THE UNIVERSITY OF MANITOBA

HYDROLYSIS OF THE FLAVONOID GLYCOSIDE RUTIN TO ITS GENOTOXIC AGLYCON QUERCETIN BY HUMAN ORAL STREPTOCOCCAL ISOLATES: AN EXAMPLE OF A POSSIBLE INVOLVEMENT OF THE ORAL MICROFLORA IN INTRA-ORAL CARCINOGENESIS

by

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A Thesis Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements for the Degree
of Master of Science

Department of Oral Biology
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ABSTRACT

Epidemiological and experimental data support the hypothesis that intestinal microflora participates in the carcinogenic process locally, and probably distally, by modifying man's intimate chemical environment. The aim of this study was to examine the potential of members of the oral microflora to activate *in vitro*, innocuous common environmental chemicals to active genotoxic agents.

Thus, streptococcal strains from the oral cavity of two healthy subjects were isolated, which can hydrolyse the common dietary and tobacco flavonoid glycoside rutin to its aglycon quercetin, a mutagenic, chromosome-damaging and carcinogenic agent. The isolates were identified as *Streptococcus milleri*. The hydrolytic enzyme or rutinase was studied in the cell-free extracts obtained from one of the isolates grown anaerobically, with the aid of a mutagenicity assay, the Ames test. The experimental findings indicate that this streptococcal rutinase is constitutive, partly inducible, cell-bound, with preference for rutin and most active at pH 6.5.

Rutin can only be hydrolysed to quercetin by certain microorganisms, and normally not by mammalian enzymes and other type culture collection strains of oral streptococci (*Streptococcus salivarius* ATCC 25975 and *Streptococcus mutans* strain 6715-10) were found unable to attack rutin.

This study examines a new aspect of the oral microflora and it may provoke research necessary to elucidate an additional novel role of the oral bacteria in a disease process other than that of caries and periodontal, namely intra-oral cancer.

Deeply dedicated to my beloved parents
Michali and Xeni
and to my dearest wife
Tasa

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INTRODUCTION

Experimental and epidemiological data have implicated the participation of endogenous microorganisms in the causation of specific cancers, e.g. colon, gastric, breast and urinary bladder (Wynder et al., 1969; Aries et al., 1969; Hill, Goddard and Williams, 1971; Hill, Hawksworth and Tattersall, 1973; Ruddell et al., 1976; Hicks et al., 1977; and Murray et al., 1980a,b) which account for a very high proportion of human cancer. The more general question, whether the microflora anywhere in the human body could mediate carcinogenic processes by modifying man's intimate chemical environment, was addressed by Hill and Williams (1973).

Although it has been established that oral microflora reduce salivary nitrate to nitrite (a precursor of carcinogenic N-nitroso compounds) in vivo, and several studies have demonstrated bacterial production of some carcinogenic N-nitrosamines in whole saliva in vitro, the authors did not discuss their findings in relation to oral carcinogenesis. (Tannenbaum et al., 1974; Ishiwata, Tanimura and Ishidate, 1975; Rao, 1978; Tannenbaum et al., 1978). This is not surprising if one considers the strong organotropic effect of N-nitrosamines to cause cancer in such distal organs as liver, lung and kidney (Druckrey, Preussmann and Ivankovic, 1969; Schmahl and Habs, 1980).

Systematic studies on the evaluation of the importance of the oral microflora in intra-oral carcinogenesis are, to the author's best

knowledge, non-existent. The present study's primary aim was to assess the ability of members of the oral microflora to activate environmental innocuous substances to carcinogenic-mutagenic ones, in vitro. Quercetin is a flavonol occurring ubiquitously in the plant kingdom and has been shown to damage DNA and chromosomes, and to cause cancer in rats (Bjeldanes and Chang, 1977; Brown, 1980; Pamukcu et al., 1980; Meltz and MacGregor, 1981). Quercetin occurs in nature almost exclusively as glycoside (Harborne, Mabry and Mabry, 1975; Wollenweber and Dietz, 1981). Rutin (L-rhamnosyl-α 1,6-D-glucosyl-β 1,3-quercetin) is the commonest glycoside of quercetin and it can be found in many common foodstuffs and beverages (Herrmann, 1976; Kuhnau, 1976; Brown, 1980). Rutin can only be hydrolysed to quercetin by certain microorganisms and normally not by mammalian enzymes (Westlake et al., 1959; Scheline, 1968, 1973; Griffiths and Barrow, 1972; Brown and Dietrich, 1979). Quercetin is suspect as a human carcinogen and is liberated from the innocuous rutin by cell-free extracts of human feces (Tamura et al., 1980).

The strong association of excessive consumption of alcoholic beverages and the practice of "smokeless" tobacco habits (i.e. chewing, snuff-dipping, etc.) with intra-oral cancer is well known (Wynder, 1957; Rosenfeld and Callaway, 1963; Hirayama, 1966; Jussawalla and Deshpande, 1971; Wahi, 1976; Binnie, 1976; Winn et al., 1981; Mehta, Gupta and Pindborg, 1981). Although there is lack of solid evidence as to the specific carcinogenic ingredient(s) involved (Bock, 1968), the presence of quercetin glycosides in alcoholic beverages and in

tobacco leaves (rutin up to 2% dry weight) (Johnstone and Plimmer, 1959; Wynder and Hoffmann, 1967; Stedman, 1968; Leake and Silverman, 1971; Tamura et al., 1980) leads one to speculate that genotoxic quercetin liberated by the action of the oral microflora may participate in intra-oral carcinogenesis.

This study reports the isolation from two human subjects of oral bacterial strains capable of activating rutin to quercetin in vitro. The enzyme system responsible for the conversion was studied in cell-free extracts prepared from one of the isolates grown anaerobically in batch cultures with the aid of a mutagenicity assay, the Ames test (Ames, McCann and Yamasaki, 1975).

LITERATURE REVIEW

Microorganisms and Cancer

The enormous body of epidemiological evidence leaves virtually no doubt that the majority of human cancers (80-90%) are influenced by environmental factors (WHO, 1964; Higginson, 1969; Armstrong and Doll, 1975; Knudson, 1977; Weisburger, Cohen and Wynder, 1977; Higginson, 1981). The recent advances in experimental carcinogenesis, the epidemiological evidence, the radiation-related cancer estimates (less than 3%) and the lack of definite proof for any common human cancer caused solely by virus, all suggest that chemical causation is most pertinent to human carcinogenesis (Doll, 1969; Selkirk, 1980; Kouri, Schechtman and Nebert, 1980; Althouse et al., 1980; Jablon and Bailar, 1980; Bierwolf, 1980; Reddy et al., 1980; Temin, 1980; Higginson, 1981; Essex and Gutensohn, 1981; Miller and Miller, 1981). Boyland (1969) even claims that about 90% of human cancers have a chemical etiology.

In view of the astonishing metabolic versatility of microorganisms, the hypothesis that human microflora participate in carcinogenesis through some metabolic effect on ingested chemicals is attractive. In fact, the thought of a parasitic and/or bacterial contribution to the disease is not entirely new (Borrel, 1910; Ferguson, 1911; Fibiger, 1913; Leyton and Leyton, 1916; Nuzum, 1925). It would even be plausible to think that with the discovery of microorganisms as specific disease agents during the golden age of medical bacteriology in the nineteenth century, the incrimination of microorganisms in cancer causation as the biological agents had to come about inevitably,

overnight. The pioneering work of Laquer (1964) and Spatz et al., (1967) produced concrete biochemical evidence, showing that intestinal microflora of animals mediate cycasin (methylazoxymethanol-β-D-glucoside) carcinogenicity by hydrolytically liberating its active aglycon.

Since then, a plethora of data both experimental and epidemiological have been accumulated supporting an involvement of the microflora (normal and infectious) in cancer causation. Thus, bacteria have been shown to produce carcinogens and their precursors, promotors or cocarcinogens and mutagens in vivo and in vitro. These substances may include N-nitrosocompounds, nitrites, secondary bile acids, phenols and hormones (Chung, Fulk and Slein, 1975; Hill, 1979; Backus and Affronti, 1981). Bacteria could also activate genotoxic agents to their biologically active form. This has also been demonstrated both in vivo (i.e. conjugated liver detoxification products of carcinogens) (Grantham et al., 1970; Goldman, 1981), as well as in vitro in cell-free extracts of pure bacteria cultures or intestinal contents i.e. activation of natural genotoxic agents, complex natural mixtures and other carcinogens (Renwick and Drasar, 1976; Mandel, Ichinotsuno and Mower, 1977; McCoy, Speck and Rosenkranz, 1977; McCoy, Petrullo and Rosenkranz, 1979; Brown, 1980; Tamura et al., 1980; Uyeta, Taue and Mazaki, 1981; Wu and Wong, 1981).

The human epidemiologic observations (population-at-risk and case-control studies) have provided invaluable information. Thus, gastric cancer has been associated with hypochlorhydric stomach com-

plicated with chronic bacterial infection (Hill et al., 1973; Ruddell et al., 1976). Chronic urinary tract infection seems also to predispose to the development of bladder tumors and the production of carcinogenic N-nitrosamines in the infected bladder has been demonstrated (Hicks, et al., 1977).

Colon cancer has been intimately associated with certain activities of the intestinal microflora (Goldin et al., 1981). Thus, excreta of people at risk or colon cancer patients were shown to contain much higher levels of bacterially produced secondary bile acids as compared to controls (Reddy and Wynder, 1973; Murray et al., 1980b). These secondary bile acids are known tumor-promoting agents (Reddy and Watanabe, 1979). The bacterially derived fecal mutagens were also higher in high risk populations (Ehrich et al., 1979; Wilkins, Lederman and Van Tassell, 1981). Several studies have also shown that the intestinal bacterial composition in the populations at different risk was different (Aries et al., 1969; Hill et al., 1971b). However, the microbial metabolic activities rather than the composition seem to correlate better with the risk (Moore and Holdeman, 1975; Mastromarino, Reddy and Wynder, 1978). Thus, cell-free fecal preparations from populations at high risk consuming the high risk diet showed higher activities for β-glucuronidase, azoreductase, nitroreductase and 7 α-dehydroxylase. These enzymes are considered responsible for the in vivo activation and/or production of carcinogens and promotors (Macdonald, Webb and Mahony, 1978; Goldin et al., 1981).

A microbial participation has also been suggested in cancer of

the breast. This may include an indirect effect of the gut flora via the production of the breast tumor promoting hormone, oestrogen (Hill et al., 1971a; Murray et al., 1980a) or through a direct association of bacteria and the target organ (Cantwell and Kelso, 1981). In fact, tumor-associated bacteria have been described in other malignancies (e.g. ovary, lymph nodes) (Backus and Affronti, 1981) and very recently in a fatal case of Kaposi's sarcoma (Cantwell and Lawson, 1981). These tumor-associated bacteria were shown to produce a human choriogonadotropin-like substance which may be involved in the progression of the tumors (Backus and Affronti, 1981).

Inhibition of carcinogenesis by bacteria has also been reported in animal studies. Thus, colons of germ-free rats were more susceptible to the direct-acting carcinogens than were those of conventional rats (Balish et al., 1977). Corynebacterium parvum (intravenous injection) inhibited methylcholanthrene-induced carcinogenesis (subcutaneous injection) in mice. Biochemical (destruction or inhibition of activation) as well as immunological mechanisms were suggested for the inhibitory effect (Woodruff, Forbes and Speedy, 1982).

Therefore, if bacteria are actually involved in carcinogenesis at these sites by producing, activating or deactivating carcinogens and other modulating agents (Hill, 1980), it would be sensible to believe that oral microflora may as well participate in the local carcinogenic process by one or another mechanism.

Quercetin - Genotoxicity and Occurrence

Regarding quercetin, besides the question of its in vivo

production and its clearance from the oral cavity (quercetin itself is also subject to extensive microbial degradation), a consideration of its genotoxicity (the ability to induce a hereditable change in the cell phenotype, e.g. by damaging DNA or chromosomes) and its possible role in intra-oral carcinogenesis, is presented. However, in the absence of direct evidence this will be cautiously attempted by discussing the available relevant data.

Although flavonoids (polyphenolic compounds), such as quercetin and its glycosides, have long been known to exhibit a wide variety of biological effects, most of which beneficial to health, (Griffith, Krewson and Naghski, 1955; Harborne et al., 1975; Kuhnau, 1976; Brown, 1980), the toxicity of some of them only recently has became apparent. Thus, several members of the subgroup flavonols were found to be strongly mutagenic to bacteria, of which quercetin proved to be the most potent (Bjeldanes and Chang, 1977; MacGregor and Jurd, 1978; Brown and Dietrich, 1979). Subsequently, the genotoxicity of quercetin was assessed in many other biological systems. More specifically, quercetin was found to induce mutation in Drosophila melanogaster (Watson, 1982), V79 Chinese hamster cells (Maruta, Enaka and Umeda, 1979), and mutation and DNA single-stand break in L5178Y mouse lymphoma cells (Meltz and MacGregor, 1981). It also caused transformation in Syrian golden hamster embryo cells (Umezawa et al., 1977), and Balb/c 3T3 cells (Meltz and MacGregor, 1981) and it was clastogenic in the mouse micronucleus test (Sahu, Basu and Sharma, 1981). The culmination of these studies was reached when Pamukcu et al.

(1980) showed quercetin to produce intestinal and urinary bladder tumors when fed to rats. However, very recent investigations undertaken in Japan produced results at variance with those of Pamukcu et al. when quercetin failed to induce increased tumor incidence in ddY mice, ACI rats and golden hamsters, although in the mice study unusual tumors were observed in those fed quercetin (Saito et al., 1980; Hirono et al., 1981; Morino et al., 1982). Many reasons were given for this discrepancy, such as the well-known species and strain differences, presence of inhibitor(s) of quercetin carcinogenesis, and differences in the composition of the basal diet and the extent of its degradation by the gut flora of different animals (Rall, 1977; Lewin et al., 1981; Ashman et al., 1982; Morino et al., 1982). There are even reports that quercetin inhibited benzo(α)pyrene carcinogenicity (mouse skin application model) (Van Duuren and Goldschmidt, 1976) and that it increased the survival time of mice inoculated with NK/Ly ascites tumor cells, which growth was also inhibited by quercetin in vitro (Molnar et al., 1981). However, it should be taken into consideration that tumorigenesis inhibitory and tumorigenesis inducing properties are not always mutually exclusive. The well-known ability of many antineoplastic agents such as Actinomycin D (Gelboin, Klein and Bates, 1965; Sieber and Adamson, 1975), as well as other carcinogenic agents (e.g. sulfur gas, several polycyclic aromatic hydrocarbons, radiation) to exhibit either action in tumorigenesis at different sets of conditions is noteworthy (Berenblum, 1935; Hill et al., 1951; Hewitt, 1962; Horm, Levij and Polliack, 1971; Prasad, 1974;

Shellabarger, 1976; Slaga and Boutwell, 1977). In fact, this is one of the paradoxical aspects of the cancer problem. Yet, these conflicting reports are disturbing as well as puzzling in assessing the genotoxic potential of such ubiquitous natural substance as quercetin. Worth considering at this point is the limited human evidence on quercetin genotoxicity. Sugimura (1979) demonstrated that quercetin increased sister chromatic exchanges in human lymphoblastoid cells (line NL3) and Ames (1979) showed quercetin could be further activated to a more potent mutagen by human (autopsy) liver.

Quercetin has not only been shown to be genotoxic, but also its inherent potential to attack DNA directly without requirement for metabolic activation, unlike most environmental carcinogens, is of a tremendous toxicological significance (Bjeldanes and Chang, 1977; MacGregor and Jurd, 1978). Furthermore, the potentiation of its genotoxicity by mammalian enzymes (Brown and Dietrich, 1979; Ames, 1979) and by other genotoxic chemicals, i.e. arecoline (Stich, Stich and Lam, 1981) or tannic acid (Stich and Powrie, 1982) in natural products such as betel nut and various beverages, increases its overall toxic potential. The induction by quercetin of tumors in the urinary bladder and intestinal wall attesting to a non-sparing effect on epithelial tissues, is consistent with its direct genotoxicity.

