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Many substances in the plant kingdom and in man's diet occur as glycosides. Recent studies have indicated that many glycosides that are not mutagenic in tests such as the Salmonella/microsome test become mutagenic upon hydrolysis of the glycosidic linkages. The Salmonella/microsome test utilizes a liver homogenate to approximate mammalian metabolism but does not provide a source of the enzymes present in intestinal bacterial flora that hydrolyze the wide variety of glycosides present in nature. This investigation was designed to study the effect of stable cell-free extracts from enteric bacteria of human feces, fecalase, which was shown to contain glycosidases which bioactivate in vitro many natural diet glycosides to compounds which are mutagenic in the Salmonella/liver homogenate test. Cantaloupe (Cucumis melo); Raspberry (Rubus idaeus), Red and Yellow Onion (Allium cepa) varieties all

contain quercetin which presumably forms mutagenic flavonol glycosides in the gut. The White Onion does not contain quercetin. Flavonol extract of Cantaloupe, Raspberry, Red and Yellow varieties of onion were mutagenic in the test when fecalase was added. Frameshift mutagenicity (TA 1537, TA 98, and TA 97) among the flavonoid extracts tests was mainly confined to the flavonols (flavon-3-ols). The base-pair mutants (TA 1535, TA 100) did not show mutagenic activity upon testing the flavonoid extracts of the samples investigated in this study. Since the flavonols are probably the single largest group of flavonoids, and the mutagenic agent detected, quercetin, is the most common flavonol aglycone, plant breeding has been suggested to reduce the amount of flavonoids present in the food we eat.

Frameshift Mutagenicity of Flavonol Glycosides Activated by Human Fecalase Enzyme

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◆◆ *DEDICATION* ◆◆

To whom I belong and love .....

MY PARENTS, Mohamed and Asha

MY WIFE, Duria

MY CHILDREN, Julinar and Mohamed

MY BROTHERS AND SISTERS

MY COUNTRY, Sudan.

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LITERATURE REVIEW

Introduction

Ordinary food has not been examined very thoroughly for mutagens/carcinogens, principally because of the belief that since they have been eaten for a long time they must be safe, and also due to the lack of reasonable cheap, short-term method to detect them. Consumers are also ignorant of the fact that many natural substances possess mutagenic/carcinogenic activity. In recent years it has become increasingly apparent that environmental factors play a dominant role in human mutagenesis/carcinogensis. Boyland [1967], Higginson et al. [1973], and Wynder et al. [1977] claim that 90 percent of cancer incidences are induced by elements originating in man's environment rather than as a result of purely genetic or viral factors. The other recognized causes of human cancer aside from certain predisposing genetic factors [Mulvihill, 1975] are radiation [Jablon, 1975] and chemicals. Among the latter are several well-documented examples from occupational sources including coal tar, benzene, auramine, vinyl chloride, bis (chloromethyl) ether, chromium, nickel, and asbestos [Bartsch et al., 1975; Cole et al., 1975; Dean, 1978]. The World Health Organization reported in 1975 that tobacco smoking is also a well-recognized

etiologic factor in cancer of the lungs, buccal cavity, pharynx, and larynx. Other environmental factors such as alcohol consumption [Rothman, 1975], air pollution [Pike et al., 1975], viruses and other microorganisms [Heath et al., 1975], chlorinated water [Simmon et al., 1977; Glatz et al., 1977], food additives [Boffrey, 1976; Brown et al., 1977], drugs [Fraumeni et al., 1972], are receiving continued attention with respect to human carcinogens.

Food, apart from the effects of dietary deficiencies or excess, has only relatively recently received increased attention as a source of cancer risk [Brown, 1980]. Lownfels et al. [1977], Miller [1973], and Munro [1976] showed man's food can contain a wide variety of naturally occurring carcinogens, but it has been particularly difficult to associate specific dietary carcinogens with a specific kind of cancer. Kassira et al. [1976] concluded cancers appear to be associated with certain dietary patterns rather than specific carcinogens.

However, the causes of most human cancers are unknown, Sugimura et al. [1977] pointed out that the studies on Japanese immigrants in the United States have clearly demonstrated that human stomach cancer is probably related to diet, and the same may be true for colon and pancreatic cancer. Ames [1979] claimed that natural chemicals present in the human diet as complex mixtures appear to be the major cause of DNA damage resulting in cancer and genetic birth defects.

Mutagenicity-Carcinogenicity Overlap

Humans are being exposed to a wide variety of environmental chemicals that are mutagens/carcinogens [Hiatt et al., 1977; McCann et al., 1977; Blum et al., 1978; Ames et al., 1977]. A number of rapid in vitro systems for detecting these chemicals have been developed [Hollstein et al., 1979; Hiatt et al., 1977; McCann et al., 1977], such as the Salmonella/Mammalian Microsome Mutagenicity Test [Ames, 1975]. A large number of investigators showed the Salmonella test to be about 90 percent accurate in detecting a wide variety of carcinogens as mutagens [McCann et al., 1975; McCann, 1976; Ames, 1979; Sugimura et al., 1977; Purchase et al., 1976; Yamasaki, 1977]. The overlapping of carcinogens and mutagens has been emphasized by Ames et al. [1976], McCann et al. [1976], Purchase et al. [1976], Nagao et al. [1978], Sugimura et al. [1976], and Yahagi et al. [1978]. Also, Sugimura et al. [1977] graphically showed the relationship between carcinogenicity and mutagenicity (see Fig. 1).

Mortelmans [1978] and Commqner [1978] indicated the Salmonella/Mammalian-Microsome as the most widely used short-term test for detection of mutagens. The test takes about three days to complete [Ames et al., 1975] and uses specially constructed strains of Salmonella typhimurium for detection of frameshift or base-pair substitution reverse mutations [Ames et al., 1973; McCann et al., 1975]. Rodent, preferably rat, (or human) tissue homogenates (S-9 mix) are used to metabolize chemicals to their active mutagenic

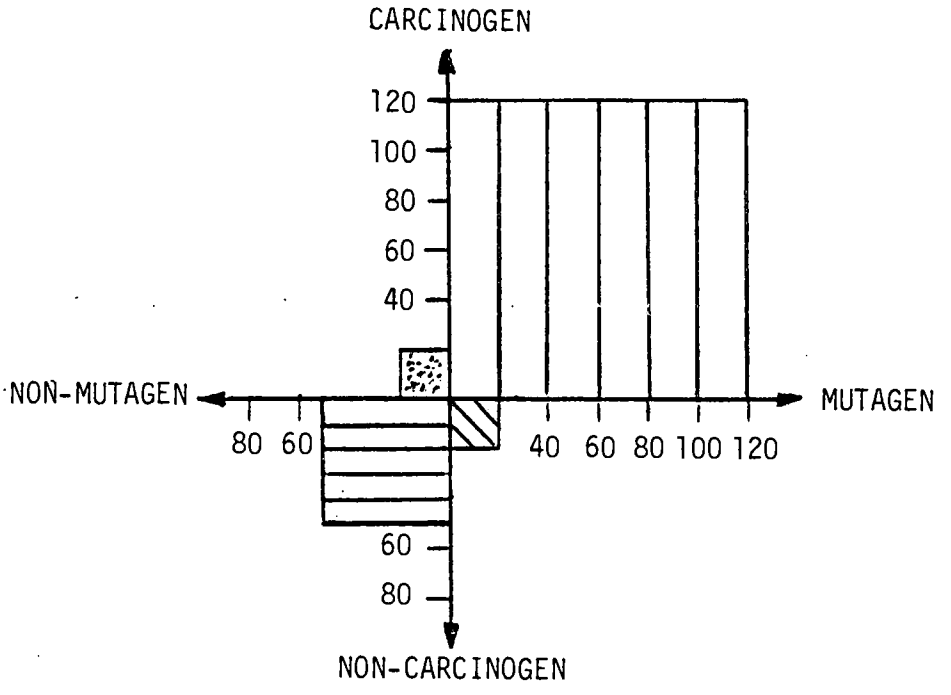


FIGURE (1). Correlation between Carcinogens and Mutagens. Abcissa indicates number of mutagens and non-mutagens; ordinate indicates number of carcinogens and non-cardinogens.

Source: Sugimura et al., 1977.

forms [Ames et al., 1973; Muller et al., 1980].

Pioneering work by P. E. Hartman and others in the genetic mapping of the enteric bacterium Salmonella typhimurium made possible the selection by B. N. Ames and colleagues, of a set of point mutations in the his operon that demonstrated a low rate of spontaneous mutation and the ability to be reverted to the wild type (his^+) upon exposure to known carcinogens [Ames et al., 1973].

In early work with E. coli, Benzer [1961] demonstrated certain chemicals elicited somatic mutations; N-methylnitrosoquanidine and other compounds induced base-pair substitutions, while others such as 9-aminoacridine induced addition or deletion mutations (also called frameshift mutations). Using S. typhimurium Strain LT-2, Ames developed three tester strains. Strain TA 1535 is a mutant in the his G gene coding for phosphoribosyl ATP synthetase, an enzyme necessary for histidine synthesis. The mutation designated his G46 confers histidine auxotrophy to the organism and is susceptible to base-pair mutations. These mutations revert TA 1535 to the wild phenotype, enabling their enumeration on histidine-free microbiological medium [Ames et al., 1975]. Strain TA 100 was developed by transferring a resistance transfer factor (R-factor) to the tester Strain TA 1535. Strains TA 1537 and TA 1538 detect various kinds of frameshift mutagens. Frameshift mutations are so named since they shift the "reading frame" of the translation template when the addition of base-pairs not divisible by three occurs in a specific location within the gene. Organisms especially susceptible to frameshift mutations frequently possess a sequence of identical bases or repetitive sequence of identical bases in mutable gene [Okada et al., 1972]. Strain TA 1537 contains a run of cytidine residues in the sequence coding for histidine aminotransferase [Ames et al., 1973], a mutation designated his C3076. Similarly, Strain TA 1538 has a repetitive GC sequence in the structural gene for histidine dehydrogenase [Isono and

Young, 1974], the mutation being termed his D3052. Strain TA 98 was developed by transferring a resistance transfer factor (R-factor) to the tester Strain TA 1538. Strain TA 97 has a sequence of A-C-A-C-C-C-C-C-C in the D gene of the histidine operon. The mutation was designated his D1242 his D6610.

Chemicals are generally tested on five strains (TA 1537, TA 97, TA 98, TA 1535, and TA 100), both with and without S-9 mix. Each tester strain contains a mutation in one of several genes governing the synthesis of the amino acid histidine, and cannot grow unless histidine is added to the growth medium. Different doses of the compound to be tested are combined directly on a Petri dish along with a bacterial tester strain and the S-9 mix. A trace of histidine, which is not enough to permit colonies to form, but which allows sufficient growth (one or two generations) for expression of mutation is added. About 10^9 bacteria reverted back to an ability to grow without added histidine are measured by counting the revertant colonies on the plate after two days incubation at 37°C. Quantative dose-response curves are obtained that, with a few exceptions [McCann et al., 1977] are linear. The method is extremely sensitive, and usually micrograms and in some cases even nanograms of mutagens can be detected. Results are almost always clear-cut, with a number of mutagens-induced revertant colonies usually two or more orders of magnitude greater than the relatively low spontaneous incidence of revertants.

Therefore, sensitivity and versatility of the Salmonella/

Ames test is due to several factors [Ames et al., 1973; Ames et al., 1980; Muller et al., 1980; Ames and McCann, 1976; McCann and Ames, 1977; Levin et al., 1982]:

1. The tester strains contain unique types of DNA damage at the sites of the mutations in the histidine operon; a base-pair substitution mutation in strains TA 1535 and TA 100, and two different frameshift mutations in strains TA 1537, TA 97, TA 1538, and in Strain TA 98. The frameshift mutations are long strings of repetitive bases (C-G-C-G-C-G-C-G-, or -C-C-C-C) that are "hot spots" for mutagenesis by certain classes of mutagens.

2. The tester strains contain a mutation deleting the μvrB gene, which causes loss of the DNA excision repair system that normally removes μv-induced thymine dimers from S. typhimurium and greatly increases the sensitivity of the test for detection of many mutagens.

3. Two of the tester strains (TA 100 and TA 98) were developed at Ames Laboratory of University of California at Berkeley, by transferring a resistance transfer factor, pkM101 (R-factor) to strains TA 1535 and TA 1538, respectively. The insertion of the plasmid, pkM101, reportedly carries an error-prone DNA repair system [McCann et al., 1975]. Error-prone DNA repair is greatly enhanced in these strains [McCann et al., 1974, 1975; Walker, 1978], and the sensitivity of the strains to

reversion with a variety of potent carcinogens that were previously weakly detected, such as Aflatoxin B_1 (AFB_1), sterigmatocystin, furyfuramide, benzo (α) pyrene, and methylmethane sulfonate [Ames et al., 1975] is greatly increased. The new Salmonella tester Strain TA 97 was developed for use in the Salmonella/Microsome Mutagenicity test by Levin et al. [1982]. The DNA sequence has shown that this strain contains an added cytosine, resulting in a run of six cytosine at the mutated site in the histidine D gene. Its mutagenic specificity is similar to that of the frameshift mutagen tester Strain 1537, which also contains an added cytosine in a run of cytosines. The Strain TA 97 also contains a mutation deleting the μvrB gene, and carries an error-prone DNA repair system through the transference of the Ampicillin-resistant R-factor by inserting the pkM101 plasmid.

4. Finally, all of the standard tester strains contain an additional "deep-rough" mutation, designated (rfa) that causes loss of the outer lipopoly-saccharide (LPS) permeability barrier. Elimination of the polysaccharide side chain of the (LPS) cell wall that coats the bacterial surface, make the cell more permeable, and thus facilitate the diffusion of large organic molecules (e.g., polycyclic hydrocarbons, aflatoxins, and many aromatic amines) into the bacteria [Schmeltz, 1977; Ames and McCann, 1975; Kier, 1974]. The latter mutation also renders the bacterium completely nonpathogenic.

Hepatic activation system, (S-9) supplemented with the co-factors NADP and glucose-6-phosphate were first used by Ames et al. [1973] to affect activation of the promutagens AFB_1, benzo (α) pyrene and others, thereby incorporating an important aspect of metabolism into the in vitro mutagen assay. Hepatic microsomes from a variety of sources have been used, including mouse, hamster, dog, pig, monkey [Muller et al., 1980], human [Ames et al., 1973]. and trout [Ahokas et al., 1976; Stott and Sinnhuber, 1978; Coulombe et al., 1982]. The most widely used system for metabolic activation is the rat liver S-9 fraction supplemented with the co-factor NADP and glucose-6-phosphate. The S-9 fraction is the supernant fraction of liver tissue homogenate obtained by centrifugation at 9000 x 9 for 10 minutes [Ames et al., 1975]. Before the S-9 fraction from rat was prepared, the animal is usually treated with polychlorinated biphenyl (PCB) [Alvares et al., 1973; Czygan et al., 1973; Ecogichon et al., 1974; Litterst et al., 1974; and Schmodlt et al., 1974]. Although some carcinogens are potent alkylating or acylating agents and can, without metabolic activations, react directly with cellular macromolecules, procarcinogens require metabolic transformation to the active compound, the ultimate carcinogen [Miller, 1970; Heidelberger, 1973]. Procarcinogens, as well as many drugs, xenobiotics and endogenous steroids are metabolized by the hepatic mixed-function oxidase system (MFO), also known as the drug metabolizing enzyme system or the monoxygenase system. The mammalian MFO system was elucidated about

25 years ago, when initial studies on hepatic microsomes revealed a CO-binding pigment [Klingenberg, 1958] which was later shown to be responsible for the dehydroxylation of steroid and the oxidative demethylation and hydroxylation of drugs [Estabrook et al., 1963; Omura et al., 1965]. This pigment, cytochrome P-450, which is identified spectrally as the CO complex, is an integral component of the NADPH-dependent O_2-requiring MFO system. This system contains two other elements known to be required for the metabolic activity and conversions: (a) phosphatidylcholine, whose function is yet to be determined; and (b) cytochrome P-450 reductase, which is thought to be the rate-limiting component [Campbell and Hayes, 1975].

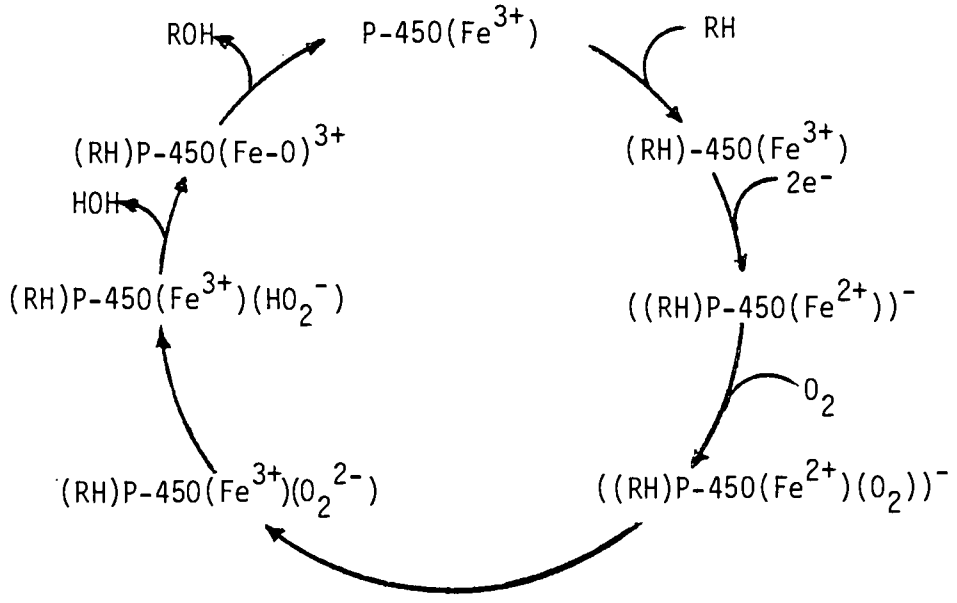


FIGURE (2). Proposed scheme for the metabolism of substrates by the cytochrome P-450-containing monooxygenases.

Source: Doull et al., 1980.

In the standard plate method described by Ames et al. [1975], the mixture of bacteria, S-9 mix, and test substance is added directly to the top agar and poured onto the minimal-glucose agar medium [Vogel, 1956]. In the method reported by Nagao et al. [1977], the mixture of bacteria, test substance, and S-9 mix were incubated for 20 minutes at 37°C before adding the top agar. With this preincubation method, Dimethylnitrosamine (DMN), a typical mutagen and carcinogen, gave positive results.

The Salmonella/Ames test detects almost all of the known organic chemicals that are human carcinogens. In several extensive studies designed to examine the ability of the Salmonella test to detect chemicals carcinogenic and non-carcinogenic in animal cancer tests, 90 percent of over 200 carcinogens tested were positive [McCann et al., 1975; McCann and Ames, 1976; Sugimura et al., 1976; Heddle and Bruce, 1977; and Purchase et al., 1976, 1978]. The Salmonella/Ames test is one of the few short-term tests in which a large number of carcinogens and non-carcinogens, comprising a wide variety of chemical classes, have been examined systematically using a defined protocol.

Generally, most classes of chemical carcinogens are detected in the Salmonella/Mammalian test, yet important exceptions are not detected as some heavily chlorinated carcinogens like dieldrin and carbon tetrachloride. Another weakness point is that metal carcinogens are negative in the Salmonella assay (dichromate is an exception: [Petrilli and DeFlora, 1977]). The standard test system

is not suitable for metals entering the bacteria because of the large amount of Mg salts, citrate, sulfate, and phosphate in the minimal medium [McCann and Ames, 1977]. Also, despite the attempts to improve correlation with carcinogenesis, it is essential to recognize that limitations exist in the use of bacterial mutagenesis systems, whether for screening or for evaluation of potential modifiers of carcinogens. Since somatic mutations do not provide the basis for all malignant transformations, microbial mutagen assays fail to detect epigenetic carcinogens, compounds which do not interact with DNA. Czygan et al. [1973] indicated that highly water soluble mutagens such as Dimethylnitrosamine (DMN) which cause carcinogenic activity in vitro are not easily detected without modifying the Ames test. It is thought that these compounds do not readily interface with the P-450 cytochrome system and may not be metabolized to the active species by the microsomal biotransformation system.

Dietary Intake and Metabolic Rate

Modern industry has introduced many mutagens and carcinogens, but nature itself also created many mutagens and carcinogens, which can be found in molds and plants. Chemical mutagens enter the body through the diet or in other ways. Several authors have recently reviewed the possible roles gut bacteria might play in potentiating promutagens or procarcinogens present in the diet [Drasar et al., 1974; Hill et al., 1975; Alcantara et al., 1976;

Brown, 1977). Development of short-term environmental carcinogens as mutagen [Ames et al., 1975; Anderson, 1978] has created a technology for identification of suspect environmental agents, possibly leading to an assessment of human risks associated with various dietary patterns. Such short-term tests have already been employed to detect possible carcinogens in human feces [Wang et al., 1978], broiled meat and fish [Sugimura et al., 1977; Nagao et al., 1977], vegetables [Kada et al., 1978], and spices [Seino et al., 1978].

Laquer [1970] and Reddy et al. [1975] showed the relationship between enteric bacteria and carcinoma of the colon, while Bueding [1975] in another observation suggested that microorganisms of the intestinal tract played a central role in the formation of the mutagenic metabolites. Also, Bridges [1976], McCann et al. [1975], McCann et al. [1977], Purchase et al. [1976] and Sugimura et al. [1976] establish that it is indeed the microorganisms in the gasteriointestinal tract rather than the metabolic machinery of the host that are responsible for the formation of the mutagenic and potentially carcinogenic metabolites appearing in the urine of animals after the ingestion of the flavonol quercitin. The flavonol glycosides, particularly of quercetin and kaempferol, are found in the edible portions of the majority of food plants, e.g., citrus and other fruits, berries, leafy vegetables, roots, tubers and bulbs, legumes, cereal grains [Harborne, 1975; Herman, 1976], herbs and spices [Seino, 1978], and red wine [Tamura et al.,

1980]. As the findings of Brown and Dietrich in 1977 and 1979 and others indicates, flavonol glycosides, while not mutagenic in themselves, are essentially promutagenic in the presence of suitable glycosideases. Recently, many studies on the mutagenicities of flavonoids have been reported by Wang et al. [1978], Hardigree and Epler [1978], Bieldanes and Change [1977], Brown et al. [1977], and Sugimura et al. [1977]. MacGregor and Jurd [1978] demonstrated that the structural features which appear essential for mutagenic activity in the Salmonella typhimurium Strain TA 98 are a basic flavonoid ring structure with (1) free hydroxyl group at the 3 position, (2) a double bond at the 2,3 position, (3) a keto group at the 4 position, and (4) a structure which permits the proton of the 3-hydroxyl group to tautomerise to a 3-keto compound. Also, they show that the free hydroxyl groups in the B ring are not essential for activity if a rat-liver metabolic activating system is employed (see Fig. 3).

The fate of orally ingested food flavonoids in mammals is similar in its stages to that of other plant glycosides, e.g., the anthraquinone glycosides [Brown and Dietrich, 1979]. The flavonol glycosides, being hydrophillic and relatively high in molecular weight, are poorly absorbed in the small intestine. Since they are β-glycosides, they are not hydrolyzed by intestinal digestive enzymes and pass largely unaltered to the lower bowel. Hawksworth et al. [1971]; Prizant et al. [1976] and Scheline [1968] reported that the microflora of the mammalian lower bowel

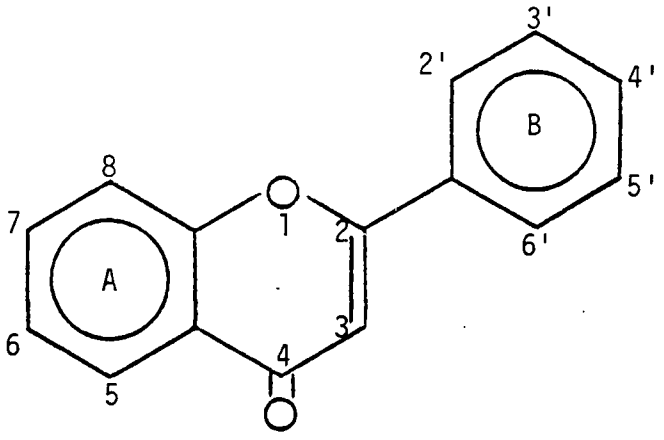
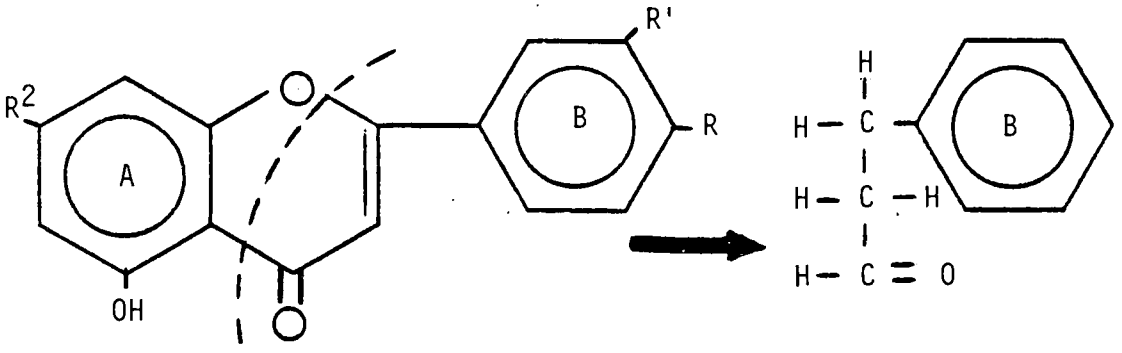


FIGURE (3). Structure and numbering of flavone is illustrated. Flavons are identical except the 2,3-bond is saturated. Flavonols are 3-hydroxyflavones.

Source: Macgregor et al., 1978.

are known to elaborate numerous glycosidase enzymes and in particular to hydrolyze many favonoid glycosides to their constituent aglycones and sugars. In addition to glycosides hydrolysis, Cheng et al. [1969]; Griffiths and Barrow [1972; Griffiths [1975]; Krishnamurty et al. [1970]; and Simpson et al. [1969] showed more extensive catabolism of many flavonoids can occur in the lower bowel. This involves cleavage of the heterocyclic pyrone ring to yield a variety of phenolic acids (phenylpropionic acid and phenylacetic acid derivatives) and phloroglucinol. This catabolism is carried out exclusively by the gut microflora. Brown [1980] illustrated that the large majority of food flavonoids



Flavonoid

m-Hydroxyphenyl
Propionic acid

FIGURE (4). Flavonoid structure, site of cleavage, and principal metabolic product from rat urine.

Source: Booth et al., 1958.

(those with 5, 7, 3^1, 4^1-hydroxylation pattern) are particularly subject to decomposition by intestinal microorganisms and will only be absorbed to a limited extent as intact flavonoids. However, since glycosidase action proceeds more rapidly than ring cleavage, free flavonols will occur in the large bowel and may be absorbed.

Observations of Chang et al. [1980] showed homogenate of human feces contain different glycosidase activities; Tamura et al. [1980] developed a model for activation of dietary glycosides to mutagens by intestinal flora. This will allow the in vitro activation of many natural glycosides to mutagens in Salmonella/liver homogenate test.

Kirby and Styles [1970] emphasize there is relatively little information on the effect of plant hybridization on flavonoid synthesis, however, the flavonol content of foods might be altered through novel applications of plant genetics.

MATERIALS AND METHODS

Samples

Samples of Fresh Cantaloupe (*Cucumis melo*), Fresh Raspberry (*Rubus idaeus*), and three varieties of Onion (*Allium cepa*) Red, Yellow, and White, used in this study were obtained from a local produce market. Those samples not used immediately were stored at -10°F until used.

Reagents

Biochemicals used were obtained from the following sources: Sigma Chemical Co. (St. Louis, MO), O-Nitrophenyl-β-D-Galactopyranoside (ONPG); lysozome (E.C. No. 3.2.1.17); β-Galactosidase (E.C. No. 3.2.1.23); Heparin Sodium Salt Grade II; d-Biotin; L-Histidine Monohydrochloride; Imidazole Grade I; β-Nicotinamide Adenine Dinucleotide Phosphate ($NADP^+$); D-Glucose-6-Phosphate; 3,4',5,7-Tetrahydroxyflavone (Kaempferol); 3,3',4',5,7-Pentahydroxyflavone (Quercitin); K and K Laboratories, Inc., Plainview, N.Y., Quercitin 3-O-β-D-Calactopyranoside (Quercetrin). Other common chemicals employed were reagent grade.

Extraction, Isolation, and Identification of Flavonoid

Two different extracts of each sample were prepared. The first was a whole extract consisting of concentrated juice; the

second was a Flavonoid pigment extract. These extracts were prepared as follows.

Whole Extract: 5 lbs. of each sample was blended for 5 minutes in a high-speed Waring blender. The slurry was covered with parafilm and stored overnight at 38°F then filtered in a Buchner funnel. The juice extracted was stored at -10°F for later use. Concentrates were prepared from the frozen samples concentrated with XAD-2 for mutagenicity test.

Flavonoid Extract: Because of the presence of active polyphenol oxidase, leucoanthocyanin, and pectic substances, which often make the preparation of an aqueous Flavonoid isolate difficult, the following modified procedure was used and found effective. A frozen powder sample was prepared by freezing the sample in liquid nitrogen then shattering it in a stainless steel blender. The powder was extracted using 1:2 (W/V) boiling acetone. The macerate was allowed to stand at 1.5°C for at least 4 hours then filtered with Whatman No. 1 filter paper. The residue was re-extracted with 1:1 (W/V) boiling acetone and filtered. The filtrate was combined and the residue discarded. The filtrate was shaken with chloroform (1.5x volume of filtrate) in a separatory funnel. The aqueous phase was retained and the bulky chloroform-acetone phase discarded. The flavonoids were isolated by extracting the aqueous phase at least six times with equal volumes of ethyl acetate. The ethyl acetate fractions were combined and concentrated nearly to dryness under 30 mm of vacuum using a

rotary evaporator at a temperature of > 30°C. Part of the concentrate was taken up in a minimal volume of methanol and used for chromatography and further identification. The remaining concentrate was taken up in dimethylsulfoxide (DMSO) in the ratio of 1:100 (W/V) for mutagenicity test.

Chromatography is a well-recognized method for separating flavonoids from plant extracts [Herrmann, 1976; Harborne, 1959; Roux and Evelyn, 1958]. Thin layer chromatography (TLC) using cellulose plates (size 20 x 20 cm on glass support with layer thickness of .25 mm) was chosen for chromatographic separation of flavonoid isolate. This method produced versatility, sensitivity, and speed required for separation and identification of these compounds. The flavonol extract was spotted with a micropipette, using a maximum of 5 µl per sample spotted 2 cm above the edge of the plate, when R_f values were determined and, in other instances, they were spotted along the base line with the idea of collecting separately the different possible fractions. The compounds thus separated were further identified using chemical Ionization/Mass Spectroscopy (CI) and Fast Atom Bombardment/Mass Spectroscopy (FAB) procedures. Further mutagenicity testing was also done with purified samples. The spotted plates were developed in all-glass developing tanks by ascending solvent. To achieve atmospheric saturation, the developing chamber was lined on three sides with Whatman No. 1 filter paper and the solvent system added to the tank one hour

before developing the chromatograms. Chromatography was carried out at room temperature. The developing solvent consisted of t-butanol, glacial acetic acid, and water (TBA) in ratios of 3/1/1, V/V. R_f values of the unknown compounds were compared with that of the standard flavonol glycoside (Quercitrin). The spots were scraped and taken up in a minimal volume of methanol and centrifuged for 5 minutes at high speed ($\simeq$ 1100 g) on a clinical centrifuge. They were respotted alone and double spotted with the standard quercitrin. The spots with the R_f value matching that of the standard was collected separately as 'A' half of the plate; the other spots were collected all together and designated 'B' part of the plate. The 'A' and 'B' isolate fractions for each sample were then dissolved in DMSO in the ratio of 1:50 for mutagenicity testing.

The 'A' part, after dryness, was subjected to further investigations. The chemical Ionization Mass Spectrometry (CI) was run using a Finnigin 4023 quadrupole mass spectrometer coupled with a computer system. Direct probe analysis was conducted with ion source temperature of 190°C. The reactant gas was methane at a pressure of .75 torr. Fast Atom Bombardment Mass Spectrometry (FAB) determinations were made using VARIAN MAT CH-7 single focusing magnetic instrument with system industries 150 data system was used. FAB with ION Tech Saddle Field source using 6 KV argon beam was employed (see Appendix for both CI and FAB charts).

