BIOCHEMICAL STUDIES IN PLANTS

I

THE ISOLATION OF TUMOR-GROWTH INHIBITORS FROM *BOLETUS EDULIS*

II

THE METABOLIC INCORPORATION OF FORMALDEHYDE INTO THE NICOTINE N-METHYL GROUP IN *NICOTIANA RUSTICA*

By

Robert Lloyd Ringler

A THESIS

Submitted to the School of Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Chemistry

1955

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VITA

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ABSTRACT

PART I

A method of preparing two purified tumor-growth inhibiting fractions (fractions IV and V) from the mushroom Boletus edulis is described. An in vivo assay employing mouse sarcoma 180 was used throughout this work. The activity of the various preparations varied from a (± -) to a (± +) effect. Toxicity was displayed by nearly all of the samples tested.

In the method of preparation the mushrooms were extracted with 0.05 per cent sodium sulfide solution. This extract was made 30 per cent acetone and centrifuged at once. The residue was discarded, and the supernatant was designated fraction I. Fraction I was made a total of 70 per cent acetone and centrifuged after standing overnight. The supernatant was discarded, and the residue was designated fraction II. Fraction II was suspended in 0.05 per cent sodium sulfite solution and dialyzed. The diffusate (outside solution) was discarded. The dialysate was designated fraction III. Fraction III was buffered at pH 6.4 with phosphate buffer (by adding the dry salts). The buffered fraction III was chromatographed on a Dowex 50 x 12 column. An activity was eluted with 0.3 M sodium chloride solution (fraction IV) and with 3.0 M sodium chloride solution (fraction V). Fraction IV was dialyzed and a solid product obtained from the dialysate. A solid product was also obtained from fraction V, however, it did not possess tumor inhibiting activity.

Fraction IV, chloride free and in the dry state, was hydrolyzed with acid and also with alkali. The hydrolysis products were studied using paper chromatography. A preliminary terminal N-group analysis was also made. On the basis of this study it was postulated that fraction IV contains a cyclic peptide composed of 10 amino acids and an unknown "basic substance". An infrared spectrum tends to support the peptide nature. The "basic substance" appears to have an absorption maximum at 276 mu, in a water solution.

PART II

Formaldehyde-C^{14} was hydroponically administered to three groups of 30 tobacco plants. The tobacco plants were of the high nicotine strain Nicotiana rustica L., var. humilis. After a 7 day growth period the nicotine was isolated and its radioactivity determined. The nicotine was demethylated and the methyl group counted as methytriethylammonium iodide. Within the limits of experimental error, all of the radioactivity of the nicotine molecule was found to be contained in the methyl group.

The postulation was made that formaldehyde or, more likely a closely related compound, is an important metabolic precursor of the nicotine N-methyl group.

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PART I

THE ISOLATION OF TUMOR-GROWTH INHIBITORS FROM *BOLETUS EDULIS*

CHAPTER I

INTRODUCTION

Chemotherapy of cancer may be considered as involving the use of chemicals for the preferential distruction of tumor cells without destroying the tumor bearing host. "It is almost -- not quite, but almost -- as hard as finding some agent that will dissolve away the left ear, say, yet leave the right ear unharmed: So slight is the difference between the cancer cell and its normal ancestor" (1).

Though early references go back a number of years (1), experimental cancer chemotherapy has largely come of age during the past decade. Greenstein (2) in 1947 found it necessary to devote approximately ten pages, out of a total of approximately three hundred fifty, to chemotherapy. In his second edition (3) published some seven years later he devotes approximately fifty-seven pages out of a total of approximately six hundred, with the added remark "No attempt will be made in these pages to cover the burgeoning area of the chemotherapy of cancer."

Of the many preparations tested as chemotherapeutic agents for cancer, plants have shown some promise of containing effective materials. Belkin, et al. (4,5,6,7) have screened a large number of plant extracts using mouse sarcoma 37 as an assay tumor. Included in this group were seventy-eight plants which are used as cathartics, diuretics and pesticides. They also tested extracts prepared from a large number of conifers. Approximately one-third of the extracts tested showed some

tumor inhibiting activity whereas only a very few produced what these authors refer to as a "strong effect".

McLeod and Ravenel (8) prepared extracts of several fungi among them Aspergillus niger and the yeast Saccharomyces cerevisiae. They report having treated about 150 cases of advanced neoplasms with improvement in practically all patients although no cures were reported. Crude filtrates of Aspergillus fumigatus were found to inhibit sarcoma 180 (9). In a continuation of the work on Aspergillus fumigatus the active principle was revealed to be a toxic protein (10).

The amino acid analogue azaserine, O-diazoacetyl-L-serine, was isolated from a culture broth of a Streptomyces (11,12,13). Azaserine is effective against sarcoma 180 at doses of 1-2 mg./kg./day (11).

Reilly et al. (14) screened thirty-three antibiotics using the sarcoma 180 assay. Of this number none displayed as marked an effect as the protein preparation from Aspergillus fumigatus mentioned above. Only the antibiotic actidione showed a slight retardation at a dose which did not elicit toxic manifestations to the host. Four other antibiotics showed a slight inhibition at a dosage level which was toxic to the mouse.

The antibiotic puromycin isolated from the mold Streptomyces alboniger (15) exhibits a medium activity against transplanted mammary adenocarcinoma of the C3H mouse (16,17) and an appreciable activity against glioblastroma cultivated in chick embryo (17). This antibiotic has been shown to have the structure 6-dimethyl-9-(3^1-p-methoxy-L-phenylalanyl-amino-D-ribosyl)-purine (18). Puromycin has been degraded

to the compound 6-dimethyl amino-9-(3^1-amino-β-D-ribofuranosyl)purine (16). This "amino nucleoside" was found to be highly effective against the transplanted mammary adenocarcinoma of the C3H mouse.

Nebularine, 9-(β-D-ribofuranosyl)purine, isolated from the mushroom Agaricus nebularis Batsch. (19,20) exhibits a greater toxicity to sarcoma 180 cells in tissue culture than to embryonic mouse skin cells (21).

Lucas (22) has screened various extracts prepared from several mushrooms grown in submerged cultures against the sarcoma 180 assay. He reports the elaboration of tumor inhibiting principles by Collybia longipes, Clitopilus abortivus, Lipiota rhacodes and Calvatia maxima.

In 1950 Lucas (22) observed that a water extract of the dehydrated fruiting bodies of the mushroom Boletus edulis would inhibit the growth of sarcoma 180. Ritchie (23) describes a procedure for the concentration of the active principle from a water extract of Boletus edulis involving acetone fractionation of the water extract. He observed lability of the active principle to both an acid and an alkaline medium and also a stability of the active principle toward dialysis.

The present work was undertaken as a continuation of the work by Lucas (22) and Ritchie (23). The scope of the problem was the isolation and possible characterization of the substance(s) responsible for the tumor inhibition observed against Crocker mouse sarcoma 180.

CHAPTER II

METHOD OF BIOLOGICAL ASSAY

Experimental Details

The tumor-growth inhibiting activity of the various samples was determined at the Sloan-Kettering Institute for Cancer Research in New York City (24,25) using an *in vivo* assay.

Preliminary to the inhibition test the mouse toxicity was determined by giving a single dose of the sample at various levels. For the inhibition test the maximum tolerated dosage was used.

Five female Swiss albino mice weighing 18-22 grams were used for each bio-assay. Uniformly cut pieces of Crocker mouse sarcoma 180 (ca. 5 mg. wet weight) were implanted by trocar subcutaneously in the right axillary region. Twenty-four hours after the implantation therapy was initiated and continued for seven successive days. The sample being tested was usually injected into the peritoneal cavity. Two injections were given daily. On the day following the last injection each mouse was weighed and the change in weight from the day of implantation was recorded. The weight change for each member of the group was averaged and the resulting figure was considered representative for that group. Tumor diameters were measured through the skin by means of calipers. The largest diameter was selected for one measurement and an axis perpendicular to it was taken for a second measurement. The two diameters were averaged for each mouse to obtain an average tumor diameter.

This parameter was averaged for the group and will be referred to as the tumor diameter of the treated mice in future discussions.

A second group of five mice were treated in the same manner except that they were given an equivalent amount of physiological saline twice daily for seven days. This second group of mice served as the controls.

The inhibition of the tumors in the treated animals was based on the development of the tumors in the untreated (saline injected) mice. The weight change of the treated animals was also compared with the weight change in the control group.

Interpretation of Assays

The single dose toxicity test results were reported as numbers between the limits of 0.5 and 5.0. The smaller number represented extreme toxicity accompanying a single dose of 0.5 ml. and 5.0 represented non-toxicity accompanying a single dose of 5.0 ml. Numbers between these two arbitrary limits represented gradations of toxicity.

The weight change was reported as the ratio of the average weight change in the treated animals to the average weight change in the control group.

In the inhibition test the following arbitrary scale was employed for grading the effects of the various samples on tumor growth. This grading was as follows:

Marked inhibition (+) -- Tumor diameter of treated group less than 25 per cent of the value for the control group.

Good inhibition ($\pm$+) -- Tumor diameter of treated group between 25 and 50 per cent of the value for the control group.

Slight inhibition (±) -- Tumor diameter of treated group between 50 and 75 per cent of the value for the control group.

No effect (-) -- Tumor diameter of treated group greater than 75 per cent of the value for the control group.

Questionable effect (?) -- This designation was used when three or more mice died during the course of the inhibition test. Most of the samples giving a questionable effect were retested at a greater dilution.

The effect and various other aspects of the assay result from the various samples tested will be given in the text. The complete assay result of the various samples will be given in the appendix.

Discussion of Assay

Sarcoma 180 was chosen as the assay tumor because of its nearly 100 per cent transplantability, low regression rate, rapid growth, lack of host strain requirement and its apparent intermediate sensitivity to several adverse agents (26). Approximately 9600 compounds and many natural products have been screened against this tumor (26). Of this number 12 have given a (+) result (26). Confidence in the assay arises from the fact that all 12 of these compounds have shown benefits to certain forms of human cancer (26).

On the other hand this assay has many objectionable features when used as a standard laboratory tool for isolation work. Although the actual assay period only extends over a period of seven days, additional time was required for toxicity studies, for shipping the samples to New York City and for mailing the results back to this laboratory.

With the very best connections, this amounts to a minimum time interval of three weeks. Such a time interval between preparation and testing eliminates the possibility of the activity being checked at various points throughout the course of a long and involved isolation procedure. It also introduces the possibility of the activity being destroyed or diminished due to such factors as time and temperature variation between the preparation and the testing of a sample.

A second objection was the fact that the assay routinely requires 80 ml. of test solution. During the experimental work involving ion exchange resins (see Chapter III) it would have been particularly desirable to have had an assay which could have been used with a much smaller volume of test solution.

A third objection was due to toxicity manifestations. With only two or three exceptions the samples prepared in this work were toxic to the mice. This necessitated a small dose being given and a subsequent effect of($\pm$) at best. The test therefore becomes merely a qualitative assay showing only the presence ($\pm$) or absence (-) of activity. This situation was remedied to a large degree when the Sloan-Kettering group consented to supply us with tumor diameters.

A fourth objection arose when it became difficult to distinguish between significant inhibition and normal biological variation. As a result of this condition a "significant trend" will be reported in a few cases although sufficient activity did not exist for the sample to be graded a ($\pm$). In this same connection, in a few cases the control tumor diameter was very small ($<<$ 1 cm.). Considerable doubt arose

as to the validity of these results since the control tumor diameter was ordinarily greater than 1 cm.

It would be very desirable to have an assay which did not include the objections mentioned above. A chemical assay would be very desirable. However, at this writing no such assay has been devised.

CHAPTER III

ISOLATION PROCEDURE

The Boletus edulis used in this work was obtained from Germany where the fungus grows naturally in wooded areas. After the mushrooms were gathered they were partially dried and packaged for shipment. Following receipt of the mushrooms they were dried at about 37° C. for about 48 hours as a safe-guard against spoilage. They were then stored in well closed metal cans until used.

The mushrooms used in this work were taken from five different mushroom crops all of which were shown to contain an active principle, but in varying amounts.

Extraction

In the early portion of this work the extraction technique developed by Ritchie (23) was used. This consisted in drying the mushrooms to the point where they could be ground to 60 mesh in a Wiley mill. They were then extracted with distilled water in a Waring blender. The ratio of sample to distilled water varied between the limits of 1:10 and 1:30.

This extraction procedure necessitated the mushrooms being dried to a very low moisture content. It also resulted in an extraction mixture which was very difficult to filter.

In an early attempt to remedy one of these difficulties the mushrooms, ground to 60 mesh, were extracted by stirring with a mechanical stirrer. This improved the filtration somewhat. However, the process was still quite time consuming, especially if a large preparation was to be carried out.

In the course of this early work it was also observed that the water extract darkened considerably during the filtration and also during the subsequent manipulations. Inasmuch as it was felt that some of the toxicity displayed by the samples in the mouse test might be due to oxidation products formed during the preparation, an attempt was made to eliminate this darkening of the extract.

The possibility of using a 1 per cent solution of ascorbic acid as an extractant was investigated briefly. This extraction medium did appear to inhibit the discoloration of the extract. However, it also lowered the pH to about 4.6. Inasmuch as it would be necessary for the activity to remain in the presence of this acidity for several hours a more neutral extractant was thought desirable in view of the acid lability observed by Ritchie (23). A 0.05 per cent sodium sulfite solution was used as the extraction medium in a subsequent experiment. Although considerable discoloration of the extract still took place this extraction medium did inhibit darkening to a significant extent.

As a result of these observations the extraction procedure used throughout most of this work consisted in extracting the mushroom pieces (without grinding) with a 0.05 per cent solution of sodium sulfite in a weight weight ratio of 1:10 by mechanical stirring for about 15 minutes

at 7° C. This extraction mixture was then filtered through cheese cloth and the residue re-extracted with the sodium sulfite solution in a ratio of 1:5. This extraction mixture was filtered the same as before. The resulting residue was discarded. The two filtrates were combined and used for future work.

Thirty Per Cent Acetone Precipitation

Ritchie (23) investigated the possibility of using acetone as a precipitant in the range of concentration from 30 to 90 per cent (v/v). The most successful acetone precipitation investigated by him consisted of making the water extract 90 per cent acetone (v/v) and filtering immediately. The residue was found to be inactive whereas the active principle was recovered from the filtrate after standing an additional 12.5 hours at 7° C.

An attempt was made to duplicate this procedure. The results are given in Table I. The mushrooms were extracted as described by Ritchie (23). Column (a) in Table I gives the sample number, column (b) the concentration of acetone used and column (c) the effect shown in the tumor assay described above.

From these results it can be seen that some of the activity was precipitated immediately upon the addition of acetone in the concentrations shown in Table I, It therefore seemed desirable to alter this portion of the technique in such a manner as to eliminate precipitation of activity at this point.

TABLE I

THE IMMEDIATE PRECIPITATION OF THE ACTIVE PRINCIPLE BY VARIOUS CONCENTRATIONS OF ACETONE

Sample Number (a)	Concentration of Acetone (b) Per Cent	Effect (c)
L101	80	(?)
L108	90	(±)
L111	95	(?)
L113	90	(±)

Acetone was therefore added slowly to the original extract until an abundant viscous precipitate was well formed. The acetone concentration was found to be 40 per cent (v/v). This viscous precipitate was removed by filtration, suspended in water and tested for activity (L129±).

Inasmuch as activity was lost at an acetone concentration of 40 per cent an experiment was carried out in which the acetone concentration was decreased still further. It was observed that a rather abundant viscous precipitate formed soon after the addition of acetone to a concentration of 30 per cent (v/v). This precipitate, when removed by filtration, suspended in water and tested also showed the presence of tumor inhibiting activity (L136±).

Due to the nature of the precipitate which formed at an acetone concentration of 30 per cent the filtration at this point was quite slow. It was therefore considered possible that if the precipitate could be

removed more rapidly the loss of activity might be decreased. An experiment was therefore carried out in which the preparation was centrifuged in a Sharples centrifuge immediately after being made 30 per cent acetone. Upon opening the Sharples centrifuge bowl a very dark colored fibrous residue was found to be present in the lower portion of the bowl and a light colored fine residue near the upper portion. These two types of residue were separated, insofar as was possible, and suspended in water. The suspension resulting from the lighter colored material was tested as sample (L319-) and that from the darker colored residue as sample (L320-). Inasmuch as there was no indication of the loss of activity in this 30 per cent acetone residue, the above precipitation technique in which the preparation was centrifuged in the Sharples centrifuge, was used throughout the remainder of this work. The supernatant resulting from centrifuging the 30 per cent acetone solution was designated fraction I.