Regarding its occurrence the case is rather interesting. Here we have a naturally occurring compound with a mutagenic potential close to that of benzo(α)pyrene (Sugimura, 1982) to which animals and humans have always been exposed. Thus, flavonol glycosides, particu-

larly of quercetin are widespread in the edible parts of many food plants such as vegetables, fruits, berries, herbs, legumes and cereal grains (Harborne et al., 1975; Herrmann, 1976; Kuhnau, 1976). Their occurrence in spices, bracken fern and in alcoholic beverages such as beer, red wine, cider and especially in tea and tobacco leaves (fresh and flue-cured) in which rutin may reach levels of 2% dry weight is of particular significance (Roberts, Cartwright and Wood, 1956; Weaving, 1958; Runeckles, 1963; Wynder and Hoffmann, 1967; Bokuchava and Skobeleva, 1969; Leake and Silverman, 1971; Beech and Carr, 1977; Seino et al., 1978; Tamura et al., 1980; Pamukcu et al., 1980). In fact, the presence of rutin in tobacco leaf is so common that its quality is directly related to its rutin content (Stedman, 1968). It should be noted here that the flavonol glycosides themselves are neither genotoxic (Brown and Dietrich, 1979) nor absorbed by the intestinal wall (Griffiths and Barrow, 1972; Kuhnau, 1976). However, small amounts of free flavonols are always present in natural products.

Oral Cancer Etiology - General

Although the exact causative agents of oral cancer are still obscure despite the enormous amount of investigation, its environmental origin is hardly questioned (Mahboubi, 1977; Smith, 1979; Pindborg, 1980; Silverman, 1981; Mahboubi and Sayed, 1982). The pathology data (approximately 95% squamous cell carcinomas and the multicentricity of the lesions) (McLaren, 1963; Krolls and Hoffman, 1976; Gluckman, Grissman and Donegan, 1980; Tepperman and Fitzpatrick, 1981), the increasing incidence with age, the association with certain

habitual practices and the site distribution, all strongly support the hypothesis that oral cancer is the result of the prolonged contact of susceptible oral tissues and unrecognized, ubiquitous noxious agents (Rowe, 1968; Vaughan et al., 1980).

Thus the causal relationship of smokeless tobacco habits (chewing and dipping), betel quid chewing (either alone or in a mixture with tobacco) and cancer of buccal-gingival mucosa in certain parts of such countries as India, Sri Lanka (Ceylon), New Guinea and U.S.A. is well established (Rosenfeld and Callaway, 1963; Atkinson et al., 1964; Wahi, Kehar and Lahiri, 1965; Hirayama, 1966; Jussawalla and Deshpande, 1971; Blot and Fraumeni, Jr., 1977; Winn et al., 1981). Although heavy tobacco smoking and excessive alcohol consumption are major factors in the etiology of oral cancer in the Western world (Rothman and Keller, 1972; Feldman et al., 1975; Williams and Horm, 1977), they are not the only ones involved in the causation (Binie, 1976). Thus, other variables which have also been universally associated with oral cancer, involve socioeconomic, nutritional and oral health status (Smith, 1979; Preston-Martin, Henderson and Pike, 1982).

Oral Health Status

The oral health is of particular interest. Thus, "dental sepsis", "dental irritation", "poor dentition", and "poor oral hygiene", have been given minor or major importance as predisposing or even syncarcinogenic factors in the causation. In fact, poor oral hygiene has been consistently associated with intra-oral cancer worldwide (Wynder, Bross and Feldman, 1957; Shanta and Krishnamurthi, 1959;

Pindborg, 1965; Wahi et al., 1965; Nelson and Ship, 1971; Smith, 1973; Khanna et al., 1975; Graham et al., 1977; Browne et al., 1977; Smith, 1979; Preston-Martin et al., 1982). The recent reduction in the incidence of oral cancer in England (in males) and other Western countries has been partly attributed to the improvement of the standards in oral hygiene (Pindborg, 1965; Binnie, 1976). However, no specific attempt has been made to explain in what manner poor oral hygiene has come to be so strongly associated with intra-oral cancer (Smith, 1979; Preston-Martin et al., 1982).

Site Distribution

The site distribution of the lesions helped (1) in establishing the causal relation of the chewing-dipping habits and intra-oral cancer ("site correspondence") (Orr and Glasg, 1933; Friedell and Rosenthal, 1941; Hirayama, 1966) and (2) in formulating hypotheses on the origin of those maligancies related to other life style factors, e.g. smoking and drinking, the ones observed in the Western world.

Several North American studies involving a large number of oral cancer cases established an apparent predilection of intra-oral carcinoma for particular sites (Anderson, 1972). These high risk areas comprise a horseshoe-shaped region that involves the floor of the mouth, anterior pillar, retromolar trygone, and ventralateral tongue. Thus, in the study by Moore and Catlin (1967) 75-80% of intra-oral lesions were located in this region. Mashberg and Meyers (1976) gave even higher figures when 97% of the lesion were found to occur in the same area. The etiologic implications for this predilection as dis-

cussed by these authors are consistent with an external causative agent. Thus, this region which involves the "lateral food channels" and the "reservoir systems" (alveolar-lingual sulcus and floor of the mouth) could concentrate food-borne and other carcinogens. The local puddling of saliva could ensure intra-oral enzymatic transformations of the noxious agents as well as their prolonged contact with the thin non-keratinized epithelium of the structures comprising this region.

Experimental Tobacco Oral Carcinogenesis

Through the years the experimental models have been improved as to the site and mode of application of the test material. More specifically, the importance of good retention, and the subjection of the test material to the intra-oral physiological conditions (e.g. continuous presence of saliva) was emphasized (Cohen and Smith, 1967; Randeria, 1974; Yamamura et al., 1975; Eveson, 1981).

Thus, snuff which was biologically inert when implanted in the hamster pouch or applied to gingivolingual fold (Peacock and Brawley, 1959; Peacock et al., 1960; Homburger, 1971), was recently shown in a new experimental model in rat to induce epithelial changes (hyperkeratotic and dysplastic lesions) (Hirsch and Thilander, 1981). In this model both requirements of maximal retention and the preservation of physiological conditions by a surgically created canal in the lower lip, were met. Tobacco was also found negative when implanted (no contact with the oral cavity) (Peacock and Brawley, 1959; Peacock et al., 1960) but it induced epithelial changes compatible to submucous fibrosis and leukoplakia and formation of dyskeratotic superficial

cells when inserted into the cheek pouch (Luthra, Bharadwaj and Wahi, 1970). Again, the different source of the tobacco products used in these studies remains a definite confounding variable for comparison and for drawing solid conclusions.

It should be mentioned here that although unburned tobacco alone has failed to induce frank carcinomas in experimental animals, its promoting or cocarcinogenic activity has long been established (Muir and Kirk, 1960; Bock, Moore and Crouch, 1964; Van Duuren, 1968; Luthra et al., 1970; Suri, Goldman and Wells, 1971; Ranadive et al., 1976). The polyphenols of tobacco have been mainly incriminated for this action (Van Duuren et al., 1966; Bock, 1968). Of a particular interest are the findings of Bock et al. (1964, 1965) who showed that extraction of unburned tobacco with aqueous $Ba(OH)_2$ produced the best recovery of the active tumor promoting agent(s). The hypothesis of whether the polyphenols rutin and/or chlorogenic acid bound to proteins (tobacco pigments) or their hydrolytic products in the alkaline aqueous extract of tobacco could account for the tumor promotion activity, was discussed by these authors. It should be added that sparingly water soluble rutin and water insoluble quercetin are soluble in aqueous alkaline solutions.

Lime

Since the original report by Orr and Glasg (1933) on the importance of lime in the etiology of oral cancer, many epidemiological studies have presented lime as a very significant contributory factor in the pathogenesis. Thus, the liberal use of slaked lime (especially

shell lime, a stronger alkali) in the quids used in parts of South-East and Central Asia, with high incidence in oral cancer including high risk areas where betel nut quid is never mixed with tobacco (parts of New Guinea and South Africa) is characteristic (Atkinson et al., 1964; Hirayama, 1966; Malhotra, 1967; Schonland and Bradshaw, 1969; Mahboubi, 1977). On the other extreme, the infrequency of oral cancer in areas such as Kubul (Afganistan) and Nigeria where tobacco is chewed without lime, and in certain regions of India where lime is hardly added to the local quid (betel leaf, area nut and coconut) is striking (Hirayama, 1966; Malhotra, 1967).

The importance of tobacco and lime mixture in the etiology of oral cancer as emphasized by the epidemiological studies is also supported by laboratory data. Thus, the incorporation of lime in tobacco quid or tobacco was shown to facilitate or even to initiate the production of carcinomatous and epithelial changes (Reddy and Auguli, 1967; Hamner, 1972; Kandarkar and Sirsat, 1977; Mori et al., 1979). Among other mechanisms proposed such as the removal of the protective mucus of the oral mucosa and the direct action on the epithelium (e.g. keratinization anomalies, hyperplasia and dysplasia), the extraction of carcinogenic or cocarcinogenic agents from the quid or the tobacco represents another interesting possibility (Orr and Glasg, 1933; Malhotra, 1967; Khanna et al., 1975).

Betel Nut Quid

On the role of betel nut in intra-oral carcinogenesis both epidemiological and laboratory data are available. Thus, the high inci-

dence rates of oral cancer in parts of New Guinea and in the female Natal Indians of South Africa where tobacco-free betel quids are used, represent the most direct evidence of the importance of the betel nut in the causation (Atkinson et al., 1964; Schonland and Bradshaw, 1969). Other epidemiological studies in India have also consistently emphasized the fact that tobacco alone cannot account for the total carcinogenic effect of the betel quid (Singh and Von Essen, 1966; Malhotra, 1967; Jussawalla and Deshpande, 1971; Jayant, 1977).

The carcinogenic effect of betel nut (DMSO extract) was established by Suri et al. (1971) in the hamster cheek pouch model and it was further supported in other studies using DMSO and aqueous extracts (Ranadive et al., 1976; Bhide et al., 1979). The recent findings by Ranadive et al. (1979) is of a particular interest. In their system, of the topical application of aqueous extracts of betel quid ingredients alone or in different combinations on hamster cheek pouch, the polyphenolic fraction of betel nut extract was found to be the most potent carcinogen. The same authors attributed this effect to the tannins found in this fraction. However, it should be noted here that flavonoids are also part of the same polyphenolic fraction (Ranadive et al., 1976).

The relevance of the aqueous extract to the in vivo situation where extraction of the toxic ingredients of betel nut and the other quid components by saliva is expected to take place, is obvious (Bock, 1968; Jussawalla and Deshpande, 1971; Umezawa et al., 1981). In fact, Stich and Stich (1982) showed that the saliva of volunteers chewing

betel quid or its individual componets exhibited chromosome-damaging activity (chromatid breaks and exchanges) in the Chinese hamster ovary cell system. The clastogenic activity seemed to be proportionate to vigorousness of chewing (extent of extraction) and it was lacking when the materials were simply kept undamaged in the mouth. Interestingly, the authors discussed their findings in relation to the known genotoxic phenolic ingredients of betel nut (besides that of the alkaloid arecoline), i.e. tannic acid and the genotoxic phenolics of the other quid components i.e. quercetin, eugenol, catechol and chlorogenic acid. These findings are consistent with epidemiological data showing that in some high risk areas for oral cancer, raw, uncured betel nut was used (Orr and Glasg, 1933; Mehta et al., 1971). It was then suggested that an unidentified carcinogen present in the raw nut could be removed during curing by boiling in water (Reddy and Anguli, 1967).

Alcoholic Beverages

The strong association of alcohol overconsumption and oral cancer in many countries of the Western world and other parts of the globe has been firmly established (Hirayama, 1966; Rothman and Keller, 1972; Bross and Coombs, 1976; Mahboubi, 1977; Feldman and Boxer, 1979; Williams and Horm, 1977; Smith, 1979; Herity et al., 1981). Because of a consistent lack of detection of any direct carcinogenic effect of ethanol even in the most sophisticated experimental models, several direct and indirect mechanisms were proposed in order to account for its largely cocarcinogenic effect (Henefer, 1966; Elzay, 1966, 1969; Schottenfeld, 1979; Lieber et al., 1979; Obe and Ristow, 1979). Thus,

besides the direct effect of alcohol which has been described at different operational levels i.e. the systemic (effects on liver and immune system) and the local (solvent effect, epithelial alterations, induction of carcinogen-activating enzymes in the regional structures, reduction of protective secretions etc.), the possibility of an indirect effect for alcohol such as serving as a vehicle for carcinogens or other promotors, is an interesting one (Kuratsune et al., 1971; Obe and Ristow, 1979; Tuyns, 1982). In fact, research in this area has been very active and it appears to be very promising.

Thus, the presence of low levels of carcinogens such as nitrosamines, polycyclic aromatic hydrocarbons and tannins has already been shown in some alcoholic beverages (Masuda et al., 1966; Warwick and Harington, 1973; Tuyns and Griciute, 1980). The recent use of short-term tests for genotoxicity yielded further supporting evidence. Direct-acting mutagens and mutagens that require metabolic activation were detected in various alcoholic spirits (Nagao et al., 1981). The mutagenicity of the beverages seems to be related to the type of the beverage and the methods of brewing. The mutagenic activity shows an inverse relationship to their degree of refinement. Thus, homemade apple brandies in west France (associated with the high incidence of esophageal cancer), were found the only samples to show mutagenicity more frequently and at much higher levels than more refined commercial brandies or other types of spirits such as rums, whiskies and cognacs (Loquet, Toussaint and Le Talaer, 1981). Seventeen of the twenty-seven Chinese spirits tested for mutagenicity were positive and all of

them were either single-distilled or contained additives, e.g. herbs. Nine of the ten negatives were of the purest triple-distilled grade. These spirits are consumed in high risk areas for esophageal cancer both in China and Hong Kong (Lee and Fong, 1979). The failure to detect or the presence of trace amounts of known carcinogens, such as nitrosamines, in beverages consumed in high risk areas for cancer of the upper alimentary tract and the detection of mutagens in the different fractions of these beverages (aqueous, alcoholic and dry residue) point to the possibility of an involvement of a diverse group of chemicals (Tuyns et al., 1979; Tuyns and Griciute, 1980). In this respect the findings of Tamura et al. (1980) are of particular significance. Using the Ames test they showed that red wine possessed considerable direct mutagenic activity which was further augmented when a mixed glycosidase preparation, "fecalase", derived from human fecal homogenates, was incorporated into the plates. In contrast, unfermented red grape juice had an absolute requirement for fecalase in order to express mutagenic activity. The mutagenicity of both red wine and grape juice were attributed to their quercetin glycoside content. In fact, flavonoids i.e. quercetin and its glycosides are regular ingredients of many beverages such as wine (especially red), apple cider and beer, originating from the starting material, the fruit juice (grape and apple) and additives (herbs), of these products.

Of interest are the relevant epidemiological data associating high incidence of oral cancer with crude homemade spirits i.e. country-made non-refined wine in parts of India (Wahi, 1976), moonsh-

ine rum in Puerto Rico (Martinez, 1969) and the downward trend of oral cancer coinciding with the prohibition of unmatured pot-still spirits containing "toxic byproducts" in the U.K. (Binnie, 1976). It has been hypothesized that types of spirits more closely to pure ethanol, such as vodka, would carry lower risk than would other "cruder" alcoholic beverages (Graham et al., 1977). In fact, many epidemiological studies emphasized the importance of beer, wine and whiskey in relation to oral cancer (Wahi, 1976; Graham et al., 1977; Williams and Horm, 1977; Browne et al., 1977). Of particular interest is the study by Mashberg, Garfinkel and Harris (1981) in the U.S.A. on 181 patients and 497 controls, which showed beer/wine drinkers had a much higher relative risk for oral cancer than did the whiskey drinkers (20.4 vs 7.3).

Esophageal Cancer Data

The geographic distribution of esophageal cancer largely coincides with that of oral cancer, suggesting common etiologic factors (Martinez, Torres and Frias, 1975; Mahboubi, 1977; Williams and Horm, 1977; Jussawalla, 1981; Day and Munoz, 1982). Furthermore, the histological similarities of the mucous lining of the risk areas of the oral cavity, pharynx and esophagus, the sensitivity to common precancerous conditions (e.g. Plummer-Vinson syndrome, submucosal fibrosis and leukoplakia sometimes complicated with candidosis), the common cancer pathology (over 90% are aquamous cell carcinomas) and the subjection to common environmental influences (i.e. foodstuffs and salivary secretions) are other aspects which along with the epidemiologi-

cal findings have often necessitated the common consideration of the malignancies of these sites as cancer of the upper alimentary tract (Day, 1975; Mahboubi, 1977; Wynder, Mushinski and Spivak, 1977; Goldstein and Zornoza, 1978; Vaughan et al., 1980; Wynder et al., 1981).