Human Feces Cell-Free Extract (Fecalase) Preparation

Stephen and Cumming [1980] showed bacterial cells make up approximately half of the human feces, and its homogenate contain different glycosidase activities originating from the gut microflora. This procedure was chosen for fecalase preparation over several other methods as it facilitates preparation of larger quantities [Tamura et al., 1980]: Two different fecalase preparations were made, one from six human subjects who were lactose tolerant, and a second preparation from six lactose intolerant individuals. For each preparation, feces were obtained on the day of the preparation. Donors were instructed to collect morning excreta in tared plastic containers. The extreta were delivered to the laboratory within the first half-hour of defecation. Samples were weighed to the nearest 0.5 g and immediately processed. Each sample was treated separately as described by Tamura et al [1980] through centrifugation. The crude preparation then was assayed for β-galactosidase activity. The individual samples with rough comparable activities were then combined to make a pooled fecalase preparation. The feces were transferred to a glass beaker and diluted with 1 mM phosphate, to a pH 7.4. Dilution was between 1:1 and 1:3 (buffer:feces), to give a consistency of a thick slurry. Homogenization was performed at room temperature in a Polytron homogenizer (Brinkman) for approximately one minute. The bacterial cells in fecal suspension were lysised by high-

pressure extrusion through a Manton-Gaulin homogenizer precooled by passing ice slush through it. Homogenate was passed through twice at a pressure of 9000 psi. Cellular debris was removed by centrifugation at 40,000 g at 32°F for 20 minutes. The β-galactosidase activity of the supernatant was determined by measuring the hydrolysis and cleavage of the glycosidic linkage of O-nitrophenyl - β-D-galactopyranoside (ONPG). The assay consisted of adding up to 100 µl of fecalase, 0.3 ml of ONPG (to ensure saturation to calculate Vmax), and 10 mM phosphate buffer (pH 7.4) to make a total volume of 2 ml. The mixture was incubated three minutes at 28°C. The reaction was then arrested by addition of 1 ml of 1 M Na_2CO_3. The reaction velocity was determined by an increase in absorbancy at 420 nm [Craven et al., 1965] resulting from the hydrolysis of O-nitrophenyl-β-D-galactopyranoside. The determination was made using Spectrophotometer 550 (Perkin-Elmer). Assays were for variable time periods (> 30 min), but results were reported for only the liear portion fo the time curve. After the fecalase activities were determined, the retained samples were pooled.

Removal of Histidine, Sterilization, and Storage of the Prepared Fecalase

Histidine (MW. 155), which would interfere with the mutagenicity assay, was removed from the fecalase preparation by passing the supernatant through a Sephadex G-50 (medium) column at a rate of

4-5 ml/min. The temperature was maintained at 4°C (~39°F) throughout the operation.

The pooled fractions were sterilized by passing them first through AP Millipore prefilter under vacuum and then successively through 0.8-μm and 0.45-μm Nalgene filters. The filtrate was then distributed in 2-ml plastic tubes, quick-frozen on dry ice, then stored at -40°F. Samples were analyzed for protein [Lowry et al., 1951], and tested for bacterial contamination on nutrient broth plates.

Salmonella typhimurium Tester Strains; Markers, Checking, and Storage

The bacterial tester strains, Salmonella typhimurium TA 1537,* TA 1535,** TA 100,** TA 98,* and TA 97,* used for screening for mutagens and were obtained from B. N. Ames, University of California at Berkeley. Throughout the study the mutant strains were propagated in OXOID #2 nutrient broth on a reciprocating shaker for 14 hours at 37°C as recommended by Ames et al. [1975]. The culture genetic markers were confirmed initially and periodically throughout the study following the procedure outlined by Ames et al. [1975]. The checking was conducted to observe the following:

1. Histidine requirement.

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*Frameshift mutant of S. typhimurium.

**Base pair substituted mutant of S. typhimurium.

2. Deep rough (rfa) character, which causes a defect in Lipopolysaccharide (LPS) and permits large molecules such as crystal violet to enter the bacteria and inhibit growth.

3. Ampicillin resistant R factor, strains which do not contain the R factor (TA 1537, TA 1535) will show a zone of growth inhibition around the ampicillin streak, whereas R factor containing strains (TA 100, TA 98, and TA 97) will not.

4. μvrB deletion, the UV-sensitive strains containing μvrB deletion (all the strains used) will grow only on the un-irradiated side of the plate.

5. The spontaneous reversion rate.

Duplicate sets of stock cultures of each tester strain were prepared from 14 hours nutrient broth culture to which dimethylsulfoxide (0.08 ml/ml of culture) was added. Four ml of culture in 10 ml sterile, plastic screw-capped vials were frozen in liquid nitrogen before storing at -40°F.* One set was used as a master copy, while the other was used routinely as a source for fresh cultures.

Induction of Rat and Preparation of Liver Homogenate Fraction "S-9"

Spraque-Dawley male rats of about 200 g each were used as a source of livers. The mixed function cytochrome oxidase system

*Ames recommends a storage temperature of -80°C. A temperature of -40°C was found equally successful in maintaining stable cultures.

of the liver of each rat was induced by a single i.p. injection of polychlorinated biphenyl (PCB) mixture (Aroclor 1254) in corn oil at a level of 500 mg/kg of body weight. The injection was given to each rat five days before sacrifice [Alvares et al., 1973; Czygan et al., 1973]. The rats were given drinking water and Purina Laboratory Chow ad libitum until 12 hours before sacrifice. On the fifth day of induction the rats were stunned by a blow to the head and decapitated and the liver aseptically excised. The liver homogenate fraction (S-9) was prepared according to the method of Garner et al. [1972]. All steps were at 0-4°C using cold sterile solutions and glassware. The livers were placed in pre-weighed beakers containing (approximately 1 ml/g wet liver) 0.15 M KCl. After weighing, livers were transferred to a beaker containing three volumes of 0.15 M KCl (3 ml/g wet liver), minced with sterile scissors, and homogenized in a Potter Elvehjem apparatus with a Teflon pestle. The homogenate was centrifuged for 10 minutes at 9000 g and the supernatant (S-9) was decanted. The protein content of the homogenate was determined by the method of Lowry et al. [1959]. The fresh homogenate was distributed in 2-ml portions to small plastic tubes, frozen in liquid nitrogen, and stored at -40°C. Fresh S-9 mix was prepared each day of an experiment. Stock solutions of $NADP^+$ (0.1 M) and glucose-6-phosphate (1 M) were prepared with sterile water and stored in sterile tubes at -40°C. A stock solution of salt (0.4M $MgCl_2$, 1.65 M CKl), and one of phosphate buffer (0.2 M, pH 7.4) were

autoclaved and stored in the refrigerator. The S-9 mix was prepared according to Ames et al. [1975].* One hundred ml of top agar (0.6% Difco agar, 0.5% NaCl) was autoclaved and stored in screw-top bottles at room temperature. Before use, the agar was melted then cooled to 50°C before adding 10 ml of sterile solution of 0.5 mM L-histidine. HCl-0.5 mM biotin was added to the molten top agar. Minimal-glucose agar medium was prepared from 1.5 percent Bacto-Difco agar, 20 ml of Vogel-Bonner Medium E** [Vogel, 1956], and two percent glucose. Degradation of glucose was minimized by dissolving it in approximately 25 percent of the total water for the medium and autoclaving it separately from the other ingredients of the medium. The two sterile components were cooled to 50°C then combined prior to plating.

Mutagenicity Assays

Thirteen to 15-hour nutrient broth (OXOID #2) cultures adjusted to 30 percent transmission at 470 nm were used in all experiments. One-tenth of the adjusted culture gave a concentration of about 10^8 cells per plate. Dimethylsulfoxide (DMSO) was used as a solvent for all compounds tested. The concentration ranged between 25 nmol and 500 nmol/plate. Each compound was screened for mutagenic activity by exposure to each of the tester

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*See Appendix for S-9 mix preparation.

**See Appendix for Vogel-Bonner Medium E preparation.

strains indicated earlier. The tester strains were exposed to the compounds both with and without the S-9 liver homogenate mix, to determine if the compounds were mutagenic per se or require bio-activation. Fecalases (tolerant or intolerant) were added in amounts ranging from 25 to 400 ml per plate. The standard test which mixes 2 ml molten top agar at 45°F; 0.1 ml tester strain; 0.1 to 0.4 ml of sample to be tested and 0.5 ml S-9 mix by vortex mixer then immediately overlapped on prepoured and solidified minimal-glucose agar, was used for samples without fecalase exposure. For samples exposed to fecalase a modification suggested by Yahagi [1977] was employed. In this procedure the fecalase, sample, S-9 mix, and culture were incubated 20 minutes at 37°C before adding top agar and overlaying the mixture on the minimal-glucose agar plates. Plates were incubated at 37°C for 48 hours and then the revertant was determined. All tests were performed in triplicate or quadruplicate and each experiment was repeated two or more times.

Concentration of Flavonol Glycosides from Juices by Adsorption with XAD-2

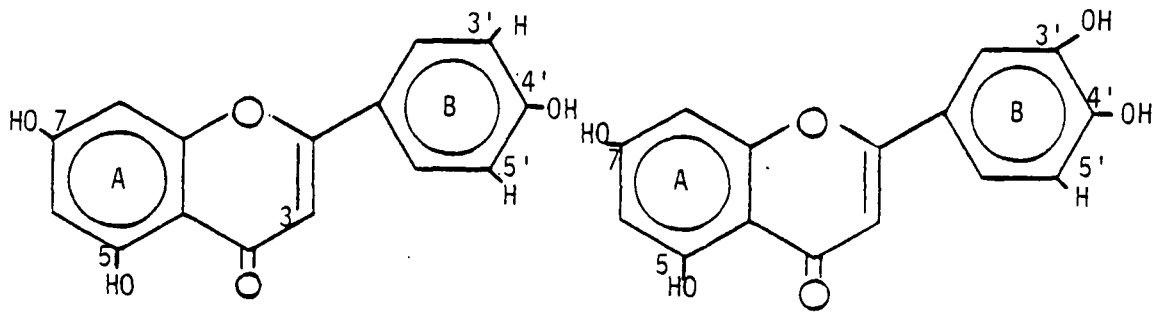
XAD-2 resin was washed by swirling and decanting several times with 10 volumes of acetone followed by absolute methanol and distilled water, and then stored in water at 4°C. Five glass columns, 0.7 cm (inside diameter) x 20 cm, were filled with distilled water before addition of sufficient washed resin (0.7 g dry weight) to

give a bed height of ≈ 8 cm. Flow was regulated with stopcock and distilled water was passed through the resin before use. Tenax GC resin, 0.5 g added per column, was washed with 20 ml of acetone followed by distilled water prior to use. Juices of Cantaloupe, Red Onion, Yellow Onion, White Onion and Raspberry in different volumes were loaded in the resin and an effluent flow rate of 2-3 ml/min. was regulated by means of the stopcock. All operations were conducted at room temperature. After loading, nitrogen was introduced into the top of the column for a few seconds to remove aqueous phase; however, drying of the resin was avoided. The adsorbed components were eluted into 18 x 150-mm glass test tubes with 10 ml of acetone. Each of the eluted fractions was then thoroughly mixed before placement of the tubes in a constant temperature heating block at 60-65°C to evaporate the solvent under an atmosphere of nitrogen. After complete dryness, 1 ml of dimethylsulfoxide was added to each tube to dissolve the acetone residue; 100 ul of each of the reconstituted juice volumes had been tested for mutagenicity with or without the addition of fecalase.

RESULTS

Identification of Flavonoid Extracts

Flavonoids [Harborne et al., 1975], which have the common skeleton of diphenylpropanes ($C_6C_3C_6$), are of widespread occurrence in plants. Flavonols can be regarded as 3-hydroxyflavones and flavones, reversibly, as 3-deoxyflavonols. Individual differences arise from the number and distribution of the hydroxyl groups as well as from the nature and extent of alkylation and/or glycosylation of these groups. In common with other flavonoids, the flavones and flavonols most frequently found in plants are those will B ring hydroxylation in the 3'- and 4'- positions, followed by those with a hydroxyl group in the 4'-position only. Kaempferol and Quercitin used as standard in this study are typical flavonols of this type:



Kaempferol

Quercitin

FIGURE (5).

Herrmann [1976] shows the preferred bonding site of the sugar radical to the flavonol is the 3 - position, much less frequently the 7- position, and only on rare cases the 4'-, 3'- or 5'- position. Also he indicated, D-glucose is the most frequent sugar residue but D-galactose, L-rhamnose, L-arabinose, D-xylose, D-apiose are also found. The sugars of D-series are bound through the β-glycosidic bond. The concentrations of flavones and flavonols, like those of all secondary plants metabolites, vary within certain limits, and are independent on a number of growing factors such as growing conditions, degree of ripeness, size of fruit and variety. Qualitative techniques for yellow flavonol pigments were developed by Puski and Francis [1967]. However, these procedures were very time-consuming and would require extensive modification before being suitable for use in quantitative determination of the flavonols. The procedure used for isolation of flavonol and flavonol glycosides have been described in the material and method chapter. According to Harborne [1959], the R_f values are sufficiently characteristic to make them of considerable value in the identification of flavonoids. He also showed an increase in the number of hydroxyl groups substituted in the favonoid molecules lowers the R_f values in alcoholic solvent system. Several combinations of sorbents and solvents were tried before selection of the system. The eluting solvents used for one-dimension Thin-Layer Chromatography (TLC) was TBA (t-butanol, glacial acetic acid, water 3/1/1; V/V). TLC was conducted for each flavonoid extract.

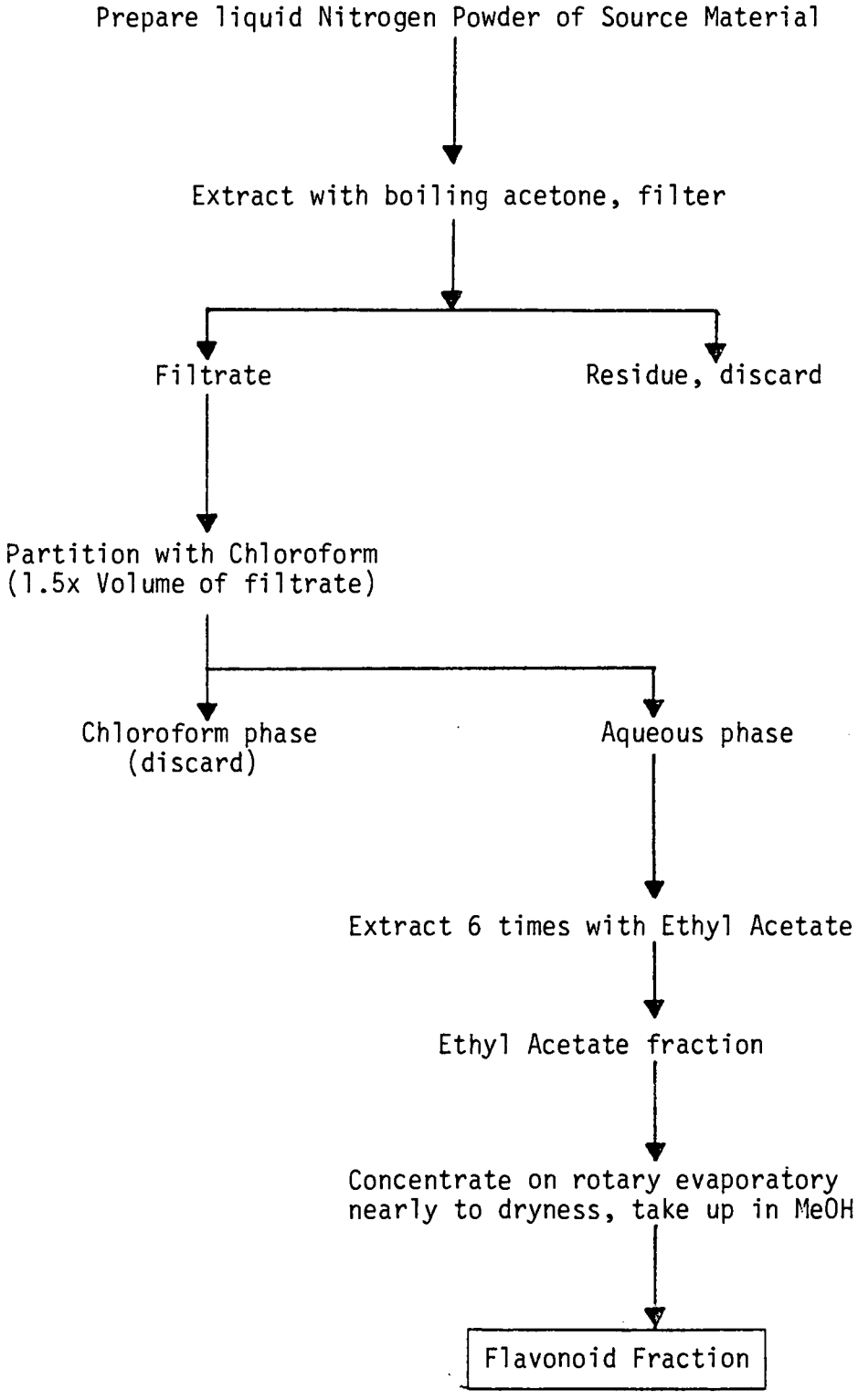


FIGURE (6). Flow Sheet for Isolation of Flavonoid Compounds

In the case of Cantaloupe, Raspberry, and the two varieties of Onion, Red and Yellow, the chromatography showed two groups of spots on the plate. The front spots of each sample mentioned were collected separately and designated 'A' isolate, while the other spots were collectively designated 'B' isolate. Part 'A' spots were again run with quercitrin as the standard for comparing R_f values. White Onion extract was also investigated. The R_f values listed in Tables 1 through 5 show clearly that the 'A' isolate flavonoid extract of Cantaloupe, Raspberry, and the two varieties of Onion (Red and Yellow) had similar R_f values to quercitrin under the specified conditions. White Onion, however (Table 5), did not show any compound with R_f values close to quercitrin. It was concluded that quercitrin as well as other flavonols were absent in this onion.

Extract separation on the TLC such that the spots were sufficiently spaced to allow collection of each spot without contamination from other compounds. Thus, spot 'A' for each sample was collected in a pure state. Approximately 0.1 to 0.2 mg of material was isolated from each item selected for study. About 0.3 mg was also collected for mutagenicity test from both parts 'A' and 'B' of the plates. For conformation of the isolated compounds, Fast Atom Bombardment/Mass Spectroscopy (FAB), and Chemical Ionization/ Mass Spectroscope (CI) were conducted. Both FAB and CI Spectra of Spot 'A' show great similarity to that recorded for by the National

Standard Reference Data System [NSRDS, EPA/NIH Mass Spectral Data Base Volume 4, December, 1978]. See Appendices B and C for FAB and CI spectra.

TABLE 1. Cantaloupe (C) Flavonoid Extract - R_f Values

Spots / Sample	1	2	3	4	5
C	.09	.25	.31	.41	.89
C_AQ	-	-	-	-	.88
Q	-	-	-	-	.88
C_A	-	-	-	-	.88

C_AQ = Spot 'A' mixed with equal amount of the Standard Quercitrin (Q).

C_A = Spot 'A' collected and plotted separately.

TABLE 2. Red Onion (R) Flavonoid Extract - R_f Values

Spots / Sample	1	2	3	4	5
R	.07	.12	.24	.36	.88
R_AQ	-	-	-	-	.87
Q	-	-	-	-	.87
R_A	-	-	-	-	.87

R_AQ = Spot (A) mixed with equal amount of the Standard Quercitrin (Q).

R_A = Spot 'A' collected and plotted separately.

TABLE 3. Yellow Onion (Y) Flavonoid Extract - R_f Values

Spots / Sample	1	2	3	4
Y	.08	.23	.39	.86
Y_AQ	-	-	-	.85
Q	-	-	-	.85
Y_A	-	-	-	.85

Y_AQ = Spot 'A' mixed with equal amount of the Standard Quercitrin (Q).

Y_A = Spot 'A' collected and plotted separately.

TABLE 4. Raspberry (RB) Flavonoid Extract - R_f Values

Spots / Sample	1	2	3	4
RB	.08	.12	.23	.84
RB_AQ	-	-	-	.83
Q	-	-	-	.83
RB_A	-	-	-	.83

RB_AQ = Spot 'A' mixed with equal amount of the Standard Quercitrin (Q).

RB_A = Spot 'A' collected and plotted separately.

TABLE 5. White Onion (W) Extract - R_f Values

Spot / Sample	1	2	3	4	5
W	.07	.26	.31	.43	-
WQ	.08	.24	.31	.43	.91
Q	-	-	-	-	.91

WQ = Mixture of White Onion Extract and equal amount of the Standard Quercitrin (Q).

Fecalase Activity

Goldin and Gorbash [1977] indicated bacteria possess a great number of inducible and repressible enzymes. They also showed gram negative bacteria to be dominant in human intestine. Goldin and Gorbash [1977] showed changes in the metabolic activity of the intestinal microflora can occur without appreciable changes of the actual numbers or types of organism in the gut. The reaction velocity was determined by an increase in absorbancy at 420 nm resulting from the hydrolysis of the glycosidic linkage, using the equation:

$$V_{max} = \frac{\Delta A_{420}}{min.}$$

Since one unit is the enzyme activity that causes 1 μmole of substrate to react in one minute, the enzyme activity is thus measured by the rate of the catalyzed reaction, preferably at substrate saturation, to achieve the highest possible rate V_{max}. In order to compare β-galactosidase enzyme activity with that of the fecalases (from lactose tolerant and lactose intolerant), both the activity per ml and the amount of protein per ml were measured. The protein content of the fecalase samples were determined by the sensitive method of Lowry et al. [1951]. Four glycosides (i, ONP-β-D-Galactopyranoside; ii, ONP-α-D-Galactopyranoside; iii, ONP-β-D-Glucopyranoside; iv, ONP-α-D-Glucopyranoside) were used as substrate to determine β-galactosidase (lactase) enzyme activity. The prepared fecalases activities were determined and compared with that of β-galactosidase. Activities for the fecalase prepared from lactose tolerant and lactose intolerant subjects are shown in Table 6. Fecalase activity was measured frequently throughout the study and showed no significant decrease in activity when stored at -40°C. The homogenization procedure described by Tamura et al. [1980], in which the cells were lysed by extruding the mixture through small holes at high pressure, was used since it was easily adapted to the reasoning for the preparation of large volumes.

TABLE 6. Glycosidase Activities in Fecalase

Glycosides*	Fecalase Activity, nmol/min/mg of Protein		
	mM	Tolerant	Intolerant
ONP-β-D-Galactoside	1.3	720	25
ONP-α-D-Galactoside	2.5	210	18
ONP-β-D-Glucoside	5	30	6
ONP-α-D-Glucoside	5	78	5

*Glycosides at saturation.

Activation of Mutagen by Fecalase

The results were interpreted according to Ames et al. [1975] as he considers a chemical to have a negative response in the test if at least 500 μg had been tested on the plate (or the maximum non-inhibitory level) and if the number of induced revertants compared to the spontaneous revertant was less than two-fold. He also suggested for compounds of low mutagenicity it is important to obtain reproducible dose-response curves. The "Ames Index" was used in this study to interpret the results. The index is the average number of revertant colonies produced by the test compound divided by average number of spontaneous revertants produced on

control plates. A positive response is a ratio of two or more.

Four major experiments were conducted to investigate the effect of fecalase on the extracted flavonoid glycosides from Cantaloupe, Raspberry, and Red, Yellow, and White Onion.

Constant Substrate with Various Amounts of Fecalase

This experiment was intended to investigate the effect of fecalase prepared from lactose tolerant (T) and lactose intolerant (IT) individuals on the flavonoid glycoside mutagenic activity. The conditions of the assay were:

a) Constant flavonoid concentration of 300 nmol.

b) Varying amounts of fecalases (T) and (IT) ranging from 50 to 400 µl.

c) Incubation at 37°C for 20 minutes before top agar addition.

Data on Figures 7, 8, and 9 show a frameshift mutagenicity as indicated by the positive response of tester strains TA 1537, TA 98, and TA 97. The mutagenic aglycone was released from the flavonoid by the fecalase preparation from lactose-tolerant individuals. The flavonoid didn't show mutagenic activity without fecalase activation nor with fecalase prepared from lactose-intolerant donors. Table 7 reflects the result obtained with the addition of various amounts of the fecalase preparations to constant amounts of Cantaloupe flavonoid extract using the base-pair mutant

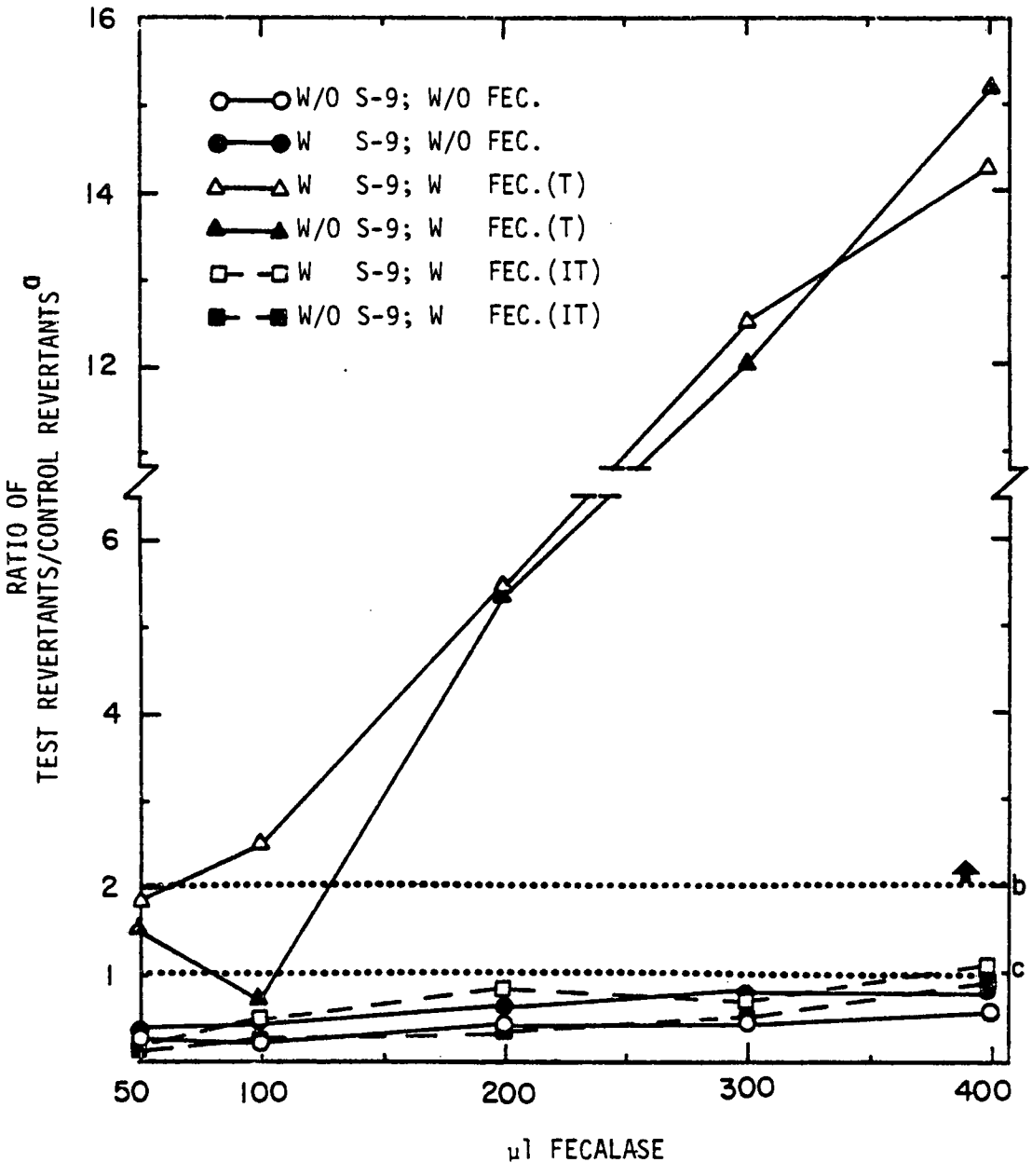


Figure 7. Mutagenicity of Cantaloupe Flavonoid Extract on Strain TA1537 with Fecalase.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA1537; WS-9 = 23, W/O S-9 = 20).

T = Fecalase from Lactose tolerant individuals.
IT = Fecalse from Lactose intolerant individuals.

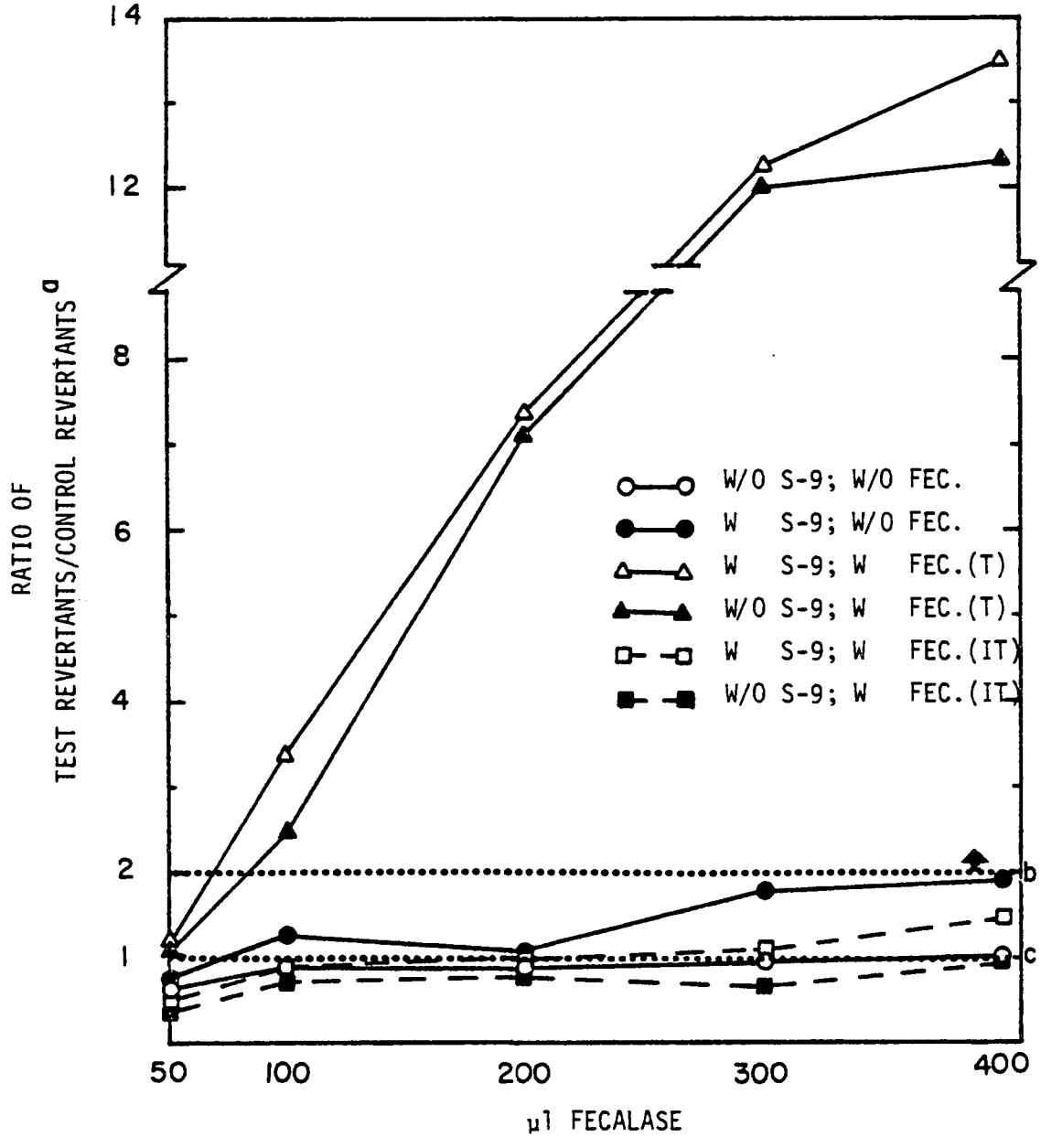


Figure 8. Mutagenicity of Cantaloupe Flavonoid Extract on Strain TA98 with Fecalase.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA98; WS-9 = 43, W/O S-9 = 41).

T = Fecalase from Lactose tolerant individuals.
IT = Fecalse from Lactose intolerant individuals.