Seventy Per Cent Acetone Precipitation

As a result of considerations mentioned below, the precipitate which forms between the limits of 30 and 70 per cent acetone was collected throughout almost all of this work.

The lower limit of 30 per cent was selected from considerations mentioned in the previous section. The upper limit was chosen partially as a result of that same study. After discarding the residue which formed at an acetone concentration of 30 per cent it was desired to selectively precipitate the active principle if possible. When the observation was made that some of the activity was precipitated almost

immediately even at an acetone concentration of 30 per cent, it became apparent that the greatest selectivity might result if a relatively low acetone concentration were used to precipitate the activity, providing sufficient time was allowed for precipitation to take place. A relatively low acetone concentration also seemed to be desirable inasmuch as the possibility of the loss of activity due to a high acetone concentration had not been investigated to any great extent. Also, the observation had been made by Ritchie (23) that the precipitation of the active principle appeared to be complete at an acetone concentration below 68 per cent.

With these considerations in mind, acetone was added to a fraction similar to fraction I to a total concentration of 60 per cent. The difference between the fraction used here and fraction I was that the first acetone precipitation was made with 40 per cent acetone rather than 30 per cent as in fraction I. At an acetone concentration of 60 per cent a precipitate seemed to be well formed. This precipitate was removed after nine hours standing by centrifugation, suspended in water and reprecipitated by adding acetone to a concentration of 60 per cent. The precipitate was collected the second time by centrifugation after 12 hours, suspended in water and tested as sample (L121$^{+}_{-}$). The preparation was repeated with one alteration, the first precipitation was allowed to take place for six hours, instead of 12 hours as used in the previous case, sample (L122?). A preparation was also carried out in which only one precipitation with 60 per cent acetone was used. The

precipitate was collected by centrifugation after only two hours standing. This precipitate was suspended in water and tested as sample (L128±).

Several preparations were also carried out in which fraction I was made 70 per cent acetone. In one of the first of these fraction I was made a total of 70 per cent acetone and the precipitate collected after two and one-half hours by filtration with suction on Whatman No. 2 filter paper. This residue when suspended in water was tested as sample (L135-)(retest±).

In a slightly later preparation the precipitate which formed after three hours was collected by filtration as before. However, a second precipitate was also collected by centrifugation after allowing the filtrate from the three hour filtration to stand a total of 64 hours at 7° C. The precipitate which was collected after three hours was not tested for biological activity at this point but was extracted with n-butanol by a technique to be described later. The aqueous layer was tested for biological activity as sample (L146±). The precipitate which formed between the time interval from three hours to 64 hours was suspended in water and tested as sample (L150-).

In a third experiment, using acetone as a concentration of 70 per cent, the preparation was allowed to stand for 20 hours at 7° C. before collecting the precipitate. The precipitate was collected by centrifugation, suspended in water and tested as sample (L195±).

The biological assay does not permit an accurate evaluation of the two methods of preparation. However, after considering Ritchie's (23)

observation mentioned above and the experiment in which the activity did not appear to be destroyed after standing in the presence of the high acetone concentration for 20 hours, it was concluded that a precipitation with acetone at a concentration of 70 per cent might be preferred.

Fraction II is therefore designated as the precipitate obtained by centrifugation after adding acetone to fraction I to a total concentration of 70 per cent and allowing the precipitation to take place overnight at 7° C.

Stability of the Activity Toward Dialysis

With but one exception, the activity was found to be non-dialyzable. Ritchie (23) also observed the stability toward dialysis.

In the one experiment in which activity might have been present in the diffusate (outside solution), the preparation of fraction II was made at the Upjohn Company at Kalamazoo, Michigan using 5 kg. of mushrooms.

An exact duplicate experiment was never carried out. However, two experiments were carried out to determine the loss of activity due to dialysis of fraction II when dissolved in 0.05 per cent sodium sulfite solution. In the first of these fraction II from 200 g. of mushrooms was dissolved in the sodium sulfite solution and dialyzed against 2200 ml. aliquots of distilled water. An aliquot of the first 2200 ml. of diffusate was tested as sample (L380-). In the second experiment fraction II from 300 g. of mushrooms was used and the first three aliquots of the diffusate collected. The diffusate was concentrated to a volume of 75 ml. on the revolving concentrator described by Craig et al. (27). The temperature of the sample was maintained at 25° C. or below

during the 24 hour concentration procedure. The 75 ml. concentrate of the diffusate was tested as sample (L389-).

In both of the experiments just described the dialysate (inside solution) contained activity (L381$^{+}_{-}$) and (L388$^{+}_{-}$).

In all future experiments fraction II was suspended in 0.05 per cent sodium sulfite solution and the mixture dialyzed. The dialysate (inside solution) was designated as fraction III.

Experimental Preparation of Fractions I, II and III

As a result of the experiments described in the previous sections the experimental procedure outlined in Figure 1 was used for the preparation of fractions I, II and III. During the experimental development of this method of preparation quite small amounts (15 to 30 g.) of mushrooms were used for each experiment. However, as the development of the method progressed larger amounts of the crude material were used to eliminate unnecessary repetition of the actual operations involved. Therefore in the experimental procedure which follows the volumes and weights of the various fractions are based on 1 kg. of crude starting material. A relatively large amount of fraction II resulted from this amount of starting material and it was used for further work on the purification of the active principle.

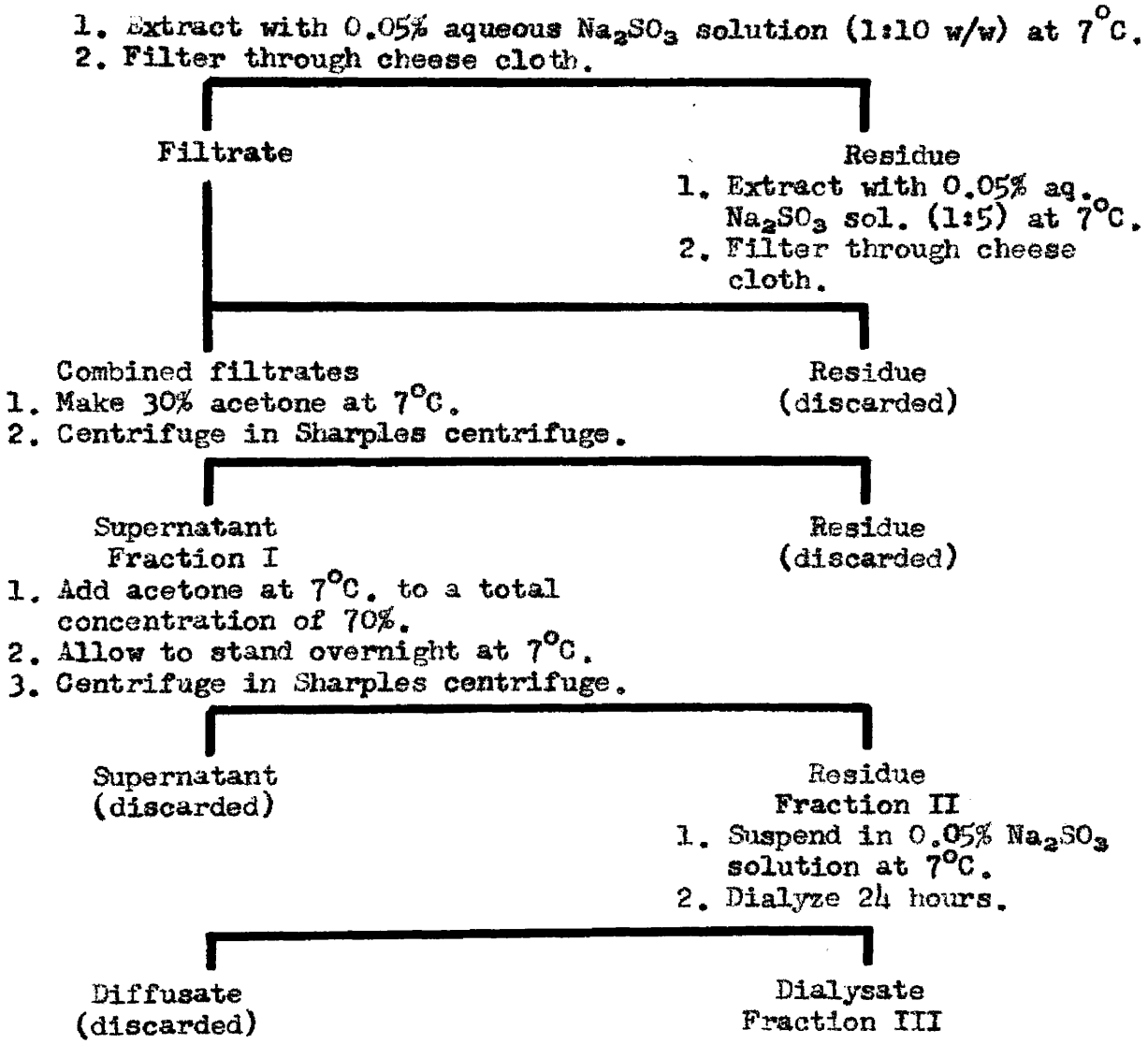
In this method of preparation all operations except the actual centrifuging were carried out at 7° C. using precooled solutions and equipment.

Ten liters of 0.05 per cent sodium sulfite solution were placed in a large glass cylinder about 30 cm. in diameter and 46 cm. high, having

FIGURE 1

PREPARATION OF FRACTIONS

I, II and III



a volume of about 24 l. The cylinder was equipped with a stainless steel stirrer attached to an electric stirring motor. One kilogram of mushrooms were added and the preparation stirred vigorously for 15 minutes.

The extraction mixture was then filtered through three layers of cheese cloth into a 10 gal. milk can. The cheese cloth, containing the residue, was kneaded to obtain as much filtrate as possible. The residue was transferred back to the large glass cylinder and re-extracted by the same technique using 5 l. of the sodium sulfite solution. This extract was also filtered into the milk can. The total volume of the combined extract was 12 to 13 l.

Acetone was added to the combined extract with stirring to a concentration of 30 per cent (v/v) and the preparation centrifuged at once in a Sharples centrifuge using a polyethylene liner in the centrifuge bowl. This liner facilitated the easy removal of the residue which formed in the bowl. The bowl and liner assembly were precooled before being used. However, the centrifuge was not located in a cold room. The flow of liquid through the centrifuge was adjusted, by means of a screw clamp on the feed line, such that a clear supernatant resulted.

At the conclusion of the centrifugation the supernatant (fraction I) was immediately returned to the cold room (7°C.). It usually required about two hours to run through the preparation up to the point where the above supernatant was returned to the cold room. The residue contained in the centrifuge bowl was discarded. Acetone was added to the supernatant to a total concentration of 70 per cent with stirring. The preparation was then allowed to stand overnight at 7°C.

The following morning the preparation was again centrifuged in the Sharples centrifuge using the technique described above. In this centrifugation the majority of the supernatant was decanted from the precipitate which had formed on standing. Using this technique a more rapid flow rate could be used throughout the first portion of the centrifugation.

Immediately upon completion of the centrifugation the polyethylene liner, containing fraction II, was removed from the centrifuge bowl and dropped into a 1 l. graduated cylinder containing 1 l. of 0.05 per cent sodium sulfite solution. The solution was stirred with a stirrer made by sealing the ends of a piece of polyethylene tubing. When turned slowly, by means of an electric stirring motor, this stirrer acted with somewhat of a whip action and was quite effective in accomplishing the suspension of the residue.

After stirring for about two hours the solution plus any solid material still adhering to the polyethylene liner was transferred to a Visking casing and dialyzed against about 10 changes of distilled water (total volume about 50 l.) for 24 hours at 7°C.

The dialysis apparatus used was of the "rotating external liquid" type described by Djang et al.(28). Using this apparatus the dialysis membrane is immersed in a cylinder eccentrically to facilitate agitation, with consequent shortened equilibrium. The cylinder was rotated by a fractional horse power motor at a speed of about 50 rpm.

At the end of this dialysis period complete solution apparently resulted inasmuch as no visible residue was obtained by filtering the

dialysate through a coarse sintered glass funnel. The dialysate, designated fraction III, varied in volume from about 1200 ml. to about 1500 ml. in the various preparations. This solution was divided into aliquots of a size depending on the expected future manipulations and stored in the deep freeze until used.

Fraction III contains about 22 mg. of solid material per gram of crude mushroom used in its preparation. This value was obtained by taking duplicate 10 ml. aliquots of fraction III and drying them to constant weight at 105°C. From the crude mushroom equivalence of fraction III in terms of g./ml. and the dry weight of the residue, the milligrams of dry matter obtained per gram of crude mushroom was calculated.

Discussion of the Preparation of Fractions I, II, and III

The original extraction presents no operational difficulties. Some small bits of mushroom did go through the cheese cloth filter. However, these are conveniently removed at a later step (the centrifugation of the 30 per cent acetone solution). The residue remaining after the second extraction has never been extracted a third time to determine the completeness of the extraction procedure. However, Lucas (22) demonstrated that activity was extracted after merely soaking the mushroom pieces, without stirring, for a period of only three minutes. As a result of this observation it would seem that the extraction should be essentially complete when carried out as described in the previous section.

The nature of the substance(s) responsible for the extensive discoloration observed during the extraction and especially during exposure of fraction II to air has not been investigated. Most mushroom species do contain polyphenol oxidases (29,30). However, Boletus edulis apparently produces a very small amount of these oxidases (31). Voinovitch et al. (32) have studied the inhibition of the oxidases present in the mushroom Agaricus campestris and found sulfur dioxide in combinations with a small amount of thiamin, nicotinic acid, cysteine or glutathione to inhibit oxidase activity. Ascorbic acid was also investigated and found to be less effective than either thiamin or nicotinic acid. Voinovitch (33) also observed a non enzymatic browning to take place in autoclaved mushroom juice on exposure to air. He found the addition of sulfite or acidifying the solution to below pH 5.2 retarded this discoloration. Grabor et al. (34) reported the inactivation of polyphenol oxidases of Agaricus campestris with ultrasonic waves. Kuttner and Wagreich (35) have studied the inhibition of catecholase activity using an enzyme preparation from the mushroom Psalliota campestris. These investigators studied the inhibition of this enzyme system by 40 organic compounds. Among others, acetone was found to inhibit the enzyme activity to the extent of 48 per cent at a concentration of 3.5 mole/l.

Inasmuch as the 30 per cent acetone and the 70 per cent acetone solutions used in the preparation of fraction II do not discolor appreciably even on long standing it might be inferred that the discoloration is at least partially due to an enzymatic oxidase activity.

If a more definite correlation between the observed discoloration in fraction II and a decrease in tumor inhibiting activity is made in a future study, further investigation of the inhibitors mentioned above would seem to be warranted.

There is still the possibility of some small loss of activity in the precipitate which forms at an acetone concentration of 30 per cent. However, inasmuch as the precipitate does not appear to form much below an acetone concentration of 30 per cent, the purification accomplished by this step would seem to warrant a small loss of activity if one does exist.

Fraction I was a very clear amber colored solution. It immediately became opaque upon the addition of acetone to a concentration of 70 per cent. The overnight period during which precipitation was allowed to take place might be longer than was necessary inasmuch as activity was obtained in samples L135 and L146 after a much shorter period. Also, the failure of activity to be detected in sample L150 suggests that precipitation of the activity was complete after three hours standing at 7° C. The longer precipitation period was used for an operational reason. It was desired to dialyze fraction II during the day so that the diffusate could be changed frequently during the first portion of the dialysis. Therefore, the preparations were normally started during the late afternoon and the precipitation in the 70 per cent acetone solution allowed to take place overnight.

Fraction II was a medium tan color when first obtained; however, it darkens on exposure to air and the resulting sodium sulfite solution

of fraction II was a very dark color. It would be interesting to carry out a preparation in which this fraction was kept under an inert atmosphere and to compare the toxicity and activity of the resulting fraction with one carried out without this precaution.