Thus, the high incidence of esophageal cancer has been associated with local betel quid (especially in areas where quid residues are swallowed) and tobacco habit practices in parts of South-East Asia and South Africa (Malhotra, 1967; Schonland and Bradshaw, 1969; Stephen and Uragoda, 1970; Jussawalla and Deshpande, 1971; Jayant et al., 1977) and with heavy tobacco smoking and excessive alcohol drinking in the U.S.A. and other parts of the Western world (Martinez, 1969; Williams and Horm, 1977; Tuyns et al., 1979; Jensen, 1979; McCoy, Hecht and Wynder, 1980). A higher incidence, particulary in females in the south U.S. has been related to snuff dipping (Fraumeni, Jr. and Blot, 1977).

It's strong association with "crude" homemade spirits has also been described in several epidemiological studies. Homemade maize beer in parts of Africa (McGlashan, 1969; Cook, 1971; Warwick and Harington, 1973), apple brandy in northwest France (Tuyns, 1979) and illicitly distilled rum in Puerto Rico (Martinez, 1969) are only some examples. The presence of flavonol glycosides in apple brandy has already been mentioned (Beech and Carr, 1977); maize husks which are included in the fermentation mixture are also known to contain flavonol glycosides (Hattori, 1962; Kuhnau, 1976). This, in conjuction

with the failure of the traces of nitrosamines, found in some of these drinks, to account for their entire carcinogenic potential, deserves special attention (Tuyns et al., 1979; Tuyns and Griciute, 1980).

Interestingly, in parts of Iran and India where the incidence is exceedingly high, esophageal cancer has been strongly associated with the excessive consumption of hot tea (Kumar and Ramachandran, 1973; Joint Iran-International Agency for Research on Cancer Study Group, 1977). Also in other parts of the world such as Curacao, the high incidence was also related to some folk medicine and refreshment plant "teas" which are widely consumed locally (Morton, 1968).

Mortality rates (esophageal cancer) in several regions in China (Linxian county) were also correlated with amount of pickled vegetables consumed. These pickled vegetables were found to contain mutagens (Miller, 1978). As flavonols had already been shown to account for the mutagenicity of Japanese pickles (Takahashi et al., 1979) these findings were also discussed in terms of flavonoid content although the Chinese pickles also contained a nitroso compound (Mingxin, Ping and Baorong, 1980).

Of significance is the epidemiologic survey by Kamon and Hirayama (1975), where the high esophageal cancer incidence in some mountainous areas of central Japan was correlated with the daily consumption of bracken fern. The risk was particularly high when a hot tea-gruel was also consumed. Notably, quercetin and other flavonoid glycosides of bracken fern have been considered by Pamukcu et al. (1980) to account for its well-known carcinogenicity in animals, exhibited both natu-

rally and experimentally (Pamukcu and Bryan, 1979).

The laboratory findings are also of parallel importance. Thus, ordinary tea (extracts from Camellia sinensis) was found to be a potent promoter of carcinogenesis when applied to the mouse skin and the tea polyphenols were considered responsible for this action (Kaiser, 1967). Characteristically the polyphenolic content of tea leaves is very high (approximately 30% dry weight) and only rutin may reach levels of 0.45% leaf dry weight but values up to 2% have also been quoted (Bokuchawa and Skobeleva, 1969; Hardigree and Epler, 1978). The carcinogenicity of tea was demonstrated by Kapadia et al. (1976) in rats when injected the tea extract subcutaneously. In the same study, extracts (teas) from five medicinal herbs indigenous to the U.S. were also found tumorigenic in the same system. The tumorigenicity of these plant materials was discussed in terms of their tannin content. Interestingly, the tannin-free polyphenolic fraction of one of these plants (Myrica cerifera) was also tumorigenic. When examined by paper chromatography, this fraction showed the presence of four phenolic compounds, one of which was identified as the mutagenic flavonol myricitrin. Other similar studies have also shown that tannin-poor extracts from plants used for home remedies and beverages in high risk areas for esophageal cancer (i.e. S. Carolina and Curacao), to be carcinogenic in over 50% of the injected rats (Kapadia et al., 1978). Experimental evidence relevent to the oral cavity, was obtained in the elegant work by Dunham, Sheets and Morton (1974) when extracts from plant materials collected from Curacao were tested in

hamsters by application to the cheek pouch, ingestion as a concentrated tea or mixing with the diet. Four out of nine plant extracts were found to induce carcinoma of the pouch epithelium and papillomas of the esophagus. The lesions were produced only after topical application on the cheek pouch. No lesions were developed when the teas were ingested. Discussing the possibilities the authors concluded: "...chemical change to a potential carcinogen may take place in materials stored in the pouch and there exposed to salivary gland secretions.". Of some relevance here are the data obtained by Uyeta et al. (1981) who showed that 70% of the total mutagenic activity exhibited by the hydrolysates (acid and enzymatic) of infusions of both green and black tea was due to the mutagenic flavonols quercetin, Kaempherol and myricetin (detected by gas chromatography and mass spectrometry) present in the infusions as glycosides. Relevant is also the study of Ranadive et al. (1979) where the application (topical, implantation) of betel quid ingredients on the hamster cheek pouch caused esophagic and gastric lesions.

Thus, the hypothesis of the involvement of food/saliva-borne extraneous carcinogenic agents in the causation of esophageal cancer is consistent with the available evidence (Stephen and Uragoda, 1970; Fraumeni and Blot, 1977; Stich and Stich, 1982). The predisposing effect of food lodging during esophagel stricture and achalasia and the site predilection (lower two-thirds of esophagus) which coincides with the areas of food stasis (analogous to the reservoir system in the oral cavity), give further support to the hypothesis (Warwick and

Harington, 1973).

What is fascinating is the etiologic implications that might have oral influences such as the associated poor oral hygiene and the saliva-treated-and-borne putative carcinogenic chemicals in esophageal carcinogenesis. In fact, it seems ironic that Chinese investigators of esophageal cancer etiology have suspected a role of the oral microflora in the causation via the production of carcinogens (Yang, 1980).

MATERIALS AND METHODS

Chemicals and Media

Rutin (trihydrate), quercetin, L-cysteine hydrochloride (monohydrate), D-Biotin and sulfamethazine were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.. Dimethylsulfoxide (DMSO) (spectrophotometric grade) was from Fisher Scientific Co., Fair Lawn, N.J., U.S.A. and L-histidine hydrochloride (monohydrate) from ICN Nutritional Biochemicals, Cleveland, Ohio, U.S.A. Polyethelene glycol (Aquacide III) was obtained from Calbiochem-Behring Corp., La Jolla, Ca., U.S.A.. Quercitrin was a gift from Dr. G.A. Jones, Dept. of Dairy and Food Science, University of Saskatchewan, Saskatoon, Canada. Chemicals were used as obtained unless otherwise indicated. The following media were used: 5 percent (v/v) sheep blood (Atlas Laboratories Co., Ltd., Winnipeg, Man., Canada) agar (Blood Agar Base No. 2, Oxoid Ltd., Basingstoke, Hants., England), TYC agar was from London Analytical and Bacteriological Media Ltd., London, England. Tryptone, Yeast Extract and Agar were purchased from Difco Laboratories, Detroit, Mi., U.S.A.; nutrient broth for the culture of the salmonella tester strain was obtained from BBL, Cockeysville, Md., U.S.A..

Bacterial Strains

Strep. salivarius ATCC #25975 and *Strep. mutans* strain 6715-10, were kindly provided by Dr. I.R. Hamilton of this department. *Salmonella typhimurium* tester strain TA98 was generously supplied by

Dr. B.N. Ames (University of California, Berkeley, Cal.).

Analytical Methods

Protein was determined by the method of Lowry et al., (1951). For the identification of the insoluble product from rutin degradation by the isolates, samples, obtained as described in text, were extracted with ethanol, crystallized and compared with chromatographically purified rutin and quercetin using the following techniques: (1) Thin-layer chromatography; the samples dissolved in ethanol were spotted on pre-coated Sil G-25 plates (Brinkmann Instruments Ltd., Westbury, N.Y., U.S.A.) and developed with chloroform:methanol:water (65:25:4 v/v) solvent; in this system rutin had an Rf of 0.50 and quercetin 0.66, (2) Spectrophotometry as described by Dowd (1959).

Isolation, Classification of Isolates and Identification of the Main Degradation Product of Rutin

Anaerobic enrichment culture technique was used for isolation of rutin-hydrolysing bacteria (Cheng et al., 1969). Pre-reduced media were prepared by the methods described by Holdeman, Cato and Moore (1977). Culture tubes with tryptone-yeast extract and resazurin medium (TYE) were reduced (by boiling, bubbling with CO_2, cooling, and adding cysteine), stoppered with red rubber serum stoppers and autoclaved for 15 min at 121^0C. Culture tubes containing KH_2PO_4-K_2HPO_4 buffer (pH 7.0) and tubes with rutin suspended in water were treated similarly. After sterilization, 0.5 ml of the buffer solution and 0.5 ml of the rutin suspension were injected aseptically into 4 ml of the

TYE broth, making 5 ml of the complete medium with a composition: 0.1% tryptone, 0.5% yeast extract, 0.4% rutin, 0.05% cysteine, 0.0001% resazurin in 50 mM phosphate buffer. In this medium precipitated rutin formed a greenish sediment. Plaque and soft tissue scrapings suspended in oral fluid were collected from a healthy individual. The complete medium was aseptically inoculated with a small portion of this "oral flora sample" and incubated for 72 hours at 37^{0}C at which time the heavy greenish sediment of rutin had changed to a lemon-yellow flocculent precipitate. A subculture was made by aseptically transfering a 200 µl sample. After six serial subcultures, isolation of the dominant organism was made on blood agar.

In order to rule out the possibility of the isolation of a unique strain, or a strain-artifact of the enrichment culture, the isolation of similar strains was attempted from another healthy individual: an oral flora sample was directly streaked onto an agar plate containing rutin (0.4%) and sulfamethazine (0.1%). Brownish colonies were formed after anaerobic incubation for 48 hours. Isolates were obtained from these colonies by serial streaking on blood agar.

Pure cultures of the isolates from both individuals were obtained in glucose-tryptone-yeast extract-phosphate broth (purity was checked by microscopy, and colonial morphology on blood agar, TYC, and agar containing rutin). The isolates identified as Streptococcus sp (Gram positive, catalase negative, facultative cocci, forming long chains in broth culture) (Fig. 1) were further characterized based on tests described by Hardie and Bowden (1976). The ability to grow on 10%

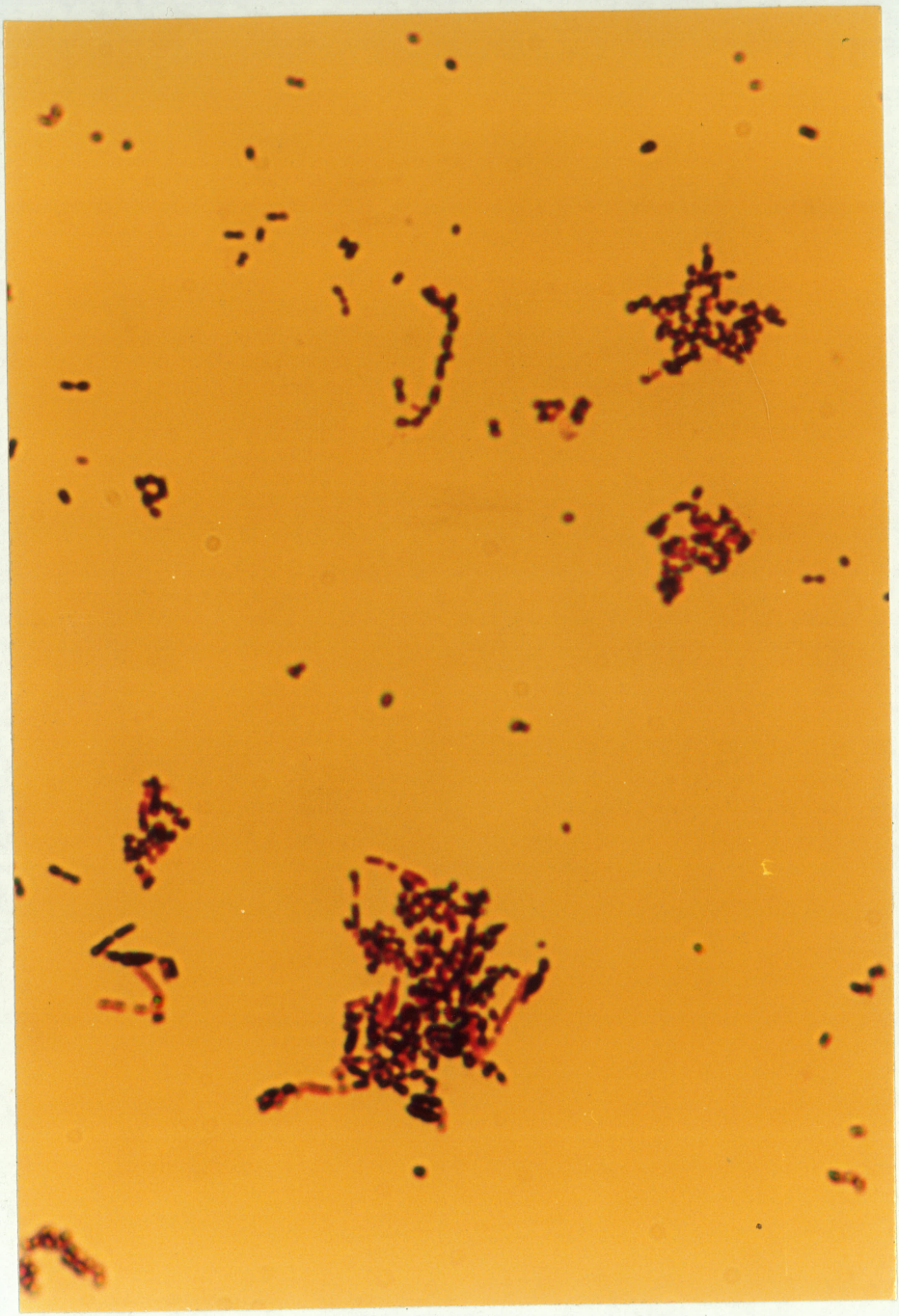

FIGURE 1: Gram stain made on a smear taken from 24-hour culture of one of the isolates on blood agar. X 1000.

bile-blood agar, MC-agar, MSB-agar and at 45^{0}C was tested. Hemolysis and fermentation of rhamnose were also included in the discriminatory tests.

Samples from the bright yellow flocculent sediment formed in pure cultures of the isolates were subjected to identification by thin-layer chromatography and spectrophotometry.

Isolates in Rutin Media

Changes in rutin broth and colonial morphology as well as medium changes in rutin agar were studied. Thus, anaerobic 48-hour cultures of the isolates were made in broth containing rutin either in suspension or in solution. The composition of the rutin broth is as described in the section Preparation of Media except where indicated. Anaerobic and aerobic 48-hour cultures of the isolates were made in agar containing rutin either in suspension or in solution. This rutin agar contained 1% tryptone, 0.5% yeast extract, 1.5% agar, 50 mM phosphate buffer, pH 7.4 and either 0.1% or 0.4% rutin. When rutin was in solution (0.1%) this agar also contained 4% DMSO. Stab cultures were also made in this agar and in TYC, both containing 0.1% rutin in solution. Uninoculated rutin media (blanks) are also included in the illustrations for reasons of comparison (Fig. 2).

It should be noted here that the study of the rutin medium changes and colonial morphology was made by inoculating with the pure isolates which had been subcultured in rutin-free media during purification, after their initial isolation on rutin media.

