of measurements on the radioactive samples and background was calculated out from the formula (Calvin et al, 1949):

$$\text{standard deviation} = \sqrt{\frac{\sum_{i=0}^{n} (V_{\text{mean}} - V_i)^2}{n(n-1)}}$$

where \( n \) = number of determinations made; \( V \) = mean value of the counts; \( V_i \) = individual counts.

Counts on any radioactive sample showing less than 3 times the standard deviation of background counts were taken as not significant of the presence of radioactivity in them.

The self-absorption correction for weak \( \beta \)-rays emitted by \( ^{14}C \), whenever needed, was done by reference to a curve obtained by counting different quantities of \( \text{BaCO}_3 \) samples of the same specific activity (Yankwich, Norris and Juston, 1947). All the \( \text{BaCO}_3 \) samples counted were either of 'infinite thickness' (about 20 mg./sq. cm.) or whenever less, were corrected to this value by reference to the aforesaid absorption curve. Only in experiments reported in section E, I (page 366) we took recourse to reducing the counts obtained to 'zero thickness' (see Yankwich et al, 1947), because the samples were very weakly radioactive due to an enormous dilution with non-radioactive \( \text{CO}_2 \) given off by the plants. This correction was also done by reference to the absorption curve.

Greatest cleanliness was observed while working with the radioactive material. All the glassware and discs were cleaned immediately after use with dilute HCl, boiling distilled water, alcohol and finally with acetone and checked for any possible radioactive contamination under the GM counter before use.

C. Metabolism of \( F. \) lycopersici in vitro

I. Effect of substrate on toxin production

It has been demonstrated by Gäumann, Naef-Roth and Miescher (1950) that the avirulent strain of \( F. \) lycopersici produces more lycomarasmin in artificial cultures than the virulent strain. Kern (1952) confirmed this observation and further showed that both lycomarasmin and fusaric acid are produced in greater quantities by the avirulent strain than the virulent one but the difference was not very significant (Kern, 1952, fig. 5) specially in
wilting tests with tomato cuttings. However, in the face of the facts, the situation is anomalous specially if we were to assume that the disease syndrome in the host is the result of the action of toxins secreted by the pathogen in vivo. If the latter contention were true, we would expect that the virulent strain produce more toxin in artificial culture than the avirulent one. This is also the line of argument taken by Dimond and Waggoner (1953). Gäumann (1951 a) has explained this situation by putting forth the argument that "the microorganisms respond in their metabolism and formation of the toxins with extreme sensitivity to nutritional minutiae. Claviceps purpurea forms ergotamine and its other alkaloids in the parasitic phase in the ovaries of the grasses, but not on the same substrate in saprophytic culture. Penicillium chrysogenum and P. notatum produce penicillin only when the nutrient solution contains Zinc: without zinc they produce notatin. Certain races of Aspergillus fumigatus form gliotoxin after a short incubation period. and helvolic acid after a longer period ... The same differential stimulation or inhibition of toxin formation evidently occurs with the various strains of F. lycopersici with other Fusarium species in the interior of the host or, respectively, in saprophytic culture."

In view of the above reasoning, experiments were conducted to arrive at an answer to the following questions:

1. Does the quantity of toxin produced by the virulent and avirulent strains vary with a quantitative change in the chemical constitution of the modified Richard's medium?

2. Are toxins produced when there is a qualitative change in the constituents of the medium? This part of the experiment was also conducted to search the possibility of a cheap source of radioactive carbon to be utilized in later experiments.

1. Influence of the quantitative composition of Richard's medium on toxin production:

Since all the known toxins (cf. Introduction) produced by F. lycopersici in artificial culture have carbon and nitrogen as chemical constituents, it was decided to vary the concentration of these two substances in the Richard's medium arbitrarily. In one set of flasks (500 ml. capacity, each containing 100 ml. liquid medium), the strains were grown on Richard's medium having only half the normal amount of sugar (2.5 %, instead of the normal 5 %) and all the other constituents in normal amounts. In the second set of flasks, only half of the normal amount of nitrogen (0.5 %, instead of normal 1 %) with the usual amounts of other constituents were provided. In the third set of flasks, only half of the usual amount of sugar and half of the usual amount of nitrogen was provided along with the normal quantities of other constituents of the Richard's medium. The fourth set of flasks served as controls and contained the unvaried Richard's medium.

Strains R-5-6 (virulent) and 257 (avirulent) were grown at 27° C in dark for five week duration. At the end of every week 10 flasks (from each
set) were removed, the contents mixed together and filtered. The filtrate was tested both with wilting (in 1/5 dilution; gradation of symptoms as in Kern, 1952) as well as with the Ustilago spore germination tests (page 336) after adjusting the pH to 6.0. pH changes in the medium were followed for the whole growth and test period to find out whether any correlation exists between the two factors.

The results of these investigations are summarized in figures 2—4.

Fig. 2. The effect of a quantitative change in the constitution of Ris medium on the toxin production by strain R-5-6 and 257 as assessed by wilting tests (concentration 1/5 dose 0.25 ml per gm test plants).

Fig. 3. Ustilago activity of the culture filtrates (in dilution units) of strain R-5-6 and 257 grown in Ris medium the constituents of which were varied quantitatively
The following generalizations can be made from a study of the figures 2–4:

a) There is no correlation between pH and the quantity of toxins secreted by the two strains of *F. lycopersici*. This is clearly borne out from a study of the corresponding curves (figs. 2–4). pH of the media employed, originally at about 4.0, rapidly increases till a seven-day-period and thence afterwards remains more or less constant (fig. 4). As will be seen later (page 354), this rise in the pH is correlated with the accumulation of ammonia and active uptake of nitrates from the medium. On the contrary, the quantity of toxins produced by both strains reaches a maximum after 14 days and thereafter gradually falls down.

b) With half the normal amount of glucose (2.5 % only) and half of the normal amount of nitrogen (0.5 % only) in the Richard’s medium, the virulent strain (R-5-6) produces more toxin than the avirulent (257) one (figs. 2–3). This is specially pronounced at the end of a growth period of two weeks, when toxin production always reaches a maximum. The curves obtained with the wilting tests (figure 2) and also with the *Ustilago* spore germination tests (fig. 3) speak for the above generalization. At the end of 2 weeks, the toxin production in both the virulent and avirulent strains takes a similar course. The above also holds true for cultures grown with either only half glucose or half nitrogen.

c) With a favourable balance of carbon : nitrogen (i.e., 2.5 % carbon : 0.5 % nitrogen), the toxin production is always more than an unfavourable balance (i.e., 2.5 % carbon : 1 % nitrogen or 5 % carbon : 0.5 % nitrogen) in both the strains. The carbon nitrogen balance of the culture medium is also known to influence the organic acid metabolism of many moulds (Foster, 1949).

d) The toxin production shows a sharp rise during the second week and a sudden fall during the third week in the case of strain R-5-6; in the case of strain 257, however, the rise and fall is not sudden (figs. 2 and 3).
2. Influence of the qualitative change in the composition of Richard's medium on toxin production:

It has been demonstrated above that a quantitative change in the constitution of the substrate causes a corresponding change in the quantity of toxins produced by F. lycopersici. It yet remained to be seen whether toxins could be produced by this organism in a substrate having other sources of carbon than glucose. Gottlieb (1944) had earlier shown that various amino-acids could be used as the exclusive carbon sources in the medium for the growth of F. lycopersici. Preliminary experiments showed that with various amino-acids as the carbon sources (e.g., aspartic acid, glutamic acid etc.) the fungus did not produce significant quantities of toxins, even though growth took place. However, when 2.5% glycine was used as the sole carbon source, toxin production was quite comparable to that produced by glucose. After preliminary experiments it was found that the best medium for the optimum production of toxins was: 25 gms. glycine, 7 gms. ammonium nitrate, 5 gms. monopotassium phosphate, 2.5 gms. magnesium sulphate, 0.02 gms. ferric chloride; distilled water to make up 1000 ml. Growth in this medium, however, was not comparable to the Richard's medium, nevertheless toxin production was optimum.

Strains R-5-6 and 257 of F. lycopersici were grown in 500 ml. Erlenmeyer flasks with 100 ml. of glycine medium in dark at a temperature of 27 °C for different lengths of time. At regular intervals of 7 days, 10 flasks from each series were removed, freed from mycelium and the culture filtrate tested for the presence of toxins by the wilting tests (in 1/5 dilution) and also by Ustilago spore germination tests. Figures 5 and 6 represent the results of this investigation. Although not included in these figures, vasinfuscarin was regularly present in the culture filtrates from the beginning of the 7th day to the end of 6 weeks.

The following conclusions can be drawn from a study of figures 5 and 6:

a) Toxins (lycomarasmin, fusaric acid and vasinfuscarin) are produced by the two strains during their growth in the glycine medium.

b) Toxin production, as assessed by the tomato wilting tests, steadily increases in the culture filtrates of both the strains till the 4th week and subsequently falls down to a minimum at the end of 6 weeks (fig. 5).

c) As assessed by the Ustilago spore germination tests, toxin production reaches a maximum at the end of a 2 week growth period and subsequently falls down in both the strains.

d) In the glycine medium, strain 257 produces more toxin than the strain R-5-6. The difference, however, is not so significant as in the Richard's medium.

With the above facts in hand, we can discuss the questions we asked ourselves at the beginning of these experiments. It has been demonstrated that, by varying the chemical environment, a corresponding variation also occurs in the quantity of...
toxin production. Thus, under certain conditions, the virulent strain produces more toxin in vitro than the avirulent strain under precisely the same conditions. This is not surprising, since the microorganisms are known to be fastidious in this regard (Gäumann, 1951a; Turel, 1952; Kern, 1952; Sauthoff, 1955). This fact, however, may have a bearing on the toxin production in vivo. The chemical environment in the plants is not the same as in the laboratory cultures, and as we have shown, by a change in the chemical environment a corresponding shift also occurs in the toxin production. Thus, the virulent strain may be able to produce more toxin in the tomato plants with the resulting severity of disease as contrasted with the less virulent strains. No doubt there are many other factors in operation apart from the toxin factor in the production of the overall disease picture in the tomato plants by the different strains of the pathogen. Resistance of the host is one such factor. This resistance may manifest itself in two ways. 1. Resistance to the spread of the pathogen and 2. resistance to the toxins produced by the pathogen (Gäumann, 1951b; Zähner, 1955). Disease in the tomato plant results only when both these obstacles have been overcome by the pathogen. Strain 257, although capable of producing some quantity of toxins cannot do so in the plants, because it cannot overcome even the
preliminary resistance of the host to the spread of the pathogen. Thus, it remains localized at the focus of infection, and since no growth occurs, toxins too are not produced in significant quantities.