The possibility that the supernatant corresponding to fraction II contains additional activity has not been investigated. If a particularly quantitative recovery of the activity were desired this supernatant might be concentrated at a low temperature and reprecipitated with acetone at a concentration of 70 per cent after removal of the precipitate which might form at 30 per cent. Inasmuch as the supernatant would be expected to contain the majority of the low molecular weight compounds which were extracted a concentration and reprecipitation at this point might not accomplish much in the way of a purification of the active principle.

The dialysis step described above was carried out quite empirically since no convenient method of detecting a "complete" dialysis was available. The major portion of any low molecular weight compounds either precipitated or coprecipitated in fraction II, by acetone at a concentration of 70 per cent, would seem to be removed by the 24 hours dialysis technique used.

Biological Assay of the Various Preparations of Fraction III

Table II contains a list of the various preparations of fraction III which were assayed for biological activity. Inasmuch as these samples were tested at various dilutions, possibly due to different toxicity levels, column (b) in Table II is an expression of the number of grams of crude mushroom represented by the daily mouse dose.

TABLE II

THE BIOLOGICAL ASSAY OF THE VARIOUS PREPARATIONS OF FRACTION III

Sample Number (a)	Crude Mushroom Equivalence (b) grams	Effect in Tumor Assay (c)	Tumor Diameter Treated/Controls (d) cm.
L292[a]	0.13	?	
L292[a,b]	0.04	±	0.49/0.98
L293[c]	0.22	?	
L293[b,c]	0.04	±	
L322[d]	0.17	?	
L322[b,d]		-	1.03/1.02
L381	0.09	±	0.56/1.11
L388	0.08	±	0.36/0.89
L388[b]	0.04	±	0.51/1.02
L393	0.05	±	0.59/0.94
L415	0.12	±	0.73/0.98
L428	0.20	?	
L428[b]	0.05	?	

[a] Fraction II dissolved in phosphate buffer pH 6.4.
[b] Retest.
[c] Original extraction 1:15, no second extraction.
[d] No sodium sulfite solution used.

By averaging the values in column (b) for those samples which gave tumor inhibition (Table II) it can be seen that the average crude mushroom equivalence is about 0.07 g. Considering that about 22 mg. of solid material was obtained in fraction III per gram of crude mushroom, a daily mouse dose of about 1.5 mg. of dry matter can be calculated. Since the daily dose is ordinarily reported in terms of milligrams of sample per kilogram mouse per day (mg./kg./day) this value becomes 75 mg./kg./day (average mouse weight 20 g. or 1/50 kg.)

Fraction III has also been tested against the Murphy-Sturm lymphosarcoma and found to be inhibitory (36).

Fractionation of Fraction III Using Paper Chromotography

In an attempt to gain some insight into the chemical composition of fraction III, a one dimensional chromatographic separation on Whatman No. 1 filter paper was attempted. The chromatograms were developed using the solvent n-butanol, ethanol, water in the volume ratio of 4:1:1 (37) by the ascending technique. A glass cylinder about 45 cm. in height and 15 cm. in diameter was used as the chromatographic chamber. After developing the chromatograms overnight at room temperature they were removed from the chamber and dried at room temperature. Three different means were used to detect the spots on the various chromatograms. The first of these was a reagent consisting of a 0.2 per cent solution of ninhydrin (triketohydrindene hydrate) in water saturated n-butanol (37). A second reagent was prepared by dissolving 0.93 g. aniline and 1.66 g. phthalic anhydride in sufficient water saturated n-butanol to make 100 ml. of

solution (37). The third means of detecting the spots consisted of merely observing the chromatograms under ultra-violet light in the dark room (37). One chromatogram was sprayed with the ninhydrin reagent and a second with the aniline-phthalic anhydride reagent. These two chromatograms were then placed in a forced draft oven at about 95° C. for approximately 15 minutes.

Seven ninhydrin positive spots appeared on the chromatogram which had been sprayed with this reagent. These spots were numbered 1 through 7 and will be referred to in future discussions by this number. The seven spots and their R_f values are given in Table III. Spots number 6 and 7 were very faint on the chromatogram.

TABLE III

NINHYDRIN POSITIVE SPOTS ON PAPER CHROMATOGRAM OF FRACTION III

Spot Number	R_f Value
1	0.00
2	0.03
3	0.10
4	0.15
5	0.33
6	0.44
7	0.56

Two spots appeared on the chromatogram which had been sprayed with the aniline-phthalic anhydride reagent. They were designated as carbohydrate spots 1, R_f 0.15 and 2, R_f 0.29.

Two spots were also visible under ultra-violet light. These were designated as U. V. spots 1, R_f 0.00 and 2, R_f 0.56.

A brown spot was also observed at the origin (R_f 0.00) of the unsprayed chromatogram under ordinary sunlight.

As a result of the observation that at least eleven spots could be detected on a paper chromatogram of fraction III an attempt was made to further purify this fraction using a technique based on this observation.

For this experiment fraction III was applied as a band about 1 cm. wide near one edge of a sheet (18 1/4 x 22 1/2 inches) of Whatman No. 1 filter paper. A total of 8 ml. of solution, equivalent to about 3 g. of crude mushroom, were applied to three sheets of filter paper by means of a pipette drawn out to a fine capillary. The papers were dried periodically between applications with an infrared lamp at very low heat. The chromatograms were developed with the solvent mentioned above in a Chromatocab (manufactured by University Apparatus Company, Berkeley, California) for about 12 hours using the ascending technique. After being developed and dried a narrow strip, about 1 cm. wide, was cut vertically from each edge and from the center of each chromatogram. The two strips cut from the edges of the sheets were treated with the ninhydrin reagent as described in the previous section. The middle strip was treated with the aniline-phthalic anhydride reagent. The bands made visible by ultra-violet light were marked on the remainder of each

chromatogram. Then, using the narrow strips as guides the various bands made visible by the two reagents were marked on the remainder of each chromatogram. It was observed that U. V. spot no. 2 was not present, however, a second U. V. spot, designated U. V. spot 3, R_f 0.19 was visible under ultra-violet light. The chromatograms were then cut into five horizontal sections, each section containing one or more of the various bands. Each section was eluted with water and the eluate from corresponding sections combined. The resulting five samples were tested for biological activity. The tumor innibiting effect as well as the various spots which would be contained in each sample are shown in Table IV.

TABLE IV

CHROMATOGRAPHIC FRACTIONATION OF FRACTION III ON FILTER PAPER

Sample Number	Chromatographic Spots Present	Effect in Tumor Assay
L159	nin. 1	±
L160	nin. 2,3,4 & carbo. 1	-
L161	U. V. 3	-
L162	nin. 5	-
L163	nin. 6	-

Discussion of Chromatographic Procedure

It is evident from Table IV that the activity did not migrate in the solvent used to develop these chromatograms. Some purification might

have been accomplished by this procedure. However, inasmuch as the activity did not migrate, it was considered questionable if the purification merited the effort involved

Perhaps other solvents should have been investigated. One such possibility would be a solvent composed of acetone and water. Inasmuch as the activity is soluble in water but relatively insoluble in a water solution containing a high concentration of acetone it might be possible to select a concentration of acetone and water which would cause the activity to migrate as a well defined spot. Reid (38) has reported the resolution of a protein fraction in a water acetone solvent on Whatman No. 1 filter paper. Bently and Whitehead (39) have reported the resolution of amino acid mixtures using an acetone water solvent and also using an acetone 0.5 per cent aqueous urea solvent. Such a technique, if applicable, might offer a convenient method of assaying for the biological activity providing a correlation between a given spot and the tumor inhibiting activity could be demonstrated.

Extraction of Fraction III With n-Butanol

Inasmuch as fraction III was found to be a rather impure fraction in the chromatographic experiment mentioned above an attempt was made to further fractionate it by means of its distribution between n-butanol and water. A solvent system of neutral pH was chosen inasmuch as Ritchie (23) had shown the activity to be destroyed if the pH of his preparation was altered for a short period of time (ca. 2 hr.) and then neutralized back to the original pH of 5.1.

The extraction of fraction III with n-butanol was carried out by four different techniques. The extraction was carried out in separatory funnels both at room temperature and in the cold room (7°C.). In both of these experiments a considerable length of time was required for equilibrium to be attained. In the last experiment carried out in the cold room, the preparation was allowed to stand for a period of three days with only poor separation of the two layers. In an attempt to facilitate the attainment of equilibrium a small amount of sodium chloride and also ethyl ether was added to the mixture without significant success.

The technique used resulted in the preparation of three fractions. After a given number of extractions the combined aqueous layer was concentrated to dryness in vacuo at a temperature of less than 50° C. The residue, suspended in water is designated fraction A. The combined organic layer was also concentrated in vacuo to a small volume at which time a precipitate was observed. This precipitate was removed by filtration and suspended in water and was designated fraction B. The filtrate was concentrated to dryness in vacuo, and was called fraction C. Table V shows the resulting activity of the various fractions when assayed in the tumor test.

From the result shown in Table V it was concluded that the activity present in fractions A and B might be due to the poor separation of the two layers during the extraction procedure. An attempt was therefore made to extract fraction III with n-butanol in a liquid liquid extraction. In one experiment the extraction was done at atmospheric pressure. In a second experiment the extraction was carried out under reduced pressure

TABLE V

DISTRIBUTION OF ACTIVITY FOLLOWING EXTRACTION OF FRACTION III WITH n-BUTANOL

Experiment Number	Number of Extractions	Fraction A	Fraction B	Fraction C
1[a]	4	(L141$^{+}_{--}$)	(L142$^{+}_{-+}$)	
2[a]	15	(L146$^{+}_{--}$)	(L148-)[c]	
3[b]	15		(L157$^{+}_{--}$)[c]	(L155-)
4[b]	4	(L391$^{+}_{-+}$)	(L390$^{+}_{-+}$)	(L392-)

[a] Extracted at room temperature.
[b] Extracted at 7°C.
[c] Sample dialyzed before being tested for activity.

(water aspirator). In each of these experiments the aqueous and organic phases were treated in the same manner as described earlier. However, all samples proved to be negative on assaying for biological activity.

Discussion of the Extraction of Fraction III with n-Butanol

At the time this work was carried out essentially nothing was known as to the chemical identity of the substance responsible for the tumor inhibition. The choice of butanol-water as a solvent system was therefore quite arbitrary. This solvent system was used by Dakin (40) for the fractionation of protein hydrolysates, however, a number of other solvent possibilities do exist (41). Craig and Craig (41) list a number of factors which should be considered when the choice of a solvent system is to be made. The fact that the particular solvent system chosen forms

an emulsion, apparently due to a surface active agent present in fraction III, renders the choice unsatisfactory. Craig and Craig (41) also mention a number of suggestions for avoiding emulsions. One of these is the addition of a neutral salt. This was tried but without apparent success. They also mention the possibility of using continuous extraction. However, using the solvent system mentioned, such a procedure apparently results in too high a temperature, even under reduced pressure, inasmuch as all samples extracted by this method were negative.

Simultaneous with this work a preliminary experiment using ion exchange resins was carried out. Since the ion exchange resins appeared to offer considerable promise as a means of further fractionation of fraction III, the possibility of distribution between partially miscible solvents was not investigated further.

Ion Exchange Chromatography

Simultaneously with the above experiments using paper chromotography and extraction with n-butanol as possible means of further fractionating fraction III, an experiment designed to determine the feasibility of using synthetic ion exchange resins was carried out. In this experiment 25 ml. aliquots of fraction III were shaken with about 2 g. portions of four different ion exchange resins in stoppered Erlenmeyer flasks. The contents of each flask were filtered and the residue washed with sufficient water to make a total of 80 ml. of combined filtrate and wash solution. The 80 ml. solution, along with a control solution treated in the same manner but without using a resin, was tested for biological activity.

The resins used are given in column (b) of Table VI. The tumor inhibiting effect of the various aliquots, after treatment with the resin as described above is given in column (c).

TABLE VI

TUMOR INHIBITION OF ALIQUOTS OF FRACTION III AFTER TREATMENT WITH VARIOUS ION EXCHANGE RESINS

Sample Number (a)	Resin (b)	Tumor Inhibiting Effect (c)
L175	Dowex 1 x 8	±
L176	Dowex 2 x 10	±
L177	Dowex 50 x 8	±
L178	Dowex 50 x 1	-
L179	None	±

From the results shown in Table VI it can be seen that the active principle was apparently removed from sample L178 by the treatment with Dowex 50 x 1. On the assumption that the activity was adsorbed by the cation exchange resin, further study was undertaken to determine the feasibility of using Dowex 50 as a means of further purifying fraction III.

Cation Exchange Chromatography--General Considerations

From the standpoint of practical considerations there are at least four variables to be noted when selecting the most desirable ion exchange

procedure for a specific problem. These may be referred to as the elution technique, degree of cross linkage of the resin, mesh size of the resin and the structure of the resin bed. The type of exchanger, e.g., strong or weak acid, is also a variable. However, in the preliminary experiment mentioned above the strongly acid Dowex 50, sulfonic acid exchanger, apparently adsorbed the active principle. Therefore, further considerations were based on the use of this resin.

The technique of ion exchange may be thought of as a two step process (42,43). The first step is adsorption by the ion exchange resin. The second step is the elution of the adsorbed ions from the resin. During the adsorption step the conditions should be such that the affinities of the resin for the ions to be adsorbed are made maximal. This is ordinarily accomplished by suitable pH adjustment (42). During the elution step conditions should be such that the affinities of the resin for the adsorbed ions are made minimal. This is ordinarily accomplished in one of two ways (42), either by suitable pH adjustment or by ionic strength adjustment. Elution by pH adjustment is achieved by altering the charge, usually a decrease, of the adsorbed ions by adjustment of the pH of the eluent (incoming solvent). Elution by ionic strength adjustment is accomplished by increasing the ionic strength of the eluent to the point where the concentration of the competing ions displace the adsorbed ions by mass action.

Elution by pH adjustment, frequently referred to as elution analysis, is frequently the method of choice especially in biochemical mixtures (42). This method possesses the advantage of producing an eluate

(outgoing solvent) of lower ionic strength and thus greater flexibility with respect to subsequent chemical steps.

Elution by ionic strength adjustment, also referred to as displacement chromotography, utilizes the maximum adsorptive capacity of the ion exchange column (44), a feature particularly desirable in preparative work. Using this method of elution "tailing" ordinarily does not occur. However, the bands are frequently close together (45). Of the two methods of elution, elution analysis tends to possess the greater resolving power (46).

Insofar as the further fractionation of fraction III was concerned, elution by increasing the ionic strength of the eluent seemed to be the method of choice for several reasons. The active principle was apparently unstable to appreciable pH adjustment (23). Due to our assay it was desired to collect the active principle in a minimum volume of eluate and a method easily adaptable to a preparative scale was desired.

A sodium chloride solution was therefore chosen as the eluent. Aside from being readily available, the presence of a small amount, physiological or below, of sodium chloride in the samples when tested for biological activity would not be expected to be toxic to the mouse. The concentration was chosen quite arbitrarily. A very concentrated solution would tend to elute the activity in a minimum volume of eluate. However, inasmuch as the active principle was apparently a macro molecule, too high an ionic strength might cause alteration of the molecule. Conversely, a very dilute solution would be desired from the standpoint of stability but would be undesirable from the standpoint of dilution of

the active principle in the eluate. A 3 M solution, approximately a half saturated solution, was therefore selected as one possibility.

In the event the active principle was loosely bound to the resin a more dilute eluent would tend to render the separation more selective with respect to the other constitutients of fraction III. Therefore, 0.3 M sodium chloride was used as the first eluent, after washing the column with water, to be followed by the 3 M solution.

A second consideration in setting up an experimental ion exchange procedure was the choice of cross-linkage to be used. The resin cross-linkage in the case of Dowex 50 is defined as the per cent of divinyl benzene added to the vinyl benzene in the polymerization of this type of resin (47). In the Dow resins the degree of cross-linkage is designated by the numeral following the name of the resin e.g. Dowex 50 x 12 is a 12 per cent cross linked resin. A resin of a very high degree of cross-linkage undergoes a minimum volume change with a change in ionic strength of the external resin phase (46,48,44). On the other hand a resin of a low degree of cross-linkage undergoes a large volume change under these same conditions. Equilibrium between the external resin phase and the resin phase, is attained most rapidly with a low degree of cross-linkage (48,49). For column work a high degree of cross-linkage would be preferred inasmuch as a minimum volume change of the resin bed is desired. However, with reference to the tumor inhibiting activity, a low degree of cross-linkage would be desired inasmuch as such a resin would be more porous and would approach an equilibrium between the external resin phase and the resin phase more rapidly (50). Ordinarily a resin with the highest

degree of cross linkage compatible with the separation to be achieved should be used (46). Inasmuch as Dowex 50 x 12 was available in the laboratory it was selected. A 12 per cent cross-linkage might be considered a high degree of cross-linkage inasmuch as Moore and Stein (51) have recently used a 4 per cent cross-linkage for an amino acid and peptide separation.