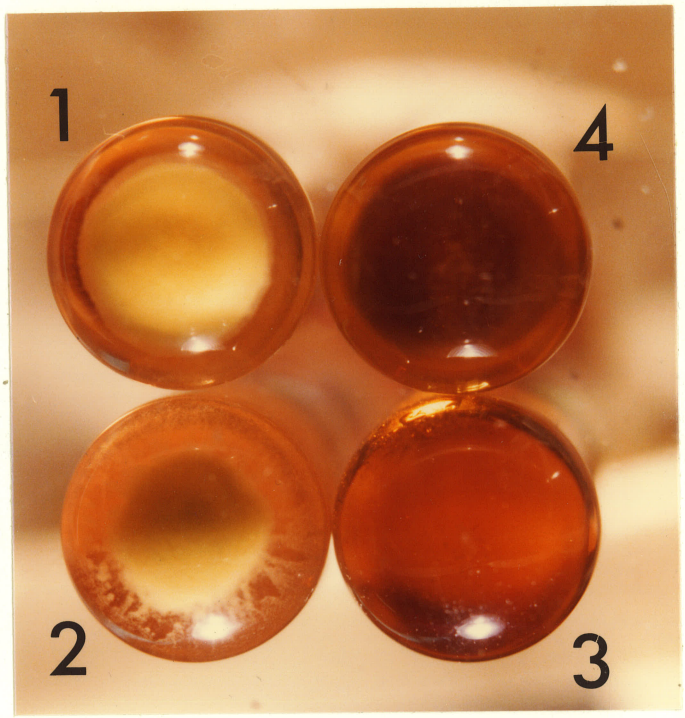

FIGURE 2: View of the bottom of uninoculated tubes containing rutin broth (Blank). Composition of the medium as described in the text (Preparation of Media) except for glucose.

(1) rutin in suspension - no glucose
(2) rutin in suspension - 0.2% glucose
(3) rutin in solution - 0.2% glucose
(4) rutin in solution - no glucose.

Induction

In order to test for an inducible enzyme system responsible for the conversion of rutin to quercetin, cell-free extracts were obtained from the isolate grown with a limiting concentration of glucose (0.3%) in (1) the presence of rutin and (2) the absence of rutin (see Preparation of Media - Bulk Cultures). The isolate was also grown anaerobically in a medium containing 0.1% rutin and an excess of 0.8% of glucose; this medium was otherwise similar to that described in the following section.

Preparation of Media - Bulk Cultures

Based on studies of the growth characteristics of the isolates, a medium giving adequate cell yield was designed. The final composition of this medium was 1% tryptone, 0.5% yeast extract, 0.3% glucose (limiting concentration), 0.05% L-cysteine, 0.0001% resazurin and 50 mM potassium phosphate buffer, pH 7.4. The buffer solution was prepared and sterilized separately from the rest of the media. Thus, 200 ml of 500 mM buffer in a filtering Erlenmeyer flask and 1750 ml of the medium in a culture bottle were prepared. The buffer and the medium were reduced and sterilized by autoclaving and then cooled. The buffer was then added aseptically to the medium in the bottle through sterile interconnecting tubing. The complete medium was aseptically flushed with CO_2 through the cotton-plugged tubing on the side-arm of the flask. Sterility of the medium was checked by overnight incubation. Bottles containing complete medium (glucose 0.2%) were inoculated with 1.5 ml of the pure culture of the strain isolated from the

first subject, and incubated overnight at 37^{0}C. Sterile glucose solution (2 g/10 ml/bottle) was then injected to give a final concentration of 0.3% glucose. In one set of bottles, 500 mg rutin dissolved in 40 ml DMSO were injected to give a final concentration of 0.025% rutin and 2% DMSO and in the other set 40 ml of DMSO were injected, after which incubation was resumed. The cultivation was discontinued and cells were harvested at a point where a yellow precipitate began to form in the medium containing rutin, about 5 hours after addition of rutin. Purity of the grown media was checked in the usual manner. The final pH was found to be around 5.8 for the medium with rutin, and around 6.0 for the medium without rutin.

Cell Collection - Cell-free Extract Preparations

Pilot experiments showed that collection of cells under aerobic conditions did not alter enzyme activity. Therefore, all procedures described below were carried out aerobically. The bright yellow precipitate formed in the culture with rutin was removed by filtration (Whatman #31). Cells from both cultures were harvested by centrifugation at 10,000 g for 15 min at 4^{0}C, washed twice with large volumes of 50 mM phosphate buffer, pH 7.4, resuspended in the same buffer at 50 mg wet weight/ml and stored immediately at -80^{0}C.

Cells from both cultures were disrupted (7.5 ml cell suspension/cup) by the use of Mickle disintegrator (The Mickle Laboratory Engineering Co., Ganshall, Surrey, England) after which cell-free extracts (CFE) were prepared by centrifugation of the cell homogenates at 10,000 g for 15 min. (Pilot experiments showed that disruption by

even mild sonication severely affected the enzyme activity). Protein concentration was adjusted to 4 mg/ml. These cell-free extracts were designated CFE_{10}. Further centrifugation of the CFE_{10} at 100,000 g for 4 h gave a high-speed supernatant designated CFE_{100}. To obtain an enzyme preparation with a higher activity, CFE_{10} from the isolate (grown in the presence of rutin) was centrifuged at fixed volumes (8 ml) at 25,000 g for 30 min. Protein concentration was determined. This 25,000 g supernatant was concentrated by dialysis against 14 vol of 30% polyethelene glycol (20,000 mol. wt.) in 50 mM phosphate buffer at 4^0C. Protein concentration was adjusted to 9 mg/ml. Nine mg of this preparation, designated CFE_{25}, corresponded to about 10 mg of the 10,000 g supernatant of the cell homogenate (CFE_{10}). Culture filtrates from the isolates grown in the presence of rutin were also prepared by centrifuging a small portion of the grown medium at 25,000 g for 30 min, followed by dialysis of the supernatant against 80 vol of buffer at 4^0C overnight in order to remove any excess of histidine in the filtrate which would interfere with the Ames test. Heated enzyme preparations were obtained by heating cell-free extracts or culture fluid at 80^0C for 10 min.

Enzyme Studies - Mutagenicity Assays

Crude cell-free extracts derived from the oral isolate grown in the presence and in the absence of rutin, were the source of enzyme in these studies. A mutagenicity assay, the Ames test, as described by Ames and co-workers (1975) was used to measure enzyme activity. However, the mammalian microsome component of the test (S-9) was omit-

ted and replaced by cell-free extracts from the isolate. In this system any mutagenic quercetin liberated from rutin by the glycosidic action of the cell-free extracts could revert the his^- mutant strain TA98 of Salmonella typhimurium to its protrophic his^+ phenotypic state, allowing it to grow on histidine deficient minimal-glucose agar plates and form visible revertant colonies.

Thus, in the plate incorporation procedure, 2 ml molten top agar, 0.1 ml of an overnight nutrient broth culture of the salmonella tester strain TA98, 200 µg rutin dissolved in 0.1 ml DMSO, and 0.5 ml filter-sterile (Millipore filter, type HA, 0.45-µm pore size, Millipore Corporation, Bedford, Mass. U.S.A.) cell-free extract (either CFE_{10}, CFE_{100} or CF) were mixed together, and immediately poured onto the plates. A positive (20 µg quercetin in 0.1 ml DMSO plus 0.5 ml 50 mM potassium phosphate buffer, pH 7.4/plate) and five other controls: (1) 0.5 ml buffer/plate, (2) 200 µg rutin in 0.1 ml DMSO plus 0.5 ml buffer/plate, (3) 200 µg rutin in 0.1 ml DMSO plus 0.5ml heated cell-free extract/plate, (4) 0.5 ml cell-free extract/plate, and (5) 0.5 ml heated cell-free extract/plate were done simultaneously. Tests were usually conducted in triplicate except where indicated. Plates were put in a dark 37^0C incubator for 2 days, after which visible colonies were counted. Background "lawn" was also checked under a binocular dissecting microscope for assessment of normal growth of the tester strain.

For the statistical analysis, rutin activation value, i.e. rutin + CFE_{10} of each experiment (Tables 2 and 3) was adjusted for day

effect and for residual activity, i.e. rutin + heated CFE_{10} value, using the following equation: adjusted rutin activation value = rutin activation value - quercetin activity + average quercetin activity - residual activity value. The adjusted rutin activation values for the two enzyme preparations were compared using a t-test.

To study the effect of protein concentration of the cell-free extracts on the activation of rutin, different concentrations of fresh and heated cell-free extracts (CFE_{10}) from the isolate of both cultures were tested similarly. The data were subjected to a factorial analysis of variance.

CFE_{10} (2 mg protein/0.5 ml/plate) from the isolate grown in the presence of rutin was used to study the effect of different concentrations of rutin (substrate) on the enzyme activity.

To study the effect of pH on the enzyme activity a different procedure was introduced in order to control the pH of the reaction mixture, that could not be stringently controlled in the standard assay, and to avoid pH effects on components of the assay other than the principal reactants, that is the cell-free extracts and rutin. The concentrated 25,000 g supernatant (CFE_{25}) of a preparation derived from the isolate grown in the presence of rutin was used in order to manipulate the pH at a specific protein concentration. Thus, CFE_{25} was incubated with rutin in citrate - phosphate buffer at the indicated pH (0.45 mg protein corresponding to 0.5 mg protein of the CFE_{10} and 0.5 µmole rutin in 10 µl DMSO in 500 µl buffer) for 1.5 h at 37^0C in a gyrotory waterbath shaker. The reaction mixtures were extracted

with diethyl ether according to the method described by Uyeta et al. (1981). The dry residues were redissolved in 0.5 ml DMSO; each solution was tested in the Ames assay in the absence of rutin or enzyme preparation (0.1 ml/plate). Another portion of the dry residues was examined by thin-layer chromatography and spectrophotometry. Pilot experiment showed that rutin or quercetin did not suffer irreversible changes in this pH range.

To examine whether a low pH (4.5) can exert any permanent deleterious effects on the enzyme activity, the following procedures were used: 0.9 mg of a concentrated 25,000 g supernatant (corresponding to 1 mg protein of the CFE_{10}) of the cell-free extract from the isolate grown in the presence of rutin, in 1 ml citrate - phosphate buffer, pH 4.5, were left at room temperature for 3 min; the sample was then split into two aliquots and 1 µmole rutin/20 µl DMSO was added to each of them; the volume was then adjusted to 1 ml by adding 0.5 ml of the same buffer of pH 4.5 in one, and 0.5 ml of 0.2 M Na_2HPO_4 in the other; the pH of the last one was found to be 6.8; another reaction mixture of the same volume and composition with a pH of 6.8 was also prepared; all three reaction mixtures were then incubated for 2.5 h at 37^0C; extraction was performed as described above, and the residues were redissolved in 1 ml DMSO; each solution was assayed in the Ames test in the absence of rutin or enzyme preparation (0.1 ml/plate).

Isolates and Quercitrin

The isolates were grown in broth cultures containing another glycoside of quercetin, quercitrin (quercetin-3-L-rhamnoside), in suspen-

sion or in solution, in the presence or in the absence of glucose, aerobically or anaerobically in a similar manner to that described above and any changes in the media were noted. The ability of the cell-free extract from the isolate (grown in the presence of rutin) to activate quercitrin in the Ames assay, was also tested; the same procedures were used (0.5 μmole quercitrin in 0.1 ml DMSO/plate; CFE_{10}, 2 mg protein/0.5 ml per plate).

Type Culture Collection Strains of Oral Streptococci in Rutin Media

Strep. salivarius ATCC 25975 and Strep. mutans 6715-10 were tested for the ability to attack rutin in liquid and solid media: (1) in tryptone-yeast extract broth in the absence or presence of limiting concentration of glucose (0.2%) and 0.05% rutin dissolved in DMSO or in suspension, aerobically and anaerobically, (2) in the same media with glucose in absence of buffer, anaerobically (to test for acid hydrolysis at low pH) and (3) in tryptone-yeast extract agar containing 0.1% rutin in DMSO or 0.4% rutin in suspension, aerobically and anaerobically. Stab cultures were also made in tryptone-yeast extract-phosphate and TYC agar, both containing 0.1% rutin in solution. Any changes in the media and the colonial morphology were noted.

RESULTS

Isolation of Oral Bacteria Capable of Hydrolysing Rutin to Quercetin

Strains of oral streptococci capable of attacking rutin were isolated from anaerobic enrichment culture (first subject) and rutin-containing agar (second subject). The isolates convert the heavy greenish sediment of rutin in anaerobic broth cultures to a bright yellow flocculent one and produce the same type of insoluble product in a similar medium containing 0.05% w/v rutin in solution (Figs 3 and 4). The conversion of rutin to the insoluble product by the isolates is also well illustrated in the stab agar containing rutin (in solution) where the formation of yellow granules is distinctly seen along the stab line (Fig. 5). Rutin was also degraded when the isolate was grown in the presence of excess of glucose, even at a higher rate than when in the presence of limiting glucose concentration. Thus, even in TYC-rutin agar which contains high amounts of sugar (5% sucrose) formation of yellow granules was also observed (Fig. 6). On tryptone-yeast extract-phosphate agar containing rutin in suspension (pale yellow, not transparent in appearance) the isolates, grown anaerobically, form small, circular, low convex, finely granular to smooth, glistening, entire, non-adherent, cohesive, opaque, brownish colonies. On the first subculture on rutin agar, clearing of this medium (0.1% rutin) occurs around the densely coalescent colonies (heavy streaks) with a concomitant deposition of a bright lemon-yellow granular precipitate (Figs 7 and 8). On a similar medium containing rutin in solution (yellow-greenish, transluscent) small, circular, low convex,

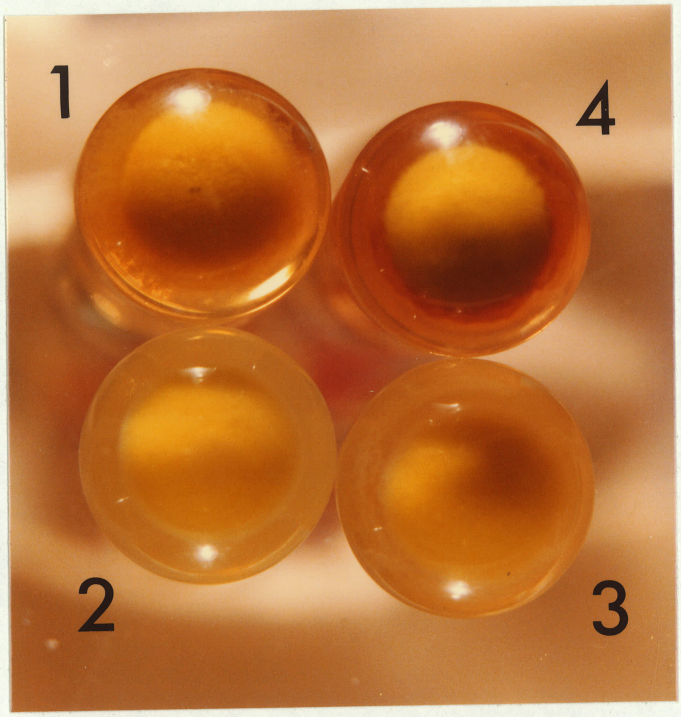

FIGURE 3: Anaerobic 48-hour culture of the isolate in rutin broth. View and composition of the medium as in Fig. 2.

(1) rutin in suspension - no glucose
(2) rutin in suspension - 0.2% glucose
(3) rutin in solution - 0.2% glucose
(4) rutin in solution - no glucose

FIGURE 4: Anaerobic 48-hour culture in broth containing 0.05% rutin in solution. Composition of the medium as described in text (Preparation of Media) except glucose was not added. Transmitted light.