The fact that *F. lycopersici* can produce its toxins from certain amino-acids (in this case glycine) as the exclusive carbon source is significant. This would mean that in the absence of optimal quantities of sugars in the host, the pathogen would be able to metabolise other constituents, like amino-acids, for toxin production in vivo. If we compare figures 3 and 6 we see that the *Ustilago* activity of the glycine medium is far greater than Richard’s medium, specially during the first two weeks. Among the known toxins of *F. lycopersici* only fusaric acid inhibits germination of *Ustilago* spores. In other words, with glycine as the exclusive carbon source, more fusaric acid is produced by the two strains of *F. lycopersici* than with glucose as the carbon source. Although not exactly ascertained, it might be mentioned in the passing, that the growth of the strains in glycine medium is approximately 1/5 less than in glucose medium, and still more fusaric acid is produced in the former medium. This is in keeping with the observations of other authors (Kern, 1952).

The preferential production of fusaric acid, as assessed by the *Ustilago* spore germination tests, in the glycine medium by the strains of *F. lycopersici* led us to think that glycine itself might serve as a distant precursor of fusaric acid. If this were true, we would expect increased fusaric acid production in Richard’s medium containing small quantities of glycine. Accordingly, the following three media were prepared and both the strains of *F. lycopersici* were cultured in them:

a) Richard’s medium + 0.3% glycine,  
b) Richard’s medium + 0.15% glycine,  
c) Richard’s medium alone as control.

The culture filtrates were assayed as usual with *Ustilago* spore germination tests after each 7 days interval. The results are given in figure 7.

The following facts are borne out from the figures:

a) In all media, fusaric acid production by strain R-5-6 is uniformly lower than strain 257.  
b) With increasing quantities of glycine in the medium, a corresponding increase in the production of fusaric acid takes place, especially at the end of 14 days.

We are naturally tempted to interpret these results as evidence for the fact that glycine, either directly or indirectly serves as a precursor of fusaric acid. The facts, however, are not so simple. It is equally possible that glycine causes, by its presence, a more efficient uptake and metabolism of the immediate precursor of fusaric acid. In keeping with this possibility, we observe that there is no significant difference in fusaric acid production at the end of a 7 day period in media with or without glycine. Only between
the 7th. and the 14th. day does the difference become visible. This might again be due to the fact that the enzyme systems, responsible for the metabolism of fusaric acid, are first produced after some days growth.

Fig. 7. *Ustilago* activity of the culture filtrates (in dilution units) of strains R-5-6 and 257 grown in R₅ medium with different concentrations of glycine

II. Chemical changes in artificial cultures during fusaric acid production

To come closer to an understanding of toxin production in vitro it was necessary to investigate the series of events that lead to the production and accumulation of the toxins in artificial cultures. Facts gathered here could be of use for us in interpreting the toxin production in vivo. In the experiments that follow, we have restricted ourselves to a study of the biosynthesis of fusaric acid alone. The reasons are obvious. For lycomarasmin and vasinfuscarin, there do not exist any chemical methods of determination. Only very recently methods have been developed for the chromatographic determination of lycomarasmin (Kern and Flück, unpublished). Fusaric acid, however, lends itself well to experimentation and methods for its determination are known (Zähner, 1954).

Fusaric acid is a 5-n-butylpyridine-2-carboxylic acid and has carbon, nitrogen, oxygen, and hydrogen in its molecule. In its biosynthesis, therefore, the carbon and nitrogen containing constituents of the medium must have a central role to play. In our preliminary experiments we found out that the common organic acids, resulting from the dissimilation of glucose and accumulating as the same time as fusaric acid in the medium, bear no relationship to fusaric acid production. Their study was, therefore, not taken
up further. The nitrogen containing constituents, however, appeared to have a much closer relationship to the biosynthesis of fusaric acid. In the following study, therefore, the metabolism of nitrate, ammonia, amino-acids, purines and glucose has been studied in relation to fusaric acid production.

Only strain 257 was used and was grown in 500 ml. Erlenmeyer flasks containing 100 of modified Richard's medium (page 336). Analysis of the culture filtrates was done every third day till 42 days. To obtain reliable results and to minimise errors due to natural fluctuations in the metabolism of individual cultures, contents of 10 flasks each time were taken for analysis. In calculating results, corrections incidental to the loss by evaporation were applied. The following constituents were analysed in the culture filtrates: 1. Dry weight of the mycelium 2. Glucose (as reducing sugar). 3. Total nitrogen 4. Nitrate nitrogen 5. Ammonia nitrogen 6. Amino-nitrogen 7. Purine nitrogen and 8. Fusaric acid nitrogen. The methods of analysis have been given earlier (section B, III). The results of this investigation are represented in figures 8, 9 and 10.

Fig. 8. Dry weight of mycelium and glucose content of the culture filtrates

Fig. 9. Amino-N and purine-N in relation to fusaric acid nitrogen in the culture filtrates

Fig. 10. The relationship between total nitrogen, ammonium nitrogen and nitrate nitrogen in culture filtrates

The following facts can be summarized from a study of figures 8, 9 and 10:

a) The dry weight of the mycelium increases steadily till the 25th day of growth and then gradually falls down. At about the time the mycelium has reached the maximum growth limit, very little glucose is present in the medium. The increase in the weight of the mycelium is proportional to the quantity of sugar consumed.
b) Fusaric acid first appears in the medium after 6 days of growth and thence afterwards steadily increases till the 18th. day, when it reaches its maximum peak. At the end of this period, the level of fusaric acid production again falls down till the 33rd. day and again rises to a maximum at the end of 36 days.

c) The amino-acids are produced in the medium from the beginning of growth period, reach a peak at the end of 12 days and except for a decrease in production between 12th. to 21st. day and again between 33rd. and 39th. day, are always present at the peak level. Towards the end of the growth period of the mycelium, free amino-acids rapidly increase in the medium. This may be due to the hydrolysis of the cell proteins, once lysis has started.

d) Purines increase in the medium till the 12th. day (at about the same time as the amino acid production reaches its peak), after which the level of production falls down, corresponding to the amino-acid level, and then remains more or less constant. The production of purines corresponds to the amino-acid production in the medium.

e) It will be recalled that Richard's medium contains ammonium nitrate as the source of nitrogen. Figure 10 shows that the organism utilizes nitrate nitrogen in preference to ammonia nitrogen. In fact, ammonia increases steadily in the medium till the end of the growth period. On the contrary, nitrates are rapidly taken up. Some of the excess ammonia may accumulate in the medium due to deamination of some of the amino-acids. These findings are in conformity with the earlier work of Luz (1934). The rise in the pH of the medium (page 348) is perhaps correlated to the accumulation of ammonia.

The total nitrogen of the culture filtrate decreases with active growth of the mycelium but this occurs very gradually.

The significance of these results is obvious. It can be said with very great deal of certainty that fusaric acid is not an autolysis product. Thus, at the time greatest quantity of fusaric acid is available in the medium, the mycelium is still in the log phase of growth. This observation is in contradiction to that observed in Gibberella fujikuroi (Stoll, 1954), causing Bakanae-disease of rice in Japan, where maximum quantity of fusaric acid is present when a lysis of the mycelium has already set in. The results with *F. lycopersici* correspond to earlier investigations with the aid of biological tests (Kern, 1952).

The most interesting point, however, is the relationship between amino-acids, purines and fusaric acid (figure 9). The production of purines in the medium parallels amino-acid production. The amino-acids, however, rapidly increase till the 12th. day, when fusaric acid production is the lowest. From 12th. to 18th. day, when fusaric acid production rapidly increases, the amino-acid production decreases. Precisely the same set of events occur between 33rd. and 39th. day. It seems plausible that fusaric acid is produced at the
cost of some of the amino-acids and, therefore, when more fusaric acid is produced, corresponding amounts of amino-acids decrease in the medium. Recent paper chromatographic studies of amino-acid metabolism of *F. lycopersici* (Flück and Richle, 1955) lead to the conclusion that in particular Alanin and Citrullin might be involved in the biosynthesis of fusaric acid.

**D. Studies on the metabolism of *F. lycopersici* in vivo**

It has been shown in the previous section that *F. lycopersici* produces the three known toxins (lycomarasmin, fusaric acid and vasinfuscarin) both on a glucose and glycine medium. It has been assumed by us, in view of the indirect evidences, that these toxins are also produced by the pathogen in vivo. The direct demonstration of these toxins and other metabolic products of the pathogen in vivo is difficult with ordinary chemical techniques. The only way we could demonstrate the metabolic products of the pathogen in the host would be by certain differential techniques, where we would be able to recognize apart the substances secreted by the pathogen from those of the host. The only possibility that suggested itself was the 'tagging' of the pathogen with a suitable radioisotope.