The size of the resin particles is also to be considered, however, it is probably somewhat less critical than the considerations mentioned previously. Dowex 50 is available in a variety of mesh sizes including a colloidal size. The exchange capacity of a resin bed increases as the particle size decreases (52). Also, elution appears to be more rapid with a finer mesh resin (53) and the resulting elution "peaks" tend to be more sharp (46). However, the use of a fine mesh resin produces a decreased flow rate (54). From the standpoint of the present considerations the flow rate was thought to be quite important inasmuch as large columns were to be used at room temperature. A rather course resin, 100-200 mesh, was therefore selected.

The shape of the resin bed influences the flow rate and the resolving power of the column (54). This factor has very little influence on the capacity of the column per unit amount of resin (54). Generally speaking the larger the ratio of the cross section area to the length of the resin bed the greater the capacity of the resin bed (55). Conversely, the smaller the ratio the greater the resolving power of the resin bed.

Of the four columns to be described in the next section, the two smaller ones were purchased commercially. The first large column constructed (resin bed 7 x 80 cm.) has a cross section area to length ratio of about 0.5. The ratio was increased from that of the smaller columns inasmuch as a large preparative column was desired. However, in the fourth (resin bed 5 x 87 cm.) this ratio was decreased to about 0.2 to gain a greater resolving power.

The flow rate perhaps is a fifth point to be considered inasmuch as the more rapid the flow rate the smaller will be the approach to equilibrium between the resin phase and the external resin phase at each section of the resin bed. However, as mentioned previously the flow rate is determined to some extent by the choice of resin and the dimensions of the resin bed. The flow rate used might be considered quite rapid considering the active principle was believed to be a macro molecule. However, the time factor due to the instability of the molecule was also considered in this connection.

With these considerations in mind the chromatographic procedure described below was used for the further fractionation of fraction III.

Preparation of Ion Exchange Columns

The Dowex 50 x 12, 100-200 mesh was purchased from the Dow Chemical Company, Midland, Michigan. The resin, as received, was slurried with a large volume of water and allowed to settle until the larger particles settled out. The supernatant, containing the fine material, was decanted and discarded. The process was repeated until a homogeneous sample

resulted, as determined by uniform sedimentation and the absence of fine material suspended in the supernatant. The resin was then slurried with 2 N hydrochloric acid and filtered in a large coarse sintered glass funnel. The washing with hydrochloric acid was continued until the resulting effluent was free of the yellow colored material which is removed by this treatment. The resin was washed next with distilled water until the effluent was neutral and then with 2 N sodium hydroxide until the effluent was strongly alkaline. After washing with distilled water until neutral again the cycle, acid-water-base-water, was repeated once more.

The columns were filled by slurring the resin with about two volumes of distilled water. A large funnel was attached to the top of the column by means of a rubber stopper and the column filled with distilled water to the level of the bottom of the funnel. The slurry was then introduced into the funnel, care being taken that no air bubbles were trapped in the system. Sufficient resin was used in making the slurry that one filling of the funnel would fill the column with resin. Columns prepared in this manner were free of sedimentation bands which otherwise result due to the non-uniform particles size of the resin. After the columns were filled with resin they were recycled through the above mentioned acid-base cycle and then washed with distilled water until no color was produced in the effluent upon the addition of phenolphthalein. The columns were then considered ready for use.

Buffering of Fraction III

The various preparations of fraction III ordinarily have a pH of 6.6($\pm$ 0.2). Inasmuch as the activity in ffaction III, without the

addition of buffer, apparently was adsorbed on Dowex 50 in the preliminary experiment mentioned above it might be concluded that the tumor inhibiting substance was in a cationic form at this pH. It was therefore thought desirable to add a buffer to fraction III to insure pH stability during the adsorption step of the chromatographic separation.

Fraction III was therefore buffered at pH 6.4 by adding mono basic potassium phosphate and di basic potassium phosphate as the dry salts immediately before chromatographing. The pH of the resulting solution was checked with a pH meter in all cases.

Chromatographic Separation on Dowex 50

In the course of this work four different sized columns were used. The preliminary work was done on two small columns. However, once the technique was worked out larger preparative columns were used.

Throughout all of this work the solvent changes were made abruptly. Before a new solvent (eluent) was admitted into the column the previous eluent was developed to within a few millimeters of the top of the resin. This technique was used inasmuch as it was desired to collect the activity in a very minimum volume of eluate. This was especially true in the early work when the stability of the resulting fractions to concentration was unknown.

The first column used contained 2 x 21 cm. (2 cm. diameter) of resin in the sodium form. Four milliliters of fraction III was admitted to the top of the column and allowed to pass onto the column to the upper level of the resin bed. The column was then developed with distilled

water until the eluate was essentially free of colored material. Two 90 ml. fractions of eluate were collected, using distilled water as the eluent, (L233- and L234-). The eluent was then changed to 0.3 M sodium chloride and three 90 ml. fractions collected, (L235-, L236- and L237-). Three 90 ml. fractions were then collected using 3.0 M sodium chloride as the eluent. These were tested for biological activity after dialysis to remove the sodium chloride, (L238±-, L239- and L240-). Four milliliters of the same buffered sample of fraction III was diluted to 90 ml. with distilled water and tested as sample (L241±-) to serve as a control.

It should be noted in the above experiment that if the elution with 0.3 M sodium chloride solution had been continued slightly longer the activity might have been recovered in the eluate from the 0.3 M sodium chloride solution rather than in that from the 3.0 M sodium chloride solution.

In the following experiment a slightly larger column (3 x 21 cm.) was used, along with a 40 ml. sample of fraction III. This volume of fraction III proved to be too large inasmuch as activity was recovered in the first aliquot of eluate collected from the column. The remainder of the activity was found to be present in the eluate resulting from the 0.3 M sodium chloride solution (L249±-).

At this point a large column (7 x 80 cm.) was constructed with the intension of carrying out the separation on a large preparative scale. In this experiment 700 ml. of fraction III buffered at pH 6.4 was placed on the column. Water was then used to develop the column until the eluate was essentially colorless, six 1 liter fractions having been

collected. Three 1 liter fractions were then collected using 0.3 M sodium chloride as the eluent, followed by four 1 liter fractions in which 3.0 M sodium chloride was used as the eluent. An aliquot of each fraction was tested for biological activity. Only the third fraction resulting from the 0.3 M sodium chloride eluent was found to contain activity (L308$^{+}_{-}$). A duplicate experiment was carried out and in this case the corresponding fraction was the only one found to contain activity (317$^{+}_{-}$).

This large column was accidently broken, probably due to the expansion of the resin during the regeneration of the column. The ordinary technique used for regenerating the resin was to pass 2 N sodium hydroxide through the column until the eluate was strongly alkaline to insure the complete regeneration of the resin in the sodium form. At this point the resin has the minimum volume in the cycle used. Therefore when water was introduced to wash the resin bed until neutral to phenolphthalein a rather large expansion took place. As a result of this experience a column was designed with a sintered glass disc at the top as well as at the bottom. A ground glass joint was inserted near the upper disc for filling the column. The technique for regenerating the resin at the end of a run was also altered in the following way. After the last fraction resulting from the 3.0 M sodium chloride eluent was collected water was forced through the column in the reverse direction. This forced the resin up against the upper disc. The sodium hydroxide solution was then admitted, also in the reverse direction, at such a rate that the resin particles would slowly fall under the force of gravity

as the sodium hydroxide solution flowed up through the column. This technique served the added purpose of collecting the finer resin particles in the upper portion of the column thus eliminating the possibility of their clogging the lower disc during an actual run. The sodium hydroxide solution was passed through the column until a strongly alkaline eluate resulted. The column was then washed with distilled water in the ordinary manner.

The newly designed column contained (5 x 87 cm.) of resin. A 415 ml. sample of fraction III was placed on the column which was then developed with the solvents described earlier. Fractions of the eluate were collected at four minute intervals using an automatic fraction collector. A total of 60 fractions of approximately 100 ml. were collected. The first fifteen fractions collected, using distilled water as the eluent, contained evidence of activity. A total of 2710 ml. of distilled water was passed through the column before the eluate became essentially colorless. Fifteen fractions were collected using 0.27 M sodium chloride solution as the eluent. The total volume of 0.27 M sodium chloride solution used was 1479 ml. These fifteen samples were combined in groups of two, except that the last three were combined and assayed for biological activity. The biological activity was found to be contained in the eluate between 617 ml. and 1042 ml. ($L337^{\pm}+$, and $L338^{\pm}-$). Fifteen fractions were also collected using 2.7 M sodium chloride solution as the eluent. Total volume of 2.7 M sodium chloride solution 1724 ml. These fractions were combined in the same manner as the previous fifteen samples were and assayed for biological activity after dialysis.

Activity was found to be contained in the eluate between the limits of 1006 ml. and 1309 ml. (L346+ and L347-[a]). It should be noted that these two activities were separated by about 1440 ml. of eluate.

In future discussions the activity contained in the eluate resulting from the 0.3 M (ca) eluent is designated as fraction IV and that resulting from the 3.0 (ca.) eluent as fraction V.

Preparation of Fractions IV and V

The following chromatographic procedure was used in the preparation of all future samples of fractions IV and V. The same column, containing (5 x 87 cm.) of Dowex 50 x 12 in the sodium form, described previously was used. The technique of preparing the resin, filling the column as well as the technique for regenerating this column were all as described earlier.

Three hundred milliliters of fraction III were buffered at pH 6.4 by the addition of 0.659 g. of mono basic potassium phosphate and 1.526 g. of di basic potassium phosphate as the dry salts. The buffered sample of fraction III was placed on the column and allowed to drain to the upper level of the resin bed under the force of gravity. This procedure usually required about 30 to 45 minutes.

The column was then developed under a positive pressure of about 19 cm. of mercury using the solvents described below. Under these conditions about 15 ml. of eluate was collected per minute. This corresponds to a flow rate of 0.77 cm. per min. on a column 5 cm. in diameter.

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[a] Note tumor diameter.

A total of 2940 ml. of distilled water was passed through the column and the entire eluate discarded. The distilled water was developed to within a few millimeters of the upper level of the resin bed before the admission of the second solvent.

The second solvent used was 1500 ml. of 0.265 ($\pm$ 0.005) M sodium chloride solution. The first 200 ml. of this eluate were discarded. The next 1200 ml. were collected as fraction IV. The last 100 ml. were discarded. This solvent was also developed to within a few millimeters of the resin bed before admitting the third solvent.

The third solvent used was 1500 ml. of 2.65 ($\pm$0.05) M sodium chloride solution. The first 600 ml. of this eluate were discarded. The next 900 ml. were collected as fraction V.

Discussion of Ion Exchange Procedure

This procedure presents no particular difficulties. However, certain aspects of it might be investigated further. The 12 per cent cross-linkage is perhaps quite high. A lower cross-linkage might be investigated. Moore and Stein (51) report the use of Dowex 50 x 4 for the resolution of peptides containing up to 8 or 10 amino acid residues and suggest Dowex 50 x 2 for larger peptides.

As is indicated by the next section, the activity is apparently more stable to acid than it was originally believed to be. This might suggest the possibility of buffering fraction III at a lower pH which would tend to increase the cationic nature of the active principles and thus their ability to be adsorbed by the resin. If such a technique proved to be

feasible a larger quantity of fraction III could be applied to the column.

The possibility of concentrating fraction III before placing it on the column might also be considered. A smaller volume of sample solution would tend to improve the resolution due to the activity being adsorbed in a narrower band near the top of the column.

It would also be desirable to collect a smaller volume of eluate representing fraction IV and also fraction V. The empirical method used necessitates the collection of a very wide band of eluate for each fraction since the active principles would not be expected to be eluted in exactly the same volume of eluate each time. Such a technique introduces the possibility of other substances also being contained in the eluate collected inasmuch as the elution bands would be expected to be very close together using the elution technique described in the previous section.

Treatment of Fraction III with Acid and Cuprous Oxide

In an early study of the hydrolysis products of fraction IV using paper chromatography some indication was given that this fraction might contain cystine. This later proved not to be the case. However, in the intervening period an experiment patterned after the precipitation of glutathione (56) was carried out.

For this experiment 63 ml. of fraction III was warmed to 50 C. and 5.5 ml. of a 3 M sulfuric acid solution added with stirring. To this solution was added a suspension of cuprous oxide, prepared by boiling

20 ml. of Benedict's solution with an excess of glucose and removing the precipitate of cuprous oxide by centrifugation. The mixture was allowed to stand for four hours in the refrigerator. After the four hour period a considerable amount of precipitate was observed to have settled out. The precipitate was removed by centrifugation, the supernatant being designated as fraction D. The precipitate, after washing with water, was suspended in 50 ml. of distilled water and saturated with hydrogen sulfide gas. The precipitate of cuprous sulfide was removed by centrifugation and discarded. The supernatant was designated as fraction E. Fraction E was aerated with nitrogen gas to remove any remaining hydrogen sulfide and tested as sample (Lh12-) after adjusting the pH to 6.4 with very dilute sodium hydroxide solution. Fraction D was adjusted to pH 6.4 with very dilute sodium hydroxide solution and tested for biological activity as sample (Lh10$^{\pm}_{+}$) (retest$^{+}_{--}$).

In a duplicate experiment an aliquot of fraction D was tested for biological activity after neutralization (Lh19$^{\pm}_{--}$). A second aliquot of fraction D was dialyzed and the dialysate tested as sample (Lh21$^{+}_{-+}$).

Discussion of Cuprous Oxide Experiment

Although the active principle was not precipitated by the cuprous oxide as might have been expected if a sulfhydral compound had been present the experiment provided some worth-while information.

The active principle was apparently stable to the acidity used in this experiment. This is in disagreement with the observation made by Ritchie (23). However, the discrepancy is possibly explained by the

technique used to neutralize fraction D. Ritchie (23) reports having acidified a preparation to pH 2.0 with hydrochloric acid and after two hours neutralizing the sample to pH 5.1 with 10 per cent sodium hydroxide solution with a subsequent loss of tumor inhibiting activity. In the preparations just described fraction D was allowed to remain at an acidity of less than pH 1 for four hours. However, the preparation was then carefully neutralized to pH 6.4 with a very dilute solution of sodium hydroxide. The pH 6.4 was selected since the pH of fraction III is ordinarily 6.4 or slightly above. In Ritchie's case the lower pH might possibly have resulted in alteration of the active principle before the preparation was tested for biological activity.

A second observation can also be made as a result of the experiment described above if samples (L421$^{\pm}_{-}$) is compared with sample (L415$^{\pm}_{-}$) (Table II). These two samples were prepared at comparable dilutions. However, sample L415 was diluted 1:5 due to its toxicity in the mouse test and resulted in a ($^{\pm}_{-}$) effect. Sample L421 was not diluted making a larger dose possible with a subsequent ($^{\pm}_{+}$) effect. This observation would seem to indicate that some of the toxicity of fraction III was either removed or destroyed by the treatment with cuprous oxide in the acidified solution.

Further Purification of Fraction IV

It was thought desirable at this point to attempt to recover a solid product from the fraction eluted with 0.3 M sodium chloride solution which still retained biological activity.

Fraction IV was therefore dialyzed in Visking casing on the rotating external liquid type dialyzer described earlier. The dialysis was carried out against frequent changes of distilled water at 7°C. The diffusate was discarded in all cases. The dialysate varied in volume from 1230 to 1255 ml. in the seven experiments carried out.

The dialysate was concentrated *in vacuo* on the revolving concentrator described by Craig *et al.* (27) using a water aspirator. This apparatus is well suited to the concentration of biological materials for at least two reasons. The sample container, an ordinary round bottom flask, is made to revolve thus increasing the surface area of the sample to essentially the surface area of the flask used. This facilitates a very rapid rate of evaporation even at relatively low temperatures. Secondly it is not necessary to pass air or some inert gas through the solution to prevent bumping. In these experiments the sample solution was maintained at a temperature not greater than 29° C. The concentration to a small volume (3-15 ml.) required about four to five hours. The concentrate was then dialyzed again to remove the last traces of chloride ion as indicated by the addition of silver nitrate solution to an aliquot of the dialysate.