(1) Blank
(2) Isolate
(3) Isolate (tube shaken)
(4) Strep. salivarius ATCC 25975

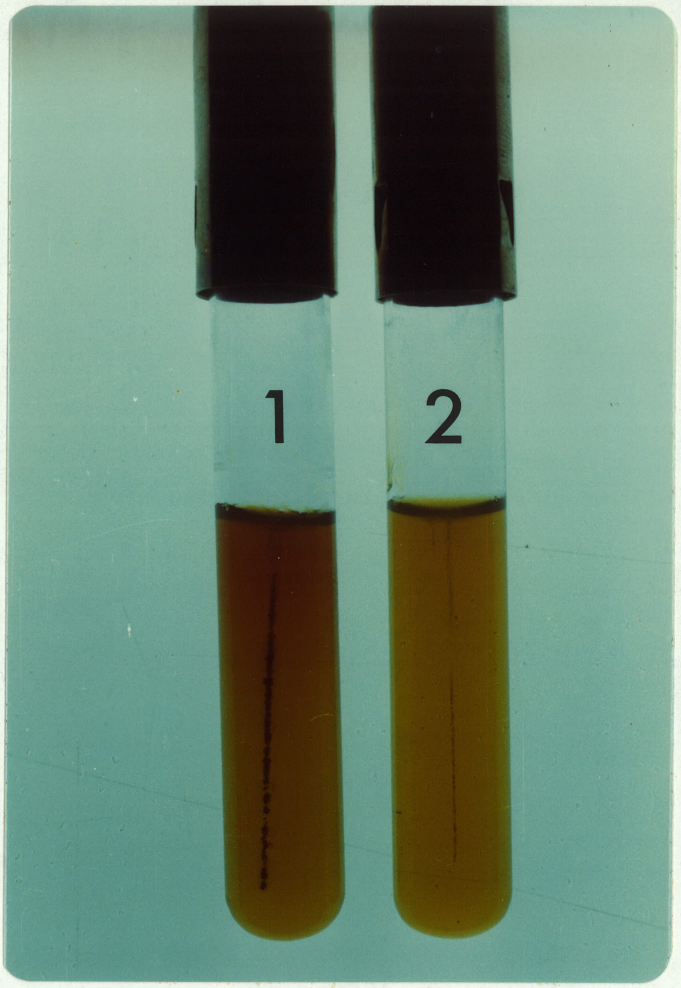

FIGURE 5: 72-hour culture of stab agar containing 0.1% rutin in solution. Transmitted light.

(1) Isolate
(2) Strep. salivarius ATCC 25975.

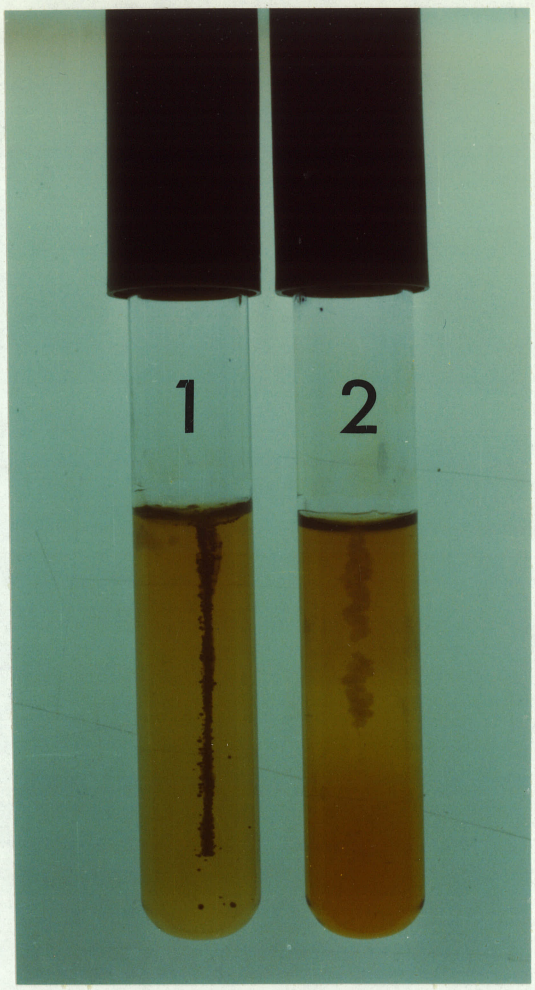

FIGURE 6: 72-hour culture of stab TYC agar containing 0.1% rutin in solution. Transmitted light.

(1) Isolate
(2) Strep. salivarius ATCC 25975.

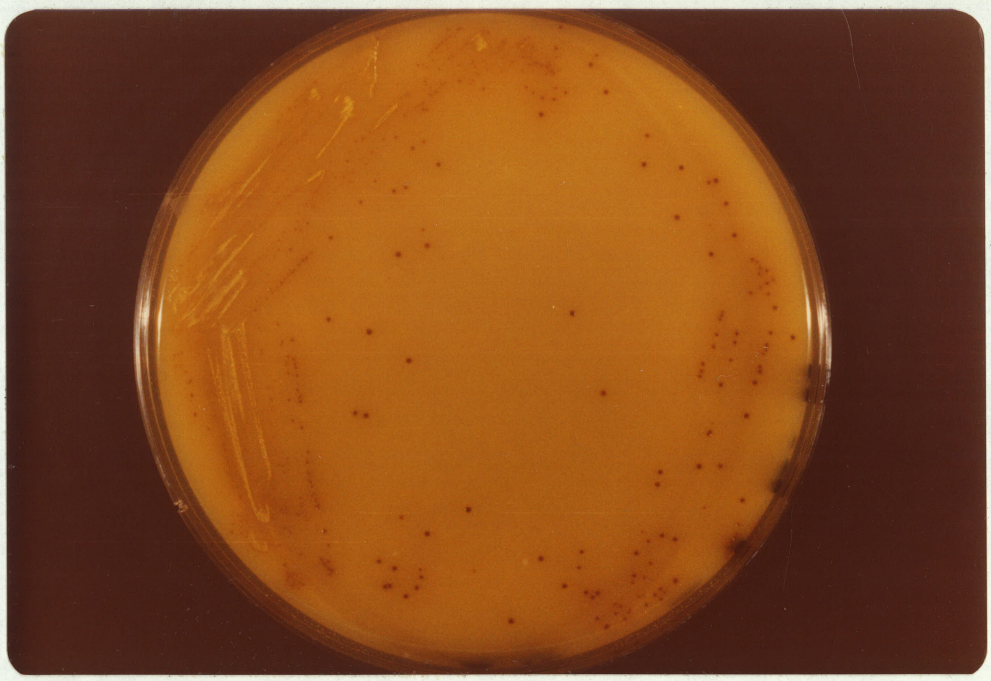

FIGURE 7: First anaerobic 48-hour subculture of the isolate in tryptone-yeast extract-phosphate agar containing 0.4% rutin in suspension. Reflected light.

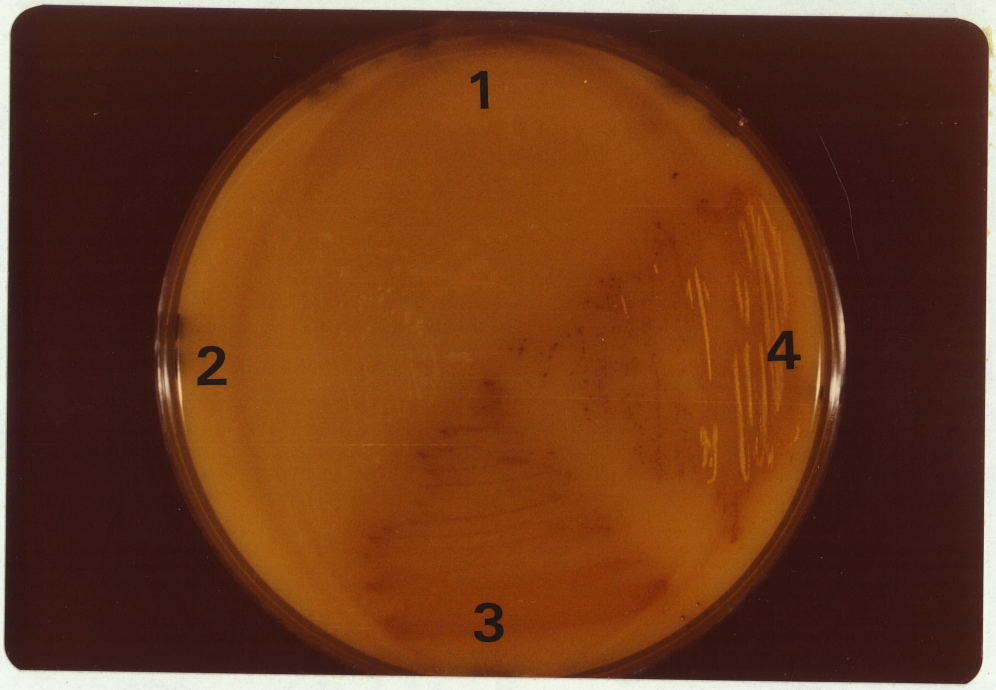

FIGURE 8: Anaerobic 48-hour culture in tryptone-yeast extract-phosphate agar containing 0.4% rutin in suspension. Reflected light.

(1) Blank
(2) Strep. salivarius ATCC 25975 (third subculture in this medium)
(3) Isolate (first culture in this medium) after subculturing in rutin-free media)
(4) Isolate (first subculture in this medium).

smooth, glistening, entire, moist, opaque colonies are formed. The same pattern of precipitate formation is also observed on the first subculture on this medium (Fig. 9). The ability to produce yellow granules on rutin agar was lost after a single passage of the rutin-subcultured isolates through a rutin-free medium e.g. blood agar (Figs 8 and 9). On aerobic incubation, basically similar colonies are formed on the corresponding media but yellow granules are not observed even after repeated subcultures.

The bright lemon-yellow precipitate, formed by the isolates in the liquid and solid media containing rutin was shown to be identical with quercetin by thin-layer chromatography and spectrophotometry (Figs 10, 11 and 12).

It is therefore concluded that the isolates are capable of hydrolysing rutin to its aglycone quercetin.

Classification of the Isolates

The isolates fermented salicin, rhamnose, sucrose and lactose, but did not ferment mannitol, sorbitol, raffinose, trehalose and inulin. They hydrolysed aesculin and arginine, and grew on MC-agar and weakly on MSB-agar. They produced neither H_2O_2 nor acetoin from glucose, did not grow in 4% NaCl, on 10% bile-blood agar nor at 45^0C. They were α-hemolytic (Figs 13 and 14) and formed soft colonies on TYC (Fig. 15). These results are consistent with those described for Strep. milleri (Hardie and Bowden, 1976) although the isolates did not produce acetoin (Table 1).

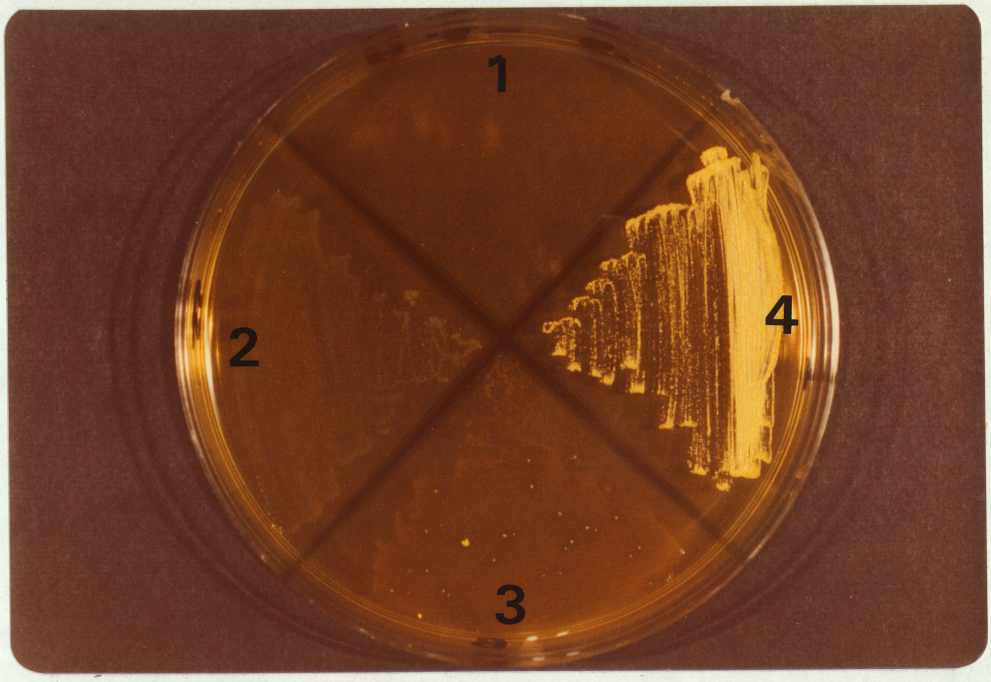

FIGURE 9: Anaerobic 48-hour culture in tryptone-yeast extract-phosphate agar containing 0.1% rutin in solution. Reflected light.

(1) Blank
(2) Strep. salivarius ATCC 25975 (third subculture in this medium)
(3) Isolate (first culture in this medium after subculturing in rutin-free media)
(4) Isolate (first subculture in this medium).

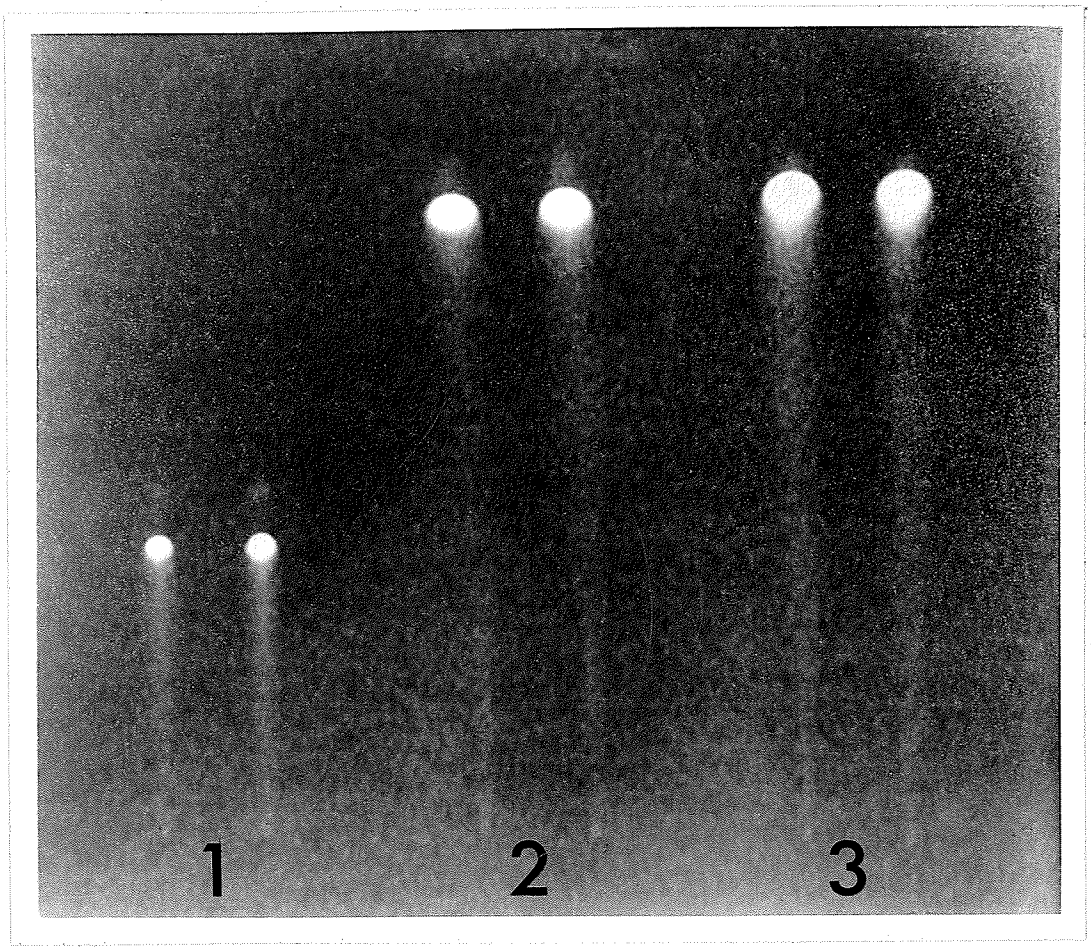

FIGURE 10: Picture of the thin-layer chromatogram developed with chloroform: methanol:water (65: 24:4 v/v).

(1) Rutin
(2) Insoluble product from rutin degradation by the isolates
(3) Quercetin.