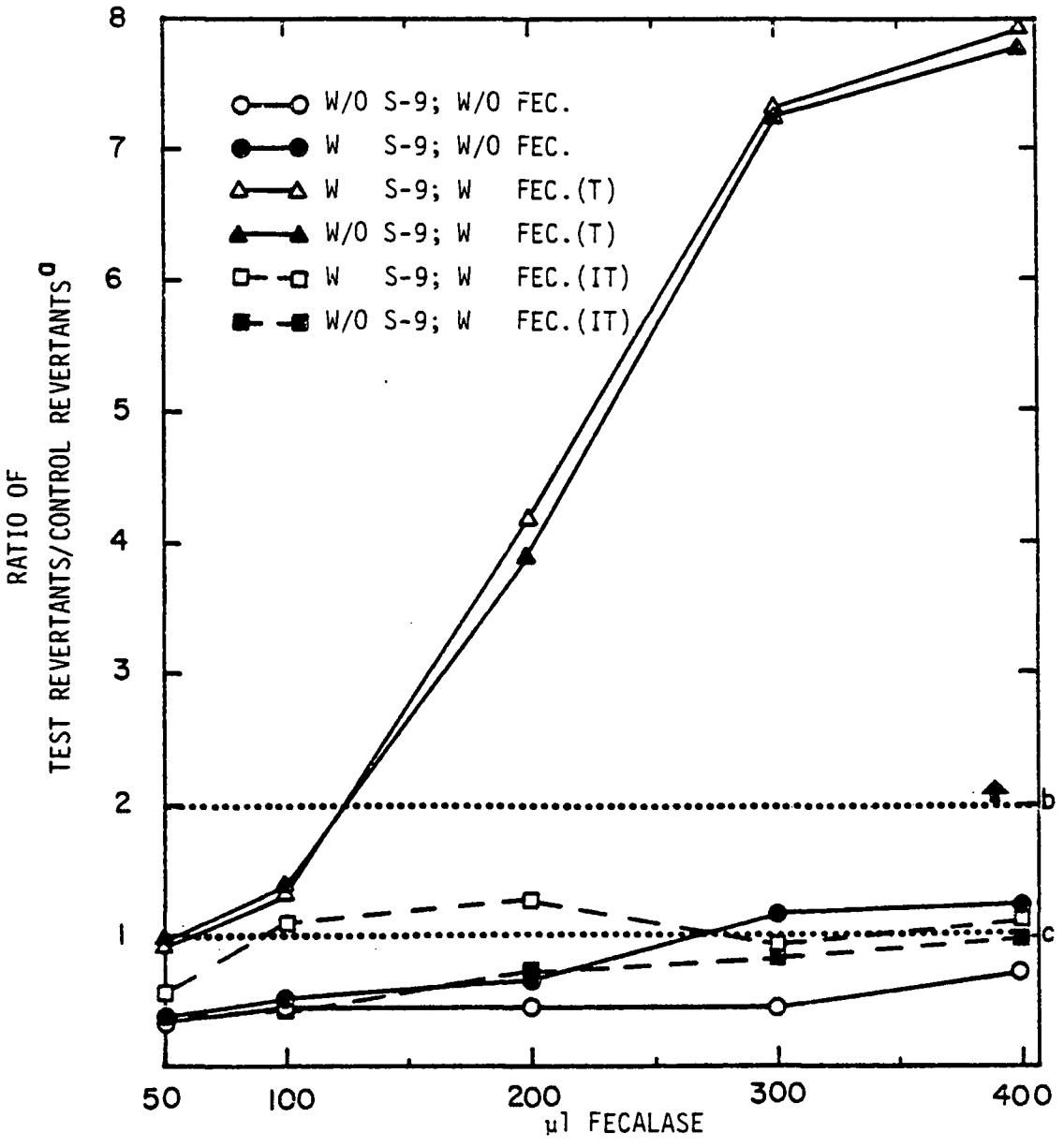


Figure 9. Mutagenicity of Cantaloupe Flavonoid Extract on Strain TA97 with Fecalase.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA97; WS-9 = 98, W/O S-9 = 95).

T = Fecalase from Lactose tolerant individuals.
IT = Fecalse from Lactose intolerant individuals.

TABLE 7. Mutagenicity of Cantaloupe (Cucumis melo) Flavonoid Extract [300 nmol] with Fecalase

		Ratio of Test Revertants/ Control Revertants					
Tester Strain	µl Fecalase	W/O S-9; W/O Fec.	W S-9; W/O Fec.	W/O S-9; W Fec.(T)	W S-9; W Fec.(T)	W/O S-9; W Fec.(IT)	W/O S-9; W Fec.(IT)
TA 1535	50	.10	.13	.48	.43	.05	.26
	100	.05	.26	.62	.65	.29	.38
	200	.15	.50	.90	.79	.36	.52
	300	.33	.57	1.06	.96	.61	.89
	400	.38	.65	.91	1.00	.58	.84
TA 100	50	.21	.29	.31	.44	.24	.49
	100	.22	.38	.47	.60	.44	.76
	200	.21	.42	.52	.61	.45	.81
	300	.25	.44	.73	.76	.50	.92
	400	.29	.45	.79	.81	.60	.86

TA 1535: W S-9 = 25; W/O S-9 = 20

TA 100: W S-9 = 184, W/O S-9 = 178

T = Fecalase from lactose tolerant; IT = Fecalase from lactose intolerant.

TA 1535 and TA 100. Neither of the two base-pair mutants showed a positive response. The data presented in Figures 10, 11, and 12 show Red Onion flavonoid extract is mutagenic to frameshift mutant strains TA 1537, TA 98, TA 97, but only in the presence of fecalase prepared from lactose-tolerant people. Data presented in Table 8, however, show no mutagenic activity on base-pair mutant TA 1535, TA 100, either with or without the addition of either fecalase preparations. Similarly, Yellow Onion flavonoid extract (Figs. 13, 14, and 15) was mutagenic on frameshift mutant strains TA 1537, TA 98, and TA 97, but not mutagenically active on base-pair mutant strains TA 1535 and TA 100 (Table 9). Data presented in Figures 16, 17, and 18 show Raspberry flavonoid extract also strongly mutagenic to frameshift mutant strains TA 1537, TA 98, and TA 97, but only in the presence of fecalase prepared from lactose-tolerant individuals. No mutagenic activity was noted either with or without fecalase prepared from lactose-intolerant donors. Raspberry flavonoid extracts were not mutagenic on base-pair mutant TA 1535, TA 100, as shown in Table 10, nor were they activated by either lactose-tolerant or lactose-intolerant fecalase preparation. White Onion extract was not mutagenic on either frameshift mutant or on base-pair mutant with or without the addition of either fecalase preparations, as illustrated in Figures 19, 20, and 21 and in Table 11.

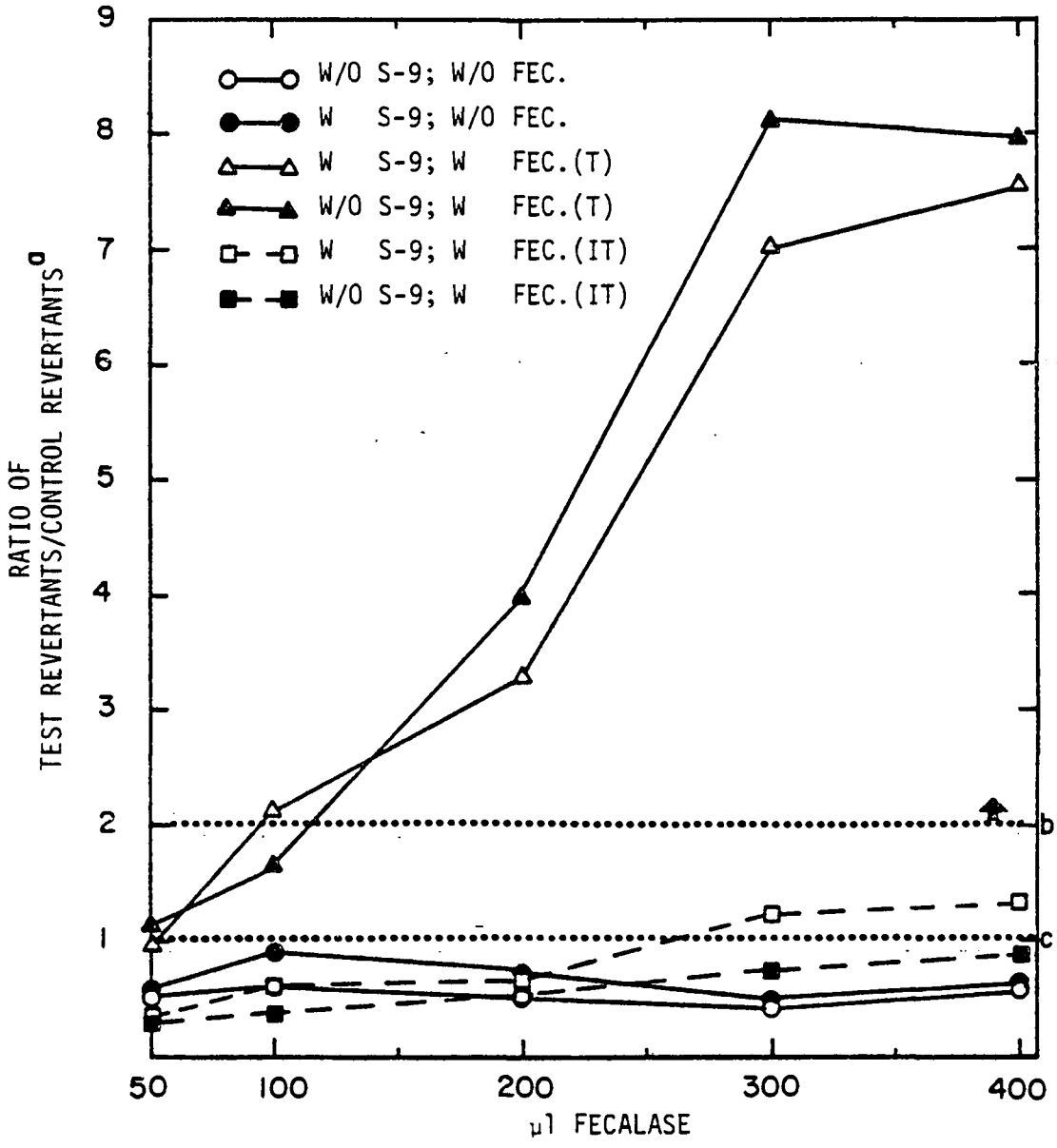


Figure 10. Mutagenicity of Red Onion Flavonoid Extract on Strain TA1537 with Fecalase.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA1537; WS-9 = 23, W/O S-9 = 20).

T = Fecalase from Lactose tolerant individuals.
IT = Fecalse from Lactose intolerant individuals.

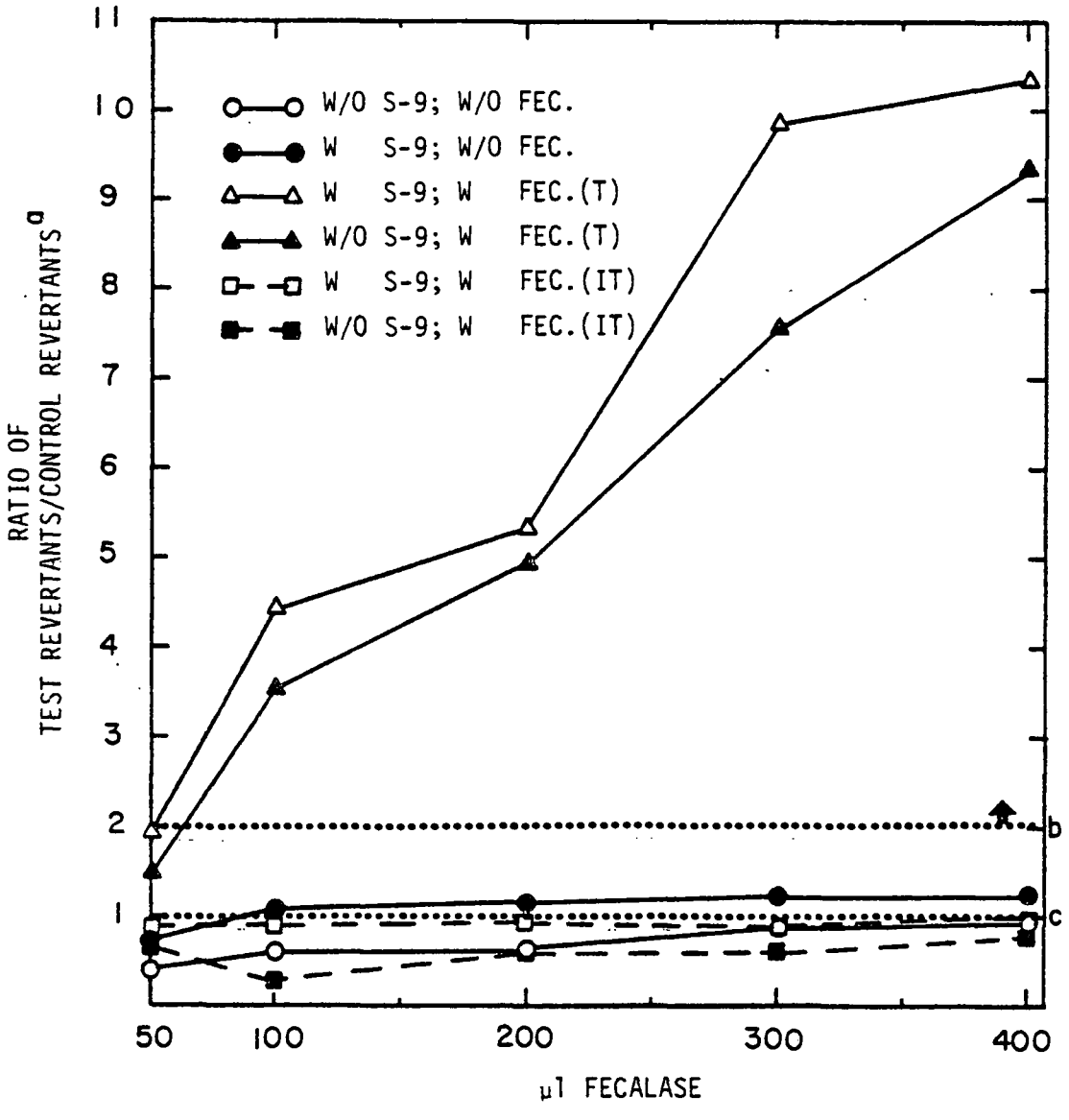


Figure 11. Mutagenicity of Red Onion Flavonoid Extract on Strain TA98 with Fecalase.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA98; WS-9 = 43, W/O S-9 = 41).

T = Fecalase from Lactose tolerant individuals.
IT = Fecalse from Lactose intolerant individuals.

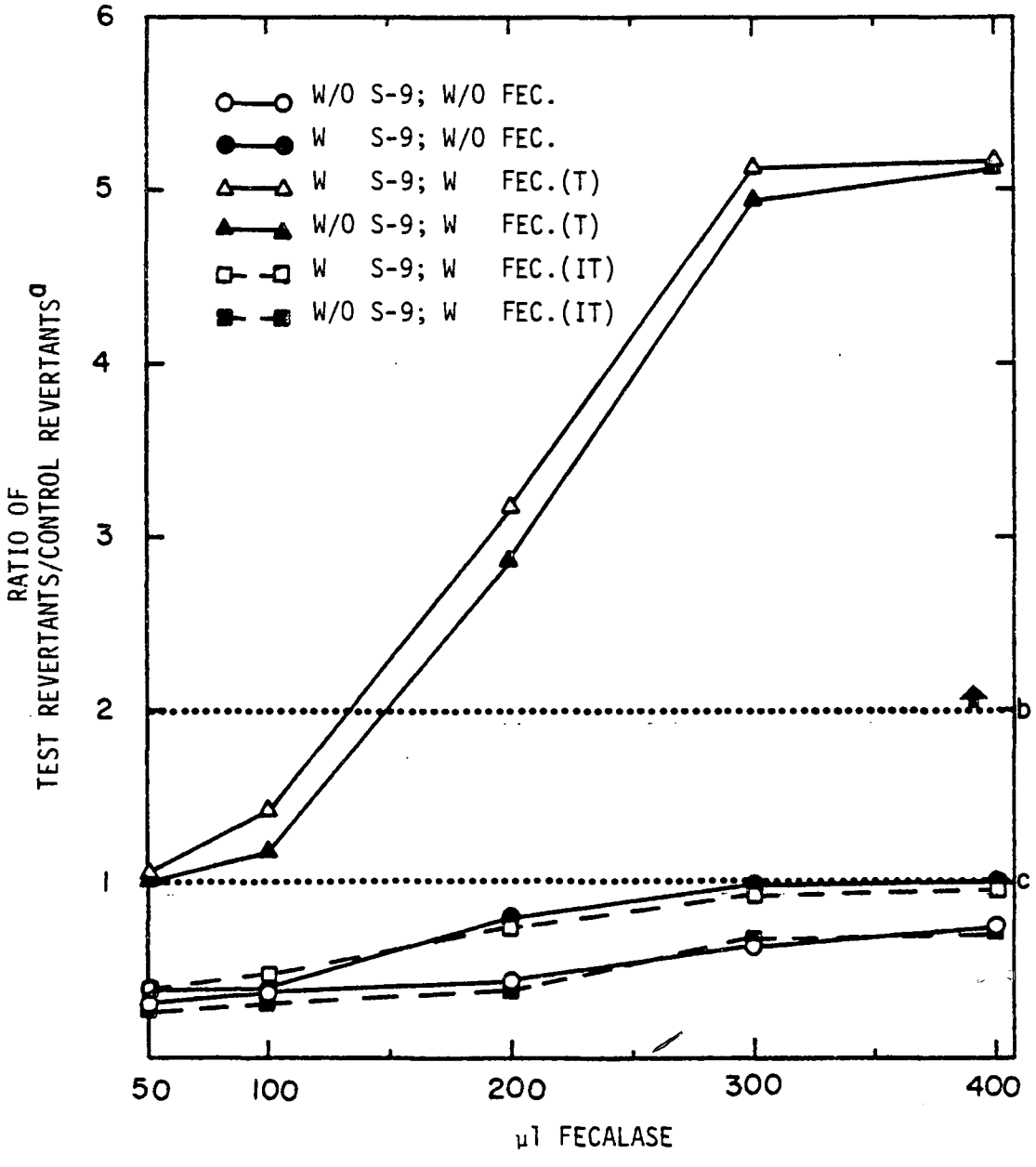


Figure 12. Mutagenicity of Red Onion Flavonoid Extract on Strain TA97 with Fecalase.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA97; WS-9 = 98, W/O S-9 = 95).

T = Fecalase from Lactose tolerant individuals.
IT = Fecalse from Lactose intolerant individuals.

TABLE 8. Mutagenicity of Red Onion (Allium cepa) Flavonoid Extract [300 nmol] with Fecalase

		Ratio of Test Revertants/Control Revertants					
Tester Strain	µl Fecalase	W/O S-9; W/O Fec.	W S-9; W/O Fec.	W/O S-9; W Fec.(T)	W S-9; W Fec.(T)	W/O S-9; W Fec.(IT)	W/O S-9; W Fec.(IT)
TA 1535	50	.29	.37	1.05	1.00	.21	.57
	100	.19	.57	1.24	1.83	.37	.52
	200	.40	.63	1.80	1.79	.59	.64
	300	.28	.30	2.22	.20	.71	.87
	400	.48	.57	2.14	2.48	.81	.93
TA 100	50	.20	.28	.36	.40	.18	.33
	100	.21	.37	.52	.59	.43	.94
	200	.26	.40	.60	.64	.62	.82
	300	.22	.53	.84	.90	.78	1.05
	400	.25	.63	.85	.87	.79	.99

TA 1535: W S-9 = 25; W/O S-9 = 20

TA 100: W S-9 = 184, W/O S-9 = 178

T = Fecalase from lactose tolerant; IT = Fecalase from lactose intolerant.

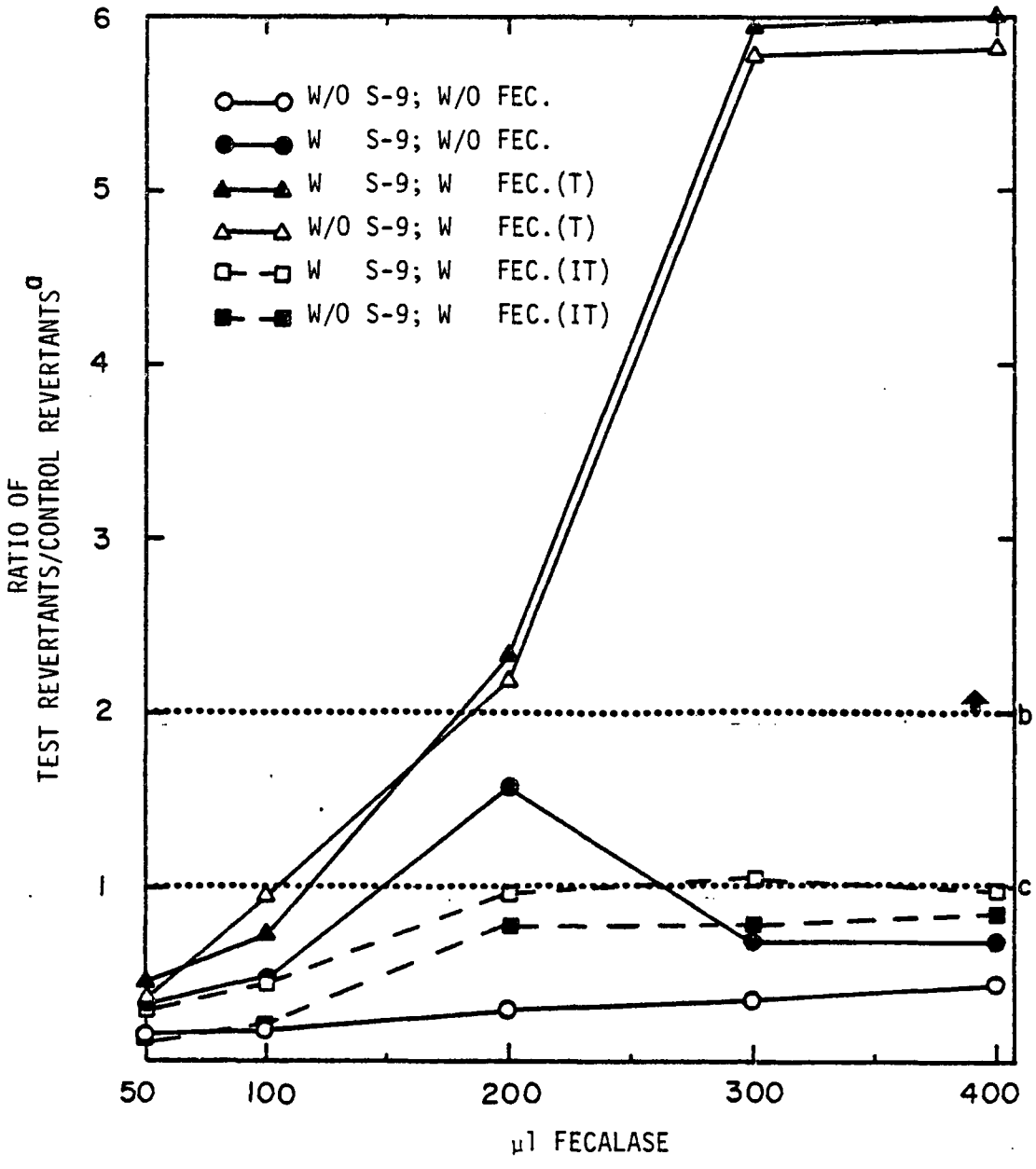


Figure 13. Mutagenicity of Yellow Onion Flavonoid Extract on Strain TA1537 with Fecalase.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA1537; WS-9 = 23, W/O S-9 = 20).

T = Fecalase from Lactose tolerant individuals.
IT = Fecalse from Lactose intolerant individuals.

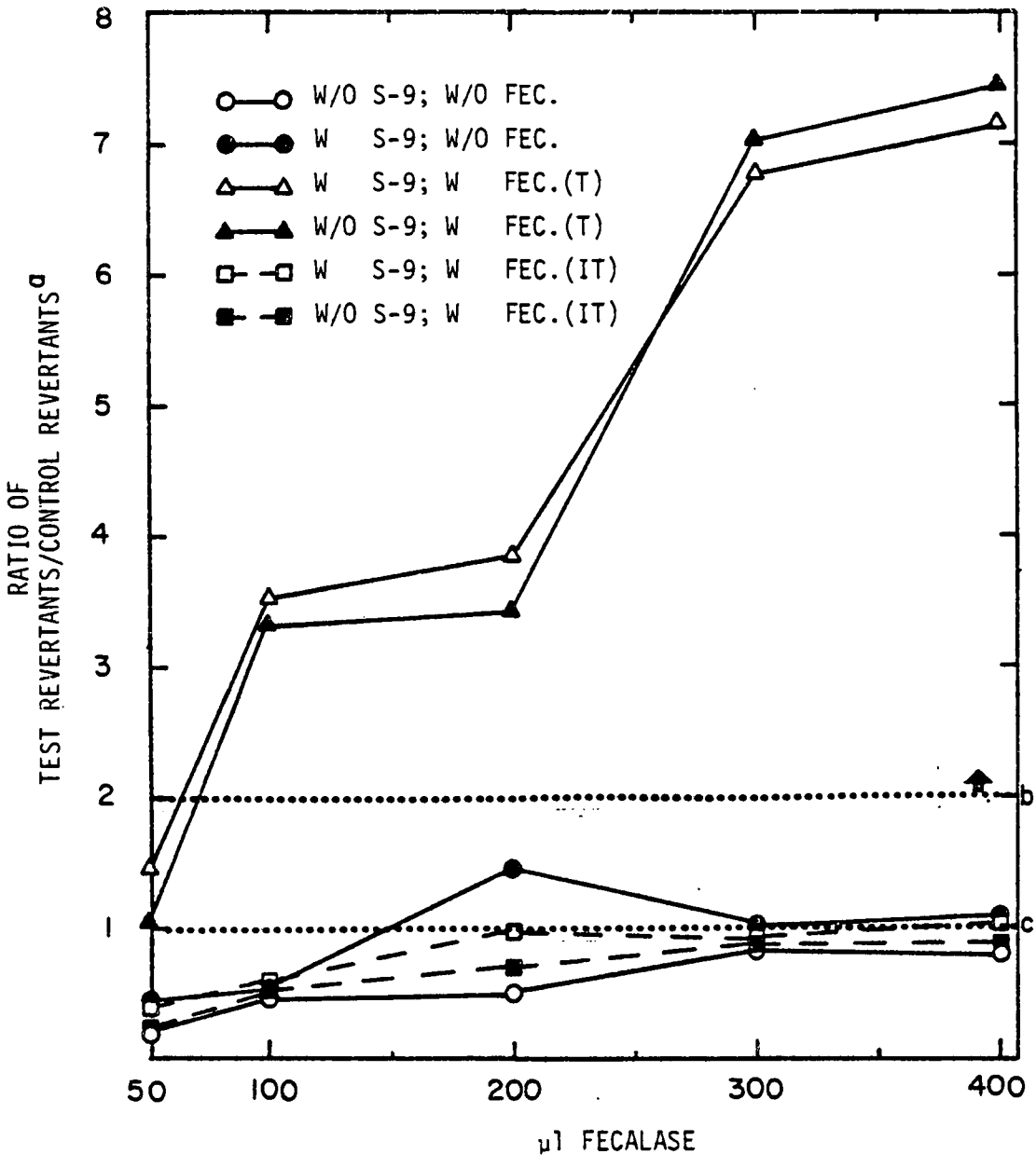


Figure 14. Mutagenicity of Yellow Onion Flavonoid Extract on Strain TA98 with Fecalase.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA98; WS-9 = 43, W/O S-9 = 41).

T = Fecalase from Lactose tolerant individuals.
IT = Fecalse from Lactose intolerant individuals.

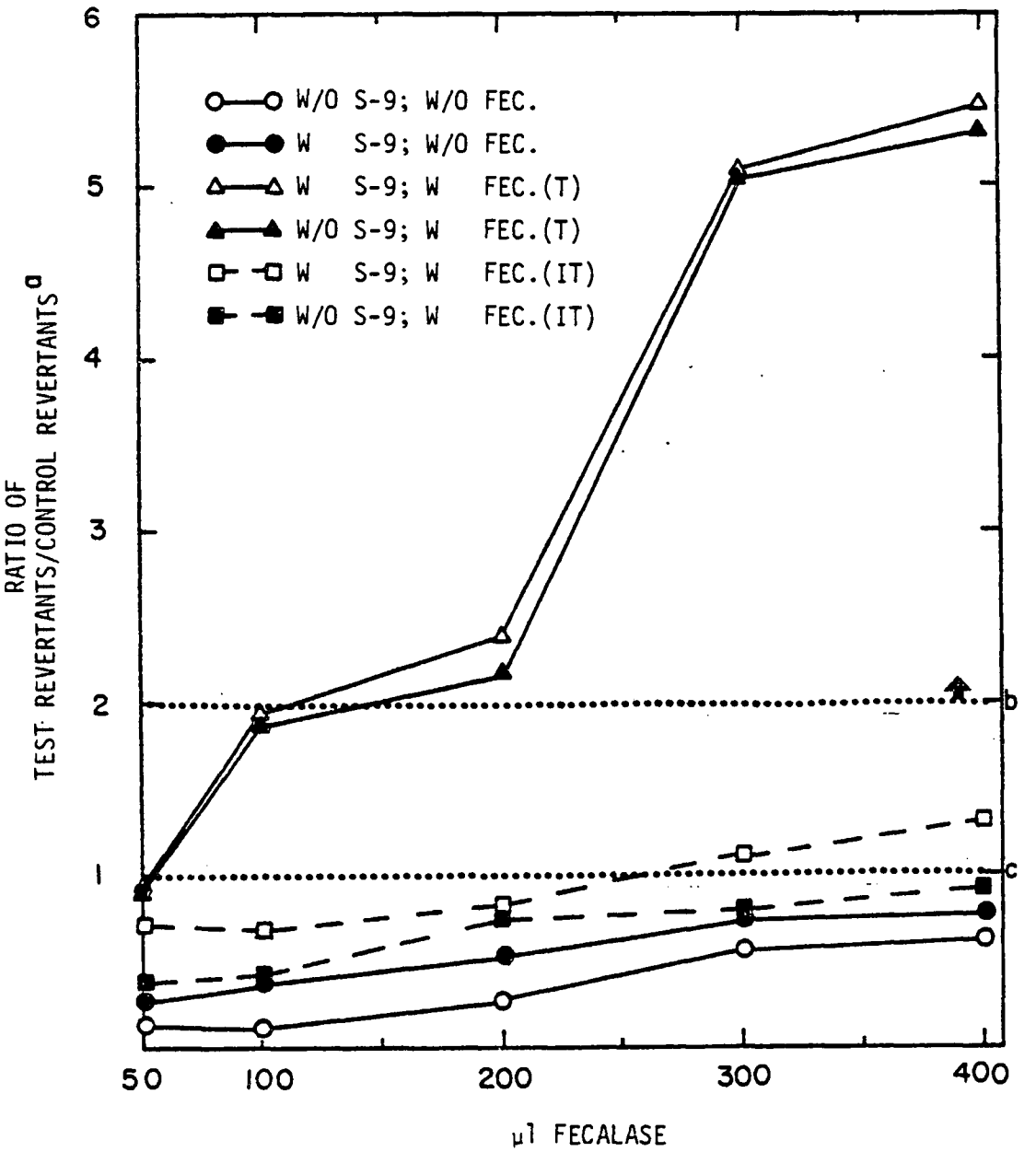


Figure 15. Mutagenicity of Yellow Onion Flavonoid Extract on Strain TA97 with Fecalase.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA97; WS-9 = 98, W/O S-9 = 95).

T = Fecalase from Lactose tolerant individuals.
IT = Fecalse from Lactose intolerant individuals.

TABLE 9. Mutagenicity of Yellow Onion (Allium cepa) Flavonoid Extract [300 nmol] with Fecalase

		Ratio of Test Revertants/Control Revertants					
Tester Strain	µl Fecalase	W/O S-9; W/O Fec.	W S-9; W/O Fec.	W/O S-9; W Fec.(T)	W S-9; W Fec.(T)	W/O S-9; W Fec.(IT)	W/O S-9; W Fec.(IT)
TA 1535	50	.19	.30	.52	.47	.18	.32
	100	.14	.61	1.05	1.22	.42	.58
	200	.20	.75	1.35	1.25	.39	.64
	300	.56	.96	1.83	1.61	.73	.90
	400	.67	.96	1.91	1.96	.91	1.18
TA 100	50	.08	.17	.46	.42	.19	.27
	100	.12	.23	.69	.69	.12	.44
	200	.19	.27	.77	.86	.16	.46
	300	.35	.41	1.00	1.08	.23	.68
	400	.34	.44	1.02	1.10	.76	.89

TA 1535: W S-9 = 25, W/O S-9 = 20

TA 100: W S-9 = 184, W/O S-9 = 178

T = Fecalase from lactose tolerant; IT = Fecalase from lactose intolerant.

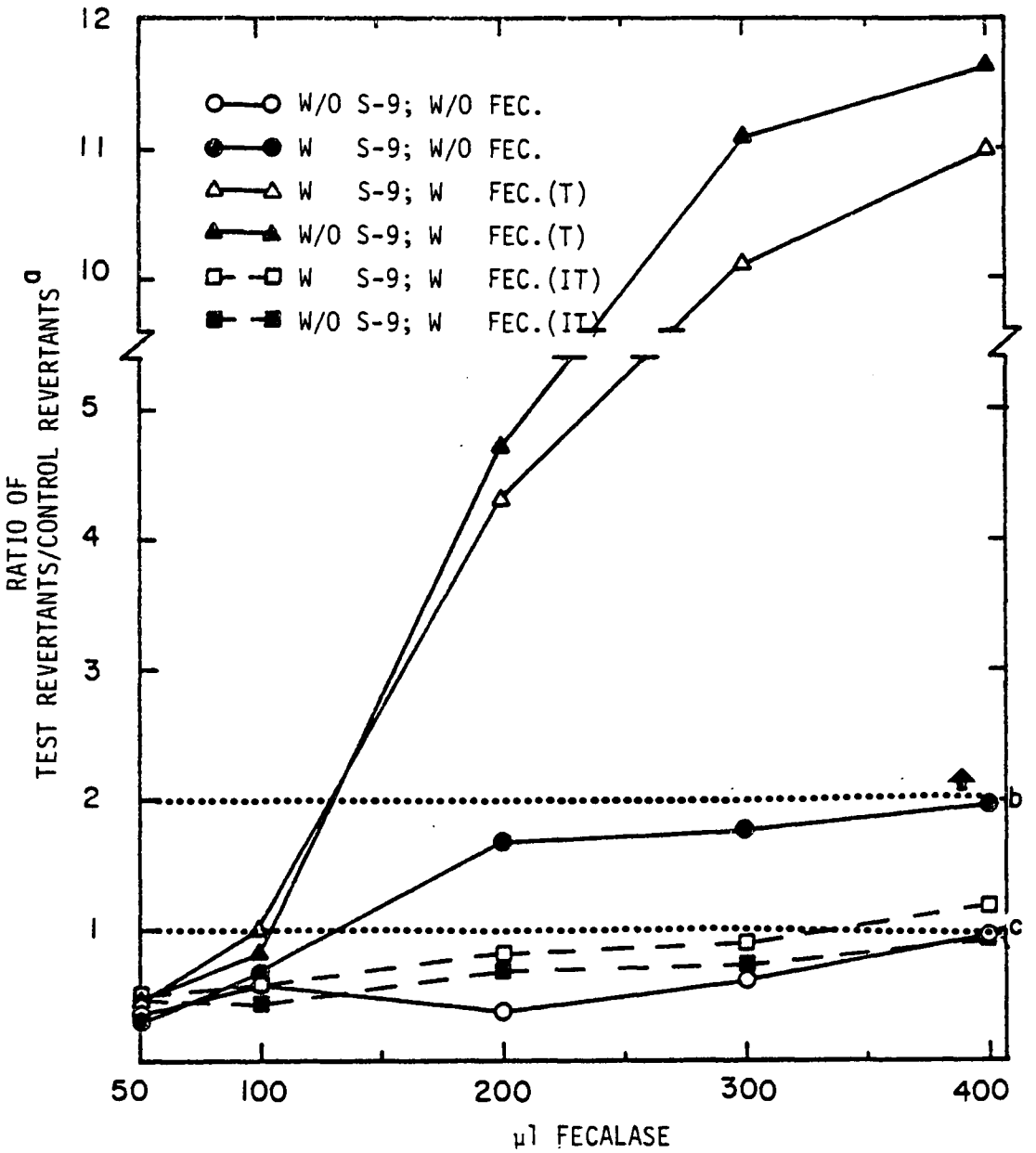


Figure 16. Mutagenicity of Raspberry Flavonoid Extract on Strain TA1537 with Fecalase.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA1537; WS-9 = 23, W/O S-9 = 20).