Three different techniques were used in the attempt to recover an active solid product from the chloride free dialysate.

In the first experiment the sample was concentrated to a volume of 3 ml. and dialyzed free of chloride ions. To this chloride free dialysate 15 ml. of acetone were added and precipitation allowed to take place overnight at 7°C. About 1-2 mg. of dry residue was recovered by

centrifugation followed by drying in a vacuum desiccator for two hours. The entire product was dissolved in 80 ml. of physiological saline (at Sloan-Kettering Institute) and tested for biological activity (L4007) (retest dil. 1:2±), (retest dil. 1:2±).

In the second experiment the chloride free dialysate was concentrated to dryness by the technique described previously. About 11 mg. of solid material was recovered. Of this material, 8.1 mg. were dissolved in physiological saline (at Sloan-Kettering Institute) and tested for biological activity (L406±).

In the third experiment the chloride free dialysate from two runs on the ion exchange column was frozen and lyophilized for 24 hours. The lyophilization apparatus was constructed by submerging a 500 ml. three necked, round bottom flask in a ethyl alcohol solid carbon dioxide bath to a level about 1 inch above the lower end of the necks. By means of ground glass joints one neck was attached to a 50 ml. round bottom flask at an angle of 90 degrees. The 50 ml. round bottom flask contained the frozen sample. A stopcock was placed in the middle neck. The third neck was connected by means of a glass adaptor and a short length of rubber tubing to a second "dry ice trap" which was in turn connected to a vacuum pump. This apparatus has the advantage of being constructed almost entirely of standard laboratory equipment. It is also quite versatile inasmuch as several samples can be attached to the one neck of the three necked flask by means of suitable adaptors.

Using the lyophilization technique just described about 86 mg. of solid material was obtained. This product was not dried in a

desiccator but was stored in the deep freeze. Fourteen and one-half milligrams of this product was sent to Sloan-Kettering Institute, however, the results of the assay were not completed when this thesis was written (L440 no result).

Further Purification of Fraction V

An attempt was also made to recover a solid product from the fraction eluted from the column with 3.0 M sodium chloride solution. The fraction was dialyzed and concentrated to a small volume by the same technique described for fraction IV.

In one experiment 15 ml. of acetone were added to 3 ml. of concentrate which had been dialyzed free of chloride ion. After standing overnight the precipitate was collected by centrifugation and dried in a vacuum desiccator. The entire product (ca. 19 mg.) was dissolved in physiological saline (at Sloan-Kettering Institute) and tested for biological activity (L403-).

In a second experiment the chloride free dialysate was concentrated to dryness. Only about 3 mg. of product was recovered. In a repeat experiment, in which the last dialysis was omitted, 32 mg. of product was obtained. The two products (3 mg. and 32 mg.) were combined and 8.5 mg. used for the biological assay (L407-).

No attempt has been made to lyophilize the chloride free dialysate from fraction V.

Discussion of the Purification of Fractions IV and V

If sample L400, in which the chloride free dialysate was precipitated with acetone, is compared to sample L406, in which the chloride free

dialysate was concentrated to dryness, several observations can be made.

Sample L400 was dissolved in 80 ml. of physiological saline and used in the first test at a dose of 0.6 ml. (see Appendix) twice daily. In terms of dry material this corresponds to approximately 1.5 mg./kg./day. In the two retests the above solution was diluted 1:2 and a total of approximately 1 ml. given daily. At the final dilution 1 ml. corresponds to about 0.4 mg./kg./day in terms of dry material.

In the case of sample L406 a daily dose, in terms of dry material, of 8 mg./kg./day can be calculated.

The wide difference in the tolerated daily dosage would suggest that sample L406 was much less toxic than sample L400. At the dosage given sample L406 also gave the greater inhibition (see tumor diameter-Appendix).

From these considerations it would seem that the technique of concentrating the chloride free dialysate to dryness was to be preferred over the technique of precipitation with acetone.

Although the results of the assay of sample L410 are not available this sample might be expected to be the least toxic of the three since the lyophilization would seem to be preferred to concentrating the fraction to dryness due to the temperature at which the operation is carried out.

The explanation for the loss of activity in both sample L403 and sample L407 is unknown. Apparently the activity present in fraction V

is not sufficiently stable to withstand the isolation procedure used. Lyophilization of this chloride free dialysate might be considered since the conditions would seem to be more desirable.

CHAPTER IV

STUDIES AS TO THE CHEMICAL COMPOSITION OF FRACTION IV

Acid Hydrolysis of Fraction IV

About 1 mg. of fraction IV was dissolved in 0.1 ml. of 6 N hydrochloric acid and the solution sealed in a capillary tube. The tube was placed in an oven at 105°C. for 12 hours (57). After cooling the contents of the tube were placed on a 2 inch watch glass made of polyethylene (57) and evaporated to dryness in a vacuum desiccator. The residue was dissolved in about 0.2 ml. of distilled water and evaporated to dryness a second time. The resulting residue, essentially free of hydrochloric acid, was dissolved in about 80 ml. of a 10 per cent aqueous solution of isopropyl alcohol which Block (58) has reported to be a good preservative for an amino acid mixture.

A duplicate hydrolysis was also carried out using about 2 mg. of fraction IV and a correspondingly increased quantity of the other materials.

These two hydrolysates dissolved in the 10 per cent aqueous isopropyl alcohol solution were used for the chromatographic study to be described later.

Alkaline Hydrolysis of Fraction IV

The hydrolysis technique used is described by Block (58). About 10 mg. of fraction IV was dissolved in 10 ml. of 14 per cent barium

hydroxide solution. The resulting solution was heated in an oil bath at about 125° C. under reflux for 20 hours.

The hydrolysate, after cooling was made very slightly acid (to litmus paper) with 1 N sulfuric acid and the precipitate of barium sulfate removed by filtration. The barium sulfate precipitate was washed with about 50 ml. of hot water, containing a few drops of acetic acid, and the washings combined with the original filtrate. The combined solution was concentrated to dryness on the revolving concentrator and the resulting residue desiccated overnight over calcium chloride. The residue was then suspended in about 0.5 ml. of a 10 per cent aqueous solution of isopropyl alcohol. This solution was used for a chromatographic study to be described in a later section and is referred to as the alkaline hydrolysate of fraction IV.

Preparation of Fractions for Terminal N-group Study

The method of preparing the terminal dinitrophenyl derivative of peptides described by Sanger (59) was used. About 3 mg. of fraction IV was suspended in 0.3 ml. of a 1 per cent aqueous solution of trimethylamine contained in a 1 ml. volumetric flask. To this solution was added approximately 50 mg. of 1-fluoro-2,4-dinitrobenzene (DNFB) (purchased from the Aldrich Chemical Company, Milwaukee, Wisconsin) contained in 0.6 ml. of ethanol. The mixture was shaken mechanically for two hours.

After being shaken the mixture was diluted with about 10 drops of the 1 per cent trimethylamine solution and then extracted with four 0.75 ml. portions of ethyl ether. The organic layer, containing the unreacted

DNFB was discarded. The aqueous layer was evaporated to dryness in a vacuum desiccator.

The residue from the evaporation of the aqueous layer was dissolved in about 15 drops of constant boiling hydrochloric acid solution and sealed in a capillary tube. The sealed tube was placed in an oven at 105° C. for eight hours. After cooling the tube was opened and its contents diluted about twice with distilled water. The hydrolysate was then extracted with three 0.75 ml. portions of ethyl ether. The combined organic layer, containing most of the dinitrophenyl amino acids (DNPAA) was evaporated to dryness on the steam bath. This fraction was designated as the "DNPAA" fraction, however, it was not used for further study (see discussion). The aqueous layer was evaporated to dryness in a vacuum desiccator and the residue suspended in water and evaporated to dryness a second time. The resulting residue, containing the unreacted amino acids for the most part, was suspended in a small volume of distilled water and designated as the "non DNPAA" fraction.

In a duplicate experiment 9 mg. of fraction IV was used with a corresponding increase in the relative quantities of the other materials.

The "non DNPAA" fraction was used for a chromatographic study to be described in a later section.

Paper Chromatographic Procedures

A chromatographic procedure very similar to that described by Sanger (60) was used to chromatograph fraction IV, the acid hydrolysate of fraction IV, the alkaline hydrolysate of fraction IV and the "non DNPAA" fraction.

Sheets of Whatman No. 1 filter paper approximately 17 inches square were used. The sample to be chromatographed was applied at one corner of the paper 4 inches from either edge. A reference spot, for comparative purposes, was also applied such that it was 4 inches from the bottom edge of the paper in the direction of the first solvent and 2.5 inches from the bottom edge of the paper in the direction of the second solvent. Both the sample solution and the reference solution were applied in 5 ul. aliquots by means of a micro pipette. The spots were dried by means of a low stream of air and an infra-red lamp between applications.

The chromatograms were developed by the ascending technique using 12 chromatograms per run in the Chromatocab mentioned previously. Before being developed the chromatograms were equilibrated for 18 hours with the vapor phase of a solution composed of 0.125 g. of potassium cyanide, 5.2 ml. of concentrated ammonium hydroxide (28.9% NH_3) and sufficient distilled water to make 500 ml. of solution. After the equilibration period a solution of phenol saturated with distilled water was added to each trough and development allowed to take place at room temperature for 24 hours. After the 24 hours development with the first solvent the chromatograms were removed from the cabinet and dried.

Two methods were used for drying the chromatograms at this point. The first method consisted in drying them in a forced draft chromatographic oven at about 40-50° C. for eight hours and then overnight with only the forced draft on. In the second method the chromatograms were dried in the forced draft oven with no heat on for about one-half hour

and then washed with ethyl ether. They were then dried in the oven with only the forced draft on for about six hours. No difference in the final result was observed between the two methods.

Before being developed with the second solvent the chromatograms were equilibrated for 18 hours with the aqueous layer resulting from the combination of 400 ml. of n-butanol, 500 ml. of distilled water and 100 ml. of glacial acetic acid. This solvent was made up 48 hours before it was to be used. After the equilibration period the chromatograms were developed with the organic phase from the above butanol-water-acetic acid solvent for 24 hours.

After developing with the second solvent the chromatograms were dried in the chromatographic oven with only the forced draft on for about one-half hour. The following techniques were used for detecting spots on the various chromatograms:

(a) The chromatogram was sprayed with a 0.2 per cent solution of ninhydrin in a water saturated solution of n-butanol followed by heating at about 90-95° C. for about 15 minutes (37).

(b) The chromatogram was sprayed with a solution composed of 0.93 g. of aniline, 1.66 g. of phthalic anhydride and n-butanol saturated with distilled water sufficient to make 100 ml. of solution. After being sprayed the chromatogram was heated for about 15 minutes at about 100° C. (37).

(c) The chromatogram was sprayed with a 0.04 per cent solution of bromcresol green in ethanol (37).

(d) The chromatogram was viewed under ultra-violet light in the darkroom (37). A Mineralight Model SL Manufactured by Ultra-Violet Products, Inc., South Pasadena, California was used as the source of ultra-violet light.

The spots made visible by the above techniques were marked by means of a small dot in the center of the region of greatest color intensity. Since a solution of the amino acid leucine was used as the reference solution the reference spot was made visible on those chromatograms not otherwise treated with the ninhydrin reagent by treating the chromatogram with this reagent as described above after previously having subjected it to one of the other techniques for detecting spots.

Inasmuch as a solution of leucine was used as the reference solution a "reference leucine" (R_L) value was calculated for purposes of comparison. The R_L value was defined as the ratio of the distance a given substance migrated to the distance leucine migrated in the same solvent system. The distances were measured to the nearest millimeter with an ordinary ruler.

The chromatographic procedure just described was designated procedure A.

A second chromatographic procedure was used to detect the presence of phenylalanine, leucine or isoleucine in the acid hydrolysate of fraction IV and also in the "non DNPAA" fraction since these amino acids were not resolved by procedure A. This second chromatographic procedure was also used to chromatograph the alkaline hydrolysate of fraction IV.

For this procedure sheets of Whatman No. 1 filter paper 7 x 22 inches were used. The general technique used here is described by McFarren (61). The papers were buffered by dipping them in borate buffer at pH 8.4 and drying at room temperature. Five samples were applied to each chromatogram.

After applying the samples by the technique described for procedure A the chromatograms were equilibrated with the vapor phase resulting from saturating the borate buffer with a 1:1 (v/v) solution of benzyl alcohol n-butyl alcohol in a 12 x 24 inch glass cylinder. After an 18 hours equilibration period the chromatograms were developed by the descending technique using a solvent composed of a 1:1 solution of benzyl alcohol n-butyl alcohol saturated with the borate buffer. Development was allowed to take place for 24 hours.

After developing, the chromatograms were dried in a forced draft oven with no heat on and sprayed with a 0.2 per cent solution of ninhydrin in n-butanol saturated with a 2 per cent acetic acid. They were then heated for about 15 minutes at 90-95°C.

For comparative purposes known samples of leucine, isoleucine and phenylalanine were run on the same chromatogram with the acid hydrolysate of fraction IV and the "non DNPAA" fraction. An authentic sample of tryptophan was used for comparative purposes on the chromatogram of the alkaline hydrolysate of fraction IV. The distances migrated by the known amino acids were compared with the distances which the various ninhydrin positive constituents of the unknown samples migrated.

The chromatographic procedure just described was designated as procedure B.

Result of Chromatographic Study

Fraction IV was chromatographed by procedure A and the chromatogram treated with the ninhydrin reagent described under detecting reagents for procedure A. No ninhydrin spots resulted.

The acid hydrolysate of fraction IV was also chromatographed using procedure A. Twelve ninhydrin spots and one blue bromcresol green spot resulted. The bromcresol green spot appeared to coincide with one of the ninhydrin spots, however, this ninhydrin spot was observed to have a very reddish color and to be quite faint making the evaluation of its R_L values very difficult. The other ninhydrin spots were of the typical blue-red color. No spots were detected with the aniline-phthalic anhydride reagent or under ultra-violet light.

The R_L value of the 12 spots in each solvent is given in Table VII. The spots are numbered in the increasing order of their R_L value in solvent no. 1 for purposes of future discussion. Spot no. 2 is the spot which produced a blue color with the bromcresol green reagent and the R_L values for this spot in Table VII are calculated on the basis of the spot given with this reagent.

Table VIII contains the R_L values of 19 known compounds which were chromatographed by procedure A and the spots detected with the ninhydrin reagent. The fourth column in this table contains the number of determinations which were used to determine the R_L values given in this table.

TABLE VII

THE R_L VALUES OF THE HYDROLYSIS PRODUCTS OF FRACTION IV

Spot Number	R_L Value	
	Solvent No. 1	Solvent No. 2
1	0.19	0.22
2[a]	0.21	0.18
3	0.32	0.30
4	0.45	0.22
5	0.51	0.24
6	0.61	0.31
7	0.69	0.35
8[b]	0.75	0.52
9	0.91	0.61
10	0.95	0.14
11[b]	1.03	0.44
12	1.04	0.22

[a] Bromcresol green spot.
[b] Very faint spot.

TABLE VIII

THE R_L VALUES OF KNOWN COMPOUNDS CHROMATOGRAPHED BY PROCEDURE A

Compound	R_L Values		Number of Determinations
	Solvent No. 1	Solvent No. 2	
Alanine	0.69	0.35	7
β Alanine	0.79	0.43	1
Arginine	1.04	0.22	6
Asparagine[a]	0.50 0.54	0.17 0.19	2
Aspartic Acid	0.22	0.22	6
Cystine	0.43	0.08	1
Glucosamine	0.74	0.27	1
Glutamic Acid	0.32	0.30	6
Glutamine	0.68	0.21	3
Glycine	0.51	0.24	5
Histidine	0.85	0.18	2
Hydroxyproline	0.79	0.25	1
Lysine	0.95	0.14	6
Methionine[a]	0.95 0.94	0.61 0.24	3
Ornithine	0.90	0.13	1
Proline	1.01	0.42	1
Serine	0.45	0.22	7
Threonine	0.61	0.31	7
Valine	0.91	0.61	5

[a] See discussion

From the results presented in Tables VII and VIII the spots found to result on the chromatogram of the acid hydrolysate of fraction IV (Table VII) may be identified as shown in Table IX.