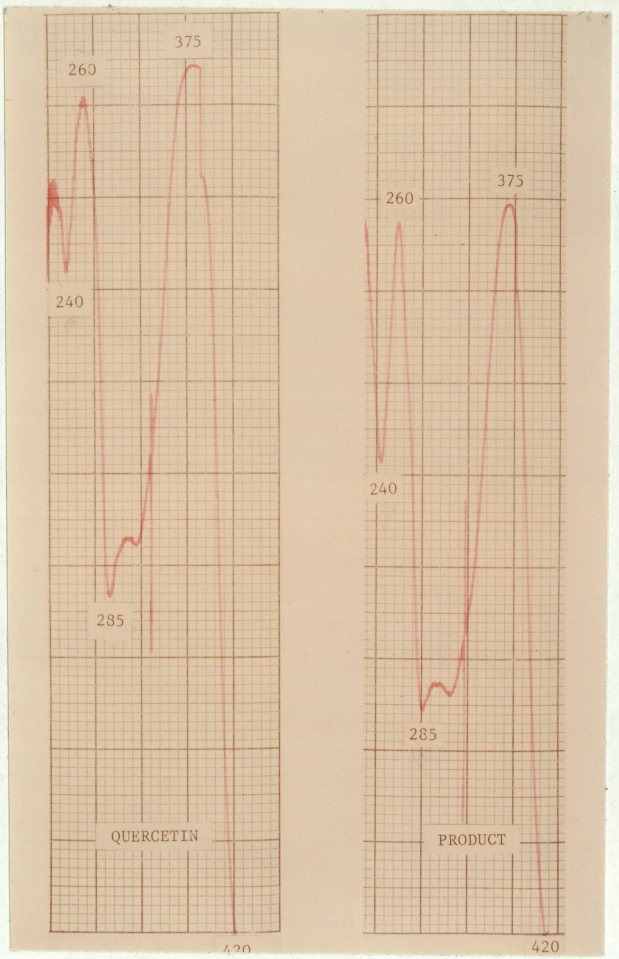

FIGURE 11: Absorption spectra of the insoluble product from rutin degradation by the isolates and quercetin in isopropanol.

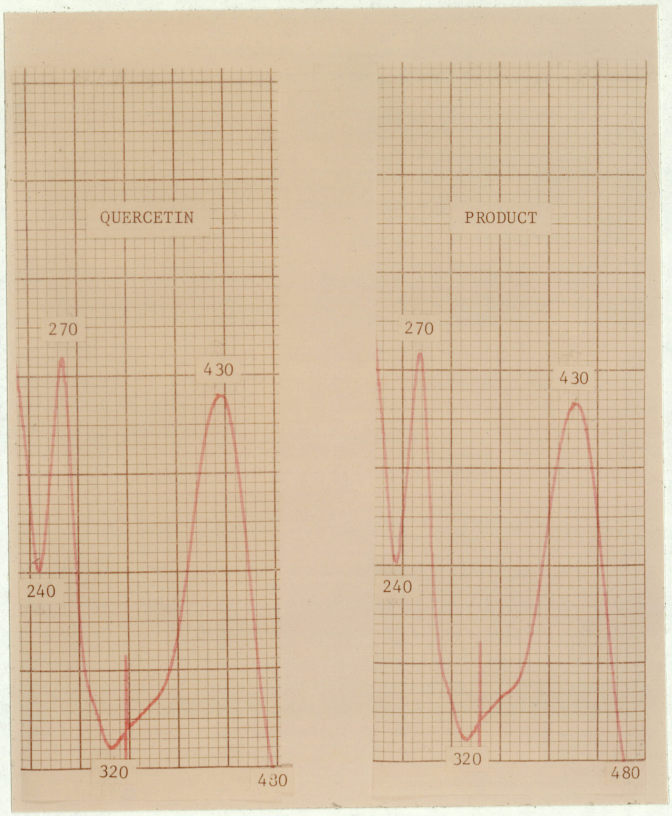

FIGURE 12: Absorption spectra of the insoluble product from rutin degradation by the isolates and quercetin in isopropanol in the presence of $AlCl_3$.

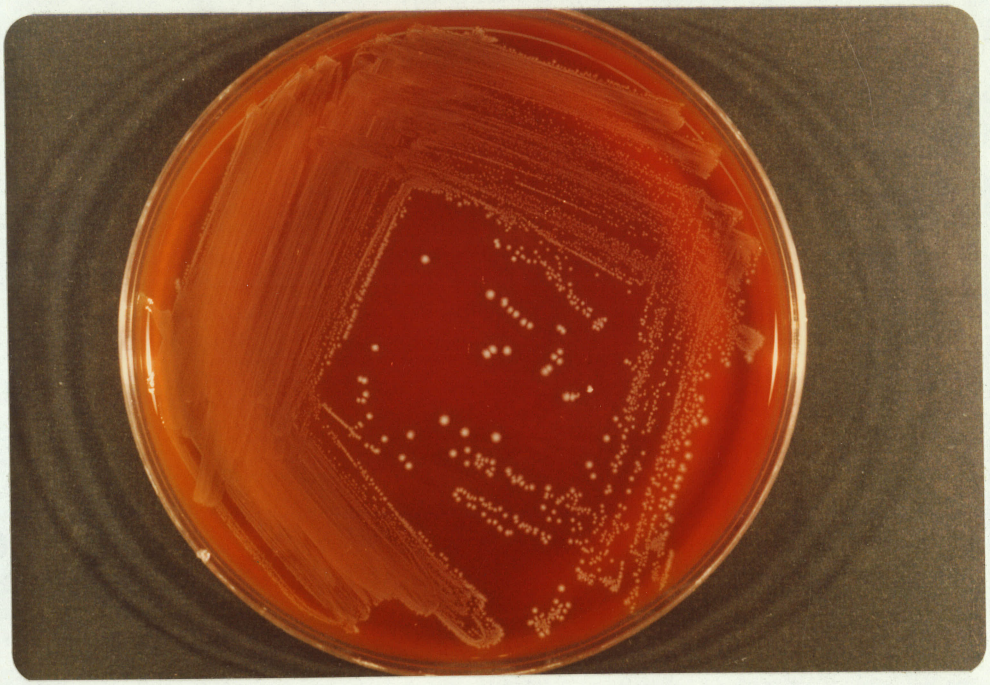

FIGURE 13: Anaerobic 48-hour culture of the isolate on blood agar. Reflected light.

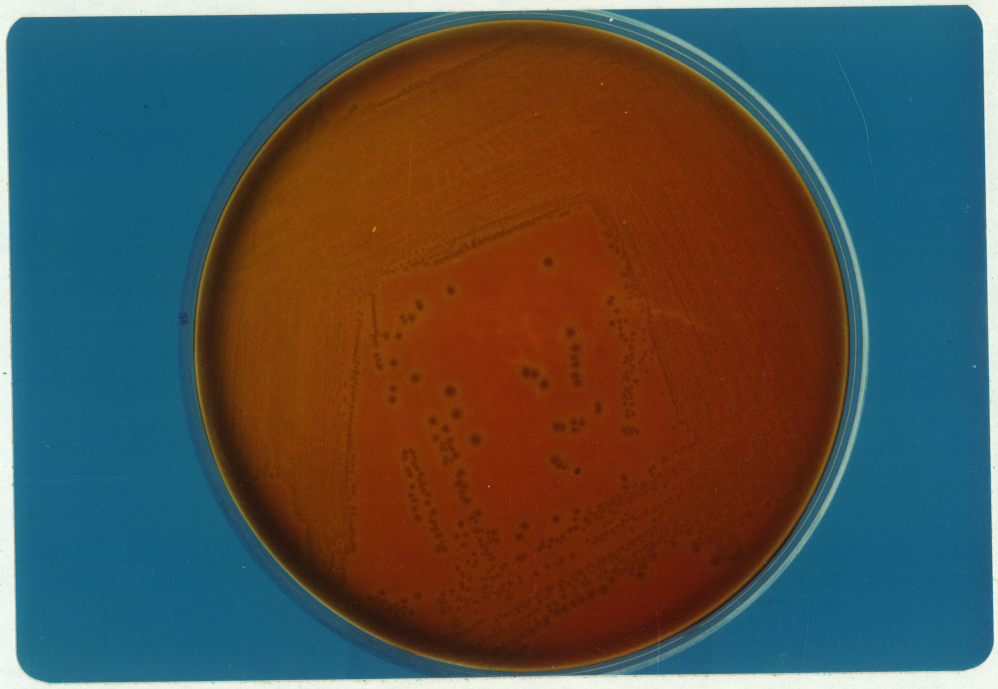

FIGURE 14: Anaerobic 48-hour culture of the isolate on blood agar. Transmitted light.

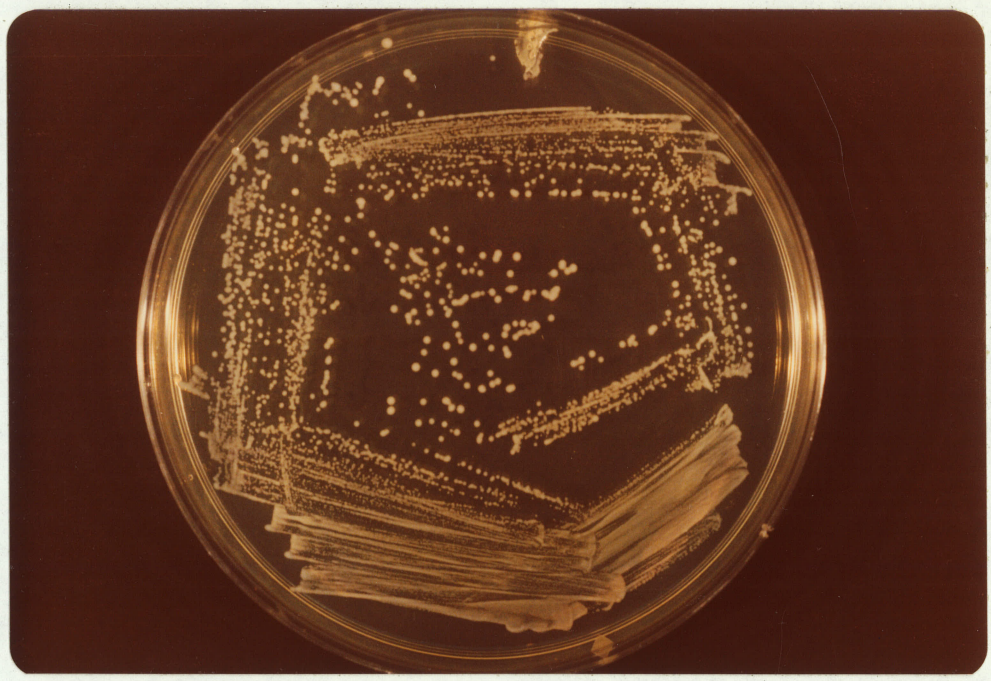

FIGURE 15: Anaerobic 72-hour culture of the isolate on TYC agar. Reflected light.

TABLE 1

Comparison between characteristics of the isolates[1] assigned to the genus Streptococcus and the "typical" Strept. milleri[2]

Test	S. milleri	Isolates
Fermentation of		
Mannitol	-	-
Sorbitol	-	-
Melibiose	-	-
Glycerol	-	-
Inulin	-	-
Sucrose	+	+
Lactose	+	+
Rhamnose	+	+
Raffinose	+	-
Trehalose	+	-
Salicin	+	+
Hydrolysis of		
Aesculene	+	+
Arginine	+	+
Production of		
Acetoin	+	-
H_2O_2	-	-
Growth at 45°	-	-
Growth in 4% NaCl	-	-
Growth on		
10% Bile-Blood Agar	±	-
MC-Agar	+	+
MSB-Agar	±	weakly
TYC Colony	soft	soft
Hemolysis	α	α

(1). Gram positive, catalase negative, facultative cocci, forming long chains in broth cultures.

(2). Mejare and Edwardsson, 1975; Hardie and Bowden, 1976.

Enzyme Studies

Since the Ames test was employed as a bioassay, enzymatic activity in this study is expressed as mutagenic activity, i.e. number of visible revertant colonies/plate. Tables 2 and 3 show that non-mutagenic rutin is converted to a mutagen by cell-free extracts (CFE_{10}) prepared from the isolate grown in either the presence or absence of rutin, as judged by the high numbers of revertant colonies formed in those plates where both rutin and cell-free extracts were incubated (Figs 16 and 17). Cell-free extracts alone had no activity (Table 2 and Fig. 16), although some CFE_{10} preparations from the isolate grown in the presence of rutin displayed a minimal mutagenic activity (Table 3 and Fig. 17), probably because of slight contamination of these preparations with quercetin produced in these cultures. No mutagenic activity was detected when rutin was incubated with heated preparations. In Tables 2 and 3 the first column gives the spontaneous mutation rate of the tester strain and the second column is the positive control with quercetin, a measure of the mutability of the tester strains.

As shown in Fig. 18, mutagenic activity is directly related to the protein concentration in the cell-free extracts from the isolate grown in the presence or absence of rutin. It is also evident that the preparation from the isolate grown in the presence of rutin demonstrates higher activity. Mutagenic activity is increased with increasing levels of rutin (Fig. 19). The activity, at higher concentrations of rutin with heated CFE_{10} is thought to be due to traces of

TABLE 2

Activity of cell-free extracts (CFE_{10}) from the isolate grown in a medium without rutin

Expt. #	Buffer	Quercetin & Buffer	Rutin & Buffer	Rutin & CFE_{10}	Rutin & Heated CFE_{10}	CFE_{10}	Heated CFE_{10}
1	28±5	362±6	30±1	361±42	58±12	37±1	27
2	32±2	297±27	61	223±18	36	-	-
3	30±4	235±13	32±9	151±2	36±4	24	31

Activity is expressed as number of revertant colonies of Salmonella typhimurium TA98 per plate. 0.1 ml of an overnight nutrient broth culture of the salmonella was mixed with the other components, as indicated, in 2 ml top agar and the mixture was poured onto the plates. Plates were incubated for 48 hr at 37°C. The buffer was 50 mM potassium phosphate, pH 7.4, and 0.5 ml was added to the plates. The CFE_{10} was prepared in this buffer at a concentration of 2 mg protein/0.5 ml. Both quercetin and rutin were dissolved in dimethylsulfoxide and 0.1 ml added to appropriate plates, at a concentration of 20 µg and 200 µg respectively. Heated preparations were obtained by heating at 80°C for 10 min. Results are mean ±SD (n=3) as indicated, remainder are mean of two plates.

TABLE 3

Activity of cell-free extracts (CFE_{10}) from the isolate grown in a medium with rutin

Expt. #	Buffer	Quercetin & Buffer	Rutin & Buffer	Rutin & CFE_{10}	Rutin & Heated CFE_{10}	CFE_{10}	Heated CFE_{10}
1	28±5	362±6	30±1	568±60	104±24	107±49	88±10
2	36±2	218±24	26±2	213±10	29	28±1	30
3	25±0	192±50	23±2	227±20	36±6	51±10	33±3
4	31±5	222±23	34±7	343±34	33	29±4	29
5	50±3	517±78	61±6	736±78	177	112	-

As in Table 2.