In phytopathological literature as yet only a few organisms have been effectively employed for tagging. The earliest work has been that of Warren (1951) who was able to tag *Bacterium stewartii* effectively with P$^{32}$ and could trace the distribution of the tagged organism in the corn plant by means of radioautographs. Fitzgerald et al (1951) working with Yeasts showed that it could be tagged by Tritium at a concentration of 100 $\mu$C/ml. At this level of radioactivity, the organism was not damaged as assessed by the retention of the budding capacity of the tagged cells. Wheeler (1952, 1953) using C$^{14}$-labeled glucose was able to tag effectively 5 different fungi at a concentration of 30 $\mu$C/ml. He observed that at this level of radioactivity some of the conidia were rendered inviable but the fungi did not lose their virulence. In a later work, using *Helminthosporium victoriae*, he demonstrated that about 100 $\mu$C/ml. of C$^{14}$-labeled glucose could be used safely, without the organism losing its virulence. At this level of radioactivity, however, a number of cells became inviable. Hoffmann and Zuckerman (1954) succeeded in tagging *Endoconidiophora fagacearum* by incorporating in the growth medium C$^{14}$-labeled sucrose at a concentration of 25 $\mu$C/ml. At this concentration the fungal cells did not suffer any damage.

For our experiments, radioactive glycine, labeled in the methyl carbon was used. The specific activity of the glycine was 14.9 microcurie per milligram.

The experimental procedure and details of preliminary work have already been described (Kern and Sanwal, 1954) and may be referred to for further information.
I. The secretion of radioactive metabolic products into the host

Previous experiments have shown (Kern and Sanwal, 1954) that the mycelium can be safely tagged with radioactive glycine at a concentration of 90 μC/ml., after a growth period of 7—12 days. In this case, although about 40% of the cells become non-viable, yet the organism does not lose its virulence and no difference in the morphology of the fungus can be discerned. It was safe to assume, therefore, that tagged mycelium introduced in the host could be used to find out whether tagged products of metabolism of the pathogen are produced in the plants. This could be investigated both by the counting techniques as well as radioautography of the tissues showing disease symptoms.

In the following experiments, the inoculum was obtained by growing strain R-5-6 in glycine medium having a specific radioactivity of 90 μC/ml., for eight days. The inoculum was washed after this preliminary period of growth with 5 changes of 2% sterile, inactive glycine solution and further few changes of sterile distilled water in a centrifuge. Tomato plants were inoculated with this mycelium as usual. After every 24 hour period from the time of inoculation, one plant at a time was cut into convenient pieces, about 2 cms. long. The leaves with their petioles were treated separately. Each two centimeter length of the stem was then surface sterilized by means of 0.2% HgCl₂, washed with sterile distilled water and by means of a sterilized knife cut into smaller bits. Pieces of each stem part were laid on poured petri dishes and incubated at 27 °C for two days. Those stems, in which the fungus appeared were not counted further. In the case of leaves, only the petiole parts attached to the stem were taken and if this showed the presence of the mycelium, the whole leaf was rejected. The tested pieces of each part of the stem were then gathered together, dried and counted.

In the case of strain R-5-6, no radioactivity was detected in plants after 24 hours of infection, but after 48 hours radioactivity was apparent in stems about 13—15 cms. from the base of the stem. Growth of the mycelium at this moment was restricted to areas adjoining the roots. Subsequent measurements at 24 hours interval for a 7 day period showed that radioactivity indeed increased in the leaves, but comparatively much more remained in the stems.

Under our green-house conditions (temp. 24—30 °C) the vein-clearing symptoms first described by Foster (1946) appeared on the host leaves on the 4th or the 5th day. However, all the leaves of the plant did not show this symptom. If this vein-clearing symptom was the result of some translocable toxins secreted by the pathogen in advance of its growth, it was logical to assume that radioactivity would increase considerably in the leaves showing this symptom. This would only be the case, however, if the metabolic products responsible for the vein-clearing were radioactive. That such is the probable case is strongly supported by the analysis presented in table 1.

Here, individual leaves showing vein-clearing symptoms on the plant and those showing no symptoms at all were cut off and their radioactivity...
measured. It will be seen from the table that except in a few cases the leaves showing vein-clearing show a relatively greater accumulation of radioactivity from the leaves showing no other symptom. In both the cases no mycelium was present in the leaves.

**Table 1**

**The relative accumulation of radioactivity in different leaves of individual plants (showing vein-clearing or no symptoms at all) after 5 days inoculation with tagged strain R-5-6 of *Fusarium lycopersici***

<table>
<thead>
<tr>
<th>Plant no.</th>
<th>Counts/mt./mg. (dry weight) of leaves showing vein-clearing</th>
<th>Counts/mt./mg. (dry weight) of leaves showing no vein-clearing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaf number</td>
<td>average counts/mt./mg.</td>
</tr>
<tr>
<td>1</td>
<td>1 2 3 4</td>
<td></td>
</tr>
<tr>
<td>890</td>
<td>846 426 314 220</td>
<td>614</td>
</tr>
<tr>
<td>646</td>
<td>542 894 334 227</td>
<td>549</td>
</tr>
<tr>
<td>428</td>
<td>430 426 391 227</td>
<td>550</td>
</tr>
<tr>
<td>371</td>
<td>929 543 385 174</td>
<td>310</td>
</tr>
<tr>
<td>1650</td>
<td>430 426 391 227</td>
<td>877</td>
</tr>
</tbody>
</table>

The difference in the radioactivity of the injured and the healthy leaves on the same plant, leads us to believe that the pathogen secretes some substances of toxic nature into the host which are carried up to the leaves and cause the characteristic symptoms there. These findings are substantially in agreement to those of Davis (1954) who reached the same conclusion through different experimental procedures.

In the case of strain 257 (avirulent), the vein-clearing symptoms do not appear at all, at least under our greenhouse conditions. It was interesting to find out whether, in the absence of immediate symptoms on the host, the fungus builds up or secretes some substances in the host.

The answer to this question was obtained by growing strain 257 in the radioactive glycine medium for 8 days and inoculating the plants with the usual techniques. Analysis of such plants showed that, in contrast to strain R-5-6 radioactivity accumulates very slowly in the leaves and very little is present in the stems.

At the first sight the results of these experiments with virulent and avirulent pathogens seem contradictory. Both appear to secrete certain chemical substances in the host plant and yet one strain can produce disease in the plant and the other not. To the author, it appeared, as a reconciliation between these two apparently contradictory facts, that there is a qualitative difference in the secretion of chemical substances by these two kinds of strains inside the host; the avirulent pathogen secreting certain substances which are not toxic to the plants and the virulent one certain substances which are toxic along with others which are non-toxic.
The verification of this hypothesis is beset, unfortunately, with difficulties and complications. It would be an exacting task to purify all the plant constituents separately for radioactive measurement.

II. The distribution of radioactive metabolic products in the host tissue

Until now, in our analysis, we have included all the tissues of the host, irrespective of the fact that within the same tissue only certain parts show the symptoms. Thus, in leaves showing vein-clearing symptoms, only the veins of the leaves show the disease and not the intervenal spaces. In stems, similarly, the vascular bundles show the browning and the rest of the tissue seems perfectly healthy. If we carry our argument, that at least some of the symptoms on the host are due to toxic metabolic products of the pathogen further we should be able to demonstrate comparatively more radioactivity in the strictly diseased portions than from the surrounding healthy areas (in our examples, more in the veins than in the intervenal portions or in the case of stems, more in the vascular bundles than in the ground tissue). Since mechanical teasing of the tissues apart from one another would introduce considerable amount of inaccuracy, we took recourse to the radioautographic techniques.

The tomato plants, at the four leaf stage, were inoculated with tagged R-5-6 and 257 strains. After 12 days infection period, the stems of the plants inoculated with strain R-5-6 showed a typical browning of the vessels about 8—10 cms. above ground level and vein-clearing on the older leaves. The plants inoculated with strain 257, at about the same time, showed slight browning of the vessels only about 0.5—1 cm. above the ground level and no vein-clearing symptoms on the leaves. For radioautography, we only took the following parts from the plants:

1. Stem portions showing browning.
2. Stem portions showing no browning.

The diseased and healthy leaves were not included here, because the radioactivity in the leaves was very much less than that required for obtaining a satisfactory image on the emulsion (see Kamen, 1951; Sanwal, 1955). Convenient pieces of each one of the above tissues were prepared for sectioning in the following ways:

1. The tissues were placed in water and air was removed from them by a water suction pump. The objects were transferred then to a vessel containing ethylene glycol cooled to $-20^\circ$ C (see for further details, Tietz, 1952, 1953). After allowing them to remain there for 3 hours, the contents of the vessel were gradually warmed to room temperature and the tissues transferred to a 1 : 1 mixture of ethylene glycol and Aquaffin$^*$) (manu-

$^*$) For kindly lending me a sample of this wax, I thank Dr. H. F. Linskens of Köln University, Germany, who also was kind enough to call my attention to some of the published literature on the use of synthetic waxes in histology.
factured by Bayer Farbenfabriken, Leverkusen) for a short time at 30 ° C and then 2 hours at 50 ° C. Next they were placed in pure Aquaffin for 6 hours and embedded in the same material for sectioning. Sections about 7 μ thick were cut and stretched on specially prepared microscope slides (see section B, IV). Before radioautography the wax was removed from the slides by laying on top of the sections a filter paper soaked in a mixture of Aquaffin (10 %) and water saturated with Thymol and allowing it to dry. The sections were then covered with the 'stripping' film emulsion and exposed for 40 days.

Fig. 11. Radioautograph (on NTB emulsion) of a transverse section of tomato stem showing vascular browning (plants inoculated with tagged strain R-5-6 of F. lycopersici and tissues fixed in methanol) × 67

2. The tissues were fixed for 12 hours in methanol, dehydrated, passed through various grades of xylol and embedded in paraffin. Sections, about 7—10 μ thick were exposed on NTB plates for 40 days.

Both the above written methods were used, because we did not know whether the metabolic products were water insoluble or alcohol insoluble. The first method would preserve water insoluble metabolic products and the latter alcohol insoluble ones.

Controls were employed for all the series of sections.

Results of this work are summarised below:

a) Only those sections of diseased stems show significant amount of radioactivity which had been fixed in methanol (figs. 11 and 12); those fixed with the other methods showed very little, if at all (optical density of the developed silver grains less than D = 0.5). This might be due to the fact that the metabolic product associated with the diseased vascular bundles were water soluble, so that, during fixing procedures, most of the radioactivity leached out from the material.
b) Sections of diseased stems (showing vascular browning), obtained either from the plants infected with strain R-5-6 or 257, show *radioactivity* only in the *vascular tissue*, after having been fixed in methanol (figs. 11 and 12).

c) Sections of normal stems (showing no vascular browning) show no detectable radioactivity in any part. This is uniformly so, whether the stems used are from plants infected with either of the two strains or the different fixing procedures.