TABLE IX

IDENTIFICATION OF SPOTS GIVEN IN TABLE VII

Spot Number	Amino Acid
1	Aspartic Acid
2	--
3	Glutamic Acid
4	Serine
5	Glycine
6	Threonine
7	Alanine
8	--
9	Valine
10	Lysine
11	--
12	Arginine

The acid hydrolysate of fraction IV was also chromatographed by procedure B using authentic samples of isoleucine, leucine and phenylalanine as references. A spot corresponding to leucine was found to be

present in the hydrolysate of fraction IV. Therefore, in addition to the nine amino acids contained in Table IX, leucine appears to be a constituent of the acid hydrolysate of fraction IV. Spots no. 8 and 11 (Table VII and IX) are tentatively assumed to be small molecular weight peptides inasmuch as these spots are scarcely visible on a chromatogram containing sufficient sample to cause the other spots to tend to streak. Spot no. 11 is quite close to the R_L values for proline. However, proline produces a yellow color with ninhydrin whereas spot no. 11 produces a typical blue-red color with this reagent.

At this point a mixture composed of the acid hydrolysate of fraction IV and the amino acids listed in Table IX was made up. This mixture was chromatographed by procedure A and the spots detected with the ninhydrin reagent. No spots other than the original 12 spots found to be present in the acid hydrolysate were observed.

The alkaline hydrolysate of fraction IV was chromatographed by procedure A and also by procedure B. This hydrolysate resulted in considerable streaking of the amino acids when chromatographed by procedure A, possibly due to the presence of salts. Therefore, the results as to the presence or absence of tryptophan were inconclusive. The alkaline hydrolysate was also not well resolved when chromatographed by procedure B. However, the absence of a ninhydrin positive spot in the region where tryptophan would be expected tends to indicate the absence of this amino acid.

Tryptophan would also seem to be absent due to two other considerations. The first of these, though somewhat inconclusive, was the

observation that no appreciable discoloration occurred during the acid hydrolysis of fraction IV. The second consideration was deduced from the spectroscopic study described in a later section. An aqueous solution of fraction IV has an absorption maximum at about 276 mu. If tryptophan were present an absorption maximum might be expected in this region (62). Spot no. 2 (Table VII), eluted from a chromatogram of the acid hydrolysate of fraction IV, also has an absorption maximum at about 276 mu. Since spot no. 2 (Table VII) is not due to tryptophan it would seem that this amino acid is absent from fraction IV inasmuch as the absorption of both fraction IV and spot no. 2 appear to have the same characteristics in this region.

The "non DNPAA" fraction was also chromatographed by procedure A. One yellow spot was visible on the chromatogram at an R_L value of 1.10 and 0.84 in solvents no. 1 and no. 2 respectively. On treating the chromatogram with the ninhydrin reagent all of the spots found to be present in the hydrolysate of fraction IV were found to be present except spot no. 10 believed to be lysine. Spot no. 10 was also completely absent on a chromatogram which contained such a large amount of the "non DNPAA" fraction that the other spots tended toward streaking. Insofar as could be determined visually the various spots produced from the "non DNPAA" fraction, with the exception of the lysine spot, appeared to be present in the same relative amounts as they were in the hydrolysate from fraction IV.

In addition to the spots already mentioned two additional ninhydrin positive spots were found to be present on the chromatogram of the

"non DNPAA" fraction. One of these coinsided with the yellow spot which was visible before treating the chromatogram with the ninhydrin reagent. The second new spot had R_L values of 1.10 and 0.92 in solvents no. 1 and no. 2 respectively.

The "non DNPAA" fraction was also chromatographed by procedure B and the resulting ninhydrin spots compared with an authentic sample of leucine. A spot corresponding to the known leucine sample was found to be present in the "non DNPAA" fraction. It was also observed visually that the spot corresponding to leucine in the "non DNPAA" fraction as well as the other spots on this chromatogram were present in the same relative amounts as they were on the chromatogram of the hydrolysate of fraction IV.

Discussion of the Chromatographic Study

In Table VIII two spots are reported for asparagine and for methionine. It is considered possible that one spot in each case represents an impurity in the sample which was used as the chromatographic standard. Since none of the spots resulting from these two compounds were found to result from any of the unknown fractions studied the identity of the four spots were not investigated further.

The R_L value of spot no. 1 (Table VII) in solvent no. 1 and the R_L value for aspartic acid (Table VIII) in this solvent do not agree as well as would be desired. However, since a new spot corresponding to aspartic acid did not result on the chromatogram on which the nine amino acids listed in Table IX and the acid hydrolysate of fraction IV were

applied, spot no. 1 is believed to be aspartic acid. The discrepancy in R_L value is possibly due to the nearness of spot no. 2 since this spot also produces a color with ninhydrin making the location of the center of spot no. 1 somewhat difficult.

From the data shown in Tables VII and VIII it may be concluded that fraction IV contains a peptide which on acid hydrolysis gives rise to the amino acids shown in Table IX plus a "basic" substance (spot no. 2) and leucine. Tryptophan is believed to be absent. Inasmuch as the amino acids appear to be present in the acid hydrolysate of fraction IV in about the same molar ratio, as judged from the color intensity of their ninhydrin spots on the chromatogram, a rough estimate of a minimum molecular weight of about 1500 might be made.

It might also be concluded that this peptide is a cyclic one for the following reasons: The chromatogram of fraction IV did not produce a ninhydrin spot. Lysine appears to be the only amino acid with a terminal amino group as judged from the absence of spot no. 10 from the chromatogram of the "non DNPAA" fraction. The presence of the yellow spot (R_L values 1.10 and 0.84) on the chromatogram which also proved to be ninhydrin positive might suggest that this spot is the N^{ϵ} DNP derivative of lysine making a cyclic structure seem quite likely.

A study of the second new spot (R_L values 1.10 and 0.92) on the chromatogram of the "non DNPAA" fraction has not been made. However, since this spot is very faint it is considered possible that it represents a small molecular weight peptide inasmuch as the hydrolysis in the sealed tube was only for a period of eight hours.

A number of possibilities for further study would seem to be suggested by this work. The N^{ϵ} DNP derivative of lysine should perhaps be prepared (63) and chromatographed by procedure A. Also the "DNPAA" fraction should be studied, possibly by the method of Blackburn and Lowther (64) involving paper chromatography.

The alkaline hydrolysis should also be repeated and the hydrolysis products identified.

An early experiment should also be conducted to determine the nature of spot no. 2 (Table VII). Since this spot has such a low R_L value when chromatographed by procedure A, other solvent systems should be investigated. Spot no. 2 has been eluted (to be described later) from a chromatogram developed by procedure A. However, this spot is so close to the aspartic acid spot that this amino acid almost certainly contaminates the eluate.

It might also be desirable to determine which isomer of the individual amino acids is present in the acid hydrolysate of fraction IV. Such a determination has been made by spraying a chromatogram of the amino acids in question with a D-amino acid oxidase preparation (65).

If the amino acid sequence is to be determined the method of Sanger and Thompson (59) might be suggested.

Spectroscopic Studies Using Fraction IV

Throughout the course of this work several attempts have been made to correlate the tumor inhibiting activity with the absorption spectrum of the various preparations. However, such a correlation apparently has not been successful.

An aqueous solution of fraction IV, which was prepared in the dry state by lyophilization was used to obtain an absorption spectrum in the region from 240 mu. to 400 mu. The measurements were made at 5 mu. intervals, except in the 250 to 290 mu. region where they were made at 2 mu. intervals, on a model DU Beckman spectrophotometer. An absorption maximum was found to occur at about 276 mu. and a minimum at about 260 mu.

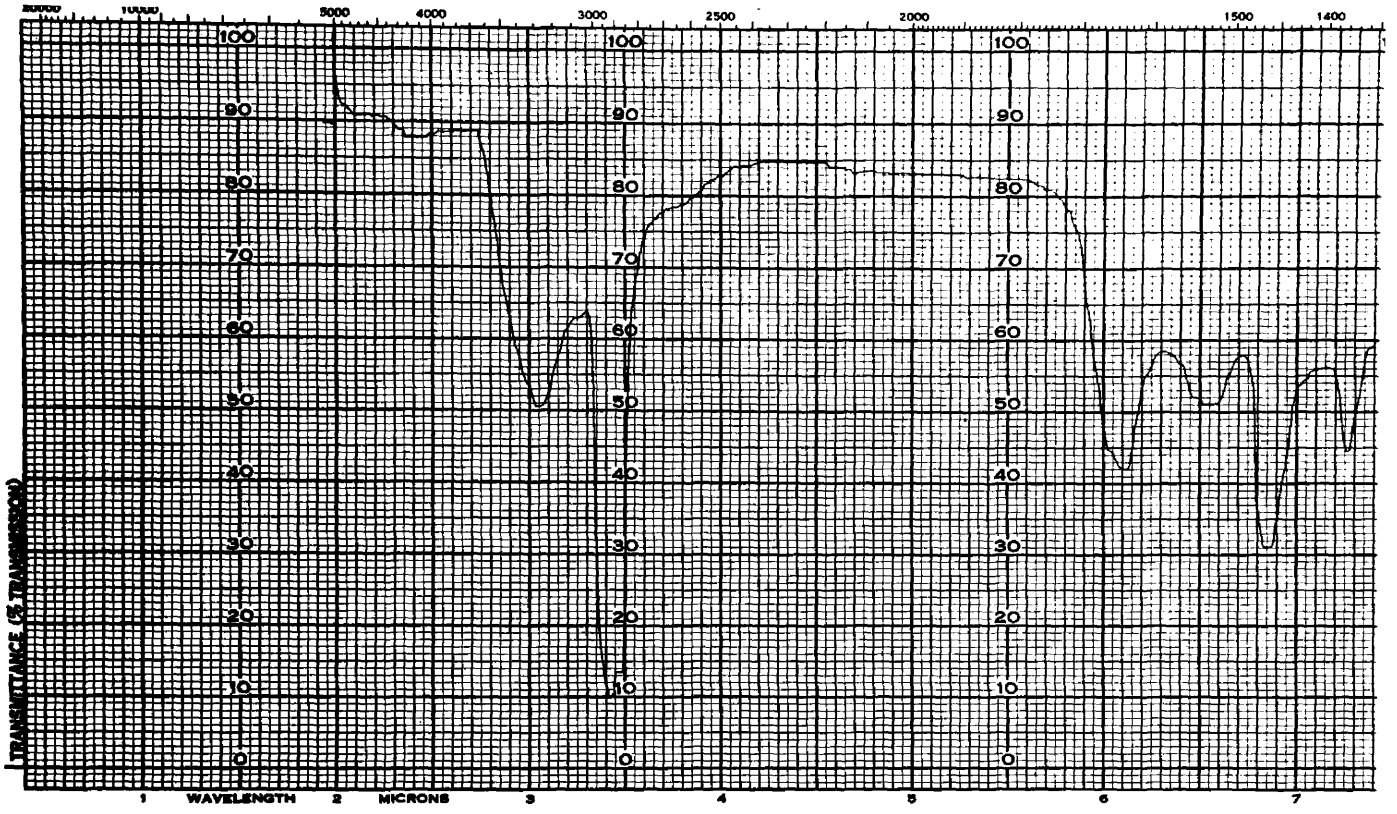
On several occasions the absorption in the 230 to 400 mu. region was measured using the various eluates from the Dowex 50 columns described previously. Inasmuch as these fractions were all very dilute no significant absorption "peaks" were observed. However, as a result of the above maximum observed at 276 mu., the optical density of the various fractions collected from the ion exchange column might be measured at 276 mu. and the resulting absorption (optical density) plotted against the volume of eluate collected at each fraction. Using this technique a correlation could be made between those fractions which exhibit a maximum biological activity and those exhibiting a maximum absorption at 276 mu. If the two "peaks" coincide a correlation might exist between the physical measurement and the biological measurement. If the two "peaks" do not coincide fraction IV must represent an impure fraction.

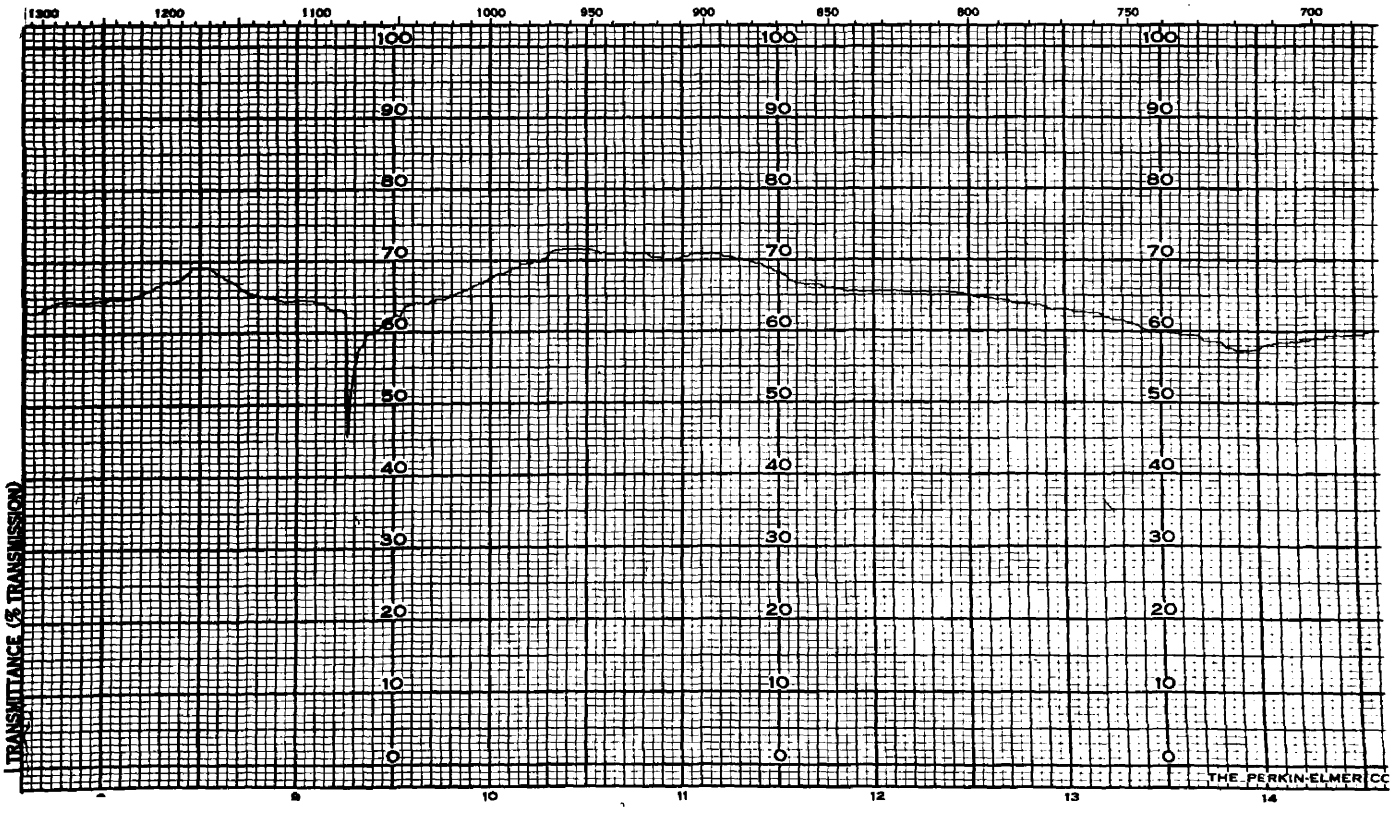
In another experiment the area represented by spot no. 2 (Table VII) was cut out from three chromatograms developed by procedure A. The spot was eluted with about 30 ml. of distilled water and the eluate evaporated to dryness, on the revolving concentrator, and the residue desiccated

overnight. After desiccation the residue was dissolved in a minimum volume of water, about 3 ml., and the absorption measured in the 240 to 400 mu. region. An absorption maximum was found to be present at about 276 mu. and a minimum at about 265 mu. Due to the nearness of the aspartic acid spot (spot no. 1, Table VII) on the chromatogram the eluate just described quite possibly was contaminated with this amino acid. However, since the absorption spectrum of aspartic acid does not show an absorption maximum at 276 mu. (66) it would appear that the absorption at this wave length is due to the "basic" substance (spot no. 2, Table VII).