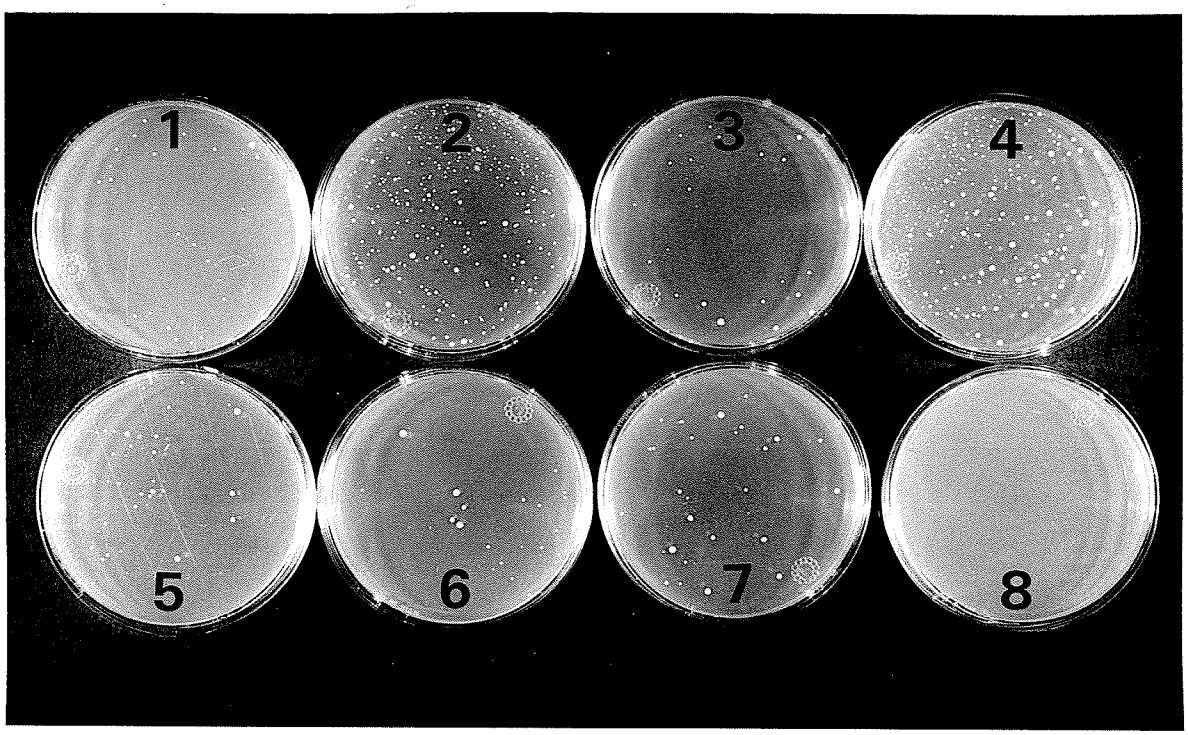

FIGURE 16: Photograph of a complete set of petri plates used in the standard Ames test in order to assess the activation of rutin to a mutagen by the cell-free extracts (CFE_{10}) derived from the isolate grown in the absence of rutin. Salmonella typhimurium tester strain TA98 was used. For more details see Table 2 or text.

(1) Salmonella & Buffer (spontaneous mutation)
(2) Salmonella & Buffer & Quercetin (induced mutation-positive control)
(3) Salmonella & Buffer & Rutin (negative control)
(4) Salmonella & CFE_{10} & Rutin
(5) Salmonella & Heated CFE_{10} & Rutin
(6) Salmonella & CFE_{10}
(7) Salmonella & Heated CFE_{10}
(8) CFE_{10} (sterility control).

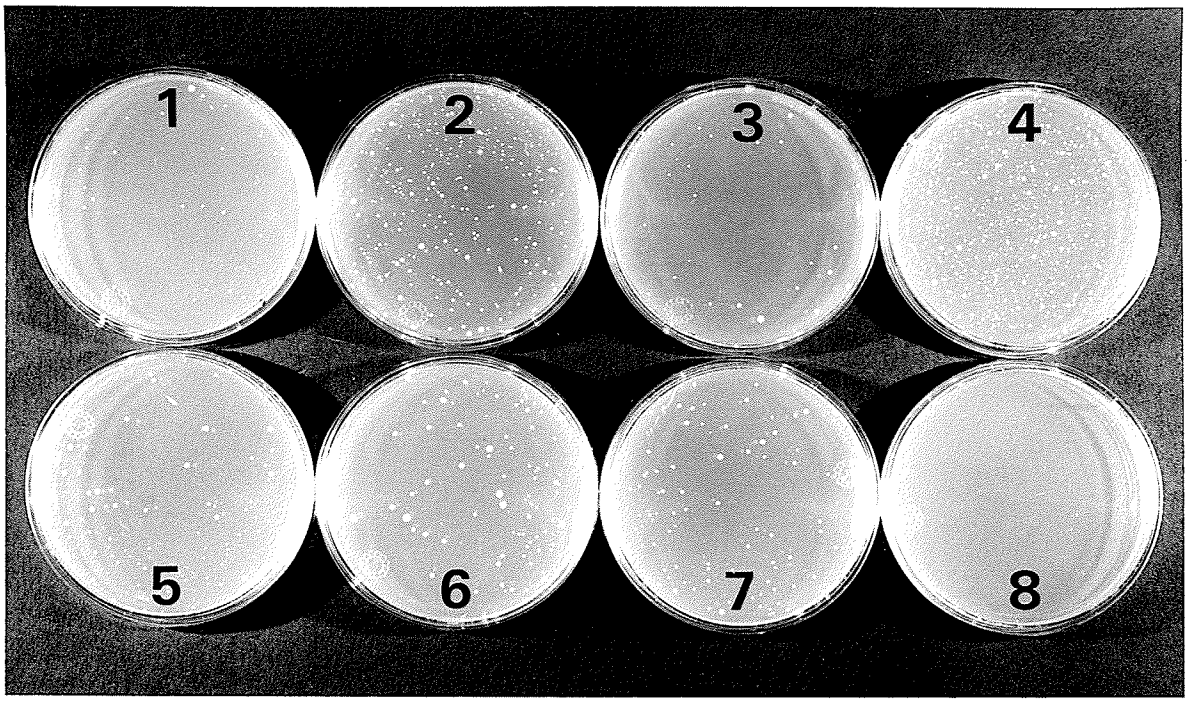

FIGURE 17: Photograph of a complete set of petri plates used in the standard Ames test in order to assess the activation of rutin to a mutagen by the cell-free extracts (CFE_{10}) derived from the isolate grown in the presence of rutin. Salmonella typhimurium tester strain TA98 was used. For more details see Table 3 or text.

(1) Salmonella & Buffer (spontaneous mutation)
(2) Salmonella & Buffer & Quercetin (induced mutation-positive control)
(3) Salmonella & Buffer & Rutin (negative control)
(4) Salmonella & CFE_{10} & Rutin
(5) Salmonella & Heated CFE_{10} & Rutin
(6) Salmonella & CFE_{10}
(7) Salmonella & Heated CFE_{10}
(8) CFE_{10} (sterility control).

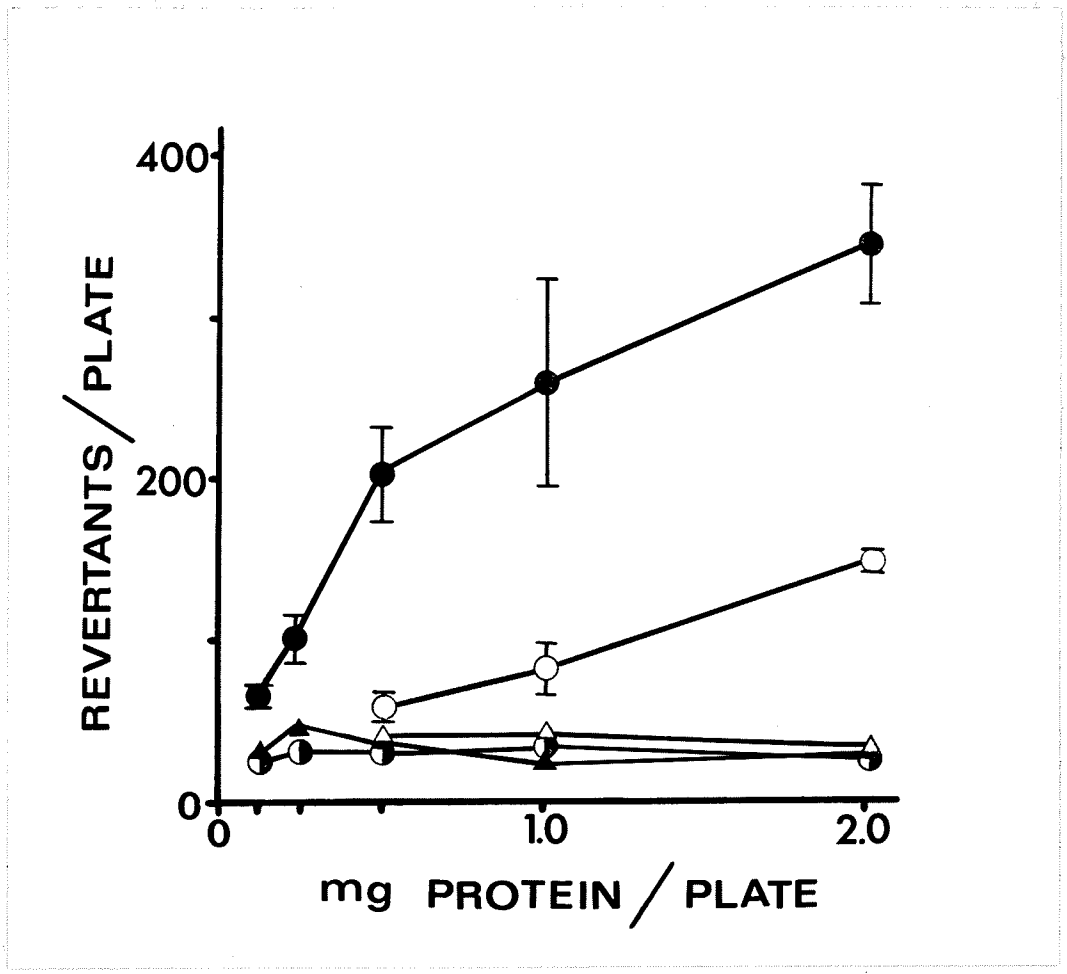

FIGURE 18: Enzyme activity as a function of protein concentration. Different concentrations of cell-free extracts (CFE_{10}) were tested in the Ames assay in the presence of 200 µg rutin in 0.1 ml DMSO/plate except where indicated. The CFE_{10} were derived from the isolate grown in the presence of rutin - either fresh (●) or heated (▲), and from the isolate grown in the absence of rutin - either fresh (○) or heated (△). (◑) indicates CFE_{10} as in (●) except rutin was absent from the test plates. Enzyme activity is expressed as numbers of revertant colonies/plate and results are mean of three plates ±SD, as indicated, remainder are mean of two plates.

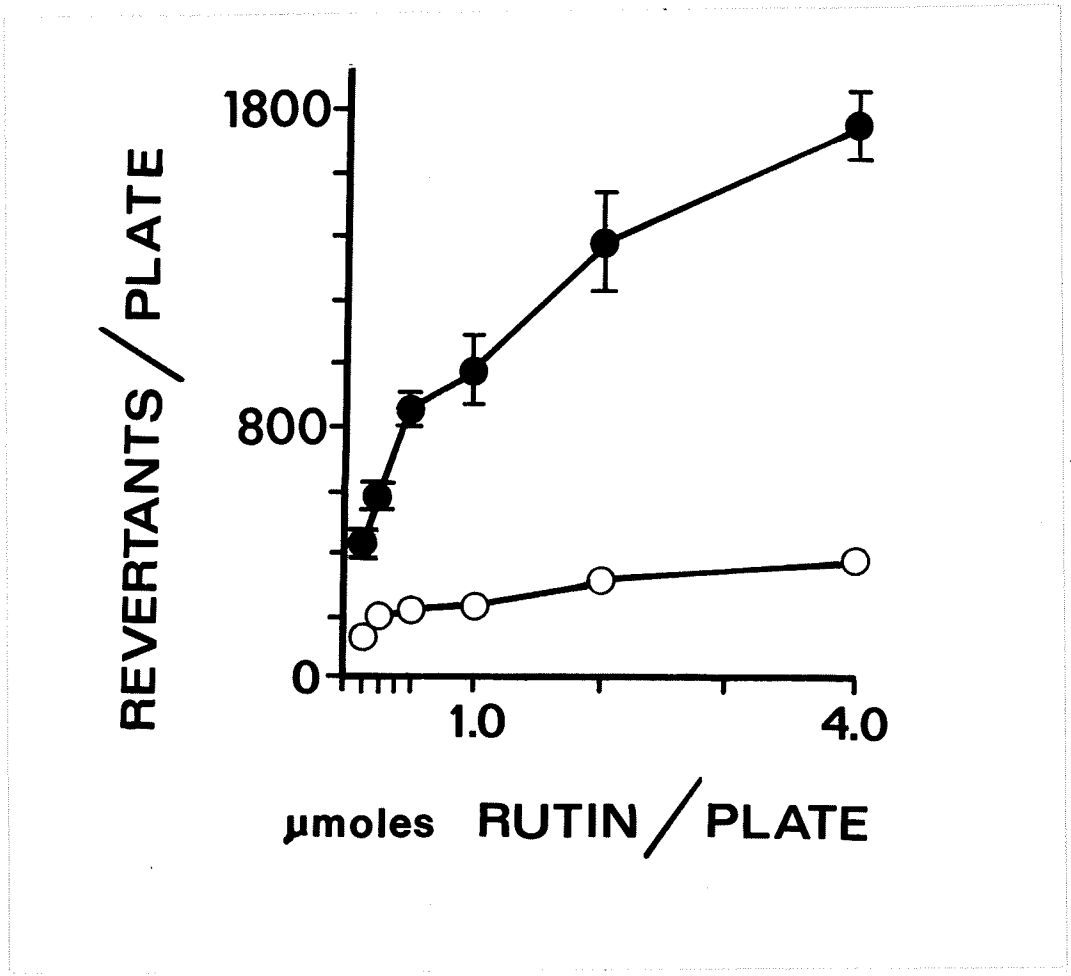

FIGURE 19: Enzyme activity as a function of rutin concentration. Different concentrations of rutin were assayed in the Ames test in the presence of cell-free extract (CFE_{10}, 2 mg protein/plate) derived from the isolate grown in the presence of rutin - either fresh (●) or heated (○). Enzyme activity as in Fig. 18.

quercetin present as impurity in the commercial preparation of rutin (Donahue, McCann and Ames, 1978). These results show that an enzyme system capable of hydrolysing rutin to its mutagenic aglycon quercetin is present in cell-free extracts prepared from the isolate grown either in the presence or in the absence of rutin.

When the 10,000 g supernatant of cell homogenates (CFE_{10}), derived from the isolate grown in the presence of rutin, was subjected to further high-speed centrifugation, the entire activity was recovered in the CFE_{100} supernatant (Table 4). The culture filtrate was inactive (Table 5).

The pH optimum was found to be around 6.5 (Fig. 20). At this pH a bright yellow precipitate was evident, which, when examined by thin-layer chromatography and spectrophotometry, proved to be quercetin. No precipitate was formed when heated preparation was incubated at this pH. Exposure of the enzyme preparation to inactivating pH 4.5 for 3 min did not cause any permanent loss of activity as the preparation showed similar activity to that expressed by the preparation not exposed to a pH lower than 6.8 (Table 6).

Isolates and Quercitrin

No changes were observed in the quercitrin media, i.e. color and appearance changes in the quercitrin sediment or precipitate formation. This is in agreement with the results of the mutagenicity assay. Thus, Table 7 shows that quercitrin, the 3-L-rhamnoside of quercetin, in contrast to rutin, was not activated to a mutagen in the presence of cell-free extracts (CFE_{10}) derived from the isolate grown

TABLE 4

Activity of the 100,000 g supernatant (CFE_{100}) of cell-free extracts from the isolate grown in a medium with rutin

Expt. #	Buffer	Quercetin & Buffer	Rutin & Buffer	Rutin & CFE_{100}	Rutin & Heated CFE_{100}	CFE_{100}	Heated CFE_{100}
1	49±7	334±49	53±5	433±59	59±1	60±1	66±6
2	38±7	358±49	55±11	641±73	70	43±1	50

As in Table 2, 0.5 ml CFE_{100}/plate is equivalent to 2 mg protein of 0.5 ml CFE_{10}.

TABLE 5

Activity of culture filtrate (CF) from a culture of the isolate grown in the presence of rutin

Buffer	Quercetin & Buffer	Rutin & Buffer	Rutin & CF	Rutin & Heated CF	CF	Heated CF
48±9	307±4	72±6	96±13	86±6	57±2	64±9

As in Table 2, 0.5 ml CF/plate with undetermined protein concentration.