Fig. 12. Radioautograph same as figure 11. One of the vascular bundles enlarged to show the developed silver grains directly above the xylem cells. Note that no mycelium is present in the vessels and no radioactivity in the ground parenchyma cells. × 270

d) In the case of diseased stems, inoculated with marked R-5-6 strain, *radioactivity is detectable in the vessels independent of the presence of the radioactive mycelium* (fig. 12), when the stems are fixed in methanol.

e) The control preparations, given the same treatment as the others above, did not affect the emulsion, thereby showing (fig. 13) that the other exposed pictures were not artefacts.

From all the observations above recorded, some significant facts can be deduced. Firstly, the pathogen during its growth in the host secretes certain metabolic substances, some of which at least are toxic to the plants. This is borne out by the fact that *radioactivity in the stems is closely associated only with the diseased portions*. Secondly, this metabolic product seems to be water soluble and alcohol insoluble (precipitable by methanol) and possibly, therefore, is of a proteinaceous nature. Vasinfuscarin, produced by *F. lycopersici* in vitro is similarly precipitable by methanol (Gäumann, Stoll and Kern, 1953). It cannot be said at this stage of work whether the precipitable substance in vitro and in vivo is one and the same, but there is evidence that such might be the case.
Fig. 13. Controls (on NTB emulsion) of a transverse section of tomato stem showing vascular browning (plants inoculated with non-radioactive strain R-5-6 of *F. lycopersici* and tissues fixed in methanol). ×165

III. Chemical substances secreted by the pathogen in the host

Elsewhere in this work, it has been demonstrated that when plants are inoculated with tagged mycelium, radioactivity is discernible in both stems and leaves of the infected plants after some time. This was irrespective of whether the inoculation was done by the virulent or the avirulent strain. The explanation given was that the metabolic products secreted by both the strains varied qualitatively from one another, i.e., the avirulent pathogen perhaps secreted certain substances which were non-toxic and the virulent pathogen substances which were both toxic and non-toxic. As we have shown with radioautographic techniques, the avirulent pathogen also secretes certain radioactive, seemingly toxic metabolic products, but they are limited to the area of infection (at the base of the plants, small portions showing vascular browning).

Incidental to the above hypothesis, the following two questions were taken up as basis for further experimentation:

1. What kinds of metabolic substances are produced by the pathogen in vivo?
2. Is there any difference, qualitative or quantitative, in the secretions of the virulent and avirulent pathogens?

The answer to these questions was given by extraction and chemical analysis of the infected plants. It must be emphasized from the very beginning that only a gross and rough characterization of the radioactive substances secreted by the pathogen could be had because of the technical difficulties involved in the work.

15 tomato plants were inoculated each by marked strains R-5-6 and 257 (tagged with 2-C\(^{14}\)-glycine at a concentration of 90 μC/ml.). After about 15 days growth (when many plants showed wilting symptoms and necrosis etc.) the plants were cut off from the soil level and divided into
The distribution of radioactivity in fractions of stem and leaf tissue 15 days after inoculation with tagged strain R-5-6 of *Fusarium lycopersici*

<table>
<thead>
<tr>
<th>Extract</th>
<th>Stem fraction (dry weight 7.9 g.) total activity = 129,162 counts per minute</th>
<th>Leaf fraction (dry weight 9.8 g.) total activity = 78,075 counts per minute</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>counts/mt.</td>
<td>percentage of total activity</td>
</tr>
<tr>
<td>Petroleum ether extract</td>
<td></td>
<td></td>
</tr>
<tr>
<td>acidic part</td>
<td>1,033</td>
<td>0.8</td>
</tr>
<tr>
<td>non-acidic part</td>
<td>1,162</td>
<td>0.9</td>
</tr>
<tr>
<td>Ether extract</td>
<td></td>
<td></td>
</tr>
<tr>
<td>acidic part</td>
<td>17,308</td>
<td>13.4</td>
</tr>
<tr>
<td>non-acidic part</td>
<td>267</td>
<td>0.2</td>
</tr>
<tr>
<td>Ethanol extract</td>
<td></td>
<td></td>
</tr>
<tr>
<td>acidic part</td>
<td>4,918</td>
<td>3.8</td>
</tr>
<tr>
<td>non-acidic part</td>
<td>12,141</td>
<td>9.4</td>
</tr>
<tr>
<td>70% ethanol extract</td>
<td></td>
<td></td>
</tr>
<tr>
<td>amino-acids</td>
<td>12,014</td>
<td>9.3</td>
</tr>
<tr>
<td>acidic subs.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>basic subs.</td>
<td>1,034</td>
<td>0.8</td>
</tr>
<tr>
<td>sugars etc.</td>
<td>10,849</td>
<td>8.4</td>
</tr>
<tr>
<td>Proteins</td>
<td>42,494</td>
<td>32.9</td>
</tr>
<tr>
<td>Tannins &amp; gums</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dextrins &amp; pectins</td>
<td>904</td>
<td>0.7</td>
</tr>
<tr>
<td>Starch and cell wall</td>
<td>16,791</td>
<td>13.0</td>
</tr>
<tr>
<td>constituents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total recovered</td>
<td>118,905</td>
<td>92.5</td>
</tr>
</tbody>
</table>

The ground up tissues were extracted (see also, Weintraub et al, 1952 a, 1952 b) exhaustively with petroleum ether for 12 hours, after which it was poured off. The solid tissues were then extracted in turn for 12 hours with ethyl ether, 12 hours with absolute ethanol and finally 12 hours with 70% aqueous ethanol. Each time the liquid was poured off and kept for a subsequent analysis. The residue was then extracted first with cold water and then with hot water by turns. In the end the residue consisted of starch and cell wall material.

The petroleum ether and ethyl ether extracts each were shaken with equal volume of sodium bicarbonate solution in a separating funnel and the bicarbonate layer separated from ether. It contained the acidic fraction and ether,
The distribution of radioactivity in fractions of stem and leaf tissue 15 days after inoculation with tagged strain 257 of *Fusarium lycopersici*

<table>
<thead>
<tr>
<th>Extract</th>
<th>Stem fraction (dry weight 12.3 g.) total activity = 85605 counts per minute</th>
<th>Leaf fraction (dry weight 14.1 g.) total activity = 111234 counts per minute</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>counts/mt.</td>
<td>percentage of total activity</td>
</tr>
<tr>
<td><strong>Petroleum ether extract</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>acidic part</td>
<td>1969</td>
<td>2.3</td>
</tr>
<tr>
<td>non-acidic part</td>
<td>257</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>Ether extract</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>acidic part</td>
<td>759</td>
<td>0.7</td>
</tr>
<tr>
<td>non-acidic part</td>
<td>7726</td>
<td>8.5</td>
</tr>
<tr>
<td><strong>Ethanol extract</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>acidic part</td>
<td>2824</td>
<td>3.3</td>
</tr>
<tr>
<td>non-acidic part</td>
<td>4879</td>
<td>5.7</td>
</tr>
<tr>
<td><strong>70% ethanol extract</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>amino-acids</td>
<td>13097</td>
<td>15.3</td>
</tr>
<tr>
<td>acidic subs.</td>
<td>1027</td>
<td>1.2</td>
</tr>
<tr>
<td>basic subs.</td>
<td>2654</td>
<td>3.1</td>
</tr>
<tr>
<td>sugars etc.</td>
<td>12583</td>
<td>14.7</td>
</tr>
<tr>
<td><strong>Proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7276</td>
<td>8.5</td>
<td></td>
</tr>
<tr>
<td><strong>Tannins &amp; gums</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1027</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td><strong>Dextrins &amp; pectins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3938</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td><strong>Starch and cell wall constituents</strong></td>
<td>15922</td>
<td>18.6</td>
</tr>
<tr>
<td><strong>Total recovered</strong></td>
<td>75488</td>
<td>88.0</td>
</tr>
</tbody>
</table>

The nonacidic fractions. The absolute ethanol extract was evaporated under vacuum and the residue was taken up in water. The pH of this solution was brought to 3.5—4.0 and it was then extracted with ethyl ether to remove the acidic constituents. The water phase, after extraction contained the non-acidic constituents. From the 70% ethanol extract, ethanol was removed under vacuum and the water solution was boiled for some minutes to remove proteins which were separated by centrifugation. The supernatant was brought to a pH of about 8.0 with alkali and extracted with ether to remove the basic substances. The extract remaining after ether extraction was adjusted again to a pH of 4.8—5.2 and treated with neutral lead acetate to precipitate amino-acids. The precipitated amino-acids were removed by centrifugation. The supernatant contained sugars etc.

The cold water extract was boiled for 10 minutes and the precipitated protein was centrifuged out. The supernatant was adjusted to a pH of 4.8—5.2 and neutral lead acetate was added to it. The precipitate consisted of tannins and gums and the supernatant of dextrins and pectins.
The hot water extract was boiled to remove all traces of proteins. The solution was then treated with neutral lead acetate to remove gums and tannins.

Each of the above fractions was counted as usual with a GM counter. Tables 2 and 3 summarize the results of this investigation.

The results obtained above show that both the virulent and avirulent strains secrete varied kinds of chemical substances in the hosts. It cannot be said for certain whether these chemical substances remain in the original form, as secreted by the pathogens in the host, or are broken down and modified further by the host enzyme systems. The salient features of the investigation can be summarized as follows:

a) The pathogen secretes a wide variety of chemical substances in the host, some of which are undoubtedly non-toxic.

b) The principle substances secreted by the pathogen are certain acidic substances, amino-acids, sugars and proteins.

c) The main difference in the secretions of virulent and avirulent pathogens is probably the protein substances; the virulent pathogen secretes more substances of protein nature than the avirulent pathogen.