T = Fecalase from Lactose tolerant individuals.
IT = Fecalse from Lactose intolerant individuals.

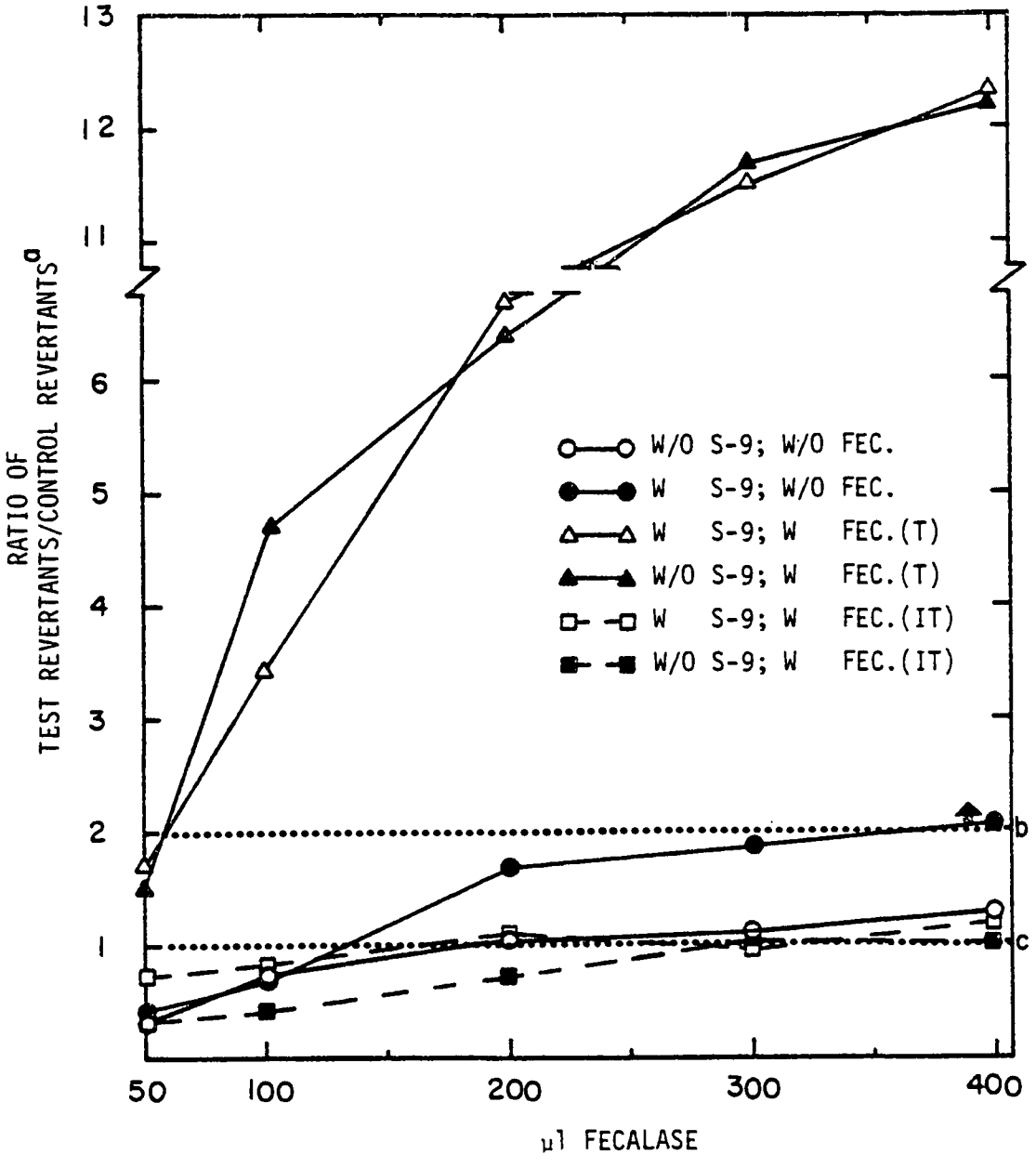


Figure 17. Mutagenicity of Raspberry Flavonoid Extract on Strain TA98 with Fecalase.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA98; WS-9 = 43, W/O S-9 = 41).

T = Fecalase from Lactose tolerant individuals.
IT = Fecalse from Lactose intolerant individuals.

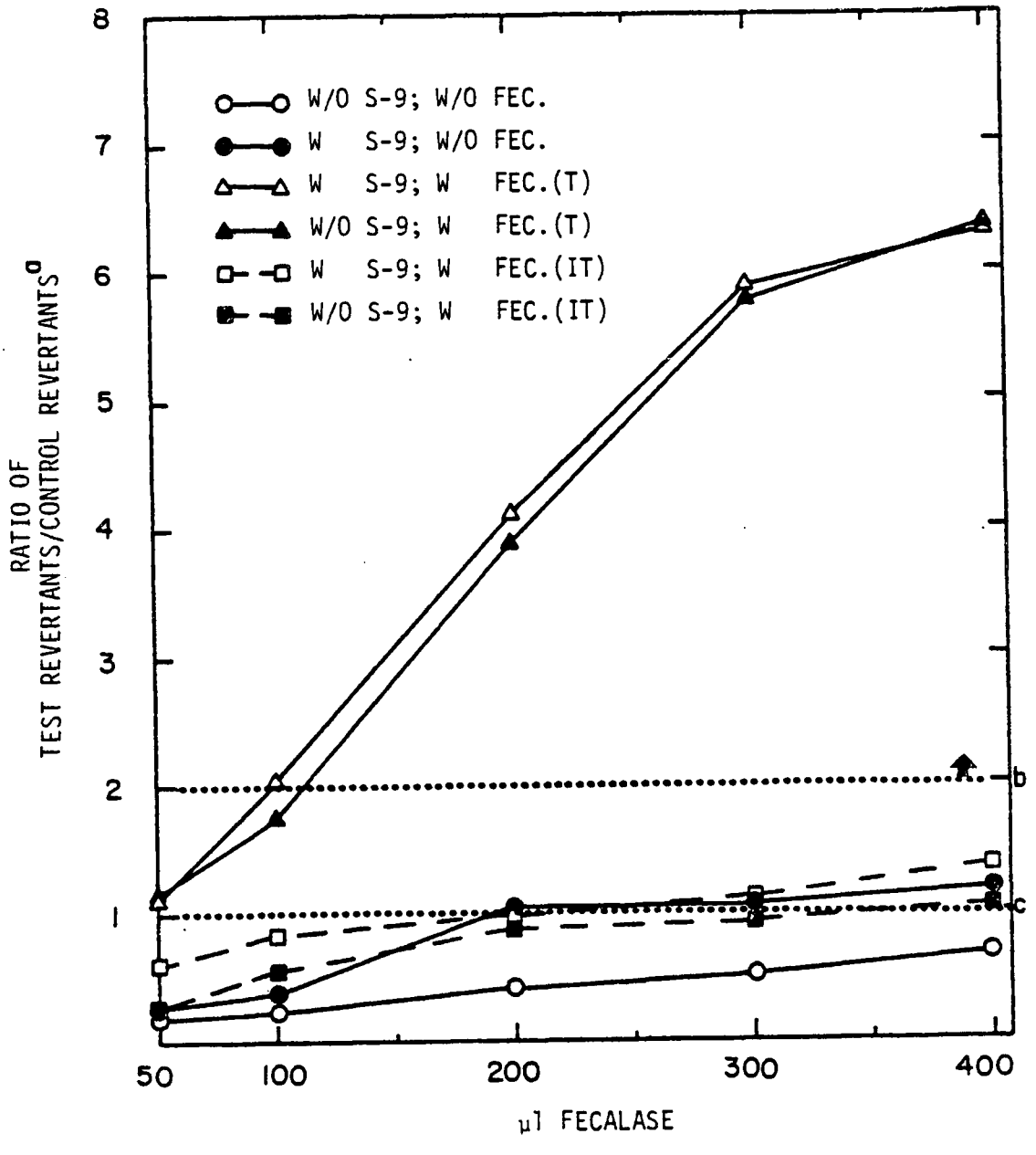


Figure 18. Mutagenicity of Raspberry Flavonoid Extract on Strain TA97 with Fecalase.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA97; WS-9 = 98, W/O S-9 = 95).

T = Fecalase from Lactose tolerant individuals.
IT = Fecalse from Lactose intolerant individuals.

TABLE 10. Mutagenicity of Raspberry (Rubus idaeus) Flavonoid Extract [300 nmol] with Fecalase

		Ratio of Test Revertants/Control Revertants					
Tester Strain	µl Fecalase	W/O S-9; W/O Fec.	W S-9; W/O Fec.	W/O S-9; W Fec.(T)	W S-9; W Fec.(T)	W/O S-9; W Fec.(IT)	W/O S-9; W Fec.(IT)
TA 1535	50	.19	.20	.38	.47	.17	.42
	100	.24	.39	.62	.74	.24	.51
	200	.30	.58	1.15	1.13	.43	.76
	300	.56	.65	1.61	1.35	.58	.81
	400	.67	.96	1.71	1.78	.76	.89
TA 100	50	.11	.18	.17	.22	.11	.24
	100	.14	.25	.24	.38	.23	.48
	200	.21	.43	.59	.64	.39	.59
	300	.27	.44	.63	.71	.43	.67
	400	.33	.43	.73	.80	.63	.79

TA 1535: W S-9 = 25, W/O S-9 = 20

TA 100: W S-9 = 184, W/O S-9 = 178

T = Fecalase from lactose tolerant; IT = Fecalase from lactose intolerant.

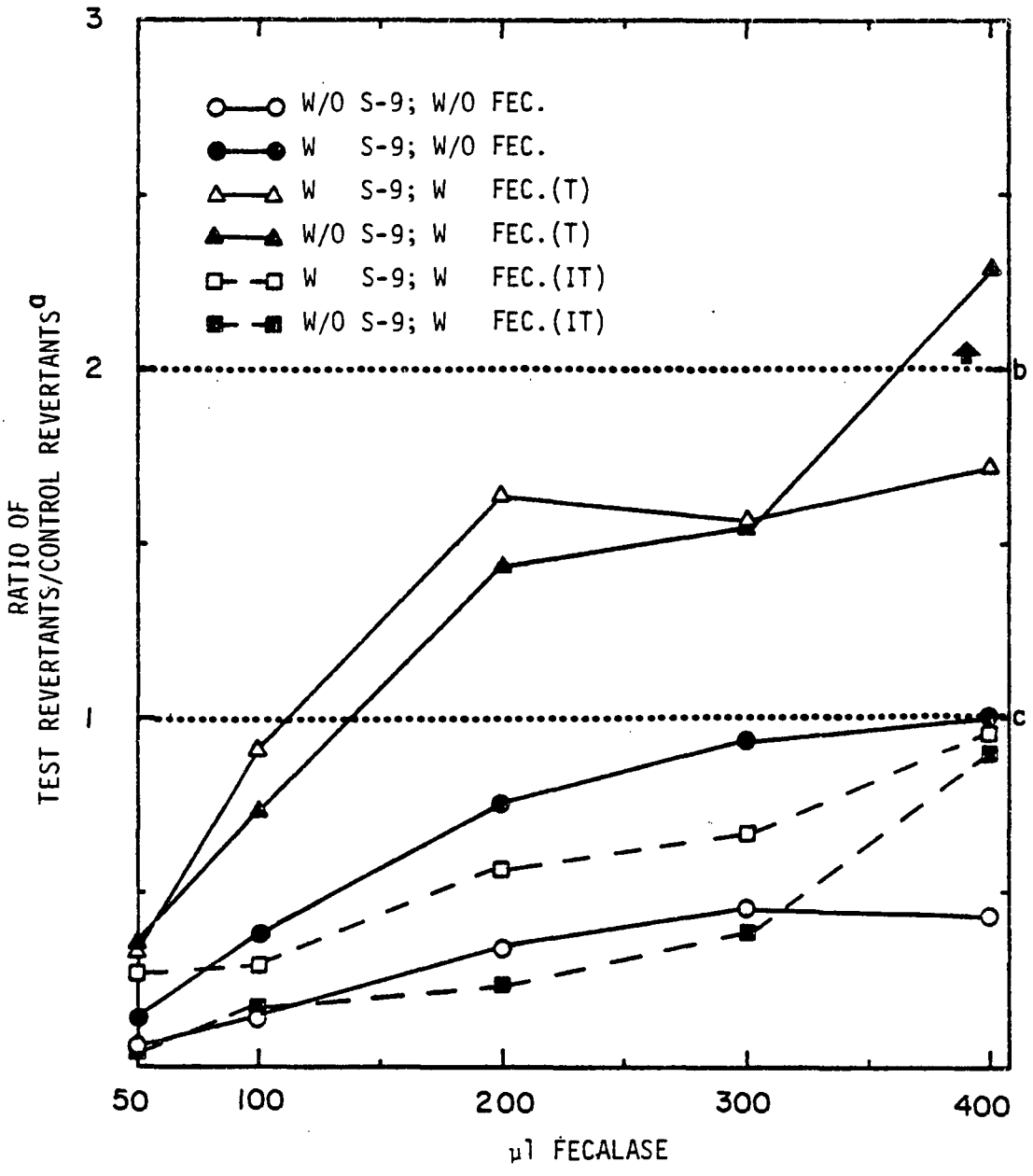


Figure 19. Mutagenicity of White Onion Extract on Strain TA1537 with Fecalase.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA1537; WS-9 = 23, W/O S-9 = 20).

T = Fecalase from Lactose tolerant individuals.
IT = Fecalse from Lactose intolerant individuals.

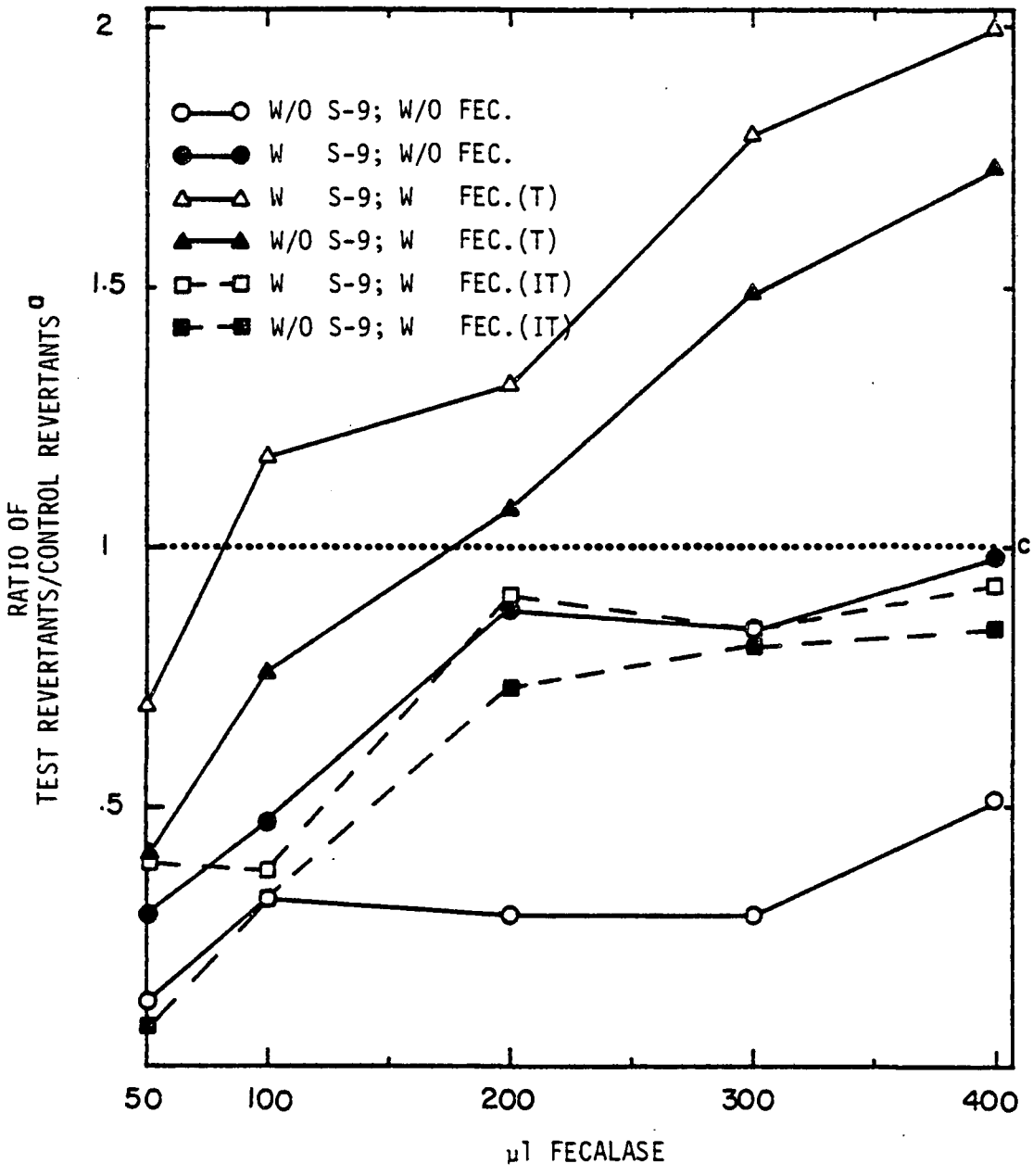


Figure 20. Mutagenicity of White Onion Extract on Strain TA98 with Fecalase.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA98; WS-9 = 43, W/O S-9 = 41).

T = Fecalase from Lactose tolerant individuals.
IT = Fecalse from Lactose intolerant individuals.

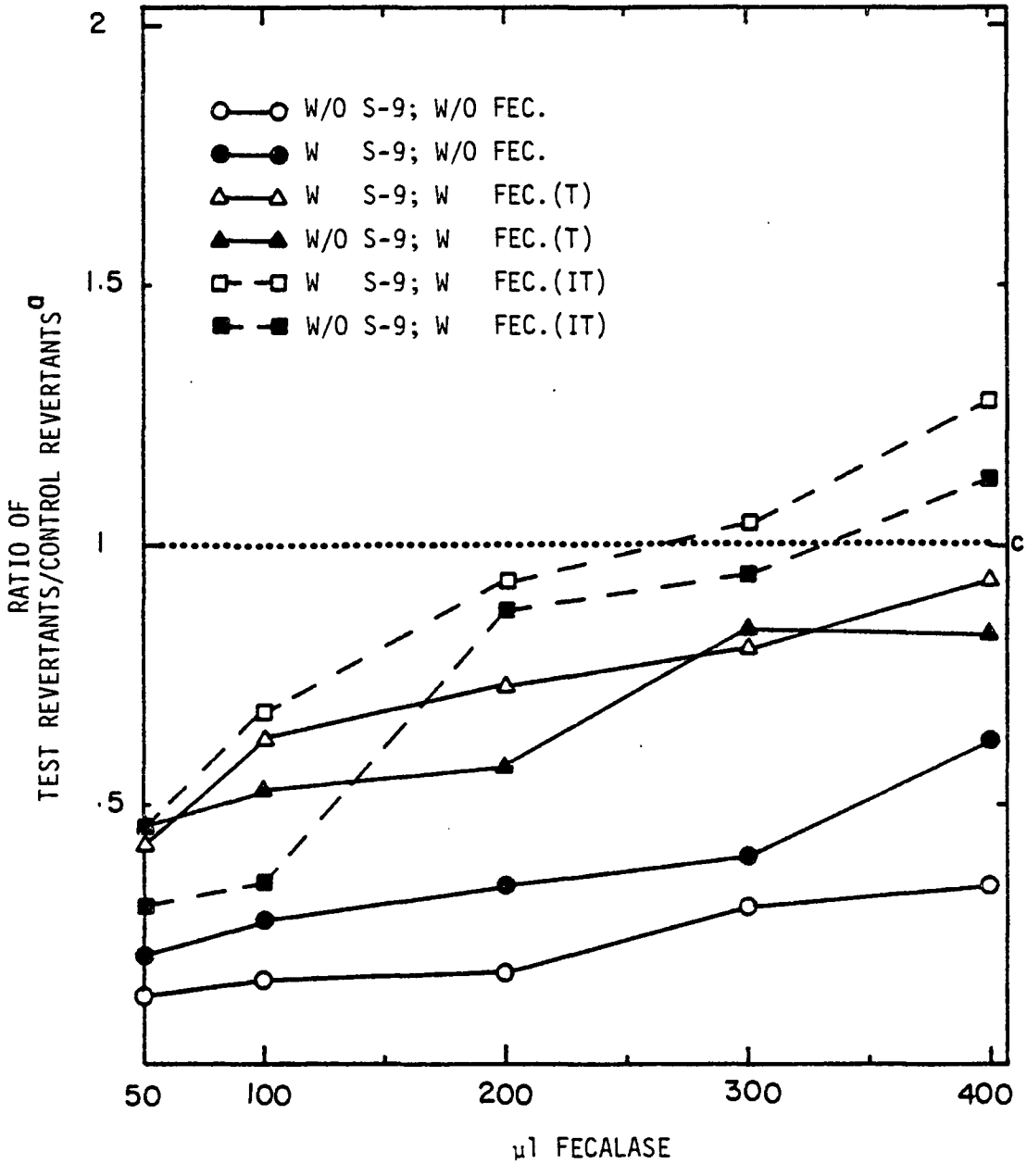


Figure 21. Mutagenicity of White Onion Extract on Strain TA97 with Fecalase.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA97; WS-9 = 98, W/O S-9 = 95).

T = Fecalase from Lactose tolerant individuals.
IT = Fecalse from Lactose intolerant individuals.

TABLE 11. Mutagenicity of White Onion (Allium cepa) Extract [300 nmol] with Fecalase

		Ratio of Test Revertants/Control Revertants					
Tester Strain	µl Fecalase	W/O S-9; W/O Fec.	W S-9; W/O Fec.	W/O S-9; W Fec.(T)	W S-9; W Fec.(T)	W/O S-9; W Fec.(IT)	W/O S-9; W Fec.(IT)
TA 1535	50	.10	.17	.29	.27	.09	.19
	100	.24	.39	.67	.57	.14	.26
	200	.30	.63	.95	1.00	.17	.38
	300	.67	.87	1.22	1.09	.24	.58
	400	.67	1.04	1.14	1.26	.74	.83
TA 100	50	.08	.13	.19	.26	.12	.41
	100	.09	.17	.29	.38	.29	.69
	200	.11	.24	.43	.51	.32	1.16
	300	.18	.29	.52	.58	.48	.77
	400	.24	.33	.61	.63	1.04	1.29

TA 1535: W S-9 = 25, W/O S-9 = 20

TA 100: W S-9 = 184, W/O S-9 = 178

T = Fecalase from lactose tolerant; IT = Fecalase from lactose intolerant.

Constant Fecalase (200 µl) with Various Amounts of Substrate

In this experiment, only fecalase from lactose-tolerant preparation and only frameshift mutant strains TA 1537, TA 98, and TA 97 were employed. The mixed function cytochrome oxidase system was incorporated but under different assay conditions. The glycosidic flavonoid substrate was tested at different levels of concentrations ranging from 50 to 500 nmol. The testing procedure was modified by incubating the fecalase enzyme, test substrate, tester strain, and mixed function cytochrome oxidase system from rat liver for 20 minutes at 37°C. This procedure employed to attain full bioactivation of the mutagen by the fecalase and the hepatic microsomal enzymes.

Amounts ranging from 200 to 500 nmol of Cantaloupe flavonoid extract showed mutagenic dose-response curve with the three frameshift mutant tester strains used, but only with the addition of 200 ml fecalase preparation from lactose-tolerant individuals.

These data are recorded in Figure 22. The addition of liver homogenate with or without the fecalase did not cause significant changes. In case of Red Onion, Yellow Onion, and Raspberry flavonoid extract, as shown in Figures 23, 24, and 25, respectively, a positive response for mutagenic activity was demonstrated between 100 and 500 nmol range, giving a soundable dose-response curve. Similarly, the addition of the S-9 mix with or without the fecalase did not reflect significant change in the overall

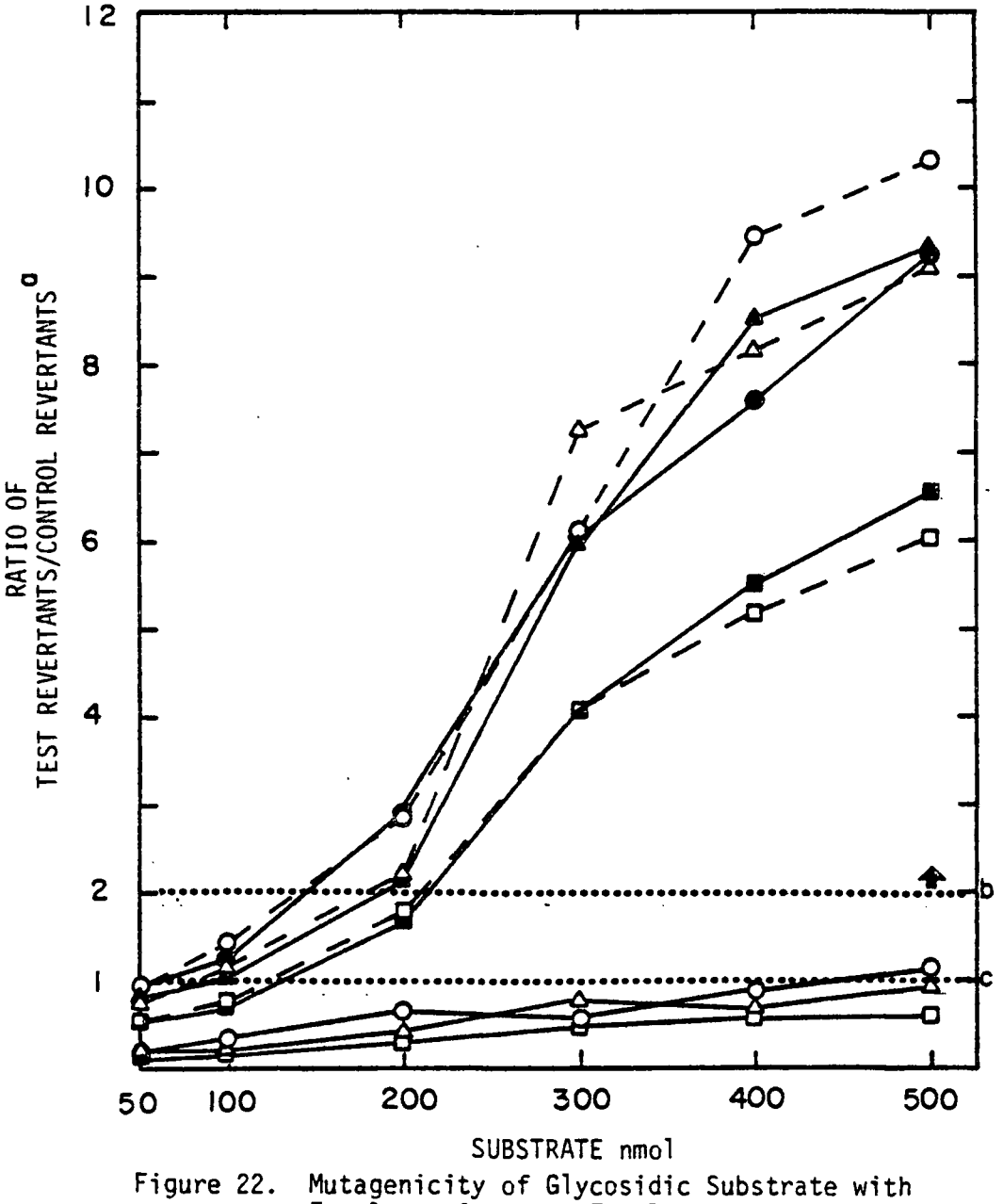


Figure 22. Mutagenicity of Glycosidic Substrate with Fecalase, Constant Fecalase with various amounts of Cantaloupe Flavonoid Extract.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA 1537; WS-9 = 23, W/O S-9 = 20), (TA 98; WS-9 = 43, W/O S-9 = 41); (TA 97; WS-9 = 98, W/O S-9 = 95).

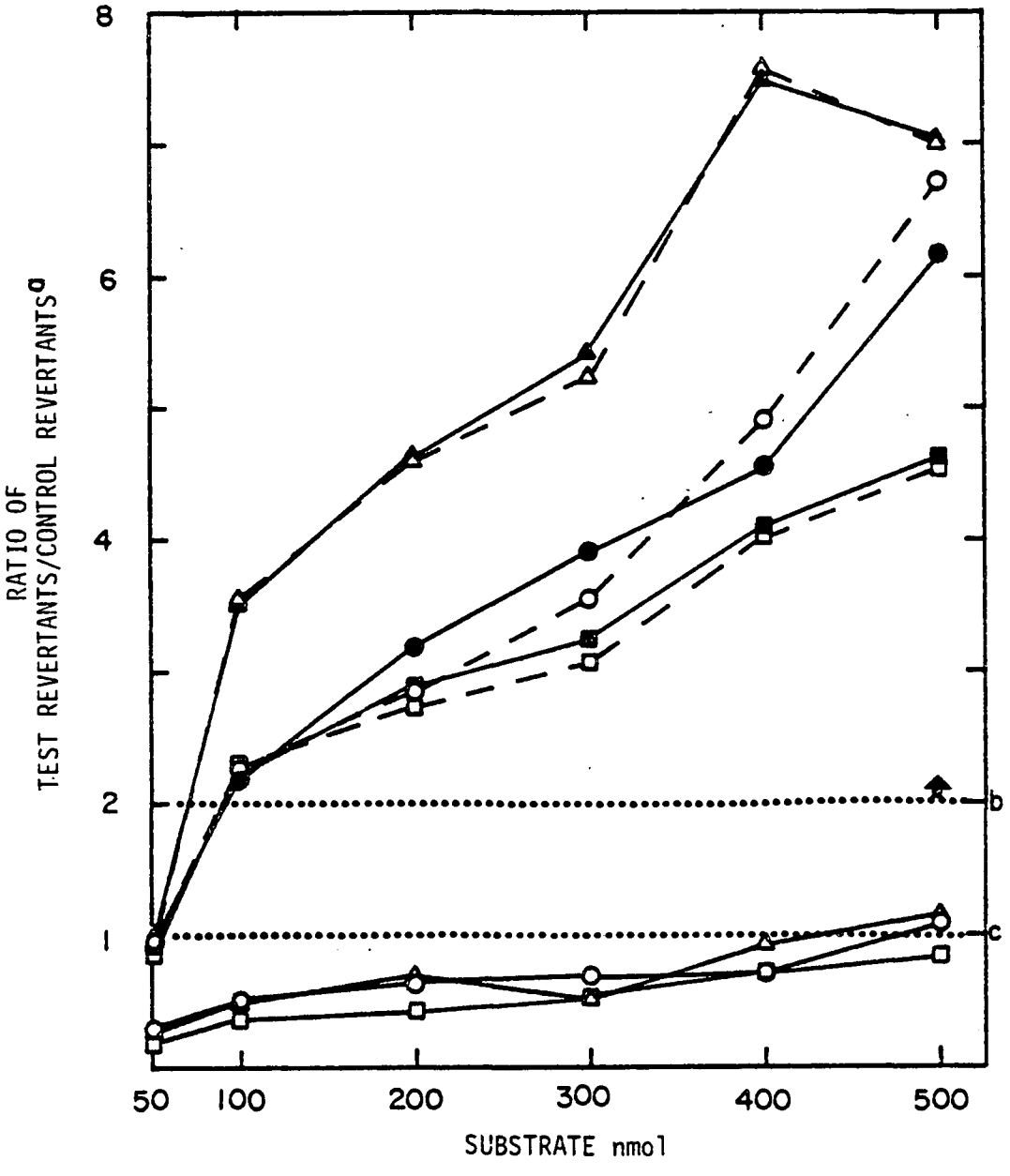


Figure 23. Mutagenicity of Glycosidic Substrate with Fecalase, Constant Fecalase with various amounts of Red Onion Flavonoid Extract.