An aliquot of fraction IV which was prepared in the dry state by lyophilization was also used to prepare a nujol mull on sodium chloride windows. The absorption spectrum was determined using the Perkin-Elmer spectrophotometer model 21 in the region from 2 u. to about 15 u. A photographic reproduction of the resulting spectrum is presented as Figure 2. Although the spectrum in itself does not appear to present too much information it was felt that it might be of value for comparative purposes and for correlation with additional chemical studies which might be conducted in the future. The spectrum would seem to be in agreement with the peptide structure postulated for fraction IV inasmuch as it appears to contain the typical band at 3.05 u. and also the amide I and amide II band at about 6.1 and 6.5 u. respectively. A number of other weak absorption bands can be observed, which if examined by an experienced spectroscopist might help to corroborate the purposed amino acid composition of fraction IV.

FIGURE 2. THE ABSORPTION SPECTRUM OF FRACTION IV IN THE INFRARED REGION





CHAPTER V

CONCLUSIONS

By referring to the assay results for the various samples (see Appendix) it can be seen that nearly all of the samples tested were toxic to the mice as judged by the weight loss of the treated mice as compared to the controls and also by the number of deaths. It would also appear that the toxicity of fraction IV (samples L400 and L406) was approximately the same as the toxicity of fraction III (see Table II and the Appendix). However, this might not actually be the case since the maximum tolerated dose was given in each case.

If the toxicity is compared to that of azaserine and amethopterin, two of the most potent inhibitors of sarcoma 180, (25) a comparable toxicity can be seen. Azaserine at a dose of 5 mg./kg./day resulted in a weight change of (-4.0/-0.5) and in a second test of (-4.5/-1.5). One or two deaths occurred during the first test. Amethopterin at a dose of 1.5 mg./kg./day resulted in a weight change of (-2.5/-1.0) and in a weight change of (-3.0/-0.5) at a dose of 2.0 mg./kg./day. In the second test one or two deaths occurred. At these dosages both of these compounds exhibit a greater inhibition than was exhibited by the fractions prepared in this work. The tumor diameters in the two tests for azaserine were (0.30/0.96) and (0.27/1.22). For amethopterin they were (0.45/0.89) and (0.22/0.92).

It would therefore seem desirable to attempt to selectively hydrolyze the peptide structure (fraction IV) to obtain a biologically active residue. Ritchie (23) attempted to detoxify one of his preparations by treatment with trypsin. Although the results were inconclusive further study on a more purified fraction (fraction IV) should be undertaken.

In the course of this work a considerable purification of one active principle (fraction IV) has been described and evidence presented to indicate that this fraction contains a cyclic peptide composed of ten amino acids and a "basic" substance. The purification of a second active fraction (fraction V) was also described, however, attempts to obtain a solid product containing activity from this fraction were unsuccessful. No studies as to the chemical composition of fraction V were carried out.

It would seem desirable at this point to study the sedimentation behavior of both fraction IV and fraction V, especially fraction IV, in the ultracentrifuge. Such a study would be expected to supply information as to the purity of these fractions and might also be used to obtain a molecular weight measurement.

All of the assay work with sarcoma 180 was carried out on a routine basis. It would be interesting to investigate the tumor inhibition somewhat further and also to screen the activity against other tumors.

SUMMARY

1. A method for the preparation of two tumor inhibiting fractions from the mushroom Boletus edulis was described.

2. Evidence indicating that one of the tumor inhibitors is a cyclic peptide containing 10 amino acids and a "basic" substance was presented.

3. Numerous suggestions for further study were presented.

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APPENDIX

APPENDIX

ASSAY RESULTS OF THE VARIOUS SAMPLES TESTED AGAINST SARCOMA 180

Sample Number	Tumor Diameter Treated/Control	Effect	Mouse Toxicity	Dose (ml.) Twice Daily	Weight Change Treated/Control	Deaths
L101		?	3.0	0.8		5
L108		±	1.0	(1:10)[a] 1.0-0.8	-5/-2	
L111		?	3.0	0.25		3
L113		±	1.0	(1:10) 1.0	-1.5/-1.5	
L121		±	3.0	0.5-0.3	-3/-1	2
L122		?	>5.0	0.8-0.4		2
L128		±	3.0	0.3-0.2	-3.0/-0.5	
L129		±	5.0	0.6-0.5	-4.0/-0.5	
L135		-	1.0	(1:2.5) 0.5	-4.0/-2.0	1
L135*	0.63/1.03	±		(1:10) 0.5	-4.0/-0.5	1
L135*	0.66/1.03	±		(1:2.5) 0.5-0.4	-3.0/-0.5	1

* Retest

[a] Values in parenthesis indicate dilution of original sample.

Continued next page

APPENDIX - Continued

Sample Number	Tumor Diameter Treated/Control	Effect	Mouse Toxicity	Dose (ml.) Twice Daily	Weight Change Treated/Control	Deaths
L136		±	1.0	(1:2.5) 0.5	-4/-2	
L141	0.70/1.12	±	1.0	(1:2.5) 0.5	-4.0/-1.5	2
L142	0.47/1.12	±	3.0	0.3-0.2	-5.0/-1.5	1
L146	0.80/1.13	±	0.5	(1:10) 0.6	-5.0/-2.5	2
L148	1.09/1.13	-	>5.0	0.8	-2.0/-2.5	
L150	1.00/1.13	-	>5.0	0.8	-2.5/-2.5	
L155	1.22/1.39	-	>5.0	0.8	-1.0/-1.5	
L157	0.86/1.39	±	5.0	0.5-0.25	-3.0/-1.5	
L159	0.91/1.27	±	3.0	0.5-0.3	-1.5/-2.5	1
L160	1.07/1.09	-	3.0	0.5	-1.5/-2.5	
L161	1.07/1.09	-	3.0	0.5	-2.0/-2.5	
L162	1.03/1.09	-	>5.0	0.8	-2.5/-2.5	
L163	1.06/1.09	-	>5.0	0.8-0.6	-1.5/-2.5	
L175	0.75/1.09	±	3.0	0.5-0.3	-2.5/0.5	1

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APPENDIX - Continued

Sample Number	Tumor Diameter Treated/Control	Effect	Mouse Toxicity	Dose (ml.) Twice Daily	Weight Change Treated/Control	Deaths
L176	0.71/1.09	±	3.0	0.5-0.4	-3.0/-0.5	
L177	0.62/1.09	±	>5.0	1.0-0.6	-3.5/-0.5	
L178	0.88/1.09	-	>5.0	1.0-0.6	-1.5/0.5	
L179	0.67/1.09	±	>5.0	1.0-0.6	-3.5/0.5	
L195	0.62/1.13	±	>5.0		-3.5/-2.0	
L233	1.04/1.00	-	5.0	0.5	-1.0/-1.5	
L234	1.09/1.00	-	5.0	0.5	-0.5/-1.5	
L235	0.99/1.00	-	>5.0	0.8	-1.0/-1.5	
L236	0.93/1.00	-	5.0	0.8	-1.0/-1.5	
L237	0.87/1.00	-	>5.0	0.8	-0.5/-1.5	
L238	0.53/1.00	±	>5.0	0.8	-3.5/-1.5	1
L239	1.00/1.00	-	>5.0	0.8	-1.5/-1.5	
L240	0.79/1.00	-	>5.0	1.0	-2.0/-1.5	
L241	0.72/1.00	±	5.0	0.5-0.4	-2.5/-1.5	1
L249	0.59/1.03	±		0.8	-2.5/-1.0	

Continued next page

APPENDIX - Continued

Sample Number	Tumor Diameter Treated/Control	Effect	Mouse Toxicity	Dose (ml.) Twice Daily	Weight Change Treated/Control	Deaths
L292		?		0.5-0.4		3
L292*	0.49/0.98	±+		(1:2) 0.5	-2.5/-1.5	
L293		?		0.5-0.25		3
L293*		±+		0.2-0.05	-4.6/-4.0	2
L308	0.58/1.05	±-		0.3	-3.5/-2.0	
L317	0.79/1.05	±-		0.3	-1.0/-0.5	
L319	1.12/1.08	-	1.0	(1:2.5) 0.5	-1.0/1.5	
L320	1.10/1.08	-	1.0	(1:2.5) 0.5	-1.0/-1.5	
L322		?	3.0	0.3		5
L322*	1.06/1.19	-		(1:3) 0.3	-2.0/-0.5	
L337	0.56/1.21	±+	>5.0	0.5-0.3	-0.5/-0.5	2
L338	0.83/1.21	±-	>5.0	0.5-0.3	-1.5/-0.5	1
L346	1.00/1.33	±-	5.0	0.5	1.0/0.5	

* Retest

Continued next page

APPENDIX - Continued

Sample Number	Tumor Diameter Treated/Control	Effect	Mouse Toxicity	Dose (ml.) Twice Daily	Weight Change Treated/Control	Deaths
L347	1.01/1.33	-	5.0	0.5	1.0/0.5	
L380	1.13/1.11	-	5.0	0.6	-1.0/-1.5	
L381	0.56/1.11	+-	0.5	(1:10) 0.5	-3.5/-1.5	1
L388	0.36/0.89	+-	0.5	(1:10) 0.5	-3.0/-1.0	
L388*	0.51/1.02	+-		(1:20) 0.5-0.4	-4.0/0	
L389	1.02/0.89	-	1.0	(1:5) 0.5	0.5/-1.0	
L390	0.72/1.07	+-	5.0	0.8-0.5	-2.5/0.5	1
L391	0.50/1.07	+-	1.0	(1:5) 0.5-0.25	-5.0/0.5	2
L392	1.20/1.07	-	>5.0	0.8	-0.5/0.5	
L393	0.59/0.94	+-		0.3	-4.0/-1.0	1
L400		?	>5.0	0.6		4
L400*	0.75/1.00	+-		(1:2) 0.5-0.4	-3.0/0	1
L400*	0.61/1.11	+-		(1:2) 0.4-0.15	-3.5/-1.0	2

* Retest

Continued next page

APPENDIX - Concluded

Sample Number	Tumor Diameter Treated/Control	Effect	Mouse Toxicity	Dose (ml.) Twice Daily	Weight Change Treated/Control	Deaths
L403	0.61/0.68	-	>5.0	0.8	-2.0/-1.0	
L406	0.54/1.06	± -	>5.0	0.8-0.6	-4.5/-1.0	1
L407	1.05/1.06	-	>5.0	0.8	-1.5/-1.0	
L410	0.41/1.00	± +	0.5	(1:10) 0.6	-4.5/1.5	2
L410*	0.89/1.41	± -		(1:10) 0.5-0.3	-3.0/-1.0	1
L412	0.97/1.00	-	>5.0	0.8	0/1.5	
L415	0.73/0.98	± -	1.0	(1:5) 0.5-0.2	-1.0/1.5	
L419	0.59/1.03	± -	1.0	(1:2.5) 0.5	-1.5/2.5	
L421	0.40/1.03	± +	3.0	0.5-0.3	-5.5/2.5	1
L428		?	1.0	0.3-0.2		4
L428*		?		(1:5) 0.5		3
L440	No result					

* Retest

PART II

THE METABOLIC INCORPORATION OF FORMALDEHYDE INTO THE NICOTINE N-METHYL GROUP IN *NICOTIANA RUSTICA*

INTRODUCTION

Transmethylation, the intermolecular transfer of methyl groups, was demonstrated in animals by du Vigneaud et al. (1). Transmethylation was demonstrated to occur in higher plants by Byerrum et al. (2,3,4).

A number of compounds have been shown to be methyl group precursors in various plants in this laboratory. Brown and Byerrum (2), using methionine labeled with C-14 in the methyl group, showed that this compound gave rise to nicotine labeled in the methyl group in tobacco plant metabolism. They also demonstrated that the carbon atom of formate could serve as a precursor of the methyl group of nicotine. However, using C-14 labeled formate, the formate was incorporated into the nicotine methyl group only to about one-tenth the extent of that resulting from the methionine methyl group. The methyl group of methionine and the carbon atom of formate were also shown to be precursors for the methoxyl group of lignin in barley plants (4). Sato (5) demonstrated that the methyl group of methionine could give rise to the methoxyl group (methyl ester) of pectin in radish plants. Sato (5) also demonstrated the incorporation of the methyl groups of glycine betaine into the nicotine N-methyl group to an extent approximately equal to the incorporation of the methyl group of methionine. Byerrum and Ming (6) showed that the methyl groups of choline can give rise to the nicotine methyl group to about the same extent as formate. Byerrum et al. (7) have also shown that the alpha carbon of glycine can give rise to the N-methyl carbon of

nicotine to a slightly greater extent than the methyl group of methionine. In this study (7) the carboxyl carbon of glycine did not give rise to the nicotine N-methyl carbon. Dewey (8) fed calcium glycolate-2-C^{14} to tobacco plants and observed a labeling of the nicotine in the methyl group to about the same extent as observed in the earlier study (2) when methionine was administered to the tobacco plants. Byerrum et al. (9) have also demonstrated that DL-serine-3-C^{14} can give rise to nicotine labeled in the N-methyl group to about the same extent as that which results from the methyl group of methionine.

Various other investigators have studied comparable metabolic reactions in other plants. Kirkwood et al. (10,11) were unable to demonstrate the incorporation of the methyl carbon atoms of choline into the methyl carbon atoms of the alkaloids hordenine of barley and ricinine of caster beans, although methionine resulted in the labeling of both alkaloids. Sribney and Kirkwood (12) were able to demonstrate that glycine betaine can donate methyl groups to the alkaloids N-methyl tryamine and hordenine of barley plants.

The above studies, especially those in tobacco plants, indicate the occurrence of an over-all reductive metabolic reaction for the synthesis of methyl groups in addition to the reaction resulting in the formation of methylated compounds by transmethylation. It was therefore of interest to study the intermediary metabolism of the reductive reaction.

The alpha carbon of glycine was not converted to the methyl carbon of nicotine to a major extent via the carbon atom of formate since the

latter is incorporated into nicotine to a much lesser extent than the alpha carbon of glycine. Similarly the alpha carbon of glycine was not converted to the nicotine methyl group to a major extent via the methyl groups of choline, betaine or methionine or via the alpha carbon of glycolate since these carbon atoms are incorporated into the nicotine methyl group at an equal or lower rate than is the alpha carbon of glycine.

In the animal serine-3-C^{14} has been shown to give rise to the methyl group of methionine to the same or a somewhat greater extent than the carbon atom of formate (13) while the alpha carbon of glycine was incorporated only to about one-sixth the extent of the beta carbon of serine. Stekol et al. (14) demonstrated that the beta carbon of serine enters the methyl groups of choline in greater amounts than does either formate or the alpha carbon of glycine. Formaldehyde and formate appear to enter methyl groups to a similar extent in animal metabolism (15,16).

In animal metabolism it has been postulated that the alpha carbon of glycine may enter methyl groups through serine (13,17). In tobacco plants, however, this does not appear to be the case since the beta carbon of DL-serine was incorporated into the methyl group of nicotine to an equal or somewhat lesser extent than was the alpha carbon of glycine.

It was therefore considered possible that the beta carbon of serine and the alpha carbon of glycine might give rise to a 1-carbon unit at the oxidation state of formaldehyde. With this postulate in mind formaldehyde-C^{14} was fed to tobacco plants and the extent of incorporation of C-14 into the nicotine methyl group determined. If the hypothesis

were correct the carbon atom of formaldehyde would be expected to be incorporated into the nicotine methyl carbon to a greater extent than either the beta carbon of serine or the alpha carbon of glycine. The present study tends to indicate that such a hypothesis might be correct.

EXPERIMENTAL

Previous studies in this laboratory (2-9,27) have shown that tobacco plants can absorb various organic compounds through their root systems from a nutrient solution. The hydroponic procedure of administering the formaldehyde-C^{14} was also used in this study since it was desired to make a valid comparison with the earlier work.

Preparation of Plants

The tobacco plants used in these experiments were of a high nicotine strain, Nicotiana rustica L., var. humilis. The seeds were planted in flats containing vermiculite* and transplanted after a two to three week period. The plants were watered twice weekly with a nutrient solution composed of 1 g. $MgSO_4 \cdot 7H_2O$, 1 g. K_2HPO_4, 5.8 g. $Ca(NO_3)_2 \cdot 4H_2O$ and 4 l. of tap water. On the remaining days of the week they were watered with ordinary tap water.