It must be borne in mind that the radioactive chemical substances, shown to be secreted by the pathogens are not the only ones. Perhaps more important are the substances elaborated by the pathogen in the host plant by transformation of the host nutrients through its enzymatic system. Thus, many important toxic substances, known to be produced by the pathogen in vitro probably cannot be demonstrated by our techniques in vivo because they may be elaborated by the breakdown of the host nutrients.

Apart from the above, our experiments have demonstrated that there are definitely some substances secreted by the pathogens which are toxic, like perhaps the metabolic product causing the vein-clearing symptoms and the browning of the vessels.

E. The role and fate of C14-marked fusaric acid in tomato plants

By the tagging techniques, described in previous sections, only those toxic components can be demonstrated in vivo which are elaborated in the mycelium and secreted to the outside, i.e., the intracellular toxins. On the contrary, extracellular toxins are elaborated by the pathogen by transformation of the host nutrients only external to the mycelium. Fusaric acid is an example of an extracellular toxin and vasinfuscarin that of an intracellular one. The terms intracellular and extracellular are here used in preference to endotoxin and exotoxin respectively, because by usage with bacterial organisms, the term endotoxins has come to signify
those toxic substances which are released after the death of the cells. Exotoxins, on the contrary, are produced by the living bacteria inside the cells and then secreted to the outside (Burrows, 1951).

It is relatively simple to distinguish between intracellular and extracellular toxins in fungi. When young, actively metabolizing mycelium of *F. lycopersici* is suspended in replacement cultures, even for a short time, the intracellular toxin vasinfuscarin is secreted in the culture medium. Alternatively, when the mycelium is disintegrated by mechanical means and extracted with water, only the presence of vasinfuscarin can be demonstrated. Using similar techniques, fusaric acid, one of the extracellular toxins, cannot be detected inside the mycelium*); it is only present in the culture filtrates. This is because of the fact that fusaric acid is only produced external to the mycelium during the course of growth and metabolism in vitro. There was no reason to doubt that fusaric acid would similarly be produced in vivo by the metabolism of the host nutrients through the enzyme systems of the pathogen. For these reasons, therefore, it was never expected that fusaric acid would be demonstrated in diseased plants by the tagging techniques. However, sufficiently sensitive methods are known for the detection of fusaric acid (Zähner, 1954). Accordingly, a number of efforts were made to extract fusaric acid from the diseased plants, but they were in vain. It was doubted, therefore, that fusaric acid is metabolised by the host as soon as it is formed by the pathogen. Evidence for this possibility exists from the work of Japanese authors (Tamari and Kaji, 1952 a, 1952 b). The following work was undertaken, therefore, to investigate the metabolic fate of fusaric acid in the host and to clarify certain questions incidental to the pH effect of fusaric acid (Gaumann, Naef-Roth and Kobel, 1952). The latter investigations shall be published elsewhere.

While the experiments on the distribution of C14-marked fusaric acid were in progress, it was noticed that the total radioactivity in the tissues was very much lower than the radioactivity originally applied, specially after the cuttings had been placed in water for 48 hours after uptake of radioactive fusaric acid. Even after reckoning with the sampling error, the loss of radioactivity from the tissues could not be explained. In case of nicotinic acid, a closely related compound, it is known that it is decarboxylated and metabolized to a number of related compounds in animals (Leifer, Roth, Hogness and Corson, 1951). Accordingly, investigations were undertaken to study the metabolism of fusaric acid in plants. The Japanese authors believe (Tamari and Kaji, 1952 a, 1952 b) that fusaric acid is changed to chelate ring complex in rice plants (see also, Deuel, 1954). The chelate complexes of fusaric acid are furthermore insoluble in water. The chelate complex may be one of the products of metabolism of fusaric acid, but whether any other products are also formed side by side with this are unknown.

*) For undertaking some of the investigations in this line the writer is grateful to Mr. D. Klüpfel.
I. Decarboxylation of C14-marked fusaric acid in plants under different conditions

If fusaric acid is decarboxylated and no other change takes place in the fusaric acid molecule, 3 n-butylpyridine is obtained:

$$\text{HOOC}_{14}\text{C}_4\text{H}_9 \xrightarrow{\text{C}_4\text{H}_9\text{C}} 3\text{ n-butylpyridine}$$

If such a series of events occurred in the tomato plants, after application of fusaric acid, we would not only get radioactive CO2 coming out from the plants, but also a product which is more toxic than the fusaric acid itself. The extent of C14O2 evolution by the plants after uptake of the minimal dose of C14-marked fusaric acid had to be known before we started isolating the hypothetical 3 n-butylpyridine from the tissues.

Tomato cuttings were allowed to take up C14-marked fusaric acid (approx. 150 mg./kg. fresh weight, at a concentration of $5.0 \times 10^{-3}$ molar) under a bell jar provided with a CO2-free air circulation devise. The CO2 evolved by the plants was collected in 2 N NaOH. Immediately after the plants had taken up by transpiration pull the required amount of fusaric acid, the cuttings were immersed in a 0.01 M NaH2PO4 (Weintraub et al., 1952 a) solution in order to minimize the retention of carbon-dioxide. CO2 was collected each 24 hour period for 3 days, converted to barium carbonate as described earlier (cf. section B, IV) and counted under a GM counter. The obtained counts were calculated to the activity of ‘zero thickness’ (page 345).

a) Effect of pH of C14-marked fusaric acid on C14O2 evolution

The tomato cuttings of approximately the same age and height were allowed to take up fusaric acid at pH 4.3, 6.0 and 7.2 respectively. In each series, six plants were included and C14O2 was collected for 3 days and analysed at every 24 hours interval. The results are given in figure 14. It will be seen that the carboxyl group of fusaric acid is broken down in the plant cells. At all the three pH values, the C14O2 evolution is at first slow and increases after 24 hours of application. At the end of 72 hours of application approximately 10% of the fusaric acid originally applied is broken down. The pH of the applied fusaric acid seems to play a very minor role in its breakdown by plant cells.
Maximum breakdown within a 72 hour period after application is achieved when the original application was at a pH of 4.3. However, during the course of these experiments it was consistently noted that the amount of decarboxylation of fusaric acid varied very much with individual plants. Even when the plants were of the same age and grown under the same conditions and handled the same way experimentally, a variation of 15—20% in the results was easily obtained.

Fig. 15. Decarboxylation of carboxyl marked fusaric acid in tomato plants kept under complete darkness and continuous light at pH 4.3.

Axis: Time in hours.
Ordinate: Percentage of applied radioactivity (as fusaric acid) in C14O2 evolved the plants

b) Effect of continuous light and continuous darkness on the breakdown of fusaric acid

As stated earlier, the experiments above reported were conducted under continuous light. Since light has an important bearing on the CO2 exchange in the plants, it might indirectly affect the breakdown of fusaric acid. The entire experimental set up was the same as above, except that the bell jar containing the plants was wrapped up on all sides with black paper. Figure 15 represents the results of this investigation. It will be seen from the histogram, that within the limits of the experimental error, the C14O2 evolved by the plants after uptake of C14-marked fusaric acid at a pH of 4.3 is about the same in light as well as in darkness. Light or darkness has, therefore, no effect on the decarboxylation of the fusaric acid molecule.

II. The intermediate products of metabolism of fusaric acid

One of the ways to understand the mechanism of action of a toxin is to find out how it is catabolized by the plants. In general, a toxin can affect the plant cells in two ways: 1. As the original molecule, e.g., by permeability distortions, coagulation of the protoplasm and so on and 2. by getting changed in tissues to substances which in their turn are more toxic to the plant cells. It is also possible that both these mechanisms simultaneously operate in causing injury to the plant cells. With reference to plant toxins, e.g., lycomarasmin, much evidence exists that they affect the plant cells mainly as chelate complexes (GÄUMANN and NAEF-ROTH, 1954 a, 1954 b; DEUEL, 1954). The fusaric acid molecule also possesses a great chelating power (TAMARI and KAJI, 1952 a, 1952 b) and naturally, a possibility exists that it is chelated in the plants with the various metal ions.

Besides the mode of action of toxins on plant cells, it is interesting to find out how the plant cells defend themselves, chemically speaking, against the action of the toxins. We know a good deal about the morphological...
defence mechanisms in the plants against the invasion by the parasite and its toxins (Gaumann, 1951 b), but we have no such knowledge regarding the chemical defence mechanisms against the penetrating toxins. One way of defence would be by detoxicating the toxins through conjugation reactions or through the action of enzymes. In animal cells, for instance, many mechanisms of detoxication are known (Williams, 1947). To give but one example, the ingested pyridine ring (along with the derivatives of pyridine) undergoes methylation in animal body and is rendered harmless. Thus nicotinic acid or nicotinamide, once introduced in the animal body undergoes transformation to N'-methylnicotinamide and trigonelline (N'-methylnicotinic acid). Again, in dogs injected pyridine, a highly toxic compound, undergoes similar methylation and in this way is excreted out of the body in a harmless form. Although not directly demonstrated, a similar possibility perhaps also exists for α-picolic acid. In certain mussels, like Arca noae, homarine (N'-methylpicolinic acid) is regularly found and this arises perhaps through the methylation of α-picolinic acid. Such mechanisms have, however, not yet been demonstrated in plants, but there is no reason why they should not occur here. The presence of numerous betaines and trigonelline in plants shows that methylation reactions play an important part here as in animals, and we should expect to find in plants also certain detoxication reactions.