TA1537		TA98		TA97	
W	S-9;W/O FEC.	W	S-9;W/O FEC.	W	S-9;W/O FEC.
W	S-9;W FEC.	W	S-9;W FEC.	W	S-9;W FEC.
W/O	S-9;W FEC.	W/O	S-9;W FEC.	W/O	S-9;W FEC.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA 1537; WS-9 = 23, W/O S-9 = 20), (TA 98; WS-9 = 43, W/O S-9 = 41); (TA 97; WS-9 = 98, W/O S-9 = 95).

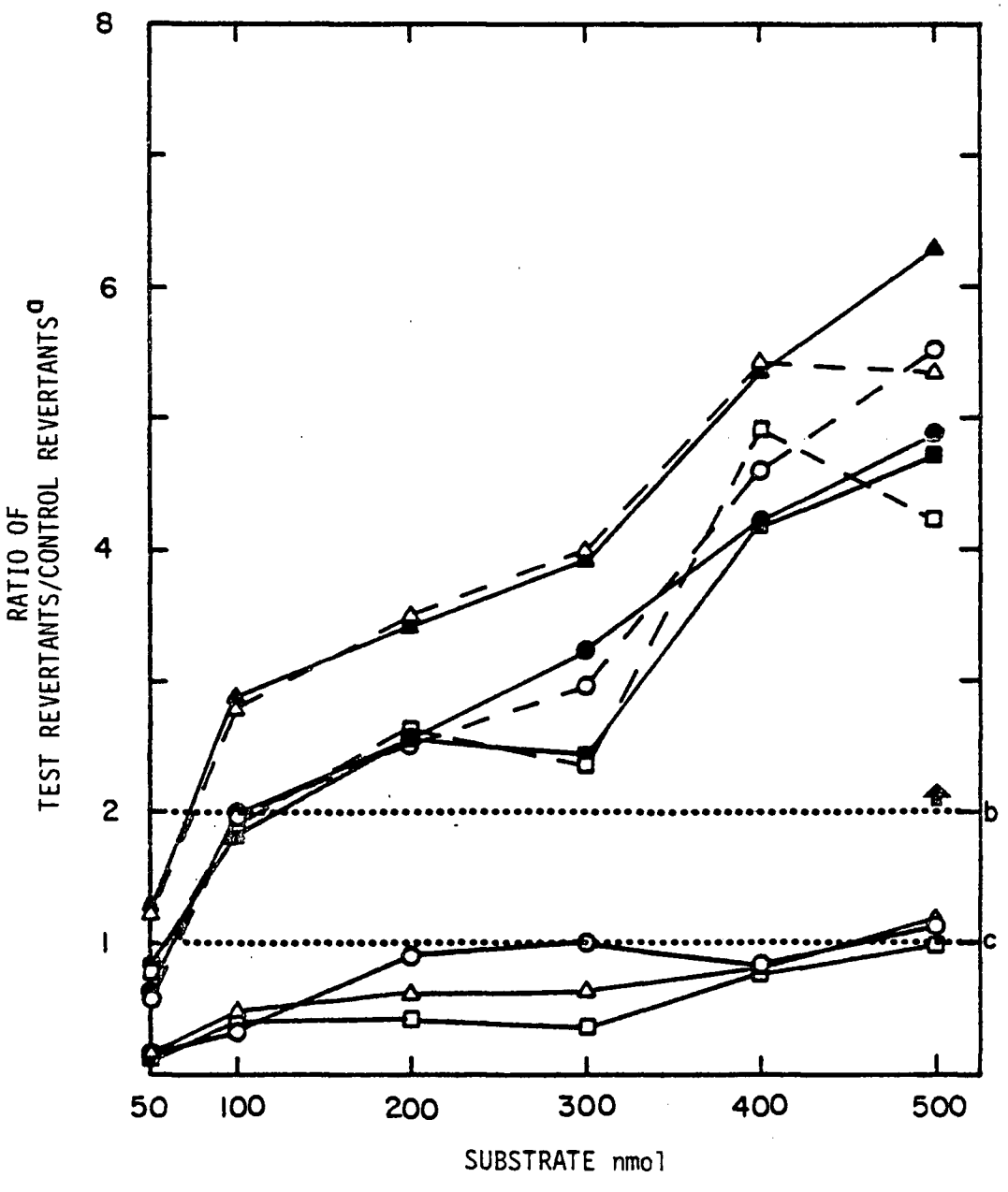


Figure 24. Mutagenicity of Glycosidic Substrate with Fecalase, Constant Fecalase with various amounts of Yellow Onion Flavonoid Extract.

TA1537	TA98	TA97
W S-9;W/O FEC.	W S-9;W/O FEC.	W S-9;W/O FEC.
W S-9;W FEC.	W S-9;W FEC.	W S-9;W FEC.
W/O S-9;W FEC.	W/O S-9;W FEC.	W/O S-9;W FEC.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA 1537; WS-9 = 23, W/O S-9 = 20), (TA 98; WS-9 = 43, W/O S-9 = 41); (TA 97; WS-9 = 98, W/O S-9 = 95).

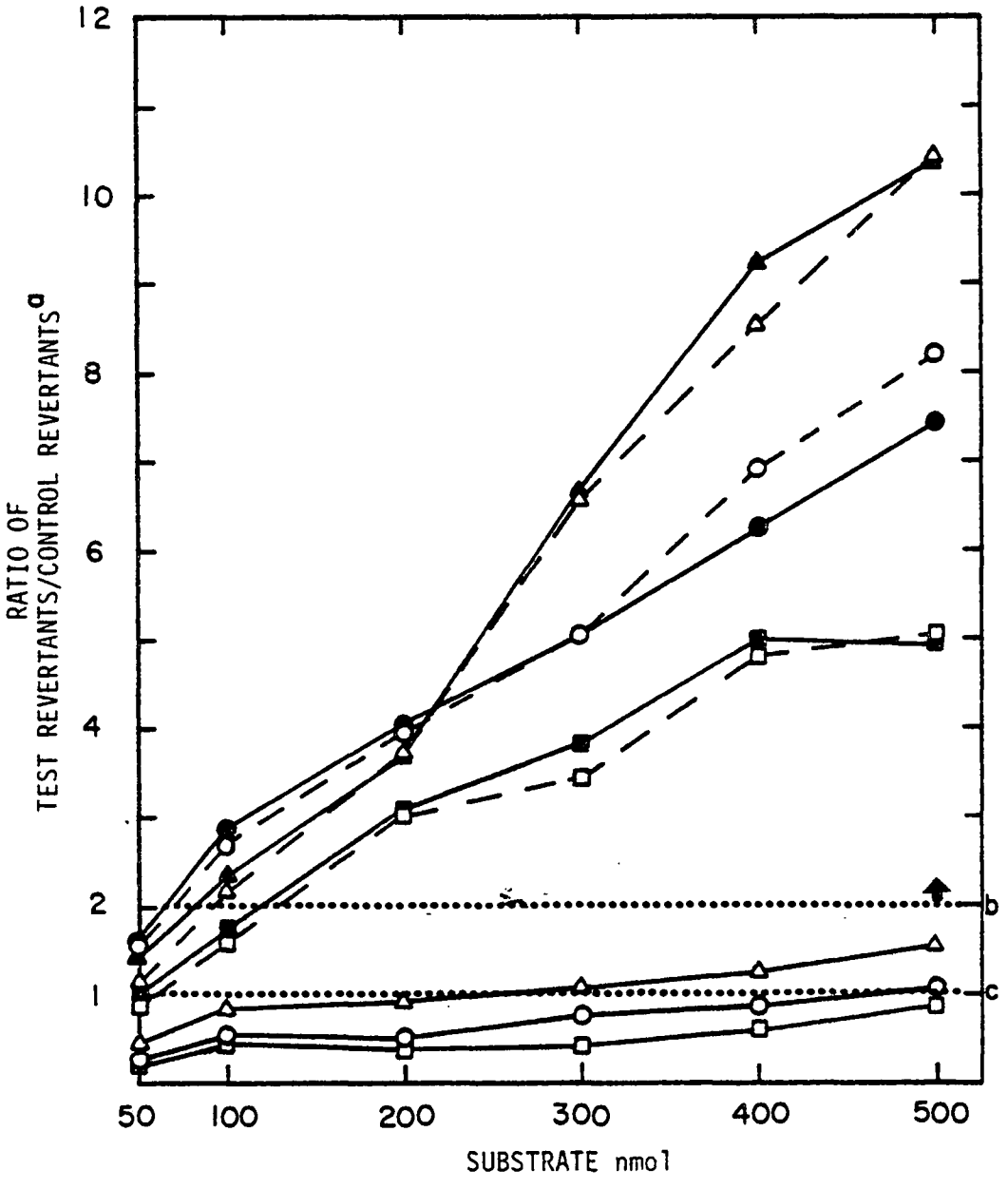


Figure 25. Mutagenicity of Glycosidic Substrate with Fecalase, Constant Fecalase with various amounts of Raspberry Flavonoid Extract.

TA1537		TA98		TA97	
W	S-9;W/O FEC.	W	S-9;W/O FEC.	W	S-9;W/O FEC.
W	S-9;W FEC.	W	S-9;W FEC.	W	S-9;W FEC.
W/O	S-9;W FEC.	W/O	S-9;W FEC.	W/O	S-9;W FEC.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA 1537; WS-9 = 23, W/O S-9 = 20), (TA 98; WS-9 = 43, W/O S-9 = 41); (TA 97; WS-9 = 98, W/O S-9 = 95).

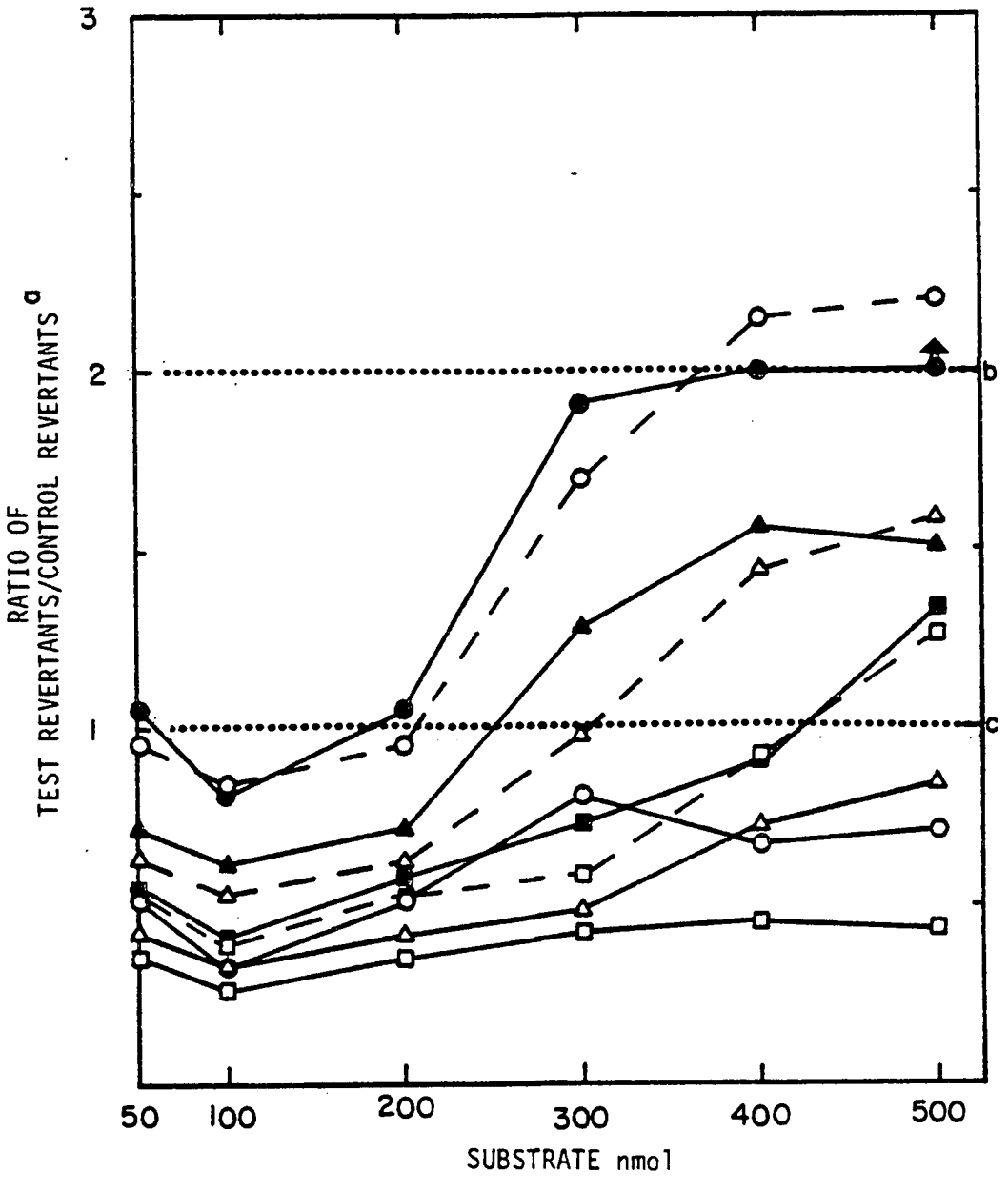


Figure 26. Mutagenicity of Glycosidic Substrate with Fecalase, Constant Fecalase with various amounts of White Onion Flavonoid Extract.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA 1537; WS-9 = 23, W/O S-9 = 20), (TA 98; WS-9 = 43, W/O S-9 = 41); (TA 97; WS-9 = 98, W/O S-9 = 95).

result. The White Onion extract as shown in Figure 26 demonstrated a very weak positive mutagenic response on frameshift mutant TA 1537 even after addition of high amounts of extract ranging from 400 to 500 nmol of the substrate. The other frameshift mutants TA 98 and TA 97 did not show positive mutagenic response even at higher extract quantities. The reason for the lack of response could be because of the high spontaneous control revertant number of TA 98=43 and TA 97=98, or very low presence of flavonoid glycosides in the White Onion extract.

Constant Substrate (200 nmol) from Spot 'A' and 'B' of TLC with Various Amounts of Fecalase (T)

According to the other experiments results, the spots designated 'A' of the flavonoid extracts of Cantaloupe, Red Onion, Yellow Onion, and Raspberry were identified as quercitrin. The spots on the bottom half of the plate were collected separately and designated 'B'. White Onion was excluded from this study since its extract did not show any quercitrin presence on the previous experiment. The different spots were scraped off the plates, taken by minimal amount of methanol and, after dryness, dissolved in dimethylsulfoxide (DMSO) to give a concentration of 200 nmol for each of the four samples of extracts tested. Frameshift tester strains TA 1537, TA 98, and TA 97 were used for mutagenic activity detection. Lactose-tolerant fecalase was used in the range of 25 to 200 ml. The mixed function Oxidase

System was added in different combinations, either with or without fecalase. The modification in the procedure mentioned earlier was employed. Data in Figures 27, 28, and 29 show that spots 'A' of Cantaloupe flavonoid extract identified as quercitrin was mutagenic on frameshift tester strains TA 1537, TA 98, and TA 97. This frameshift mutagenic activity was due to the ability of fecalase in splitting the mutagenic aglycone and the sugar moeity of the flavonol glycoside. Spots 'B' collected from the bottom half of the plate did not show any mutagenic under the same assay conditions. Both spots 'A' and 'B' show no mutagenic activity without fecalase addition. The hepatic microsomes also show no significant variation in the number of induced revertants.

Data in Figures 30, 31, and 32 show mutagenic activity for spots 'A' of Red Onion upon the addition of fecalase in the range of 25 to 200 µl, on frameshift mutant strains. Spots 'A' samples were not mutagenic without the addition of fecalase spots 'B' samples of the Red Onion were not mutagenic either with or without the addition of fecalase. The liver homogenate addition was not of significant effect on the number of induced test revertant colonies. Data presented in Figures 33, 34, and 35 show similar mutagenic results for both spots 'A' and 'B' of the Yellow Onion extract on the induced reversion rate of frameshift mutant strains, with the addition of fecalase. The only exception was the weak mutagenic activity of Spot 'B' of Yellow Onion extract on tester Strain TA 1537. This may be caused by the low spontaneous

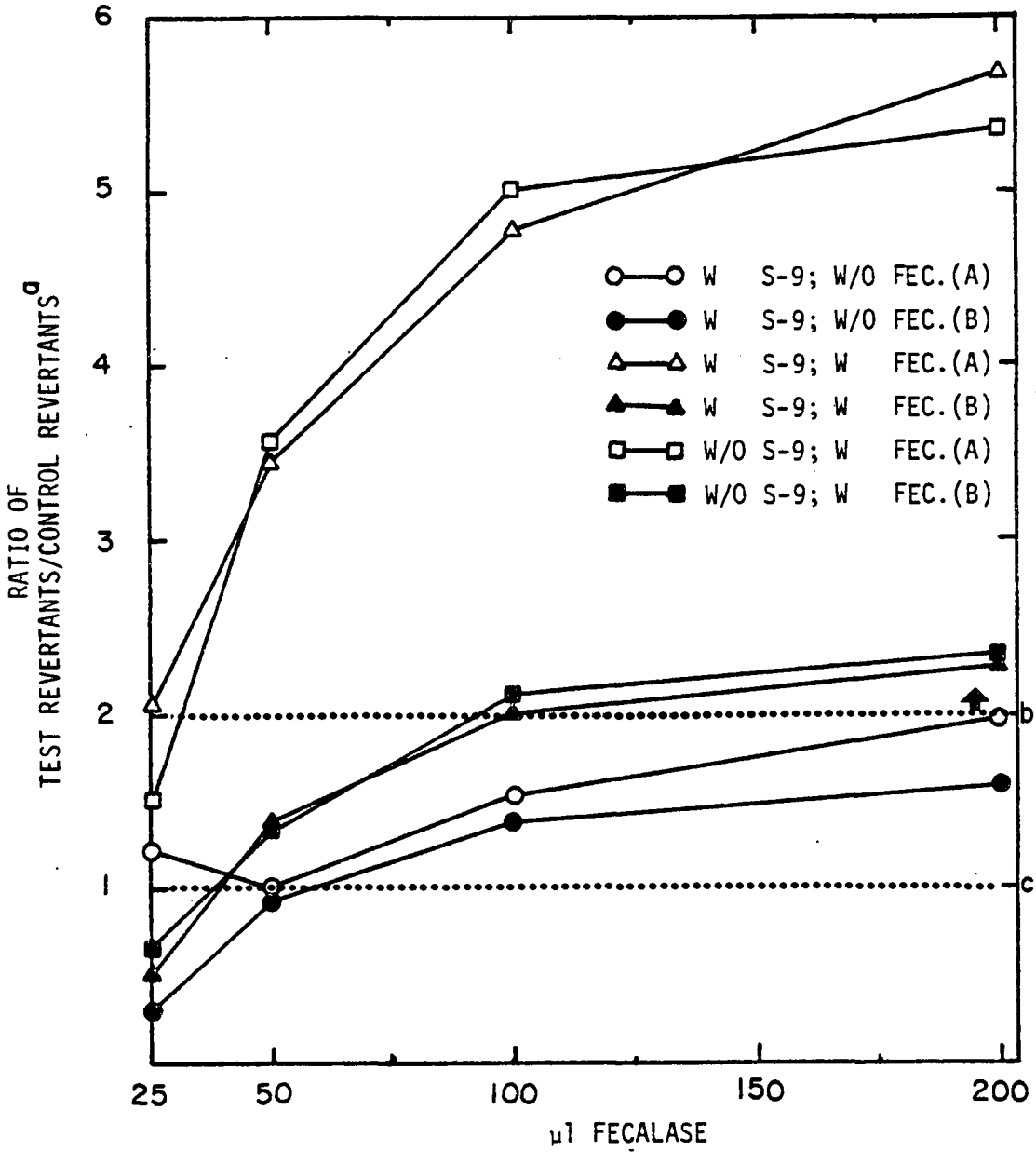


Figure 27. Effect of various amounts of fecalase on reversion of TA 1537 with TLC scrapped spots of Cantaloupe Flavonoid Extract.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA 1537; WS-9 = 23, W/O S-9 = 20)

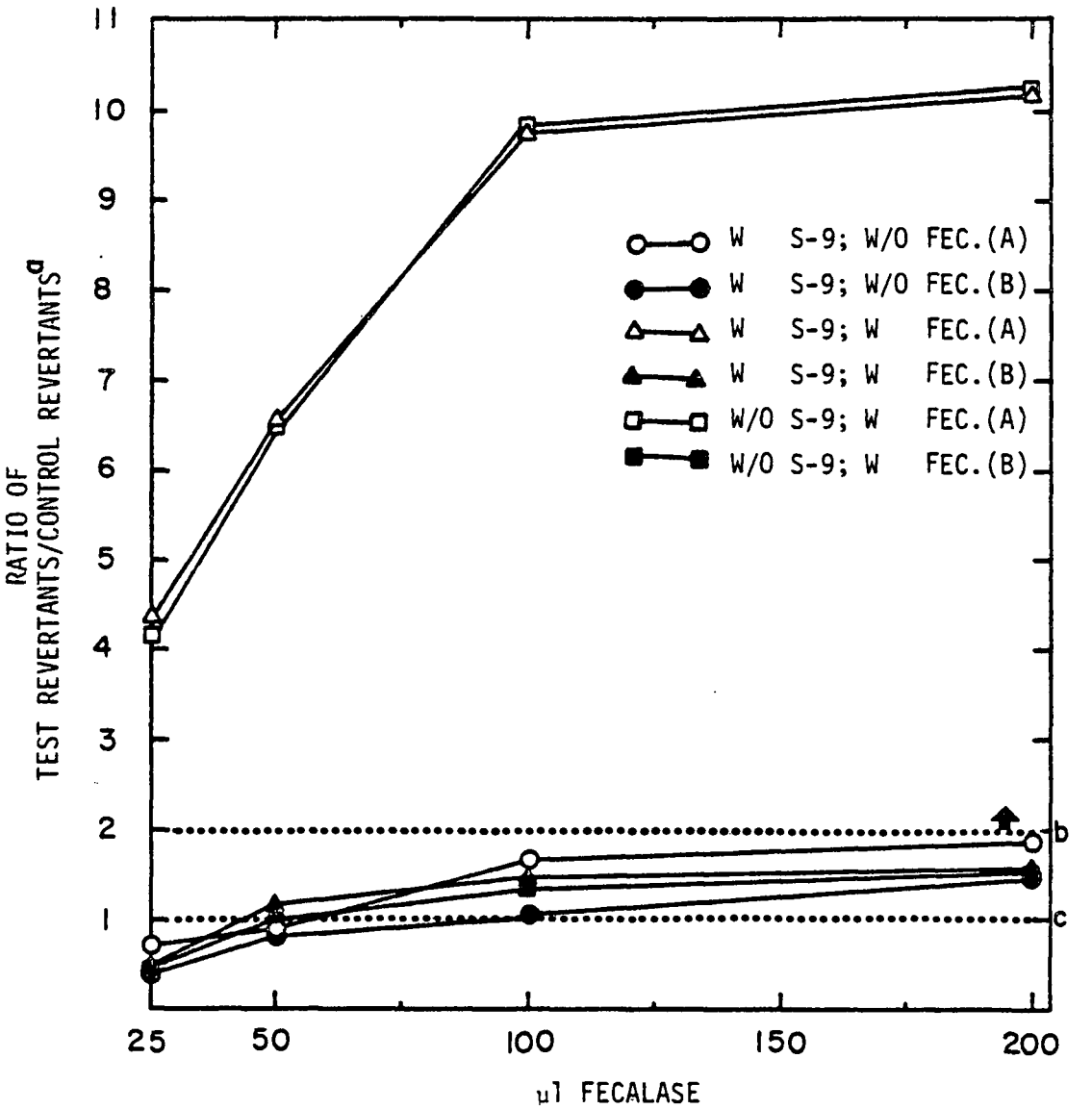


Figure 28. Effect of various amounts of fecalase on reversion of TA98 with TLC scrapped spots of Cantaloupe Flavonoid Extract.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA 98; WS-9 = 43, W/O S-9 = 41).

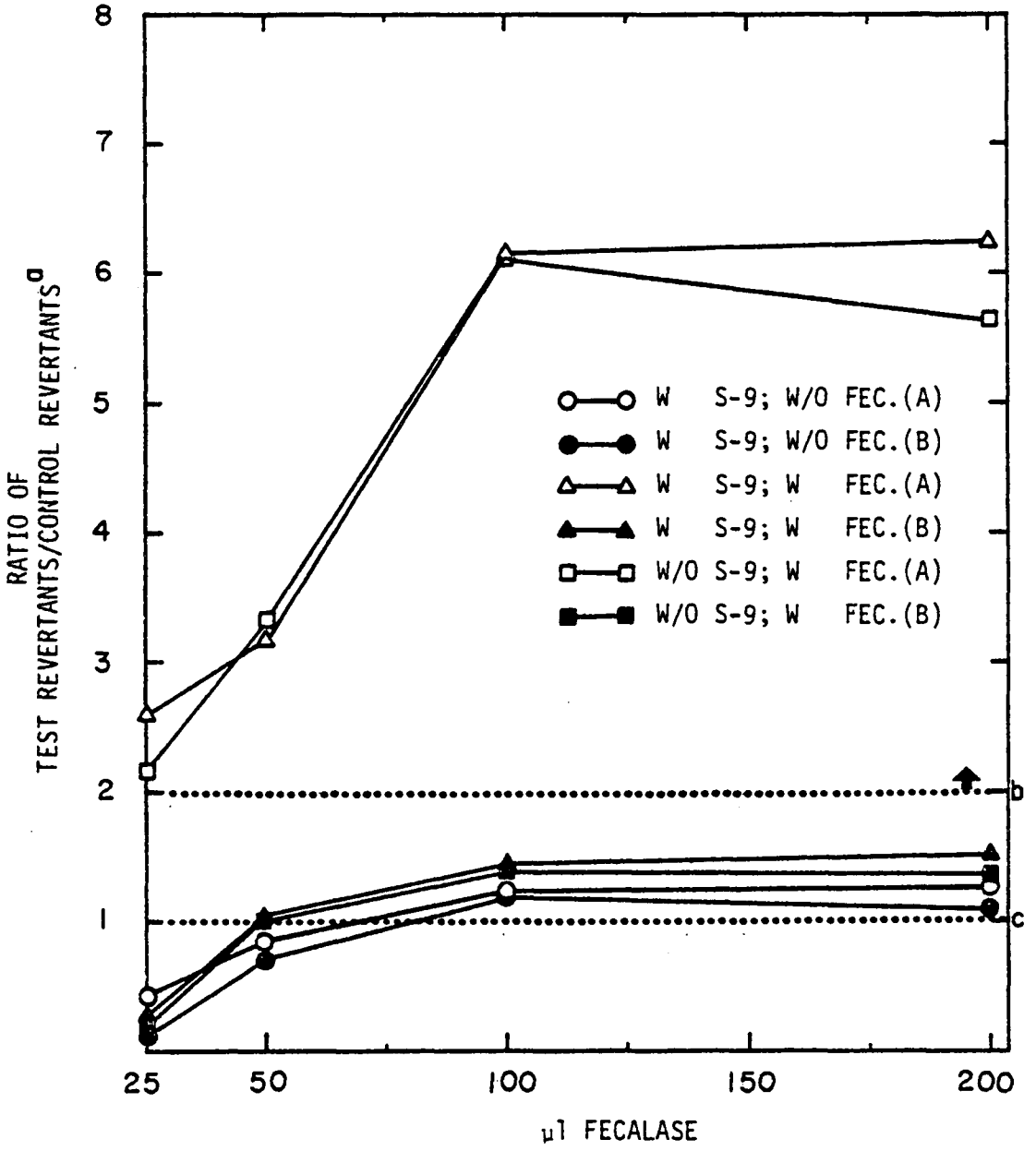


Figure 29. Effect of various amounts of fecalase on reversion of TA97 with TLC scrapped spots of Cantaloupe Flavonoid Extract.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA 97; WS-9 = 98, W/O S-9 = 95).

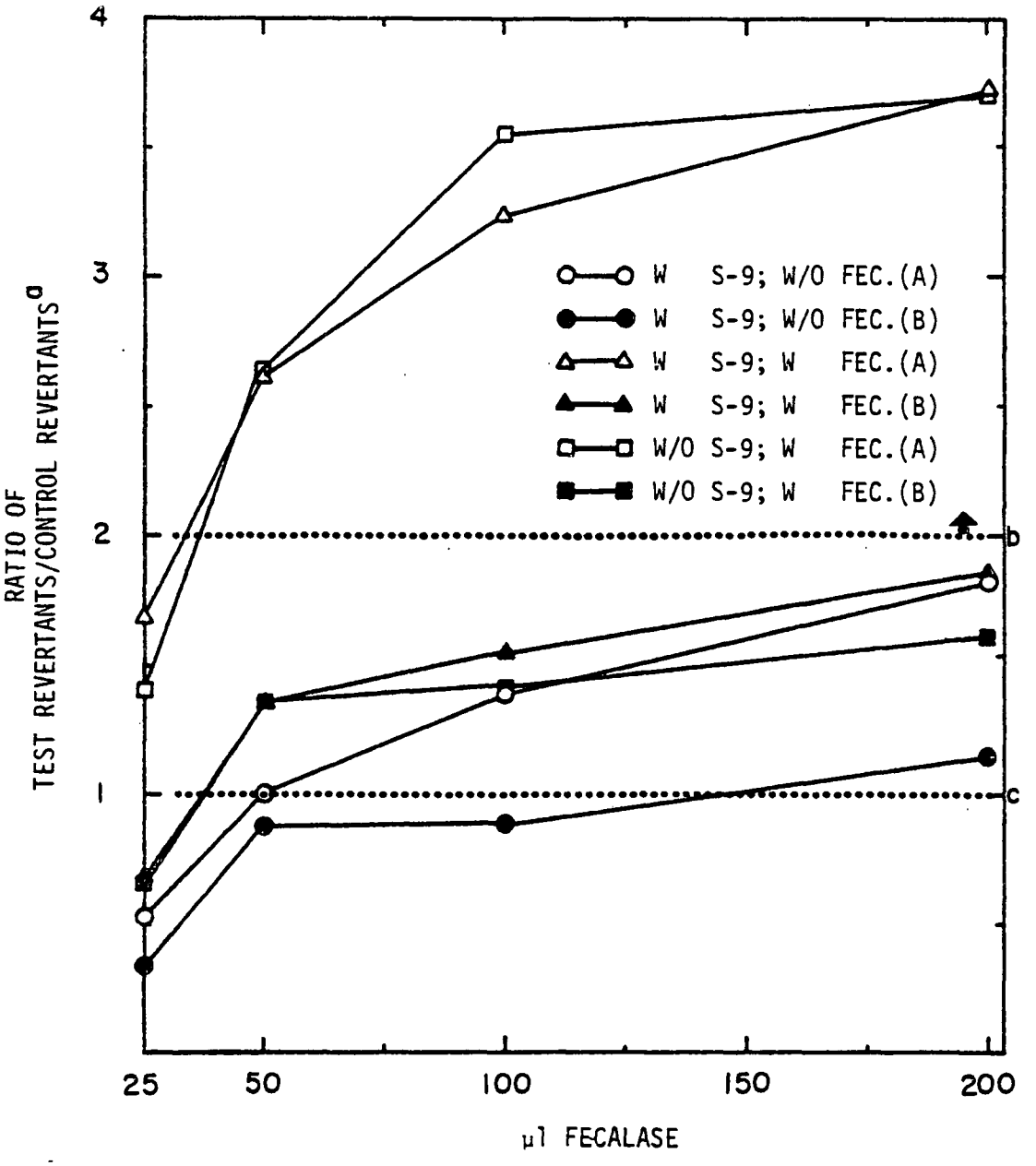


Figure 30. Effect of various amounts of fecalase on reversion of TA1537 with TLC scrapped spots of Red Onion Flavonoid Extract.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA 1537; WS-9 = 23, W/O S-9 = 20)

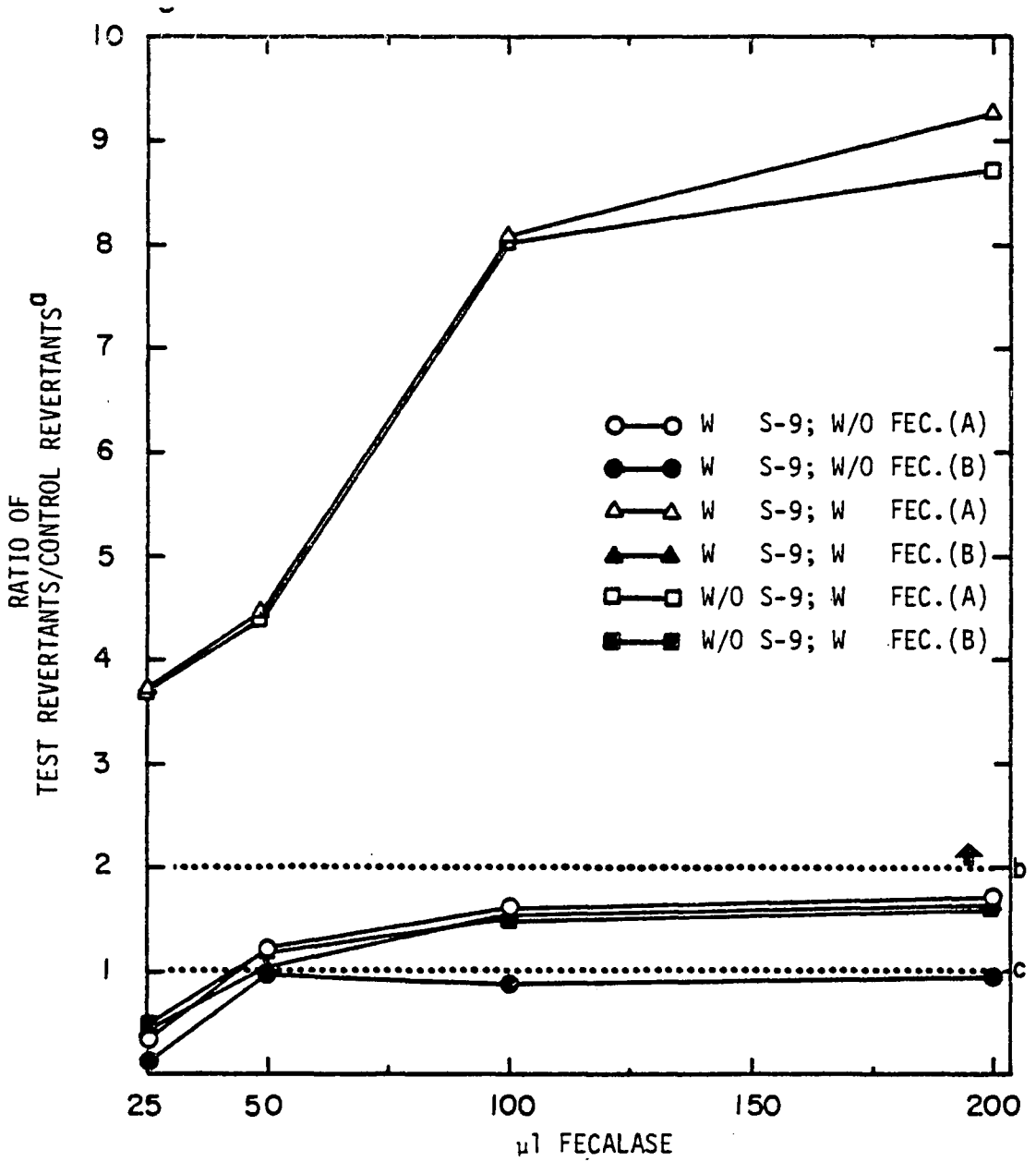


Figure 31. Effect of various amounts of fecalase on reversion of TA98 with TLC scrapped spots of Red Onion Flavonoid Extract.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA 98; WS-9 = 43, W/O S-9 = 41).