The plants were grown in the flats in the greenhouse for about three months. Although the extent of growth varied with seasonal conditions, the plants were approximately six inches in height after this period of growth. Some budding and flowering was noted in the first experiment during the experimental growth period, which was during the summer months. However, budding and flowering were absent during the second experiment, conducted in the autumn, and during the third experiment, conducted during the early winter months.

* A commercial brand of heat expanded mica.

To prepare the plants for hydroponic administration of the formaldehyde they were first removed from the flats and the vermiculite carefully removed from the roots as completely as possible. The roots were then washed with water to free them of any remaining extraneous material. After washing the roots the plants were immersed in 50 ml. of an inorganic nutrient solution contained in a 125 ml. Erlenmeyer flask. The nutrient solution was prepared by diluting a stock nutrient solution 1:3 (v/v) with distilled water. The composition of the stock nutrient solution is given in Table I. The various weights of the inorganic salts shown in Table I refer to the anhydrous compound. One milliliter of an aqueous solution containing 0.5 mg. of aureomycin was also added to each flask to reduce the population of microorganisms.

TABLE I

COMPOSITION OF THE STOCK NUTRIENT SOLUTION

Water	1 l.	Magnesium sulfate	250 mg.
Calcium nitrate	1 g.	Ammonium sulfate	250 mg.
Potassium chloride	250 mg.	Potassium dihydrogen phosphate	250 mg.
Ferric chloride	2 mg.		

In the actual feeding experiments the formaldehyde-C^{14} was then added to the Erlenmeyer flask and a cotton plug placed in the neck of the flask around the stem to decrease the loss of formaldehyde through volatilization. The plants were allowed to grow for 7 days in a special

fume hood to avoid any health hazard that might arise due to the use of radioactive material.

Artificial lighting was used during the 7 day growth period. The source of light consisted of two 36 inch 30 watt fluorescent tubes and one 100 watt incandescent bulb. The lights were placed about 14 inches above the top of the plants and were found to produce a light intensity in the range of 200-250 foot candles at the top of the leaves. The lights were left on for 12 hours each day.

During the 7 day period water was added to the Erlenmeyer flasks as necessary to maintain the volume of solution at approximately 50 ml.

Preparation of Formaldehyde Solutions

A standard formaldehyde solution was prepared by diluting about 1 ml. of a 36 per cent formaldehyde solution to about 1 l. The resulting solution was standardized by the dimedon method as described by Yee and Reid (18). Triplicate 25 ml. aliquots of the formaldehyde solution were used. The formaldehyde solution was added to a solution containing 100 ml. of the sodium acetate-hydrochloric acid buffer pH 4.6 and 25 ml. of a saturated solution of dimedon. After standing about 18 hours with occasional shaking the dimedon derivative was filtered off on a tared sintered glass crucible and dried at 60°C. The weight of the precipitate times the factor 0.1027 gave the weight of formaldehyde contained in the solution used for the determination.

The formaldehyde-C^{14} solution (purchased from the Isotopes Specialties Co., Inc., Glendale, Calif.) was also standardized as to formaldehyde content and radioactivity by diluting an aliquot of it with the

standard formaldehyde solution mentioned above and the formaldehyde content determined by the dimedon method. The specific activity (c.p.m./mM) of the dimedon derivative of formaldehyde was determined by the method described below.

Determination of Radioactivity

All radioactivity measurements were made by counting an "infinitely thin" layer of the compound contained in an aluminum counting disc having an area of 2.83 sq. cm. The counts were made with a thin end window Geiger-Müller tube and a Nuclear Instrument and Chemical Corporation scaler. The efficiency of the tube as used was about 2 per cent. (For calculations see Appendix)

Uptake of Formaldehyde by the Plants

Before administering the formaldehyde-C^{14} to the plants it was necessary to establish that formaldehyde would be taken up by the plants from a nutrient solution and also whether it might be toxic when administered in low concentrations.

Two tobacco plants were therefore placed individually in 125 ml. Erlenmeyer flasks containing the nutrient solution and aureomycin as described previously. To each flask was added a volume of the standard formaldehyde solution equivalent to 1.7 mg. of formaldehyde. The cotton plug was inserted in the neck of the flask and the plants were grown for a two day period.

At the end of the two day period the plants were removed from the flasks and the roots washed with a stream of distilled water from a

wash bottle. The wash solution was combined with the nutrient solution remaining in the flask and the resulting solution analyzed for remaining formaldehyde using the colorimetric method involving reaction with phenylhydrazine and potassium ferricyanide described by Tanenbaum and Bricker (19). Using this method 89 per cent of the formaldehyde was found to have disappeared from the flasks.

It was considered possible that this disappearance of formaldehyde represented a reaction of the formaldehyde with the proteins of the root surfaces and not true absorption. That this was not the case was demonstrated in an experiment in which about 6 root fragments 1 cm. in length were added to each of two Erlenmeyer flasks containing the nutrient solution, aureomycin and 1.7 mg. of formaldehyde as described previously. After a two day period no loss of formaldehyde was detected. In a control experiment in which the formaldehyde was incubated with only the nutrient solution and the aureomycin no detectable loss of formaldehyde due to volatilization was observed.

That the formaldehyde was metabolized by the plant was demonstrated by the observation that radioactivity spread rapidly throughout the plant when formaldehyde-C^{14} was used during the actual feeding experiments. The concentration of formaldehyde used had no adverse effect on the plants as judged by normal growth and appearance during the 7 day growth period.

Administration of Radioactive Formaldehyde

The molar quantity of formaldehyde used per plant was calculated to be 1.3×10^{-5} moles in all three experiments. This molar quantity was

used so that a comparison could be made with the earlier experiments carried out in this laboratory. In experiments 1 and 2 the 1.34×10^{-6} moles of formaldehyde contained 2.39×10^{6} c.p.m. In experiment 3 the radioactivity was 1×10^{6} c.p.m. Thirty plants were used for each experiment.

Isolation and Purification of Nicotine

After the 7 day growing period the plants were removed from the flasks and the roots washed with distilled water. The excess water was blotted off with cheese cloth. The plants were then cut into small pieces with scissors and dried under infrared lamps as rapidly as possible. The temperature was kept at 80°C. for an hour near the end of the drying period.

The dried material was finely ground in a mortar with 20 per cent of its weight of calcium hydroxide. The resulting mixture was steam distilled until the distillate gave no precipitate with silicotungstic acid, indicating that the alkaloids were no longer present in the distillate. The distillate was collected in 5 ml. of 6 N hydrochloric acid and concentrated *in vacuo* on the revolving concentrator described by Craig *et al.* (20). The resulting residue was azeotropically distilled from an alkaline medium, the distillate being collected in the hydrochloric acid solution. The concentration followed by azeotropic distillation was repeated a second time to purify the nicotine (21). The distillate from the second azeotropic distillation was collected in hydrochloric acid and concentrated to dryness and desiccated overnight.

The resulting nicotine hydrochloride was dissolved in a small amount of water (about 1 ml.) and a saturated methanolic solution of picric acid added in excess. After standing a short time (about 0.5 hr.) the precipitate of nicotine dipicrate which forms was removed by filtration on a sintered glass funnel and washed with methanol. The dipicrate was then recrystallized from hot water and desiccated over calcium chloride.

The dried nicotine dipicrate was plated on aluminum discs and its radioactivity determined as described previously.

The radioactivity observed to be present in the dipicrate in the three experiments is shown in Table II. Since in the previous studies in this laboratory a radioactivity of 1×10^5 c.p.m. per plant was used, the radioactivity expressed in Table II for experiments 1 and 2 was corrected to correspond to the previous studies by dividing the radioactivity by the factor 2.39. In experiment 3 a radioactivity of 1×10^5 c.p.m. was fed to each plant therefore no correction was necessary. It should be noted that although a different radioactivity was used in experiments 1 and 2, the same molar quantity of formaldehyde was used in all three experiments.

Demethylation

Since the nicotine dipicrate was found to be radioactive it was desired to determine the extent of radioactivity contained in the nicotine N-methyl group as a result of feeding the formaldehyde-C^{14}.

Since the nicotine dipicrate was found to be quite insoluble and unsuited for demethylation (2) the nicotine was recovered from the dipicrate. About 200 mg. of the nicotine dipicrate was dissolved in sodium hydroxide solution and the nicotine isolated by azeotropic distillation through a Widmer column. The acidified distillate was concentrated to dryness *in vacuo*, the concentration being completed in the flask from the demethylation apparatus.

The demethylation procedure followed was essentially that of Pregl (22) as modified by Simmonds *et al.* (23) and Brown and Byerrum (2). The apparatus described by Brown and Byerrum (2) was used. Using this procedure the methyl group is isolated as methyltriethylammonium iodide, a white crystalline compound suitable for counting.

The reaction flask containing the nicotine hydrochloride, recovered from the dipicrate, was attached to the remainder of the demethylation apparatus and the following compounds added based on 50 mg. of nicotine: 45 mg. of ammonium iodide, two drops of a 5 per cent solution of gold chloride and 3 ml. of hydroiodic acid. The gas washing apparatus contained 0.75 ml. of a 5 per cent solution of cadmium sulfate and 0.75 ml. of a 5 per cent solution of sodium thiosulfate to remove iodine and hydrogen iodide. The receiver contained a 5 per cent ethanolic solution of triethylamine cooled in a solid carbon dioxide-methyl cellosolve bath. A stream of nitrogen was passed through the reaction train during the entire demethylation.

The reaction flask was placed in a copper oxide bath and heated to 200°C. in 20-25 minutes. The temperature was then slowly raised to

350-360°C. and maintained at that temperature for 45 minutes. After the heating period the apparatus was allowed to cool. The stream of nitrogen was continued during the cooling period. The delivery tube was then rinsed with ethanol into the receiver which was then stoppered, shaken and allowed to stand overnight at room temperature. The next morning the major portion of the ethanol and excess triethylamine were evaporated over an infrared lamp. The last of the ethanol and triethylamine were removed in a vacuum desiccator. The methyltriethylammonium iodide recovered was a white crystalline compound.

The quarternary compound was dissolved in a small amount of ethanol and plated on tared aluminum counting discs. The ethanol was evaporated over an infrared lamp and the disc reweighed to obtain the sample weight. The discs were counted as described previously. The results are expressed as counts per minute per millimole (c.p.m.) in Table II. The observed radioactivity in the quarternary compound was divided by the factor 2.39 to correct for the larger radioactivity used in experiment 2.

RESULTS

TABLE II

INCORPORATION OF FORMALDEHYDE INTO THE N-METHYL GROUP OF NICOTINE

Experiment Number	Maximum Specific Activity (counts per minute per millimole)		Per cent Radioactivity Recovered in Methyl Group
	Nicotine Dipicrate	Methyltriethyl-ammonium iodide	
1	1.64×10^4	- - -	- - -
2	2.39×10^4	2.34×10^4	98
3	0.87×10^4	0.80×10^4	92

These results show that the carbon atom of formaldehyde is incorporated into the nicotine N-methyl group. They also show that, within the limits of experimental error, none of the formaldehyde was utilized for the bio-synthesis of the nicotine ring system under the conditions of this experiment.

DISCUSSION

The reason for the variation in radioactivity of the nicotine isolated in the three experiments is unknown. Since the experiments were conducted during different seasons of the year it is considered possible that the variation is explained by a seasonal variation in growth and metabolism. A comparable variation was also observed in the previous studies conducted in this laboratory.

Comparing the results shown in Table II to the previous studies conducted in this laboratory, formaldehyde appears to enter the nicotine N-methyl group in about 3-4 times the quantity of the beta carbon of DL-serine. The alpha carbon of glycine was incorporated to about twice the extent of the methyl carbon of methionine and only slightly greater than the beta carbon of serine. Methionine was incorporated to about the same extent as glycolate which was about 10 times the extent of formate. From this comparison it is obvious that formaldehyde was incorporated to the greatest extent of any of the precursors studied.

It would therefore appear that in the synthesis of the nicotine N-methyl group neither formaldehyde nor the alpha carbon of glycine is metabolized to a major extent by way of the beta carbon of serine. In animal metabolism it has been postulated that the alpha carbon of glycine can enter methyl groups through serine (13,17).

Based on studies in this laboratory, a more reasonable suggestion would be that the reverse reaction occurs with serine giving rise to

glycine plus a 1-carbon unit closely related to formaldehyde. The possibility of such a reaction is in line with the observation by Elwyn et al. (24) that serine is not oxidized to formate in the formation of methyl groups in the rat.

The incorporation of the alpha carbon of glycine into the methyl group of nicotine to a lesser extent than formaldehyde might suggest at least three possible mechanisms. The glycine might be hydrolytically deaminated to give glycolate which could then split to form two 1-carbon units. The 1-carbon unit arising from the carboxyl group might be expected to be at the oxidation state of carbon dioxide and the 1-carbon unit arising from the alpha carbon at the oxidation state of formaldehyde. Such a pathway would be in line with the observation that the carboxyl carbon of glycine did not enter the methyl group of nicotine (7) since there is no indication that carbon dioxide can be reduced to any one carbon compound (15,25,26). However, if such a pathway, from glycine to glycolate, does exist glycolate would be expected to be incorporated into the nicotine N-methyl group to a greater extent than is the alpha carbon of glycine. Since the reverse order of incorporation was observed, glycine is apparently not hydrolytically deaminated to produce glycolate in the metabolic pathway from the alpha carbon of glycine to the methyl group of nicotine.

A second possible pathway from glycine to the nicotine N-methyl group might be through glyoxylate. Such a reaction pathway would involve oxidative deamination of the glycine followed by cleavage into two 1-carbon units, one at the oxidation state of carbon dioxide and a

second at the oxidation state of formate (27). Such a pathway would also seem to be unlikely since formate is incorporated into the methyl group of nicotine to a much lesser extent than is the alpha carbon of glycine.

The third possibility for the conversion of the alpha carbon of glycine to the nicotine N-methyl group might involve reaction of the glycine as such, e.g. with the nitrogen and alpha carbon intact. Such a possibility has been suggested by Hamill (28). If such a pathway does exist it would suggest that the incorporation of the alpha carbon of glycine and the carbon atom of formaldehyde take place by a different pathway. Such a possibility might be investigated by using glycine labeled with N-15 in the amino group and C-14 in the alpha carbon. If the N-15 to C-14 ratio remains the same in the isolated nicotine the possibility of such a mechanism would seem to exist.

In conclusion, if the present work is compared to the previous studies it would seem to indicate that the beta carbon of serine, the alpha carbon of glycine and the alpha carbon of glycolate can give rise to a 1-carbon unit which would appear to be closely related to formaldehyde. Such an "active formaldehyde" has been postulated by Berg (29) and also by Kisliuk and Sakami (30). More recently Kisliuk and Sakami (31) have presented the results of an additional study which would seem to indicate the existence of an "active formaldehyde" in animal metabolism which appears to result from the combination of formaldehyde and tetrahydrofolic acid. If such a compound does exist in the metabolism of higher plants it might explain the failure of radioactive

formaldehyde being detected after giving plants radioactive carbon dioxide (32), since such an observation would tend to indicate that formaldehyde does not exist free, as such, in the plant. Obviously a final decision as to the nature of the "active formaldehyde", if such a compound does exist, in tobacco plants must await the result of additional study.

SUMMARY

1. Formaldehyde-C^{14} was administered to a high nicotine strain of tobacco, Nicotiana rustica L., var. humilis. The nicotine isolated from the tobacco plants was found to possess radioactivity. Demethylation experiments showed that within the limits of experimental error all of the activity was located in the methyl group.

2. The results show that the carbon atom of formaldehyde is incorporated into the nicotine N-methyl group to the greatest extent of any of the methyl group precursors studied in this laboratory.

3. A comparison and a discussion of the results in terms of the previous studies is given.

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APPENDIX

APPENDIX

The formula used in correcting the observed count to zero sample thickness was:

$$A_m = \frac{C_o \cdot M}{W \cdot b}$$

where A_m = maximum specific activity (counts/minute/millimole)

C_o = observed count (counts/minute)

M = molecular weight of compound

W = weight of sample counted

b = fraction of maximum activity at the sample thickness used (T)--obtained from self-absorption curve.

Sample calculation:

Nicotine dipicrate -- C_o = 1102 c.p.m.
W = 62.8 mg.
M = 620
T = 22.2 mg./cm.^{2}
b = 0.285

$$A_m = \frac{1102 \times 620}{62.8 \times 0.285} = 3.82 \times 10^4 \text{ c.p.m./mM}$$