To investigate the fate of fusaric acid and numerous other questions raised above, the following procedure was adopted:

4 tomato cuttings (fresh weight, 5.7 gms.), were allowed to take up with the transpiration stream a required dose of C14-marked fusaric acid at a pH of 4.3 (approximately 150 mg./kg. fresh weight). The cuttings were then left in tap water for 48 hours and at the end of this period, ground very thoroughly in a mortar in order to break the plant cells. The ground up material was placed in a percolator and extracted for 24 hours with about 800 ml. 85 % ethanol. The extract was concentrated to about 100 ml. under reduced pressure at room temperature and the solid particles, pigments etc., removed by centrifuging the water extract. Radioactivity was measured in the solid material by oxidizing the samples as described elsewhere. The dirty-white extract was alkalinized with NaOH and extracted in a Kutscher-Steudel apparatus for 72 hours with ether (referred to further as extract I). The original water extract was then adjusted to a pH of 4.0 with NHCl and again extracted with ether in a Kutscher-Steudel apparatus for 72 hours (referred to as extract II). Extract I, II and the original water extract (extract III) were then evaporated to dryness, oxidized by van Slyke wet oxidation and the radioactivity measured. The results are given in table 4.

It will be seen from table 4 that a large fraction of the applied radioactivity appears in the ethanol extract (94.5 %), but a large amount is also converted into an alcohol-insoluble fraction (28.3 %) which cannot be, therefore, extracted from the plants. That some fusaric acid remains unconverted is also indicated from the above figures. We would expect to recover all the
The radioactivity in various fractions of an alcoholic extract of tomato plants which had taken up a minimal dose of C\(^{14}\)-marked fusaric acid at a pH of 4.3 (Total radioactivity applied = 73,525 counts/mt. as barium carbonate)

<table>
<thead>
<tr>
<th>Extract number</th>
<th>Total radioactivity corrected to infinite thickness. Counts/mt.</th>
<th>percentage of applied radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissues after extraction</td>
<td>20,808</td>
<td>28.3</td>
</tr>
<tr>
<td>Pigments and small solids after removal of ethanol</td>
<td>312</td>
<td>0.4</td>
</tr>
<tr>
<td>Extract I</td>
<td>5,367</td>
<td>7.3</td>
</tr>
<tr>
<td>Extract II</td>
<td>24,851</td>
<td>33.8</td>
</tr>
<tr>
<td>Extract III</td>
<td>9,852</td>
<td>13.4</td>
</tr>
<tr>
<td>Percentage of total radioactivity recovered</td>
<td>61,190</td>
<td>83.2</td>
</tr>
</tbody>
</table>

unchanged fusaric acid, if any present, in extract II, and as the results show, this fraction shows the greatest activity. The basic and neutral substances (extract I and III) also contain a small amount of radioactivity. It is thus clear from the table, that after 48 hours of application, the fusaric acid is metabolized by the plants and converted into different metabolic products.

It will be seen (table 4) that only 82.2% of the radioactivity originally applied is recovered by our methods. A part of the applied radioactivity no doubt escapes as C\(^{14}\)O\(_2\) and the rest may be due to sampling and experimental errors. The results given in the table are roughly quantitative because the fractionation procedures involve a certain amount of error.

The next step in the above investigation was naturally to identify the various products of metabolism. Since the isolation of the metabolic products of fusaric acid involved highly empirical procedures, we took recourse to the following method for a rough identification:

Three tomato cuttings weighing 4.2 gms. (fresh weight) were allowed to take up 0.6 mg. of C\(^{14}\)-marked fusaric acid in a concentration of 5.0 \times 10^{-3} molar at a pH of 5.0. The total radioactivity of the applied fusaric acid was approximately 1.2 \times 10^{6} disintegrations per minute. After 48 hours of application, the plants were gathered together and along with a little sand were thoroughly ground up in a mortar. The whole mass was extracted in a percolator as elsewhere described and the extract divided into basic, acidic and neutral components by alkali partition and extraction with ether. Each of the extracts was concentrated to a volume of 0.5—1.0 ml. (depending upon the radioactivity of the extracts) at room temperature under reduced pressure. Approximately, a total of 0.1 ml. of each of these concentrates was applied on the starting point of Whatman no. 1 chromatographic paper by successively applying each time a 0.001 ml. drop and drying. Components of each one of these three extracts were separated unidimensionally by allowing the chromatogram to run in a
Butanol, acetic acid and water solvent (45 : 5 : 50). After separation, the chromatograms were dried at room temperature for 12 hours and strips of the chromatogram, containing the separated components, scanned with a GM counter (see section B, IV). Figures 16, 17 and 18 represent the radiochromatograms of the separated components of the plant extracts obtained after 48 hours of introduction of radioactive fusaric acid in the tomato cuttings.

It will be seen from the figures, that the acidic ether soluble fraction contains but one radioactive constituent with a $R_f$ of approximately 0.84. This is most probably the unchanged fusaric acid. The neutral extract contains two radioactive constituents with $R_f$ of 0.20—0.22 and 0.51—0.54 respectively, and the basic extract contains only one radioactive spot having a $R_f$ of 0.61—0.63. The small spot with a $R_f$ of 0.21—0.22 on this chromatogram is probably the same as the spot occupying the same position on the chromatogram of neutral constituents of the plant extract.

It is very likely that many more products of fusaric acid metabolism occur in the plant extract, but they are not demonstrable with our techniques. Only those metabolic products of fusaric acid are demonstrable here which still retain the C$^{14}$ of the carboxyl group originally marked in the fusaric acid molecule. We yet do not know how the butyl-chain of the fusaric acid molecule is metabolized.

A further identification of these metabolic products of fusaric acid is difficult. Work in this direction is still being carried on in this Institute and will be reported elsewhere.

Now we can summarise the results of our investigation as follows:

Fusaric acid, after introduction in the tomato plants, is metabolized and probably the following products are formed:

1) Converted into alcohol insoluble fraction in the plants (chelated with metal ions?).
2) Carboxyl group of the molecule broken down with the production of CO₂ and perhaps 3n-butylpyridine (if we assume that the butyl-chain remains intact after decarboxylation).

3) Converted into water soluble, neutral substances with a Rₚ of 0.20—0.22 and 0.51—0.54 in butanol-acetic acid-water solvent.

4) Converted into very small amount of basic, ether soluble substance with a Rₚ of 0.61—0.63 in butanol-acetic acid-water solvent.

5) A large amount remains unchanged as fusaric acid. This might represent the excess beyond the quantity of fusaric acid required to cause damage in the plants.

The above is only true for the carboxyl group of the fusaric acid molecule. We yet do not know how the side chain of the molecule is metabolized and what products are formed.

III. Quantitative breakdown of fusaric acid in plants

It has been demonstrated above that some fusaric acid remains unchanged in the plants after uptake of a minimal dose (approx. 150 mg./kg. fresh weight). If fusaric acid affects the plant cells as such, i.e., unchanged, we should expect to recover a major part of the applied fusaric acid from the plants which have been allowed to take up this substance. Pertinent here is also the question that we raised in the introductory part of this problem, viz., if fusaric acid is secreted by the invading pathogen in the host, it may not always be possible to detect it if it is quickly metabolized by the plant cells. According to the working hypothesis we propose for the injurious effect of the fusaric acid is that it is metabolized into some substance which is much more toxic to the plant cells than the original fusaric acid molecule, and this substance is of major importance as regards the production of damage; the remaining fusaric acid, after uptake by the plants, either remains as such or is converted into a number of substances which we have already demonstrated above, and which might be the products of detoxication reactions in the host cells. If our concepts were true, we would recover from the plants only that amount of fusaric acid which represented the excess beyond that required for causing damage. Thus, if we were to give the plants lesser and lesser quantities of fusaric acid, we would expect to recover correspondingly lesser quantities of free fusaric acid till the time a minimum dose is reached, after which no more free fusaric acid could be recovered from the plants after application. That in substance, at least, our hypothesis is valid is shown below.

The quantitative breakdown of fusaric acid in tomato cuttings was investigated by the help of isotope dilution technique (Calvin et al, 1949). The procedure adopted was as follows:

3 tomato cuttings were allowed to take up C¹⁴-marked fusaric acid at a concentration of 2.5 × 10⁻³ molar in the required dose. After 48 hours of uptake, the plants were ground up thoroughly with sand in a mortar.
The material was extracted in a percolator at room temperature with about 800 ml. of 80% ethanol for approximately 24 hours. The extract so obtained was concentrated to about 100 ml. under reduced pressure at room temperature. The pigments and other solids were removed by filtration. To the water extract was added a known quantity of pure inactive fusaric acid as carrier. The pH of the solution was adjusted to 8.0 and it was extracted with ether in a Kutscher-Stæudel apparatus for 72 hours to remove fatty substances, pigments and oils etc., which otherwise interfere in the final crystallization of fusaric acid. The water extract was again adjusted to a pH of 4.0 and extracted with ether for 72 hours. This ether extract was evaporated to dryness under reduced pressure at room temperature. The remaining dry solids were extracted 4 times, each time for two hours with 30 ml. quantities of petroleum ether under a reflux condensor. The petroleum ether extract was again evaporated to dryness under reduced pressure. The remaining solids were then extracted with 40 ml. of boiling Hexan under a reflux condensor for about 1 hour and the fusaric acid crystallized out from this solution at 0 °C. The fusaric acid crystals so obtained were purified by repeated crystallizations from boiling Hexan till the time constant radioactivity was obtained. This required generally two crystallizations. A further check on the purity of the isolated fusaric acid was made by determination of the melting point. The pure isolated fusaric acid was divided in three lots (each 2—4 mg., depending upon the quantity isolated) and each lot oxidized by the VAN SLYKE wet combustion method and radioactivity determined by counting the barium carbonate samples. A mean of three readings was taken. The amount of fusaric acid isolated from the plants was calculated from the following formula:

\[ a = \frac{b}{Z - 1} \]

where \( a \) = mg. of C¹⁴-marked fusaric acid remaining in the extract (unknown); \( b \) = mg. of inactive fusaric acid added as carrier; \( Z \) = ratio of the specific activity of C¹⁴-marked fusaric acid (counts per minute/mg. of radioactive substance) to the specific activity of the isolated fusaric acid sample (counts per minute/mg. isolated fusaric acid).

Table 5 shows the results of this investigation.