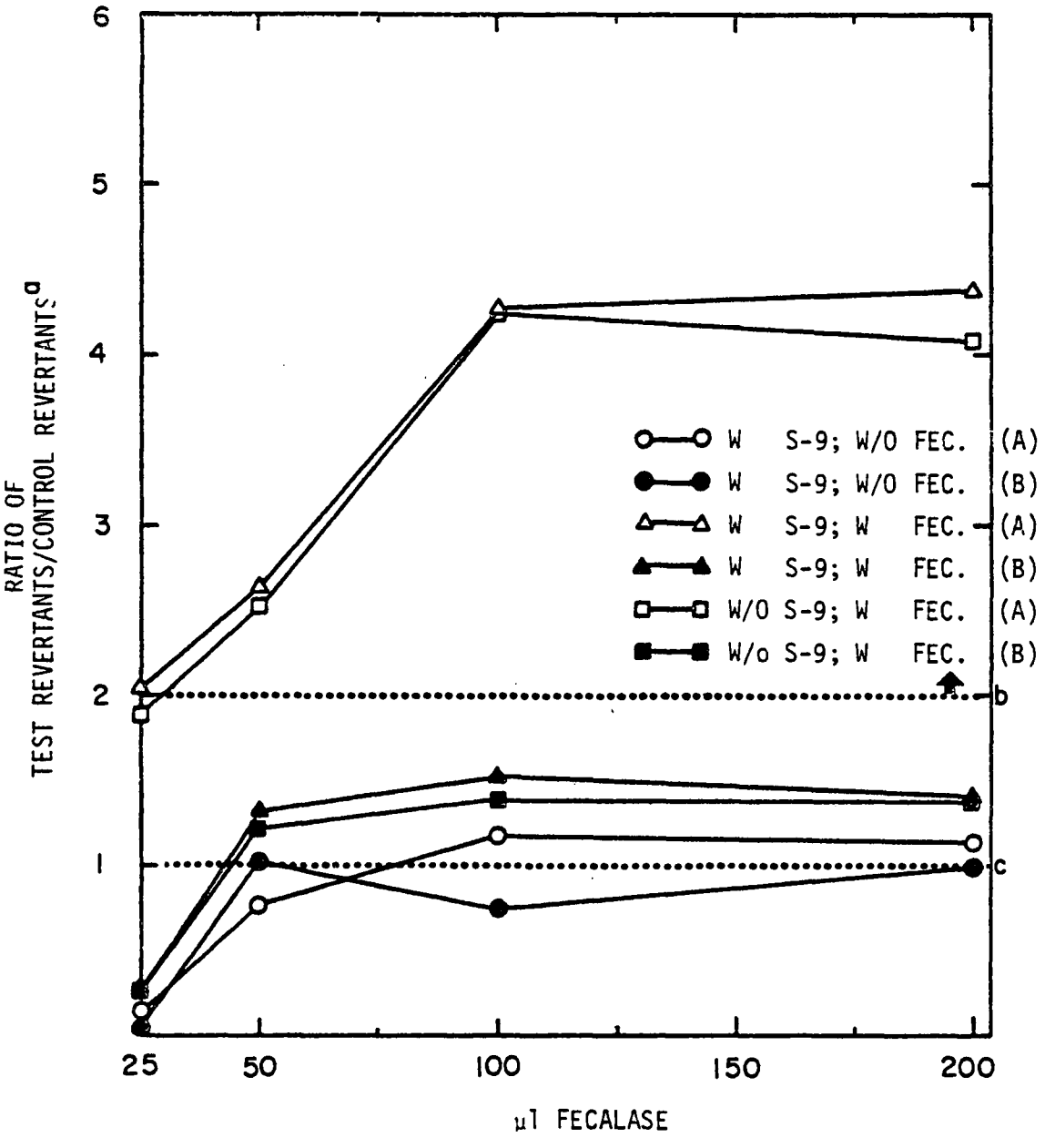


Figure 32. Effect of various amounts of fecalase on reversion of TA97 with TLC scrapped spots of Red Onion Flavonoid Extract.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA 97; WS-9 = 98, W/O S-9 = 95).

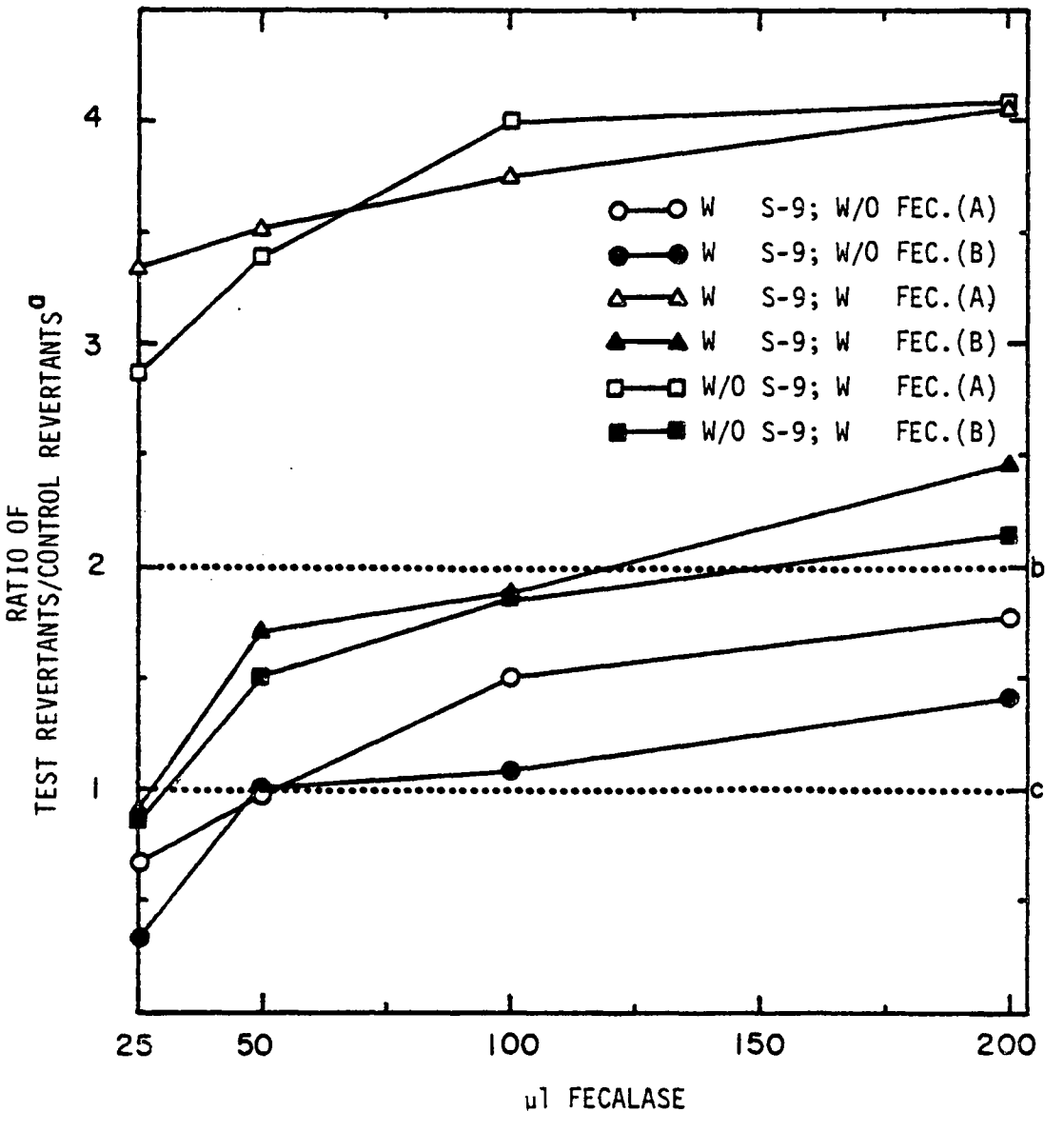


Figure 33. Effect of various amounts of fecalase on reversion of TA1537 with TLC scrapped spots of Yellow Onion Flavonoid Extract.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA 1537; WS-9 = 23, W/O S-9 = 20)

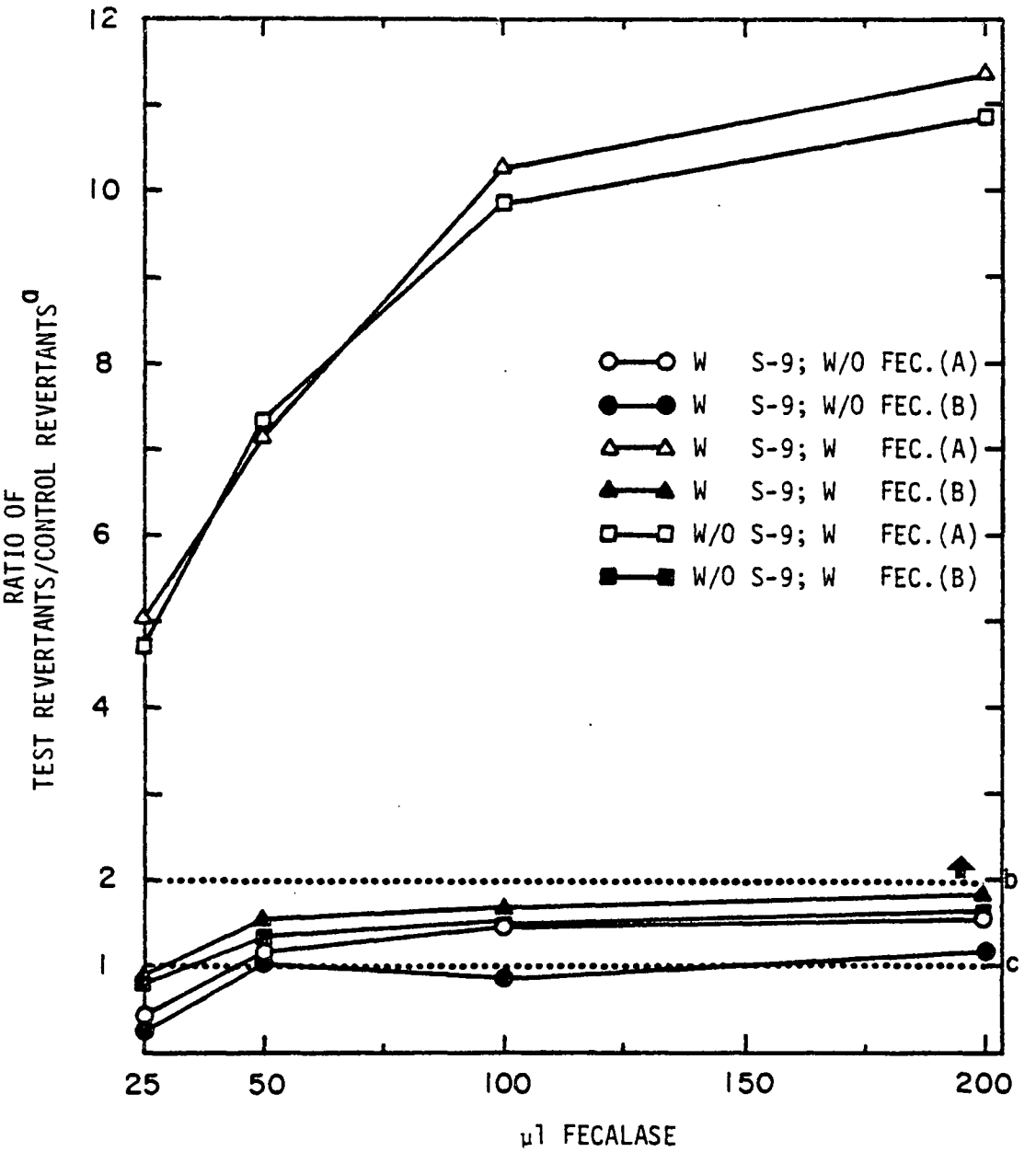


Figure 34. Effect of various amounts of fecalase on reversion of TA98 with TLC scrapped spots of Yellow Onion Flavonoid Extract.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA 98; WS-9 = 43, W/O S-9 = 41).

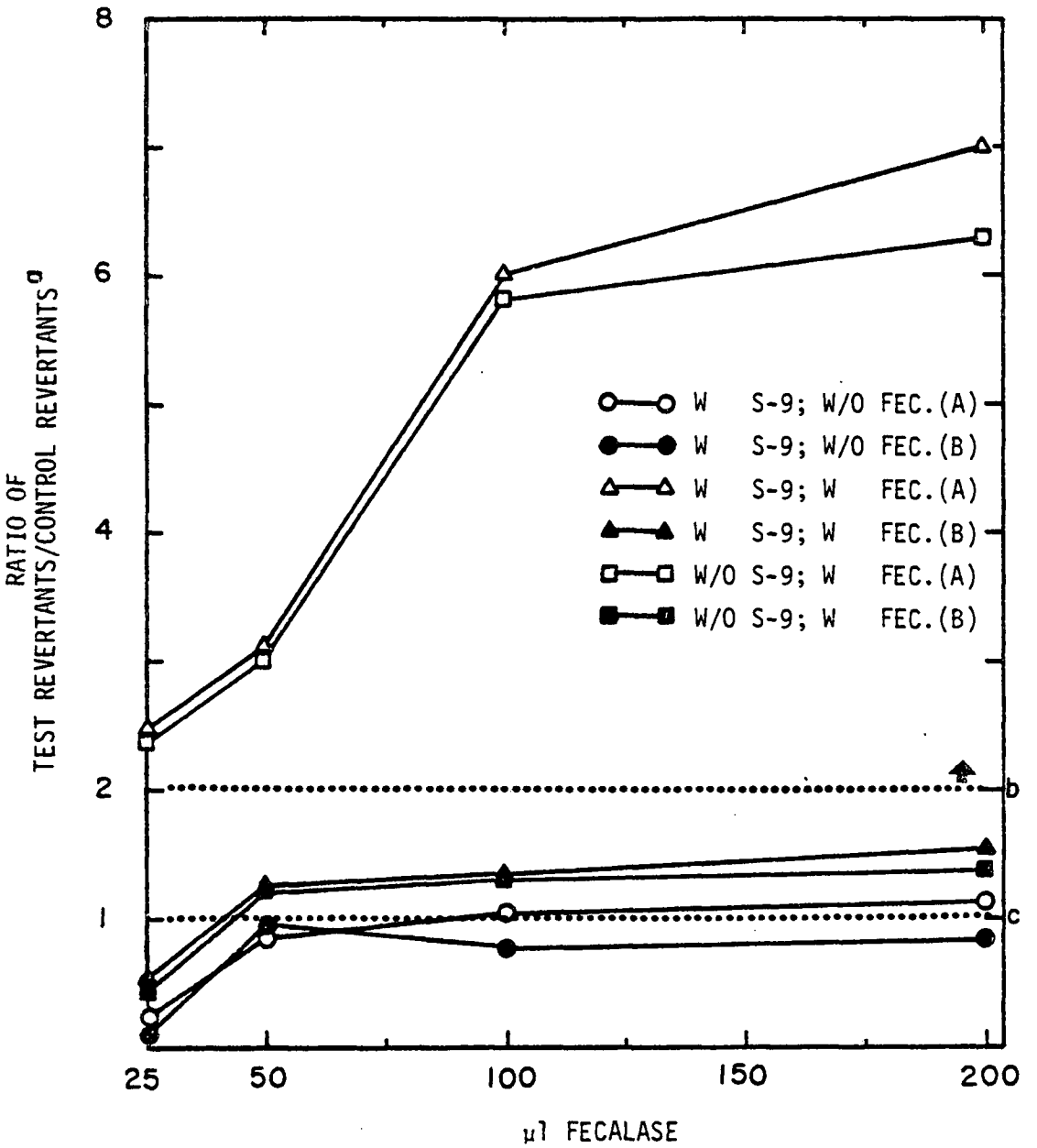


Figure 35. Effect of various amounts of fecalase on reversion of TA97 with TLC scrapped spots of Yellow Onion Flavonoid Extract.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA 97; WS-9 = 98, W/O S-9 = 95).

revertant control number of this strain (TA 1537 = 23).

Data of Figures 36, 37, and 38 show that Spots 'A' of Raspberry extract identified as quercitrin had mutagenic positive response on the frameshift mutant strains TA 1537, TA 98, and TA 97 upon the addition of tolerant fecalase while Spot 'B' of the Raspberry extract was not mutagenic upon fecalase addition. Neither Spot 'A' nor Spot 'B' were mutagenic without the addition of fecalase. The microsomal enzymes incorporation did not show significant variation in the number of the test revertant controls.

Concentration of Flavonol Glycosides from Juice by Adsorption with XAD-2

Data show reconstituted volumes 200, 400, and 800 ml of Cantaloupe juice were mutagenic on frameshift mutant strains with fecalase treatment (Appendix D (Table 12)). Also, Raspberry and the two varieties of Onion (Red and Yellow) with reconstituted volumes ranging from 400 to 800 ml were mutagenic on the frameshift tester strains with the addition of fecalase (Appendix D (Tables 13, 14, 15)). Data show in Appendix D (Table 16) that White Onion reconstituted juice at any volume was not mutagenic under any of the assay conditions.

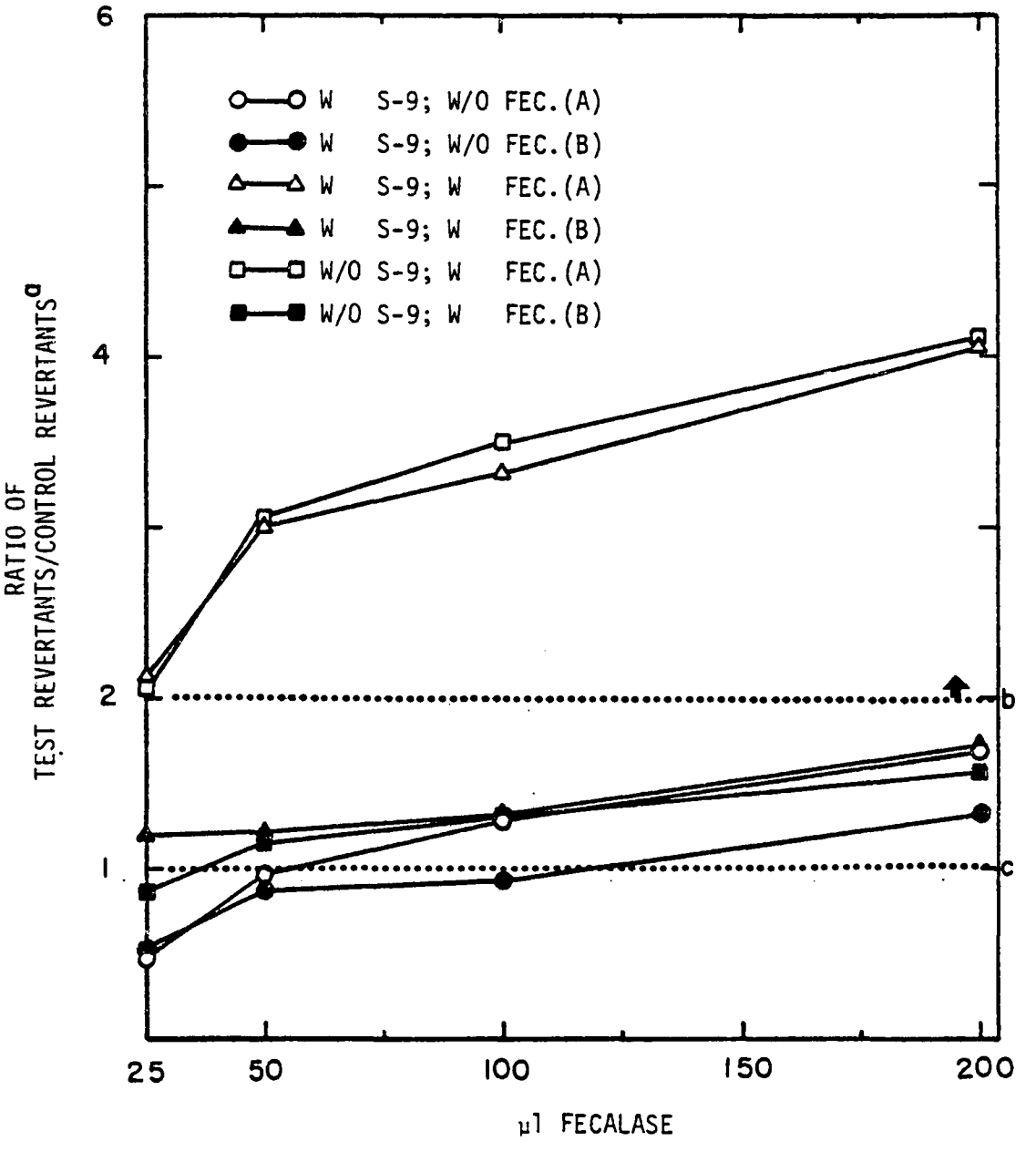


Figure 36. Effect of various amounts of fecalase on reversion of TA1537 with TLC scrapped spots of Raspberry Flavonoid Extract.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA 1537; WS-9 = 23, W/O S-9 = 20)

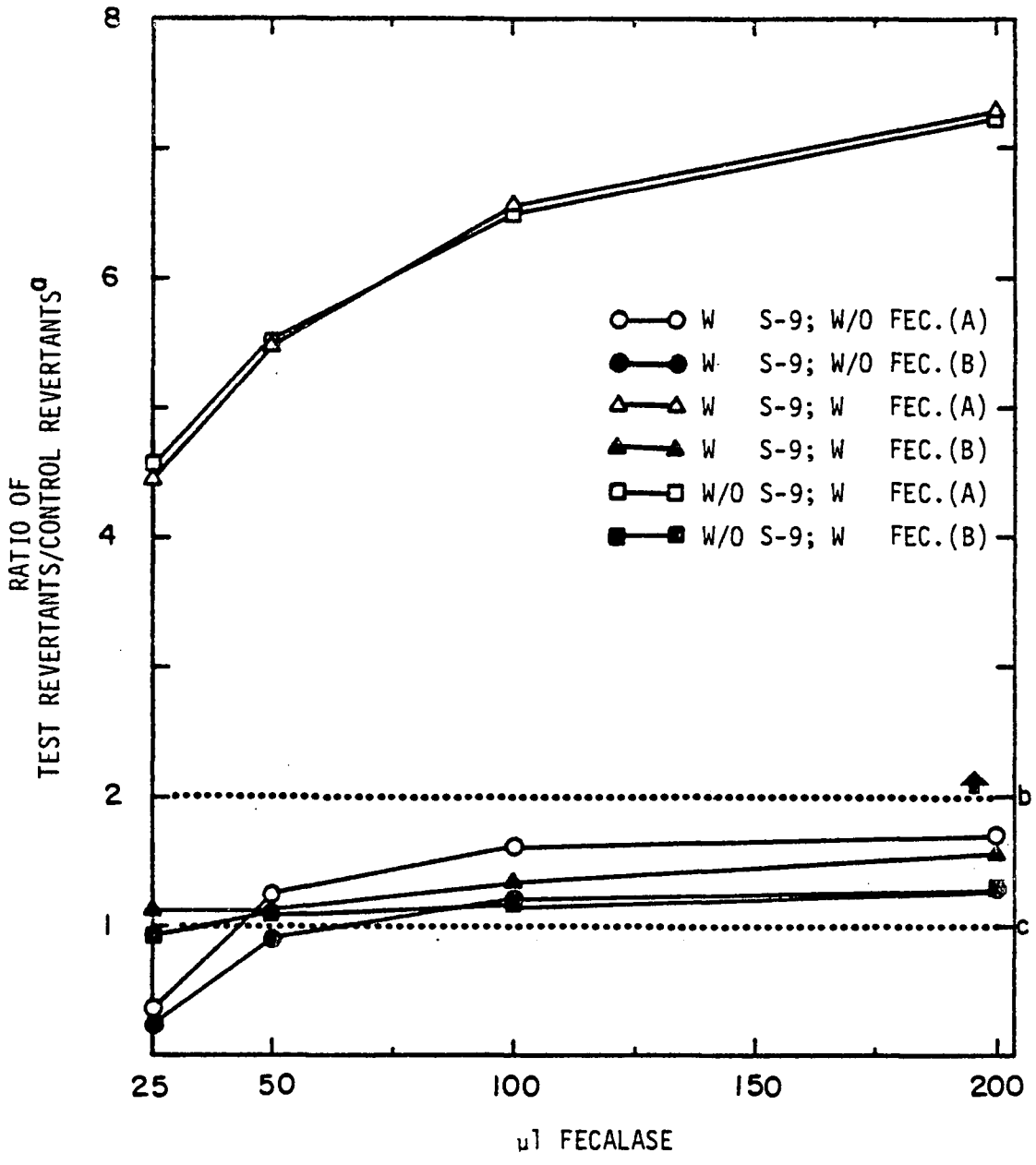


Figure 37. Effect of various amounts of fecalase on reversion of TA98 with TLC scrapped spots of Raspberry Flavonoid Extract.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA 98; WS-9 = 43, W/O S-9 = 41).

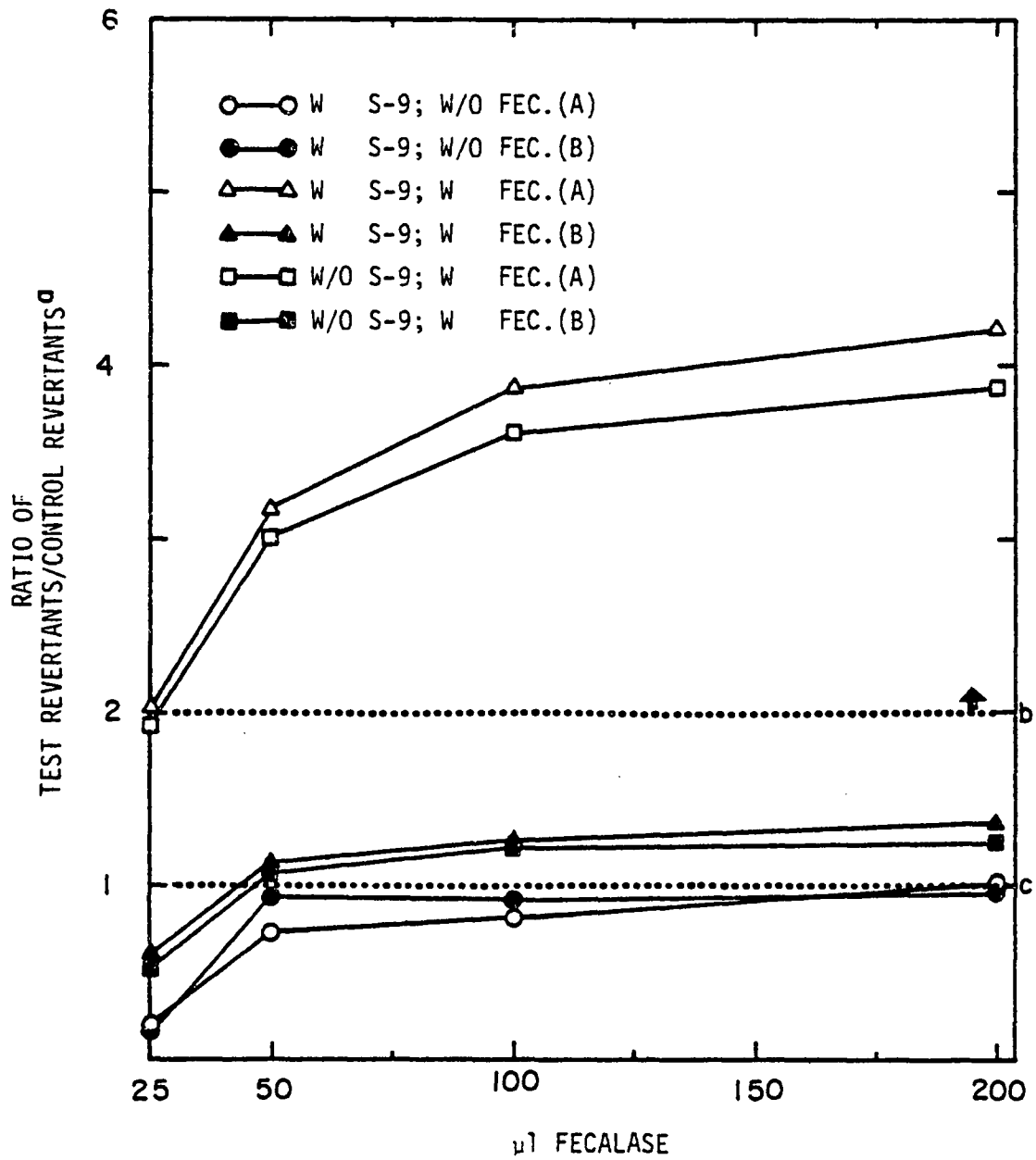


Figure 38. Effect of various amounts of fecalase on reversion of TA97 with TLC scrapped spots of Raspberry Flavonoid Extract.

a. Ratio number = The average number of revertant colonies produced by test solution divided by average number of normal revertant produced on control plates.

b. Positive response: A ratio number of 2 or more is considered a positive response. The ratio is based upon minimal induced revertants described by Ames et al., (1975).

c. Control response average: (TA 97; WS-9 = 98, W/O S-9 = 95).

DISCUSSION AND CONCLUSION

The method for preparation of the flavonol extract was easy to conduct and resulted in high yields of extract whose major constituents were easily separated and isolated using Thin-Layer Chromatography (TLC). The one-dimension TLC separation of the flavonol extract in the solvent system used gave excellent resolution of the flavonols. Thin-Layer Chromatography provides a rapid method of separation and identification of constituents of complex mixture requiring only milligram quantities of material. Chromatography also provides a reliable method of rapidly surveying plant material for the presence of flavonoids. It must be recognized, however, that appropriate analytical procedures must be used to confirm the identification of the isolates. Phenolic compounds in plants are significant in several respects. It has been known for a number of years that flavonols and flavones can act as antioxidants and can, for example, protect Vitamin C from oxidation [Herrman, 1973]. Pratt et al. [1965], as well as Pratt and Watts [1964] have shown quercitrin, which occurs most ubiquitously in natural products, to be a better inhibitor of the auto-oxidation of ethyllinoleate than the usual antioxidants, ethyl and propyl gallate and the tocopherols (Vitamin E). It has been shown also that quercetin in concentrations of 15 mg/100 gm is a very active antioxidant for butter fat and lard [Richardson et al., 1974; Morris and Riemenschneider, 1949]. Other

flavonols show similar effects although less actively than quercetin [Kaufmann and el Wahab el Baya, 1967]. The position and the number of free hydroxyl groups in flavonols are responsible for their antioxidative activity. It is believed that the 3-hydroxyl group in conjunction with the 2,3-double bond are of decisive significance. If the 3-OH group is bound glycosidically or if it is absent, as in the case of flavones, the antioxidative activity is weakened [Kaufmann and el Wahab el Baya, 1967]. Pratt and Watts [1964] and Pratt [1965] concluded the antioxidative activity which has been observed in a number of vegetables and spices may be due partly to flavonols, probably derivatives of quercetin.