**Table 5**

The quantity of fusaric acid metabolized by the tomato plants during 48 hours after uptake of a dose of C¹⁴-marked fusaric acid

<table>
<thead>
<tr>
<th>No.</th>
<th>Amount of fusaric acid given per gm. plant material</th>
<th>pH of solution</th>
<th>Amount of inactive fusaric acid added (b)</th>
<th>Ratio of sp. activities (Z)</th>
<th>mg. unchanged fusaric acid/gm. plant (a)</th>
<th>percentage metabolized in plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.2454 mg.</td>
<td>6.0</td>
<td>48.8 mg.</td>
<td>199.2</td>
<td>0.0456</td>
<td>81.4 %</td>
</tr>
<tr>
<td>2.</td>
<td>0.2432 mg.</td>
<td>4.3</td>
<td>29.7 mg.</td>
<td>73.1</td>
<td>0.0557</td>
<td>77.6 %</td>
</tr>
<tr>
<td>3.</td>
<td>0.1207 mg.</td>
<td>4.3</td>
<td>32.9 mg.</td>
<td>671.8</td>
<td>0.0085</td>
<td>93.1 %</td>
</tr>
</tbody>
</table>
Table 5 shows clearly that fusaric acid is quickly metabolized by the plants and at the end of a 48 hour period, only a very small quantity of the applied fusaric acid can be extracted out of the plants. Within the limits of experimental error, the quantity of fusaric acid broken down is the same even if the pH of the applied fusaric acid is varied. The quantitative breakdown of fusaric acid by the plant tissues is, therefore, not pH dependent.

Another fact we note from the table is that with decreasing doses of fusaric acid, correspondingly more is metabolized by the plant cells. Thus, when 0.24 mg. of fusaric acid is allowed to be taken up per gram of plant material, then 77.6% of the applied quantity is metabolized by the plants at the end of a 48 hour period. When only half of this amount (0.12 mg.) is applied per gram of plant material, correspondingly more, i.e., 93.1% of the applied quantity is metabolized. Thus a stage would be reached, when with still smaller quantities of fusaric acid, no more would be isolated in the free state, i.e., all would be metabolized by the plants.

The results obtained with fusaric acid are approximately the same as with patulin (clavacin), a toxin produced by certain Aspergillus species and other fungi (Miescher, 1950). Patulin combines in the tomato plants with the free -SH groups of biological importance and this accounts for its toxicity. Miescher (1950) has demonstrated that any quantity of patulin below 300 mg. per kg. fresh weight, once introduced in the tomato plants cannot be recovered back. Only quantities beyond this range remain unchanged in the plants because no more free -SH groups are available in the cells to combine with patulin in excess of the 300 mg./kg. limit.

In case of fusaric acid, however, such a simple relationship does not exist. Fusaric acid is metabolized by the plant cells and converted into numerous related substances. In our opinion some of the metabolic products are non-toxic but others are perhaps much more toxic than fusaric acid itself. To find out which of the metabolic products of fusaric acid are responsible for injury to the plant, we shall have to purify each one of the metabolic products and then test its activity with tomato plants. Such experiments are in progress and shall be reported on later.

F. Discussion

Dealing with wilt-diseases in plants, the toxin theory of disease has been emphasized for some years (Gottlieb, 1944 b). According to this theory, part of the symptoms on the host are directly ascribable to certain toxic metabolic products secreted by the pathogen in vivo. One of the most investigated organism in this regard has been Fusarium lycopersici, causing the wilt of tomato. Most of the symptoms naturally present on the diseased tomato plants can be reproduced when the cell-free culture filtrates of the pathogen are applied to the tomato cuttings (see, Introduction).
A very strong indirect evidence exists that all the wilting-toxins are also secreted by the pathogen in vivo (Gäumann, 1951 a). However, they have never been isolated from the diseased plants. This is perhaps due to the fact that toxins, once secreted by the pathogen in the host, are metabolized by the plant cells or converted into entirely different substances. For this reason it might not be possible to isolate these toxins from the diseased tomato plants in their original form. Evidence of other kind was, therefore, to be had to ascertain whether the metabolic products of the pathogen in vivo bear any relationship with the symptoms on the host. This necessitated the need for a clear understanding of the mode of toxin production in vitro in order to correlate such studies with in vivo metabolism of the pathogen.

We succeeded in demonstrating that the toxin production varies quantitatively from one strain to another under the same set of environmental conditions (Gäumann, Naef-Roth and Miescher, 1950; Kern, 1952). However, when these environmental conditions are changed, the quantity of toxin produced correspondingly changes. Thus, in Richard's medium strain 257 (avirulent) produces much more toxin (fusaric acid and lycomarasmin) than strain R-5-6 (virulent), but when the constituents of the Richard's medium are varied (specially carbon and nitrogen), the strain producing less toxin (R-5'6) produces more than the strain 257 (avirulent). When glucose is replaced by glycine as the exclusive carbon source, toxins are still produced. There is no reason why the virulent strains accordingly may not secrete more toxins in the host, as compared with the avirulent ones, where the chemical environment is different from the laboratory.

Restricting ourselves to fusaric acid, one of the known toxins produced by F. lycopersici, evidence is presented to show that it is not a direct product of sugar dissimilation, but is synthesized by the pathogen from some other secondary dissimilatory products, like amino-acids. Growth, amount of sugar consumed and pH bear no relationship with fusaric acid production in vitro. When various nitrogen containing compounds in the cultures are analysed, it is found that fusaric acid production bears a relationship with the amino-acid formation in culture medium. A strong probability exists that fusaric acid is produced from some amino-acid precursors. Flück and Richle (1955) as a result of paper chromatographic studies have suggested that alanin and citrullin may be involved in the biosynthesis of fusaric acid. Further, fusaric acid is produced by actively metabolizing, young mycelium and the greatest amount is produced with 12—18 days time (compare Kern, 1952). This is at about the same time that the disease symptoms are visible in the infected tomato plants.

The studies on the metabolism of F. lycopersici in vivo with the help of 2-C14-glycine demonstrate that certain symptoms on the host, viz., vein-clearing (Foster, 1946) are directly related to the secretion of metabolic toxic products of the pathogen. Thus, when tagged mycelium of F. lyco-
Investigations on the metabolism of *Fusarium lycopersici* Sacc.

*Fusarium lycopersici* is introduced into tomato plants, it secretes certain marked substances which reach in the leaves far away from the infection centers in the stems. The leaves showing vein-clearing accumulate more than 4 times the radioactivity present in the healthy leaves on the same plants. Significant here are also the results obtained by Davis (1954) who demonstrated by grafting techniques that non-tomato scions show injury from the toxins of the pathogen present in the tomato stocks, although no mycelium is present in the scions.

By the help of radioautographic techniques, it is demonstrated that a metabolic product, perhaps similar to vasinfuscarin, is secreted by the pathogen in vivo. This toxic metabolic product is closely associated with the vessels showing browning and is precipitated on the walls of the vascular elements, when tissue sections are fixed in methanol. Since it is not dem-onstrable in healthy vessels of the diseased plants by methanol fixation, it is assumed that this toxin is not transportable to greater distances in the stems and is perhaps adsorbed on the walls of the vascular elements just beyond mycelium.

Apart from the toxins, the pathogen secretes a number of substances which are seemingly non-toxic to the host. A complete chemical analysis of the plants inoculated with tagged mycelium (both virulent and avirulent strains) shows that the principle substances secreted are proteins, amino-acids, sugars and various other uncharacterized acidic and basic substances. With our techniques, only the substances formed from the methylene group of glycine can be demonstrated because only the carbon of the methylene group was marked. There is no doubt that with another source of carbon used for tagging the mycelium (like glucose and sucrose) many other substances would be detected. There is apparently no qualitative difference in the substances secreted by the different strains (virulent as well as avirulent) of the pathogen. The main difference, keeping in view the limitations of our technique, is perhaps of a quantitative nature. Thus the virulent strain secretes more substances of protein nature in vivo than the avirulent strain. This is perhaps quite an expected fact, since both strains are capable of producing toxins in vitro but the avirulent strain cannot do so in vivo because it is kept in check in the vicinity of the focus of infection by the unknown resistance factors of the host (Gäumann, 1951 b; Kern, 1952).

All the facts described so far lend support to the toxin theory of disease in the case of tomato wilt. According to Scheffer and Walker (1953), who studied the mechanism of wilt production in tomato plants due to *F. lycopersici*, the pathogen is present in all parts of the plant showing symptoms, specially vascular browning, leaf necrosis etc. They concluded therefrom that translocable toxins are not produced by *F. lycopersici* and majority of disease symptoms are caused by an impediment to water movement in the diseased stems. In contradiction to these authors, experiments conducted in this Institute by Dr. Stephi Naef-Roth (unpublished) demon-
strate that from many tissues of the plants, showing very severe disease symptoms, the pathogen cannot be isolated out except from the vessels showing vascular discolouration. However, it is an undeniable fact that physiological disturbances contribute to the presence of some symptoms on the plant, but it is also equally undeniable that many other symptoms (like vein-clearing and vessel browning etc.) can be ascribed to the secretion of certain translocable toxic substances by the pathogen in vivo. Davis (1954) has further demonstrated that the association of the mycelium to the regions showing symptoms on the plants does not conflict with the idea of toxins also bringing about the symptoms in question.

Fusaric acid, after application at a minimal dose of 150 mg. per kg. fresh weight, is metabolized by the plants in various ways. Some of the applied fusaric acid is decarboxylated, some is bound up by the plant cells, some is converted into closely related substances and some remains unchanged. Approximately, up to 30% of the fusaric acid is converted in the plants into an alcohol-insoluble fraction. Since fusaric acid forms chelate ring complexes with metal ions (Tamari and Kaji, 1952 a, 1952 b, 1953 a), a possibility exists that this alcohol insoluble fraction represents such complexes of fusaric acid with metal ions in the cells. About 10% of the applied fusaric acid is decarboxylated with the production of CO₂ and perhaps 3-n-butylpyrididine, if we assume that the butyl side-chain remains intact in this process. 3-n-butylpyrididine has a very toxic effect on the plant cells and, if our assumptions are correct, part of the toxicity of fusaric acid could be attributed to this substance. 20—25% applied fusaric acid is converted into certain basic and neutral substances, which have not yet been completely identified. From the experience of other authors on animal organisms (Williams, 1947; Leifer, 1951) it is presumed that some of these substances arise through N-methylation of the fusaric acid molecule and may eventually represent the end products of certain detoxicating mechanisms on the part the plant cells. About 30% of the initially applied fusaric acid remains in a free form and may represent the excess beyond that required for causing injuries to the plant cells.

Perhaps there are a number of other products of metabolism of the side-chain of fusaric acid, but they could not be demonstrated with our techniques, because the fusaric acid was labeled only in the carboxyl group.

When fusaric acid is introduced in smaller doses in the tomato cuttings, comparatively much more is metabolized by the plants. Thus, at a concentration of about 200 mg. per kg. fresh weight, about 77% fusaric acid is metabolized, while at 100 mg. per kg. fresh weight nearly 95% is metabolized. In this way, with still lower doses of fusaric acid, a stage would be reached when no more free fusaric acid would be present in the plants. This gradual increase in the total quantity of fusaric acid metabolized by the plants with decreasing quantities of applied fusaric acid is perhaps due to the fact that with decreasing doses, fusaric acid is converted preferentially into some substance or substances, which are then responsible for
causing injury. For verification of this hypothesis, the plant material will have to be fractionated everytime after uptake of diminishing doses of C14-labeled fusaric acid and demonstration of preferential conversion into some definite substance. Such studies are in progress. The situation with fusaric acid would, therefore, be materially similar to patulin (MIESCHER, 1950; GAUMANN, 1951 a). Patulin combines with thiol groups of biological importance and when it is applied to the plants, only that amount can be extracted back which is in excess of the amount required to saturate all the -SH groups.

The question of in vivo production of toxins can now be discussed in the light of the above facts. It has been demonstrated with a very great degree of certainty that Fusarium lycopersici produces toxins in the plants. These toxins are difficult to isolate from the diseased plants because they are partly metabolized and converted into other substances by the host (formation of chelate complexes of lycomarasmin, decarboxylation of fusaric acid etc.). DIMOND and WAGGONER (1953), discussing the role of toxins in disease production, have distinguished between two category of toxins. 1. Those produced in vitro and 2. those produced in vivo. The latter have been called 'vivotoxins'. Among the criteria laid down for the demonstration of vivotoxins, is their extraction from the diseased plants in a pure form and production of symptoms on healthy plants when this extracted toxin is applied to them. As an example of a true vivotoxin, DIMOND and WAGGONER cite the case of the toxin produced by Pseudomonas tabaci (BRAUN, 1950) which can be isolated from the diseased plants. The arguments given by DIMOND and WAGGONER are substantially correct with the restriction that some toxins can be isolated only in a metabolized form and not in their original state. In these cases, of course, direct evidence for the presence of toxins in diseased plants will be possible only with an exact knowledge of the mechanisms of action of these toxins.

Summary

1. It has been shown that a quantitative change in the constitution of Richard’s medium causes a corresponding change in the quantity of toxins produced by the various strains of F. lycopersici Sacc. Thus, the virulent strain, producing lesser amounts of toxins (used in the widest sense of the word) in Richard’s medium, produces more than the avirulent strain (257) when the quantities of carbon and nitrogen are varied in the medium.

2. With glycine as the exclusive carbon source in the medium, F. lycopersici produces all the three known toxins (vasinfuscarin, fusaric acid and lycomarasmin).

3. In the biosynthesis of fusaric acid the amino-acids seem to play a significant role and it is possible that some of them (like alanine and citrulline) serve as precursors of fusaric acid.
4. *F. lycopersici* can be tagged after growth for 5—8 days in a medium containing 90 μCi/ml of 2-Cl4-glycine. At this level of radioactivity about 40% of the fungal cells become inviable but the fungus does not lose its virulence.

5. The tagged mycelium, when inoculated into healthy tomato plants, secretes certain radioactive toxic components responsible for 'vein-clearing' symptoms on the leaves and vascular browning on the stems.

6. All the metabolic products of the tagged pathogen in vivo are not of a toxic character; some apparently are non-toxic.

7. The tagged virulent and avirulent pathogens secrete, among other substances, proteins, amino-acids, sugars and certain uncharacterized acidic and basic substances. The virulent pathogen secretes more than 4 times the protein substances secreted by the avirulent pathogen in vivo.

8. C14-labeled fusaric acid is metabolized when introduced into the tomato cuttings in the minimal dose (150 mg./kg. fresh weight). About 10% is decarboxylated with the production of C14O2 and perhaps 3 n’butylpyridine. About 30% is converted into alcohol insoluble fraction (chelated with metal ions?) and about 20% into certain neutral and basic substances. In paper chromatograms, the neutral substances have a Rf of 0.20—0.22 and 0.51—0.53 in butanol, acetic acid and water solvent and the basic substances an Rf of 0.62—0.65. Approximately 30% of the applied fusaric acid remains unchanged. This latter perhaps represents the quantity in excess of that required for causing injury to the plants.

9. A possibility is shown to exist that some of the metabolic products arise by N-methylation of fusaric acid, representing perhaps the end products of detoxicating mechanisms possessed by the host cells.

10. With decreasing doses of fusaric acid less and less amount of free fusaric acid (unmetabolized) remains in the plants. Thus, at about 200 mg./kg. fresh weight, about 77% of the applied fusaric acid is metabolized, while at 100 mg./kg. about 93% is metabolized by the host cells.

11. Fusaric acid causes injury perhaps by getting converted into some more toxic substances in the plants.

12. From a careful study of the results obtained in this work arguments are given for the role of toxic substances secreted by the pathogen in vivo in the production of an over-all disease picture on the host.

**Zusammenfassung**

1. Auf Richard-Nährösung (S. 336) bildet der in dieser Arbeit verwendete nicht pathogene Stamm von *Fusarium lycopersici* Sacc. mehr Toxine (im weitesten Sinne des Wortes) als der pathogene Stamm. Wird jedoch in der Nährösung das Mengenverhältnis von C und N verändert, so kann die
Toxinproduktion des pathogenen Stammes größer werden als diejenige des nicht pathogenen Stammes. Die Toxinbildung eines bestimmten Stammes ist also nicht konstant, sondern von der Ernährung abhängig.

2. *Fusarium lycopersici* vermag mit Glykokoll als einziger C-Quelle alle drei von ihm bis jetzt bekannten Toxine (Vasinfuscarin, Fusarinsäure und Lycomarasmin) zu bilden.


4. Wächst *Fusarium lycopersici* während 5 bis 8 Tagen in einem Medium, das radioaktives Gly kokoll enthält (mit C$^{14}$ markiert; 90 μC/ml), so wird das Myzel radioaktiv markiert. Unter diesen Versuchsbedingungen bleiben etwa 60% der Konidien keimfähig, und der Pilz behält seine Pathogenität bei.


7. Die einem Tomatensproß verabreichte markierte Fusarinsäure wird zum größten Teil chemisch verändert. Bei einer Toxindosis von 150 mg je kg Lebendgewicht wurden in der hier verwendeten Versuchsordnung folgende Ergebnisse erhalten:

   a) Rund 10% der verabreichten Fusarinsäure werden decarboxyliert. Die Pflanze gibt C$^{14}$O$_2$ ab; als erstes Spaltprodukt wäre 3-n-Butylpyridin zu erwarten.

   b) Rund 20% der Fusarinsäure werden in neutrale und basische Verbindungen umgewandelt. Im Papierchromatogramm (mit einem Gemisch von Butanol, Essigsäure und Wasser als Lösungsmittel) wurden für die neutralen Substanzen $R_f$-Werte von 0,20 bis 0,22 und 0,51 bis 0,53, für die basischen Substanzen solche von 0,62 bis 0,65 gefunden.

   c) Rund 30% der verabreichten Fusarinsäure lassen sich mit Alkohol nicht mehr herauslösen; ihr Schicksal konnte noch nicht untersucht werden.

   d) Rund 30% der verabreichten Fusarinsäure konnten in unveränderter Form zurückgewonnen werden. Sie sind möglicherweise für das Zustandekommen der Schädigungen nicht von Bedeutung.
8. Wird die Dosis der verabreichten Fusarinsäure herabgesetzt, so sinkt auch der Prozentsatz der in der Pflanze nicht abgebauten Fusarinsäure. Bei einer Dosis von 200 mg je kg Lebendgewicht konnten in einem bestimmten Versuch 22 % der eingebrachten Fusarinsäure in unveränderter Form zurückgewonnen werden, bei 100 mg/kg dagegen nur noch 7 %.


The above investigations were carried out at the Department of special Botany of the Swiss Federal Institute of Technology under the guidance of Prof. Dr. E. Gaumann to whom the writer is grateful for his consistent encouragement, advice and the most intimate concern he showed in problems, both financial and scientific, arising from this work. For numerous suggestions, criticisms and a careful study of the manuscript, the writer is very thankful to Dr. H. Kern.

The investigations were financed by a generous grant by the Trustees of "Fritz Hoffmann-La Roche-Stiftung zur Unterstützung biologischer Arbeitsgemeinschaften in der Schweiz", to whom our thanks are due. The work was carried out during the tenure of a fellowship first from the Swiss Coordination Commission for Technical help and later from the "Volkart-Stiftung", Winterthur (Switzerland). Grateful thanks are expressed to the authorities concerned.

Some parts of the work were carried out in the Organic Chemistry Laboratories of the Swiss Federal Institute of Technology under the direction of Prof. Dr. V. Prelog and Dr. J. Würsch, to whom the writer is grateful. Thanks are also due to PD. Dr. E. Hardegger and Mr. E. Nikles for synthesizing the radioactive fusaric acid used in this investigation and many other suggestions. Last, but not the least, the writer is thankful to all his colleagues in the Laboratory, specially Dr. St. Naef-Roth, Miss E. Bachmann and Miss M. Keckeis for help in many ways.

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