The development of rapid, sensitive in vitro assays for mutagens such as the Salmonella/Mammalian microsome test [Ames et al., 1975], and the demonstration that about 90 percent of chemical carcinogens can be detected by this system, makes it possible to detect the variety of mutagens/carcinogens in the environment that are contributing to cancer, genetic birth defects, and other diseases caused by DNA damage. One approach is to test the reasonably pure chemicals to which humans are exposed, both those on the market and those under development. Another approach is to test complex mixtures that people ingest, or inhale, or absorb through their skin.

Many recent studies have pointed out the importance of gut flora in biotransforming a wide variety of naturally occurring glycosides to active mutagens [Brown and Dietrich, 1979; Brown,

1980; Tamura et al., 1980]. These studies indicate that the rat liver homogenate presently used in the Salmonella test should be complemented by the addition of microbial enzymes to bioactive complex food materials which potentially contain mutagens/carcinogens and provide a more complete assessment of hazards in the human diet. Many compounds eaten by man exist as glycosides that are easily cleaved by bacteria and the bioactivated compounds likely absorbed by the intestine. Yamasaki and Ames [1977] indicated for most carcinogens and mutagens to be effective they must penetrate the cell membrane. Carcinogens and mutagens as a group tend to be nonpolar unless they are taken up by specific transport systems.

Many mutagens are commonly found in foods. Nagao et al. [1979] have shown the mutagenic activity of tea on Salmonella Strain TA 98 to require glycosidase bioactivation. Brown and Dietrich [1979] have shown many flavonol glycosides are bioactivated to mutagens by mixed glycosidases from rat cecal bacteria and other sources. The fecalase, an enzyme extract from human feces, is a simple and physiologically relevant model for metabolism by human gut bacteria and should be used in conjunction with the Salmonella test when complex mixtures such as foods are investigated. An enormous variety of glycosides (as to both the sugar and the aglycone moieties) are present in edible plants [Brown and Dietrich, 1979]. Among those glycosides included in this study and split by fecalase was quercitrin (quercetin 3-O-

β-D-galactopyranoside). Fecalase is effective in bioactivating nonmutagenic flavonol glycoside to mutagenic aglycone with the various natural substrates tested.

Fecalase is very active in hydrolyzing the glycosidic linkage of the quercitrin identified in the samples examined. The activity of fecalases are of concern for the following reasons: (i) It is most closely associated to human condition, (ii) People eat a much more varied diet. Fecalase was effective in bioactivating some naturally occurring complex mixtures containing flavonols glycosides to mutagen [Tamura et al., 1980]. Flavonol glycoside of mutagens such as quercitrin was found in Cantaloupe (Cucumis melo), Raspberry (Rubus idaeus), and two of three varieties of Onion tested. It has been shown in this study that flavonol glycosides extract of Cantaloupe and Raspberry are mutagenic in the presence of fecalase. We also showed that Red and Yellow Onion varieties extracts are mutagenic. It was also shown that the White Onion did not contain flavonol glycosides which yield mutagenic aglycone in the presence of fecalase. Separation of flavonols with a resin such as XAD-2 was a particularly convenient method since the resin quite effectively adsorbed relatively nonpolar compounds.

The Salmonella/microsome test in the past has been shown to be about 85 percent ± five percent in detecting carcinogens as mutagens [McCann et al., 1975; McCann and Ames, 1977; Ames and McCann, 1980], but compounds tested in their studies were usually

synthetic substances to which man has been exposed. The ubiquity of the flavonols in edible plants in nature raises the possibility that during mammalian evolution methods for dealing with these mutagens may have evolved. Quercetin is among the most common and is very mutagenic according to this study. The findings are in analogy of what Brown [1980] discussed about the significance of mutagenic flavonoids and anthraquinones in detail.

The mutagenic activity of the identified quercitrin from Cantaloupe, Raspberry, Red and Yellow varieties of Onion was clearly shown on the frameshift mutant strains (TA 1537, TA 98, TA 97), but not noticeable on the base-pair mutant strains (TA 1535, TA 100). Therefore, frameshift mutagenicity among the flavonoid tested was mainly confined to the flavonols (flavon-3-ols). The flavonols are probably the single largest group of flavonoids, and the most mutagenic agent detected, quercetin, is the most common flavonol aglycone. The mutagenic activity of the aglycone for the frameshift tester strains increased 5- to 15-fold by incorporating gut bacterial enzymic extracts (Fecalase) in the assay procedure. It is still uncertain as to what mutagenic intermediates the aglycone are converted. Very recent evidence shows quercetin is a carcinogen for rats. Of 25 rats fed quercetin at 1000 ppm in their diet for 14 months, 20 developed multiple tumors of the ileal section and five developed bladder transitional cell carcinoma, while none of the 19 control rats showed any such tumors [Pamukcu et al., 1980].

Two factors may reduce the mutagenicity and carcinogenicity of these compounds in humans. First, quercetin does not seem to be absorbed. A study on quercetin [Gugler et al., 1975] found that after oral dosage, free quercetin was present in the intestines but no quercetin, either free or conjugated, was detected in the blood stream. Second, microbial degradation of flavonoid is extensive. The quercetin study by Gugler et al. in 1975 found that although less than one percent of the quercetin was absorbed by the intestines, only about half of an oral dose of 70 mg/kg of body weight was recovered from the feces, indicating extensive microbial degradation.

In spite of this evidence, flavonoids still may conceivably play a role in the etiology of cancer of the stomach and other areas of the gastrointestinal tract, and they remain an area of potential interest. Mutagenic flavonol aglycones, such as quercitin, are present in some foods. They have been reported in pickles [Sugimura, 1980; Takahashi et al., 1979] and red wine [Tamura et al., 1980]. From the results reported in this work, Cantaloupe, Raspberry, Red and Yellow Onions are now included. It is becoming quite clear that the digestive tract of humans from the mouth to the anus are routinely exposed to these naturally occurring agents and the colon is in particular jeopardy because of the increased mutagenic activity of these compounds by fecalase.

If flavonols are proven to have mutagenic/carcinogenic effect

in vitro, plant breeding has been suggested by Brown [1980] to reduce the amount of flavonoids present in the food we eat. From the studies conducted and the observations noticed fecalase should aid in the detection of potentially dangerous natural substances.

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:

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APPENDICES

APPENDIX A

"S-9" Mix and Media Preparation

1. Preparation of S-9 Mix (1 ml)

   20 µl of salt solution containing 8 µmole $MgCl_2$ and 33 µmole KCl

   5 µl of 5 µmole solution glucose-6-phosphate

   40 µl of 4 µmole solution NADP

   500 µl of 100 µmole sodium phosphate buffer

   395 µl of H_2O

   40 µl of S-9 liver homogenate.

2. Vogel-Bonner Medium E (VBE) at 50x for one litre

   Ingredients must be added in the order listed. Do not add another chemical until the previous one is completely dissolved.

   670.0 ml of distilled H_2O

   10.0 gm of $MgSO_4 \cdot 7H_2O$

   100.0 gm of citric acid $\cdot H_2O$

   500.0 gm of Anhydrous K_2HPO_4

   175.0 gm of $NaNH_4HPO_4 \cdot HH_2O$

   Make to 1000.0 ml - store at room temperature. Add 1-5 ml of chloroform as preservative. Stopper tightly in flask.

3. Minimal-Glucose Medium (1 litre)

   Solution (A)

   15.0 gm of Difco Agar

   780.0 ml of distilled H_2O

Solution (B)

20.0 ml of Salt Solution (VBE)

Solution (C)

20.0 gm of Glucose

200.0 ml of distilled H_2O

Sterilize each solution in a separate flask for 30 minutes. Immediately after autoclaving combine (A), (B), and (C), mix by use of magnetic stirrer. Cool to 45°C, dispense into 15 x 100 mm plastic petri dishes.

APPENDIX B

Chemical Ionization Spectra

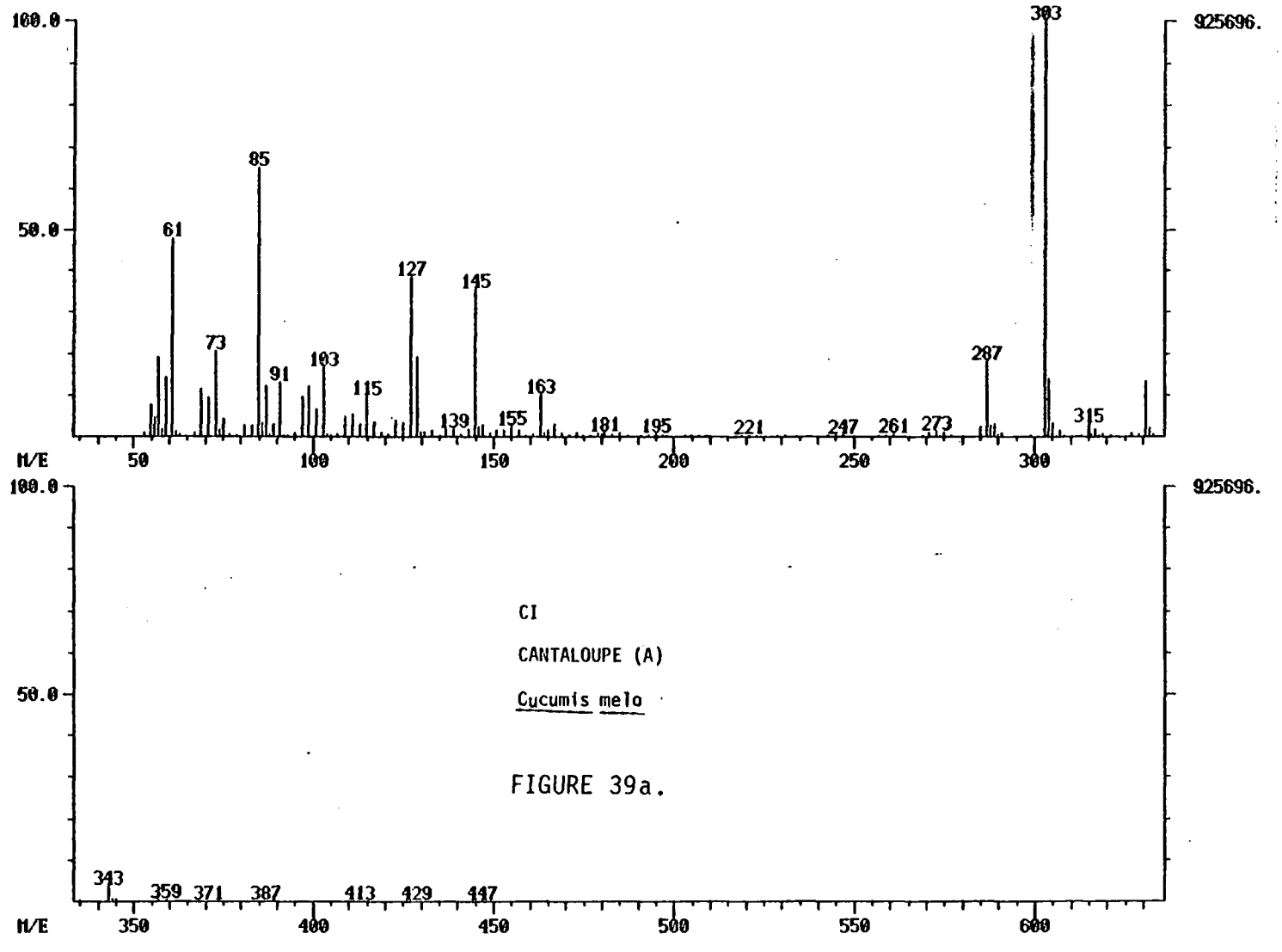
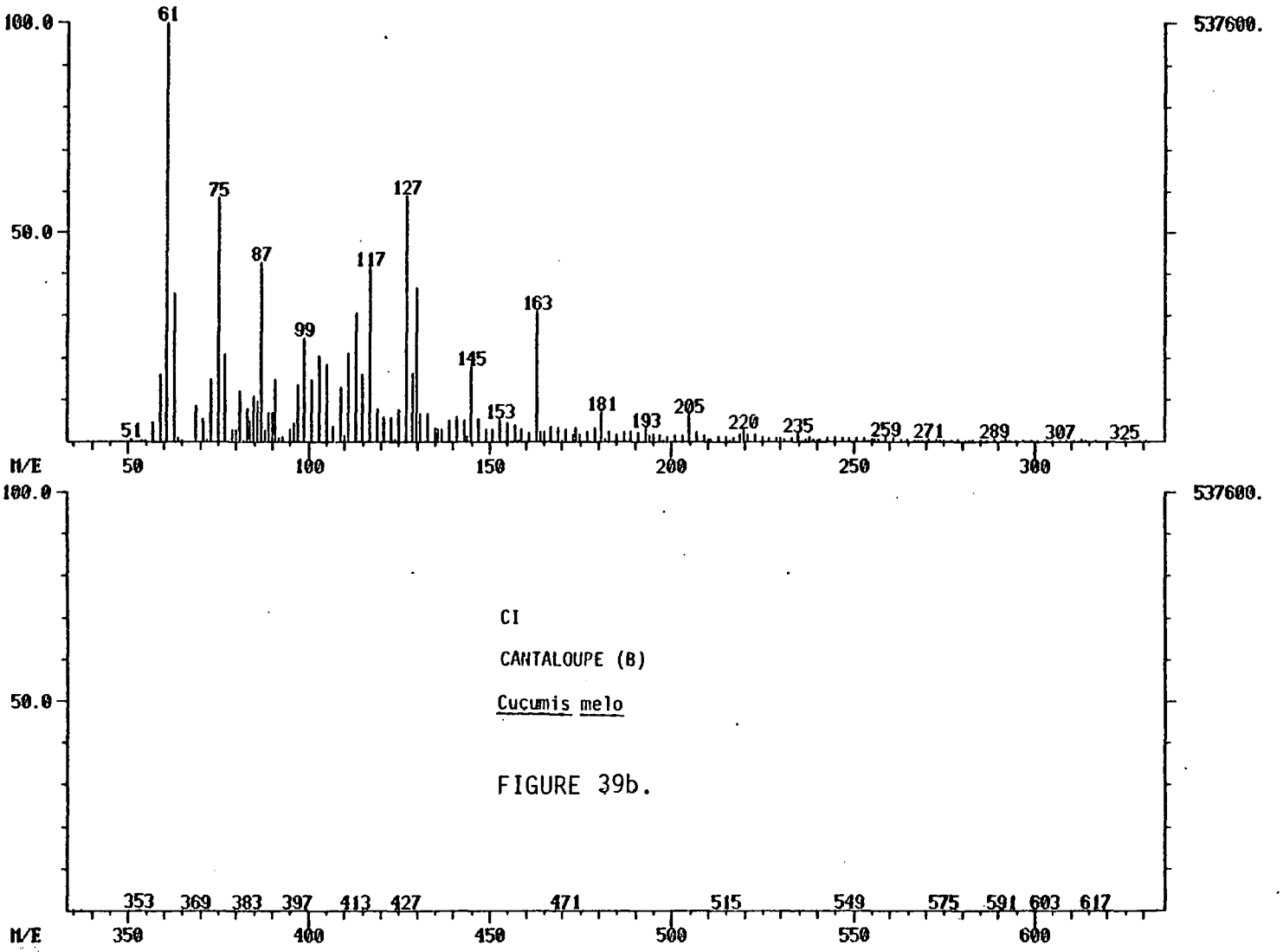

100.0
50.0
925696.
303
85
61
127
145
73
91
103
115
287
163
139
155
181
195
221
247
261
273
315
M/E
50
100
150
200
250
300
100.0
50.0
925696.
CI
CANTALOUPE (A)
Cucumis melo
343
359
371
387
413
429
447
M/E
350
400
450
500
550
600

FIGURE 39a.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50.0
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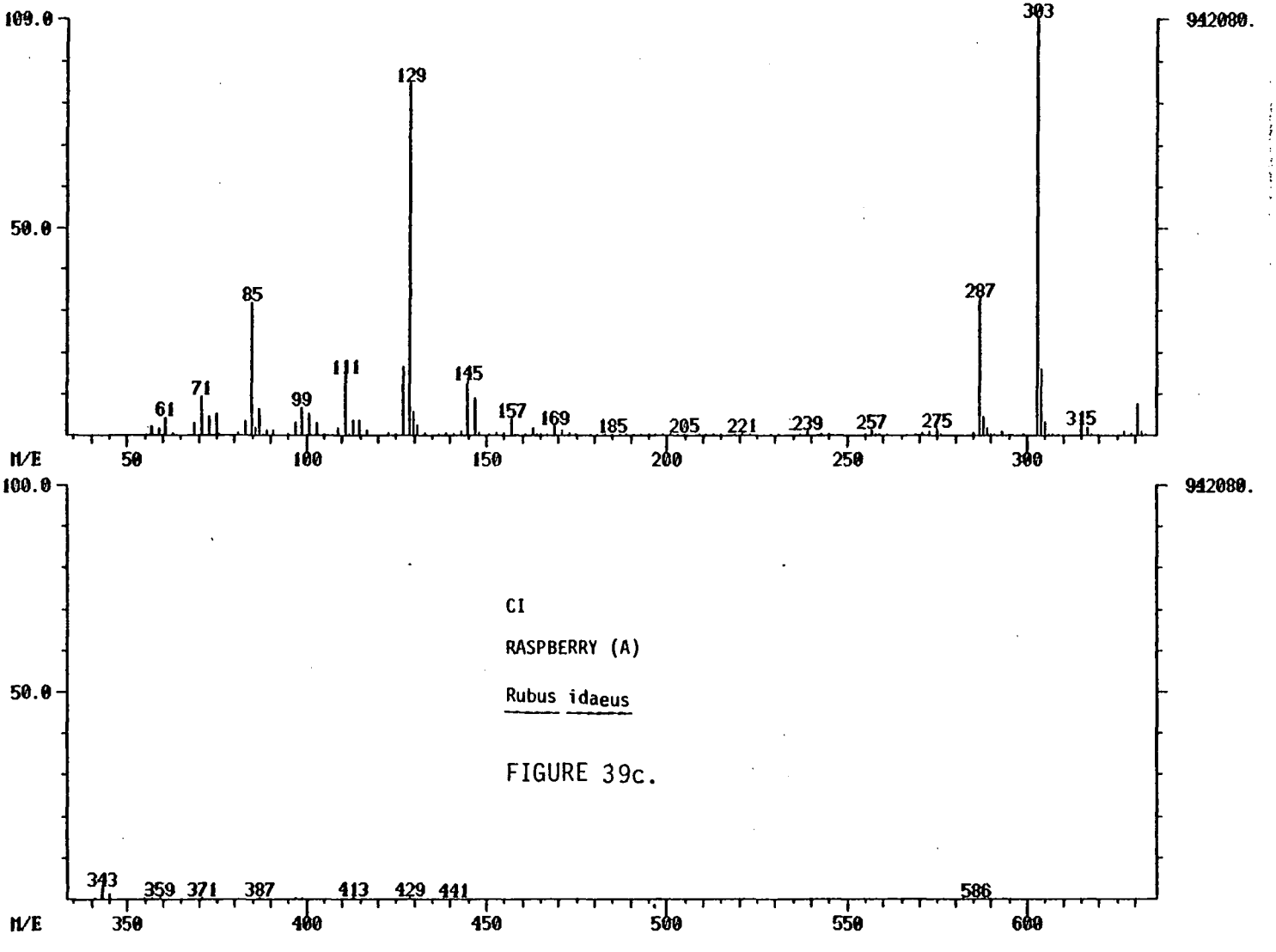
61
75
87
99
117
127
145
153
163
181
193
205
220
235
259
271
289
307
325
51
M/E
50
100
150
200
250
300
100.0
50.0
537600.
CI
CANTALOUPE (B)
Cucumis melo
353
369
383
397
413
427
471
515
549
575
591
603
617
M/E
350
400
450
500
550
600

FIGURE 39b.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50.0
M/E
50
100
150
200
250
300
303
129
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111
145
71
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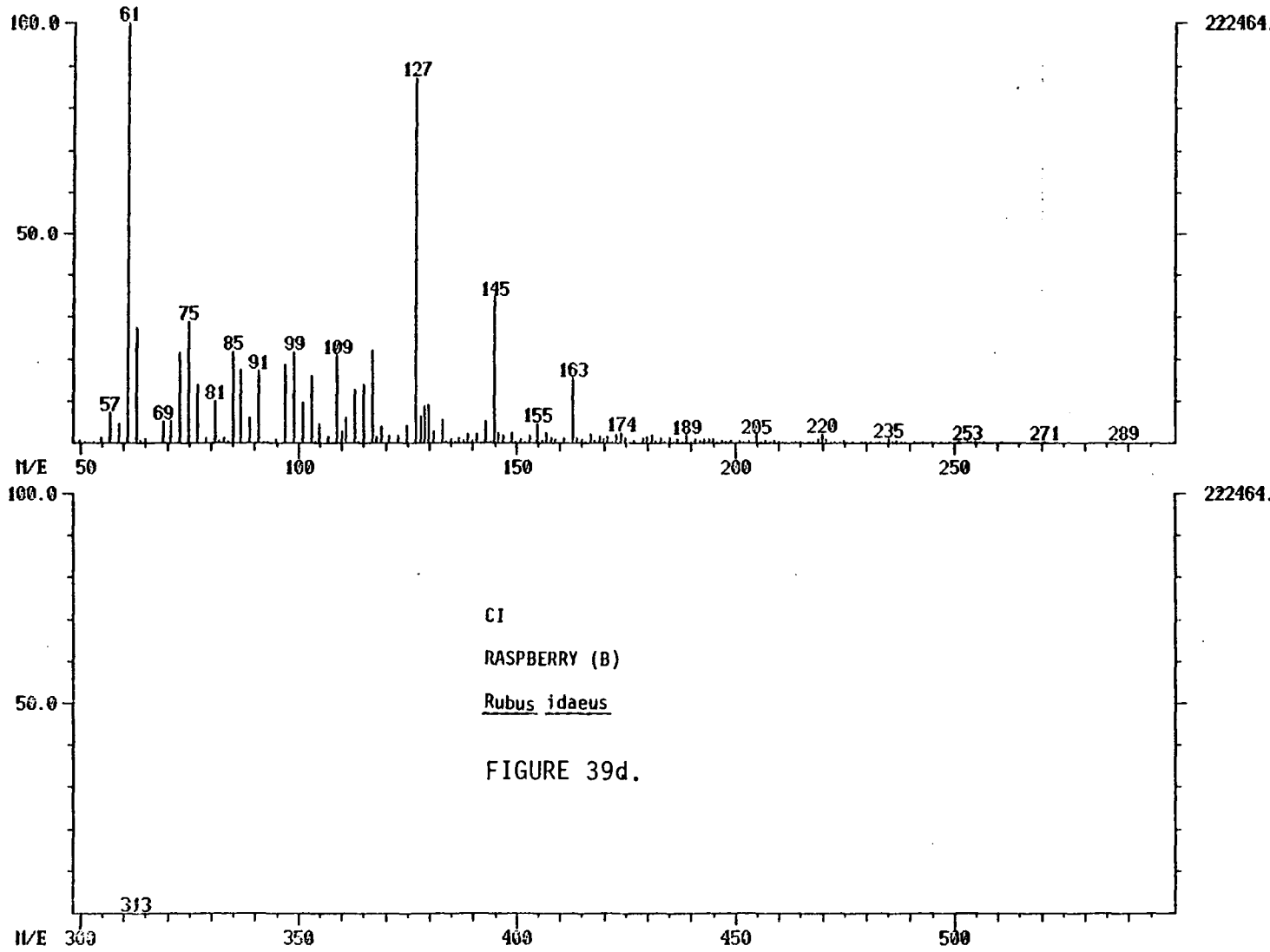
99
157
169
185
205
221
239
257
275
315
942080.
100.0
50.0
M/E
350
400
450
500
550
600
343
359
371
387
413
429
441
586
942080.
CI
RASPBERRY (A)
Rubus idaeus

FIGURE 39c.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50.0
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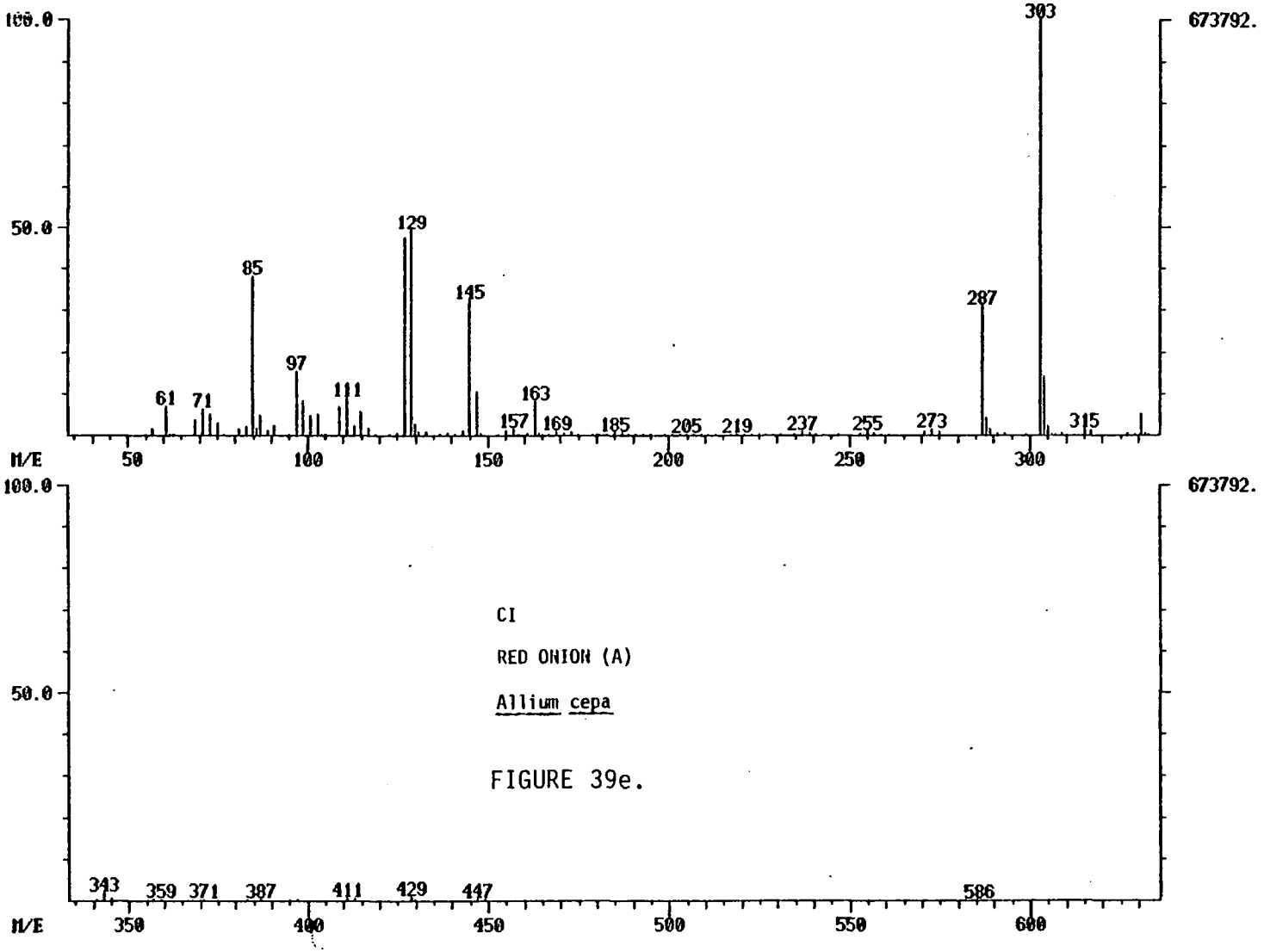
61
127
145
75
85
99
109
91
163
57
69
81
155
174
189
205
220
235
253
271
289
M/E
50
100
150
200
250
100.0
50.0
222464.
313
M/E
300
350
400
450
500
CI
RASPBERRY (B)
Rubus idaeus

FIGURE 39d.

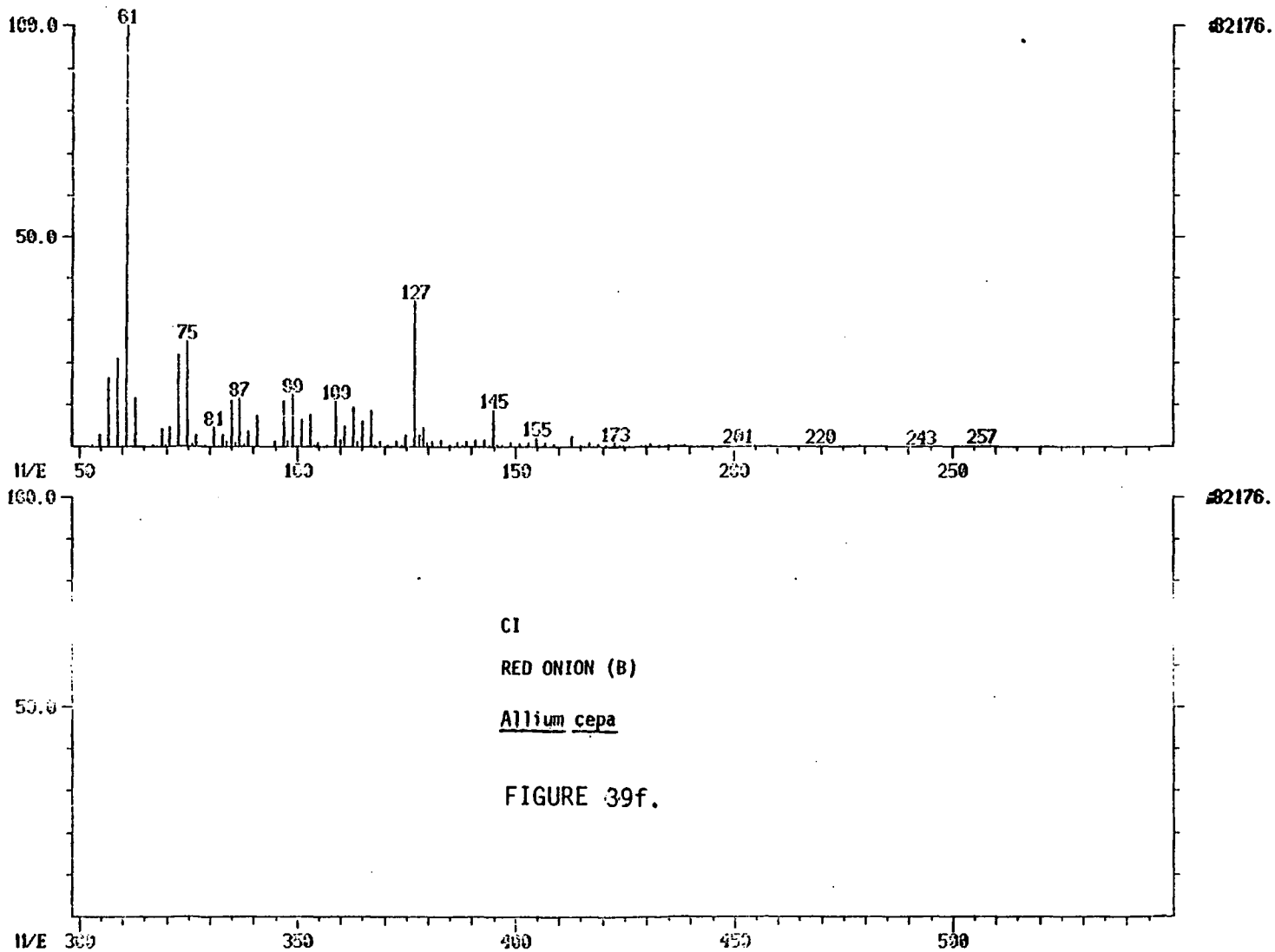


CI

RED ONION (A)

Allium cepa

FIGURE 39e.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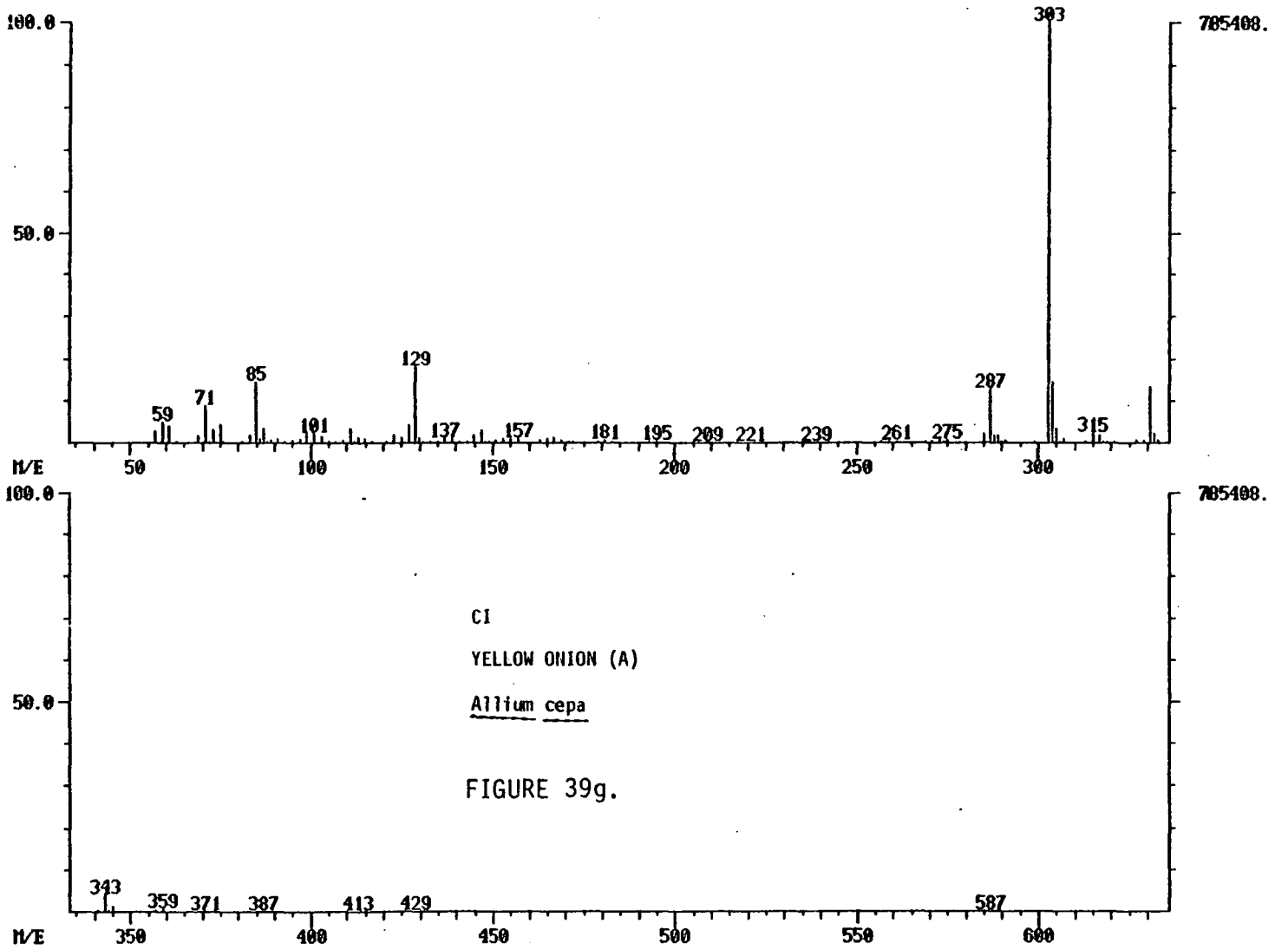
61
75
81
87
99
109
127
145
155
173
201
220
243
257
M/E
50
100
150
200
250
#82176.
100.0
50.0
M/E
300
350
400
450
500
#82176.
CI
RED ONION (B)
Allium cepa

FIGURE 39f.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M/E
785408.
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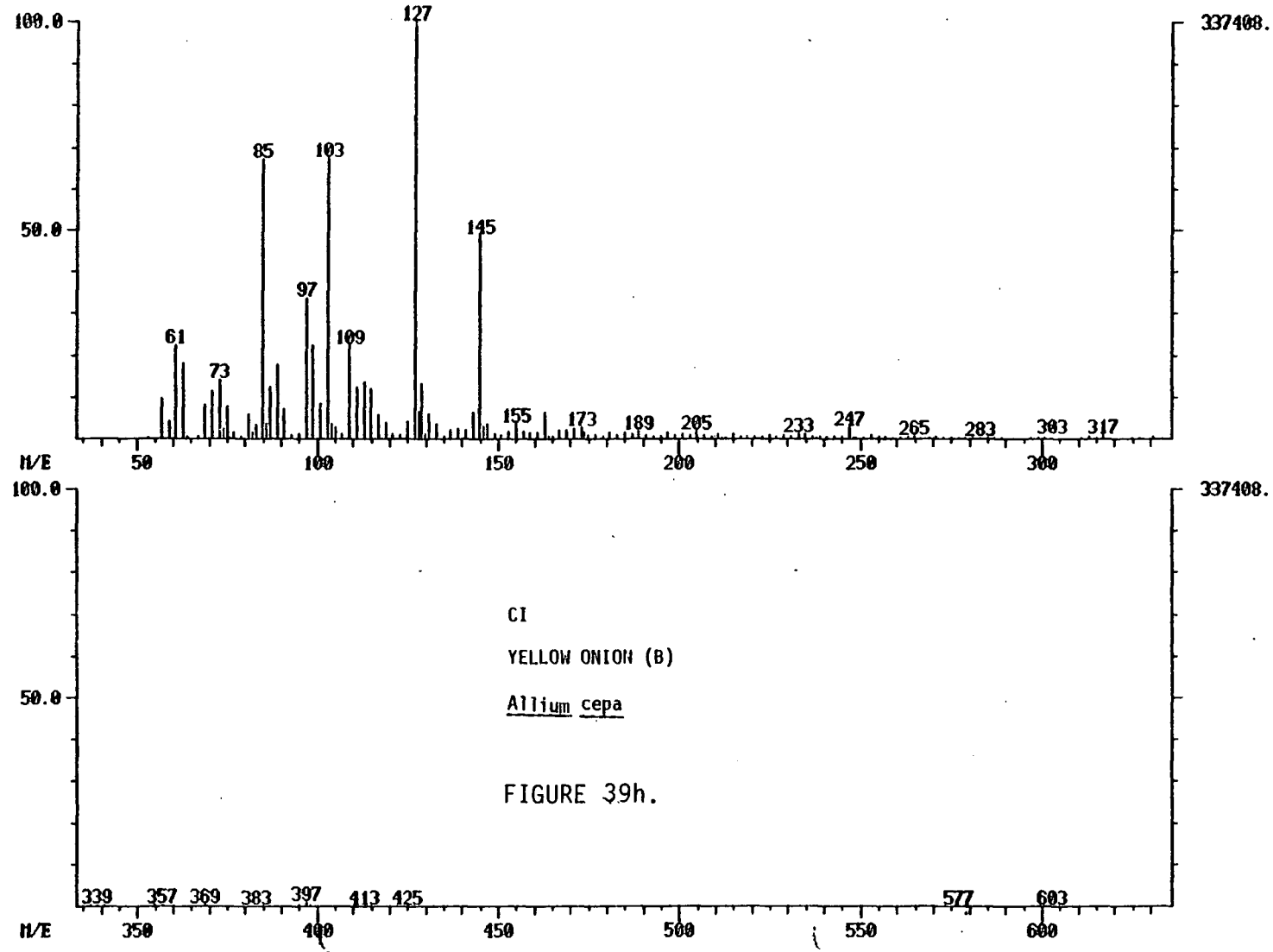
129
85
71
59
101
137
157
181
195
209
221
239
261
275
287
315
50
100
150
200
250
300
343
359
371
387
413
429
587
350
400
450
500
550
600
CI
YELLOW ONION (A)
Allium cepa

FIGURE 39g.

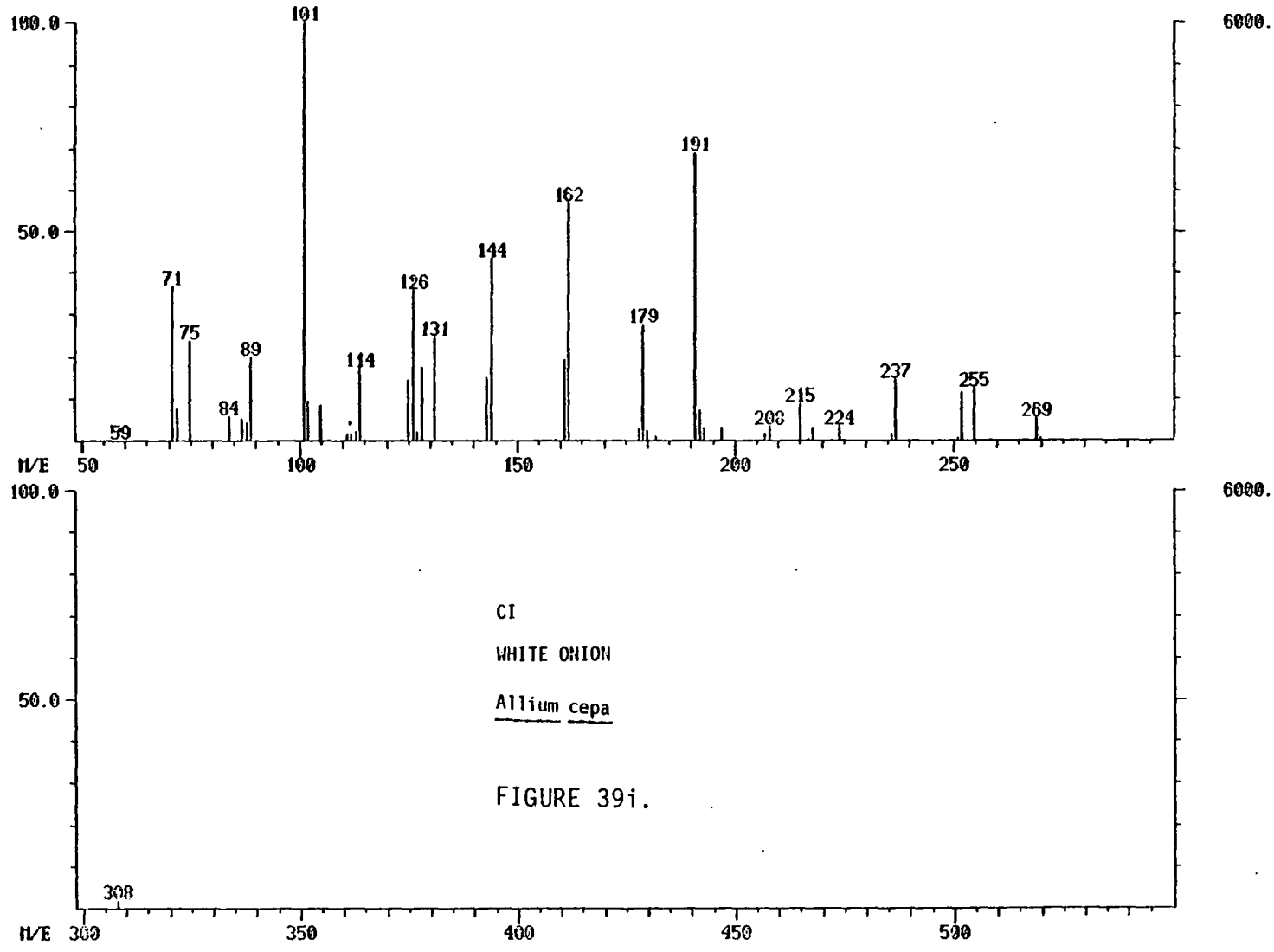


CI

YELLOW ONION (B)

Allium cepa

FIGURE 39h.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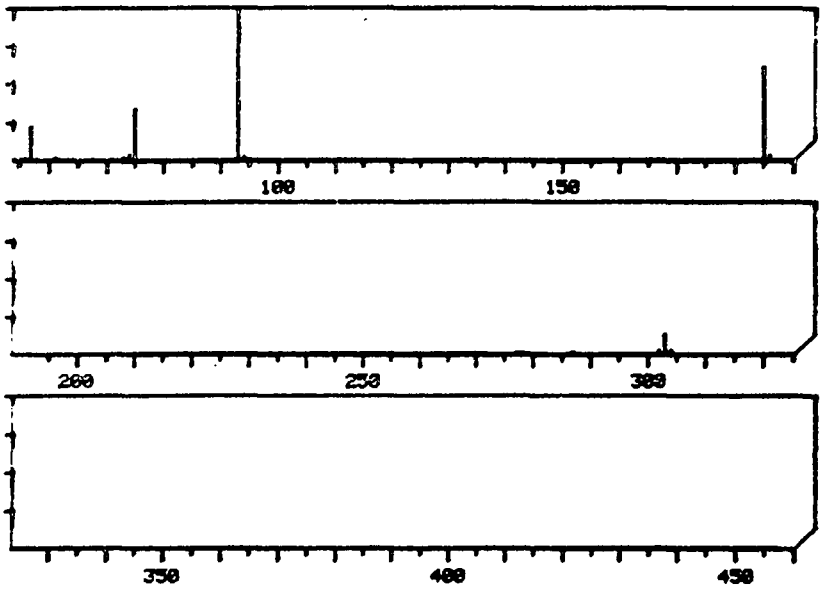
50.0
6000.
101
191
162
144
126
179
71
75
131
89
114
237
255
215
269
84
208
224
59
M/E
50
100
150
200
250
100.0
50.0
6000.
CI
WHITE ONION
Allium cepa
308
M/E
300
350
400
450
500

FIGURE 39i.

APPENDIX C

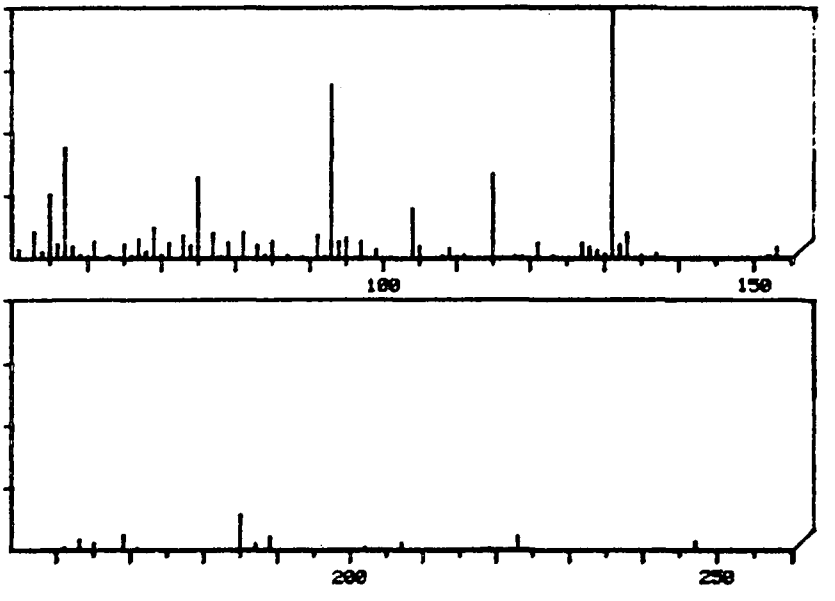
Fast Atom Bombardment Spectra



FAB

CANTALOUPE (A)

Cucumis melo

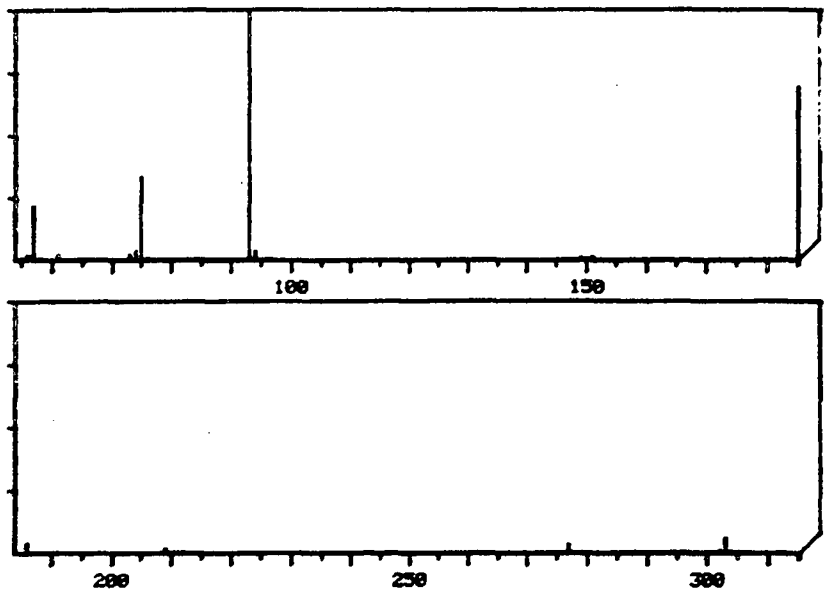


FAB

CANTALOUPE (B)

Cucumis melo

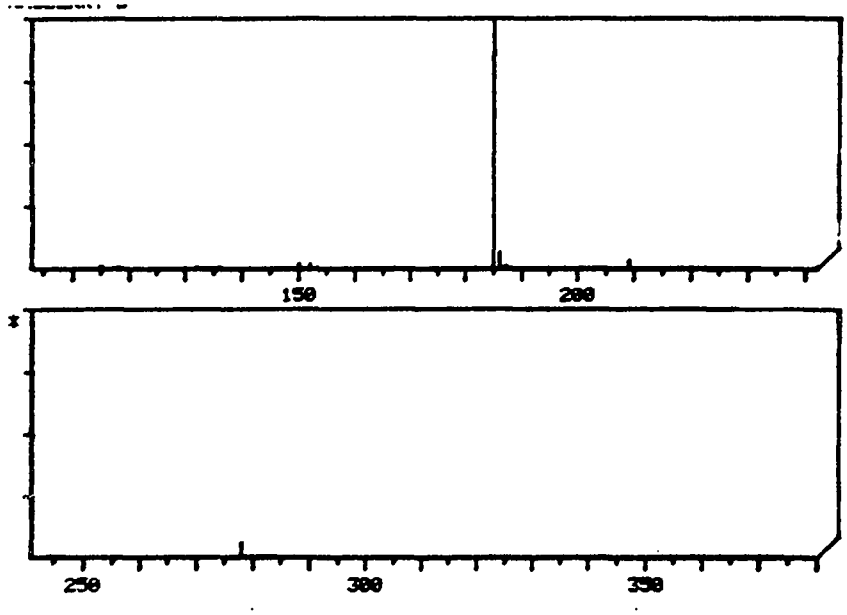
FIGURE 40a.



FAB

RASPBERRY (A)

Rubus idaeus

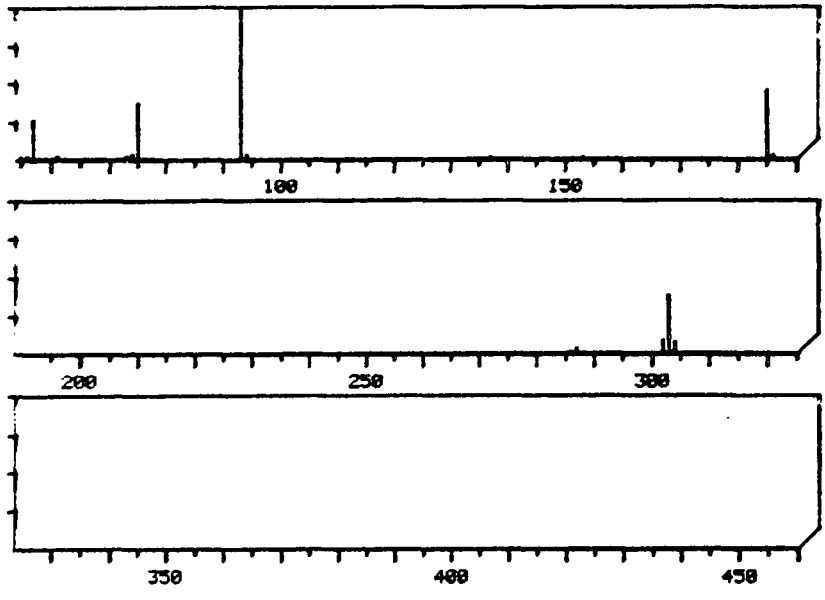


FAB

RASPBERRY (B)

Rubus idaeus

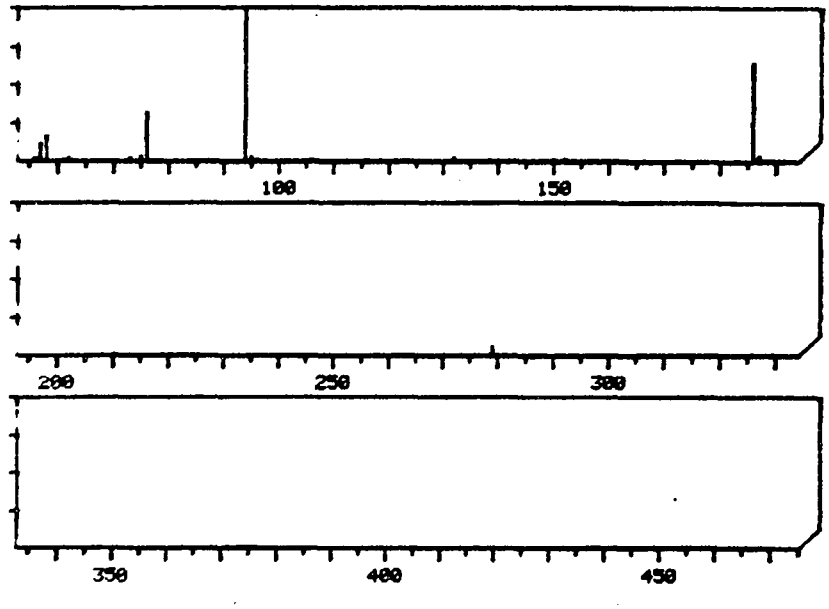
FIGURE 40b.



FAB

RED ONION (A)

Allium cepa

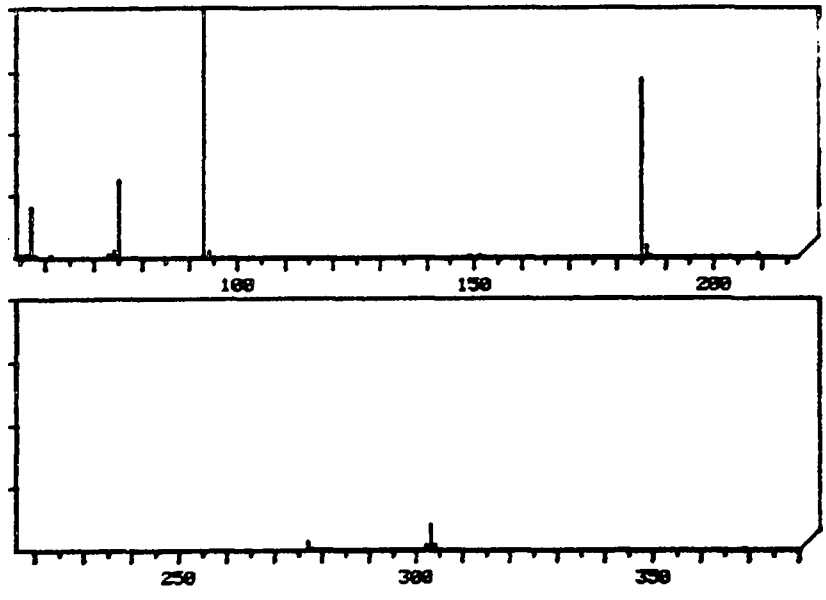


FAB

RED ONION (B)

Allium cepa

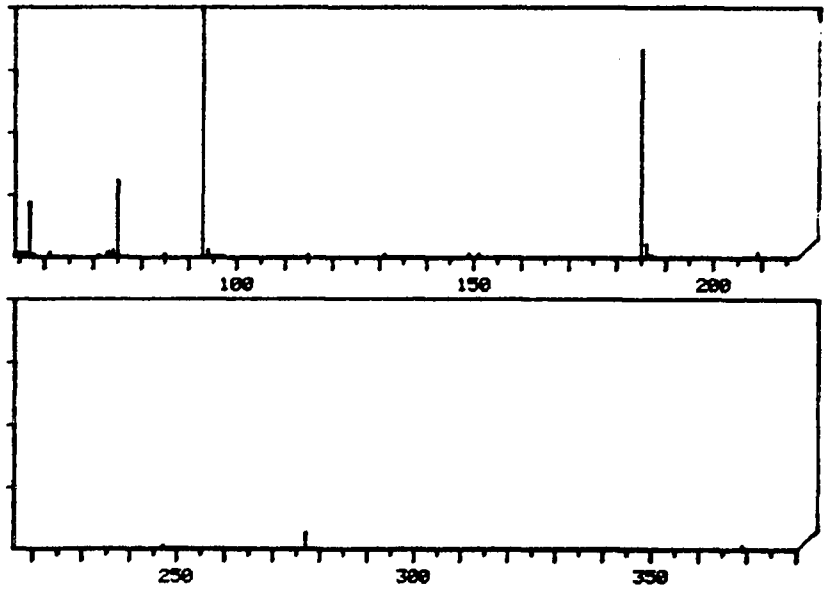
FIGURE 40c.



FAB

YELLOW ONION (A)

Allium cepa

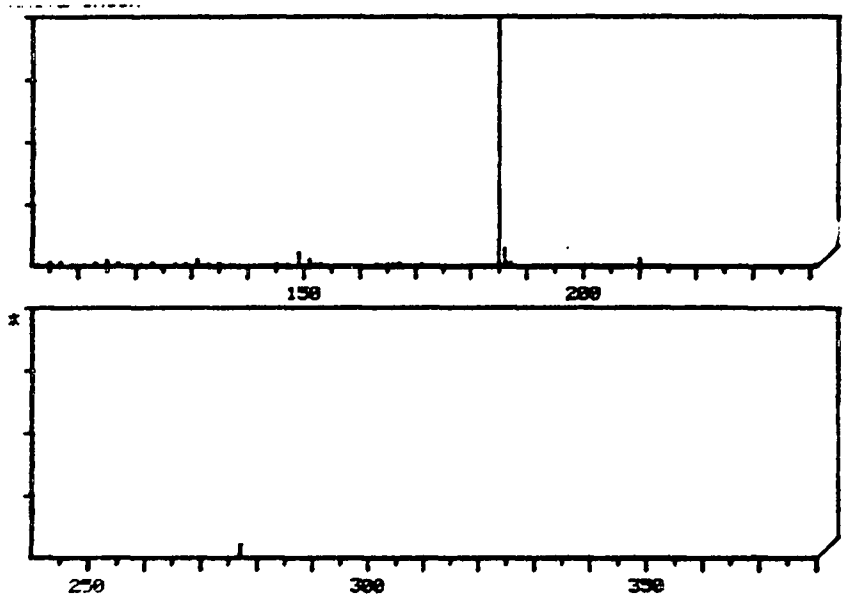


FAB

YELLOW ONION (B)

Allium cepa

FIGURE 40d.



FAB

WHITE ONION

Allium cepa

FIGURE 40e.

APPENDIX D

Mutagenicity Testing of Flavonol Glycosides Concentrated from Juice by Adsorption with XAD-2

TABLE 12a. Mutagenicity of Cantaloupe (Cucumis melo) Juice Concentrated with XAD-2

		Ratio of Test Revertants/Control Revertants						
Tester Strain	Relative Conc.** / Treatment	.05	.10	.50	4.00	2.00	4.00	8.00
TA 1537	W S-9; W/O Fec.	.09	.55	.68	.81	.76	1.00	1.29
	W S-9; W Fec.	.18	.68	.82	1.14	2.33*	3.91*	9.81*
	W/O S-9; W Fec.	.19	.70	.90	1.16	2.32*	3.55*	9.50*
TA 98	W S-9; W/O Fec.	.12	.45	.57	.48	.67	.70	.55
	W S-9; W Fec.	.19	.69	1.05	1.19	1.86	3.40*	3.74*
	W S-9; W Fec.	.18	.66	.95	1.13	1.93	3.43*	3.53*
TA 97	W S-9; W/O Fec.	.04	.26	.30	.32	.42	.91	.56
	W S-9; W Fec.	.11	.41	.46	.98	2.24*	6.91*	5.19*
	W/O S-9; W/Fec.	.08	.35	.47	.86	2.23*	7.00*	4.93*

*Positive response.

**The adsorbed components were eluted and washed with minimal amount of acetone, taken to dryness and dissolved in DMSO.

(TA 1537: W S-9 = 23, W/O S-9 = 21)

(TA 98: W S-9 = 43, W/O S-9 = 41)

(TA 97: W S-9 = 98, W/O S-9 = 95)

TABLE 12b. Mutagenicity of Raspberry (Rubus idaeus) Juice Concentrated with XAD-2

		Ratio of Test Revertants/Control Revertants						
Tester Strain	Relative Conc.** / Treatment	0.05	0.10	0.50	1.00	2.00	4.00	8.00
TA 1537	W S-9; W/O Fec.	.27	.59	.68	.95	.81	1.05	1.24
	W S-9; W Fec.	.32	.96	1.23	2.19*	2.91*	4.33*	7.29*
	W/O S-9; W Fec.	.29	1.05	1.15	2.21*	2.90*	4.55*	6.65*
TA 98	W S-9; W/O Fec.	.30	.60	.83	.79	.86	1.28	1.56
	W S-9; W Fec.	.42	.88	1.19	1.64	2.02	3.56*	4.14*
	W/O S-9; W Fec.	.38	.85	1.15	1.60	1.90	3.45*	4.10*
TA 97	W S-9; W/O Fec.	.18	.31	.47	.50	.59	.62	.83
	W S-9; W Fec.	.23	.58	.79	1.09	1.96	4.10*	4.62*
	W/O S-9; W Fec.	.21	.56	.79	1.04	1.86	3.68*	4.51*

*Positive response.

**The adsorbed components were eluted and washed with minimal amount of acetone, taken to dryness and dissolved in DMSO.

(TA 1537: W S-9 = 23, W/O S-9 = 21)

(TA 98: W S-9 = 43, W/O S-9 = 41)

(TA 97: W S-9 = 98, W/O S-9 = 95)

TABLE 12c. Mutagenicity of Red Onion (Allium cepa) Juice Concentrated with XAD-2

		Ratio of Test Revertants/Control Revertants						
Tester Strain	Relative Conc.** / Treatment	0.05	0.10	.50	1.00	2.00	4.00	8.00
TA 1537	W S-9; W/O Fec.	.23	.55	.73	.67	.76	.91	1.24
	W S-9; W Fec.	.55	.68	1.27	.95	1.76	2.91*	5.14*
	W/O S-9; W Fec.	.52	.75	1.25	1.05	1.79	2.75*	4.90*
TA 98	W S-9; W/O Fec.	.26	.48	.64	.50	.83	1.16	.83
	W S-9; W Fec.	.37	.74	1.12	1.19	1.52	3.44*	3.11*
	W/O S-9; W Fec.	.35	.63	1.10	1.10	1.53	3.35*	3.02*
TA 97	W S-9; W/O Fec.	.15	.26	.37	.43	.52	.93	.56
	W S-9; W Fec.	.16	.41	.56	.94	1.60	8.91*	4.10*
	W/O S-9; W Fec.	.12	.40	.53	.88	1.47	8.70*	3.90*

*Positive response.

**The adsorbed components were eluted and washed with minimal amount of acetone, taken to dryness and dissolved in DMSO.

(TA 1537: W S-9 = 23, W/O S-9 = 21)

(TA 98: W S-9 = 43, W/O S-9 = 41)

(TA 97: W S-9 = 98, W/O S-9 = 95)

TABLE 12d. Mutagenicity of Yellow Onion (Allium cepa) Juice Concentrated with XAD-2.

		Ratio of Test Revertants/Control Revertants						
Tester Strain	Relative Conc.** / Treatment	0.50	0.10	0.50	1.00	2.00	4.00	8.00
TA 1537	W S-9; W/O Fec.	.27	.50	.68	.81	.91	.95	1.38
	W S-9; W Fec.	.59	.68	1.00	1.33	2.05*	3.38*	4.95*
	W/O S-9; W Fec.	.52	.70	1.00	1.26	2.16*	3.00*	4.55*
TA 98	W S-9; W/O Fec.	.40	.48	.48	.67	.86	.81	1.19
	W S-9; W Fec.	.47	.55	1.02	1.31	1.69	2.35*	3.16*
	W/O S-9; W Fec.	.45	.59	.98	1.25	1.65	2.30*	3.30*
TA 97	W S-9; W/O Fec.	.19	.23	.38	.47	.56	.77	.99
	W S-9; W Fec.	.14	.36	.52	.84	1.80	2.38*	4.83*
	W/O S-9 W Fec.	.17	.34	.50	.78	1.75	2.35*	4.26*

*Positive response.

**The adsorbed components were eluted and washed with minimal amount of acetone, taken to dryness and dissolved in DMSO.

(TA 1537: W S-9 = 23, W/O S-9 = 21)

(TA 98: W S-9 = 43, W/O S-9 = 41)

(TA 97: W S-9 = 98, W/O S-9 = 95)

TABLE 12f. Mutagenicity of White Onion (Allium cepa) Juice Concentrated with XAD-2

		Ratio of Test Revertants/ Control Revertants						
Tester Strain	Relative Conc.** / Treatment	0.05	0.10	0.50	1.00	2.00	4.00	8.00
TA 1537	W S-9; W/O Fec.	.41	.46	.55	.57	.67	.76	.81
	W S-9; W Fec.	.59	.64	.73	.81	.95	1.81	2.29
	W/O S-9; W Fec.	.57	.65	.75	.95	1.00	1.65	2.20
TA 98	W S-9; W/O Fec.	.26	.33	.41	.50	.64	.72	.84
	W S-9; W Fec.	.42	.48	.52	.67	.79	1.33	1.51
	W/O S-9; W Fec.	.45	.46	.49	.68	.70	1.25	1.63
TA 97	W S-9; W Fec.	.14	.24	.33	.35	.42	.45	.43
	W S-9; W Fec.	.23	.31	.39	.51	.49	.85	1.34
	W/O S-9; W Fec.	.21	.30	.37	.50	.46	.80	1.26

** The adsorbed components were eluted and washed with minimal amount of acetone, taken to dryness and dissolved in DMSO.

(TA 1537: W S-9 = 23, W/O S-9 = 21)

(TA 98: W S-9 = 43, W/O S-9 = 41)

(TA 97: W S-9 = 98, W/O S-9 = 95)