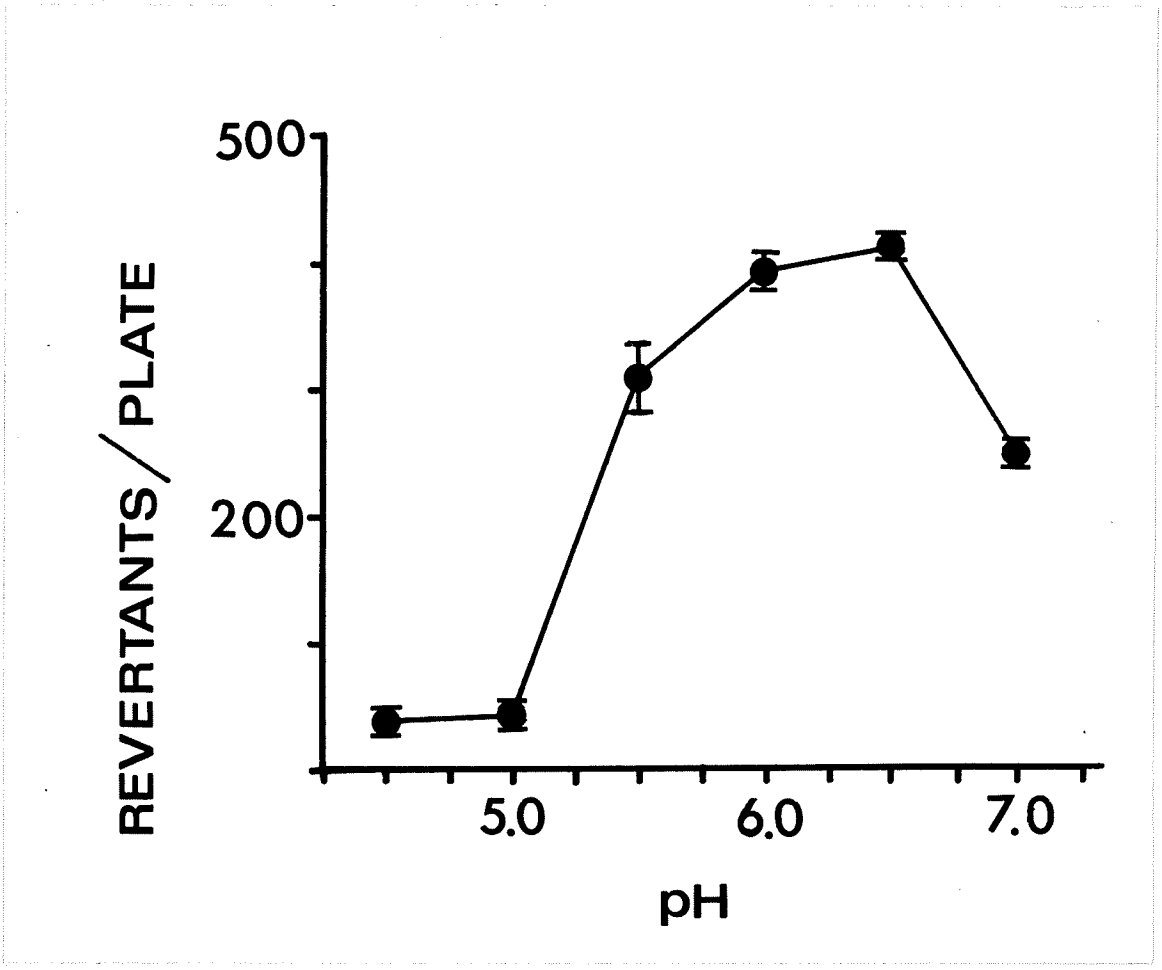

FIGURE 20: Effect of pH on enzyme activity. Cell-free extract (CFE_{25}, 0.45 mg protein) from the isolate grown in the presence of rutin, were incubated with rutin (0.5 µmole) in 0.5 ml citrate - phosphate buffer of indicated pH for 1.5 h at 37°C. Reaction mixtures were extracted with ethyl ether, reduced to dryness, redissolved in 0.5 ml DMSO and assayed in the Ames test (0.1 ml/plate). Enzyme activity as in Fig. 18.

TABLE 6

Effect of low pH on the enzyme activity of the cell-free extract (CFE_{25}) from the isolate grown in a medium with rutin

No Chemical	Quercetin (20 µg)	Sample[1] (pH 4.5)	Sample[2] (pH adjusted from 4.5 to 6.8)	Sample[3] (pH 6.8)
51±5	367±13	68±7	189±9	193±19

Activity as in Table 2.

(1). CFE_{25}, 0.225 mg protein and 0.5 µmole rutin in 0.5 ml citrate-phosphate buffer, pH 4.5 were incubated for 2.5 hr at 37°C; reaction mixture was extracted as described in the text; the residue was dissolved in 0.5 ml DMSO; 0.1 ml of this solution was added in the test plates.

(2). CFE_{25} was left in the same buffer, pH 4.5 for 3 min; pH was then adjusted to 6.8 by adding 0.2 M Na_2HPO_4; final volume, composition, treatment and testing as in (1).

(3). As in (1) except pH was 6.8 and CFE_{25} was never exposed below the pH indicated.

TABLE 7

Activation of different quercetin glycosides by a cell-free extract (CFE_{10}) from the isolate grown in a medium with rutin

Compound	Buffer	CFE_{10}	Heated CFE_{10}
None	43±2	145	151
Quercetin	337±13	-	-
Rutin	63±3	541±128	184
Quercitrin	82±2	154±15	178

As in Table 2, rutin and quercitrin at a concentration of 0.5 μmole/plate.

in the presence of rutin. Quercitrin itself showed a minimal mutagenic activity probably due to traces of quercetin present as impurity. The enzyme preparation also showed a residual mutagenic activity believed to be due to the contamination by quercetin produced in the culture.

Type Culture Collection Strains of Oral Streptococci in Rutin Media

On culturing and subculturing Strep. salivarius ATCC 25975 and Strep. mutans 6715-10 in media containing rutin, under varying conditions, no changes were observed in the media (i.e. quercetin formation and/or disappearance of rutin sediment in broth; formation of quercetin granules and/or clearing of the medium on agar plates, stab agar and TYC) (Figs 4, 5, 6, 8, 9, 21, 22 and 23). On the agar containing rutin in suspension, these strains form small, circular, effuse (umbonate for S. Salivarius), smooth, glistening, entire, moist, opaque colonies both aerobically and anaerobically (Fig. 8). On the agar containing rutin in solution, small, circular, effuse, entire, moist, opaque colonies are formed both aerobically and anaerobically (Fig. 9). It was then concluded that these strains are not capable of degrading rutin to any appreciable extent.

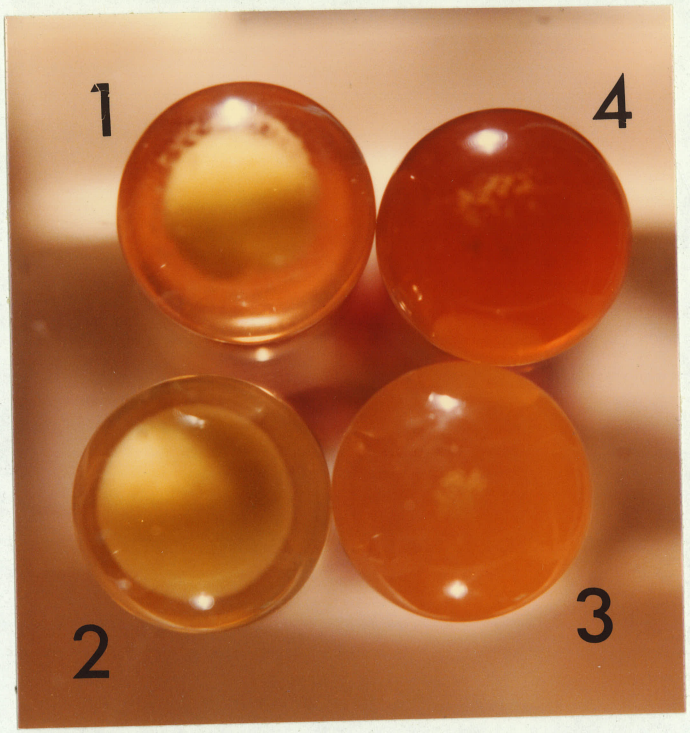

FIGURE 21: Anaerobic 48-hour culture of Strep. salivarius ATCC 25975 in rutin broth. View and composition of the medium as in Fig. 2.

(1) rutin in suspension - no glucose
(2) rutin in suspension - 0.2% glucose
(3) rutin in solution - 0.2% glucose
(4) rutin in solution - no glucose.

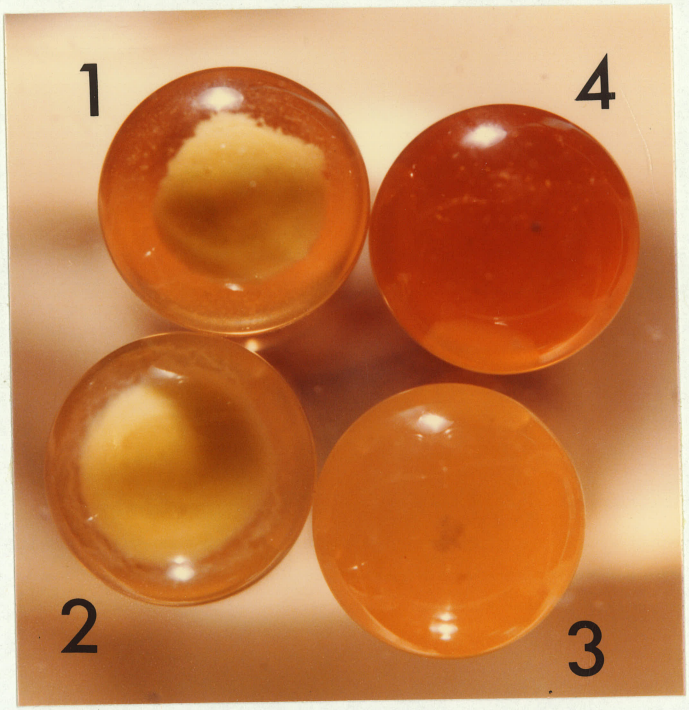

FIGURE 22: Anaerobic 48-hour culture of Strep. mutans 6715-10 in rutin broth. View and composition of the medium as in Fig. 2.

(1) rutin in suspension - no glucose
(2) rutin in suspension - 0.2% glucose
(3) rutin in solution - 0.2% glucose
(4) rutin in solution - no glucose.

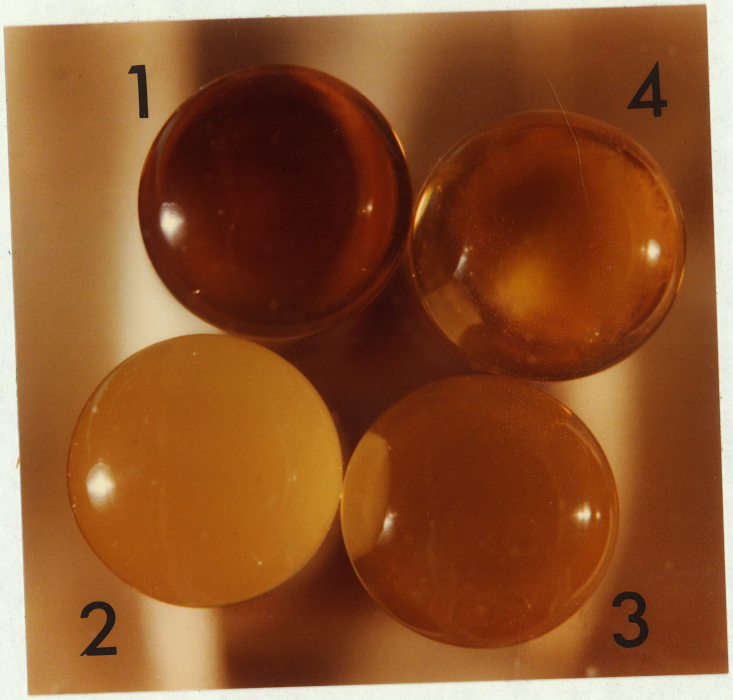

FIGURE 23: Anaerobic 48-hour culture in broth containing 0.05% rutin in solution. Composition of the medium as described in the text (Preparation of Media) except no buffer was present and 0.2% glucose was added. View as in Fig. 2.

(1) Blank
(2) Strep. salivarius ATCC 25975 (final pH 4.0)
(3) Strep. mutans 6715-10 (final pH 4.5)
(4) Isolate (final pH 5.0).

DISCUSSION AND RECOMMENDATIONS

This is the first report showing that human oral streptococcal strains can hydrolyse the flavonoid glycoside rutin to quercetin *in vitro*, a reaction which is not commonly performed by microbes and has never been reported in mammalian enzyme systems (adult animal) such as intestinal wall and intestinal secretions (Westlake *et al.*, 1959; Cheng *et al.*, 1969; Griffiths and Barrow, 1972; Kuhnau, 1976).

The isolation of oral bacteria capable of degrading rutin was not surprising in view of its very nature, i.e. a natural product and hence biodegradable, its potential nutritive value (glycoside) and its ubiquity in foodstuffs and beverages (oral flora has always been challenged by this substrate) (Kuhnau, 1976; Brown, 1980).

The streptococcal isolates were classified as *Strep. milleri* (Table 1). This does not imply, of course, that all *Strep. milleri* strains will hydrolyse rutin nor that other members of the oral microflora will not. Strain differences, species similaries and the difficulties in rigidly classifying the viridans streptococci cannot be overemphasized (Lamanna and Mallette, 1965; Guggenheim, 1968; Colman and Williams, 1972; Hardie and Marsh, 1978).

The ability to degrade rutin is not universal in the microbial world and this was well illustrated in the study by Cheng *et al.* (1969) where only three out of fifty-three strains of rumen bacteria from a laboratory collection representing 12 genera, were found positive in degrading rutin anaerobically. The present study also exem-

plifies this fact when two randomly picked laboratory strains of oral streptococci (Strep. salivarius 25975 and Strep. mutans 6715-10) were found unable to degrade rutin (Figs 21 and 22). Noteworthy is the fact that repeated subculture of these laboratory strains in rutin media failed to select mutants able to degrade rutin (Figs 8 and 9).

Although the ability to hydrolyse rutin seems to be an inherent attribute of the isolates, the overall effects of mutagenic quercetin on the isolates themselves cannot be assessed here. In other words, the mutability of the isolates by their own metabolic product remains an intriguing question. Despite this, the results from different experiments were consistent and reproducible, although some quantitative variability was observed due to the very nature of the study and the method used, i.e. bacteriological. In fact, the inter-day variability of the Ames test even under the most rigid experimental conditions is well known (Cheli, 1980; Peak, Hass and Dornfeld, 1980).

Thus, using the Ames test, it was possible to study easily the hydrolysis of rutin as mutagenesis, based on the mutagenic properties of its aglycon quercetin (Figs 16 and 17). The results (Tables 2 and 3, Figs 18 and 19) clearly show that cell-free extracts prepared from one of the isolates, grown anaerobically, were capable of activating rutin in a dose-related manner to the mutagenic quercetin. The activity was due to a glycosidic enzyme system in the extract as the heated preparations were found inactive.

Rutin (L-rhamnosyl-α 1,6-D-glucosyl-β 1,3-quercetin) but not quercitrin (L-rhamnosyl-α 1,3-quercetin), was hydrolysed by the iso-

lates (Table 7) which indicates that this glycosidase is a β-D-glycosidic enzyme (not an α-rhamnosidase/β-glucosidase system) which has a high affinity for the β-D-glucosyl bond to quercetin. It might be reasonable to expect that all quercetin glycosides wherein glucose is directly linked to quercetin would be susceptible to hydrolysis by the isolates (Harborne, 1964). Whether this glycosidase is specific for the quercetin aglycon is not known. It should be noted that such rutinolytic enzymes differ from the most common β-glucodisase which hydrolyses only terminal glucose units, by their ability to hydrolyse also in-the-chain-glucose, as in rutin (Mabry, Markham and Thomas, 1970). Therefore, this streptococcal rutinolytic enzyme will be called rutinase, by analogy of the glycosidase synthesized by Aspergillus flavus, which hydrolyses rutin to quercetin and the disaccharide rutinose (Hay, Westlake and Simpson, 1961).

The results show that this streptococcal rutinase is not an exoenzyme (Table 5) and is present in the cytosolic fraction of the preparation from the organism (Table 4). However, the possibility that it might have originally been loosely associated with the cell membrane, an association which would be disrupted by treatment with the Mickle disintegrator, cannot be excluded. The cell-bound nature of this rutinase is also consistent with its constitutivity (Eveleigh and Montenecourt, 1979). Extracellular acid hydrolysis is also ruled out at these levels of pH of the grown media (Harborne et al., 1975). The negative results with the acid producing type culture collection strains (even when grown in the absence of buffer) is consistent with

this (Fig. 23). In this respect the streptococcal rutinase resembles the glycosidase system described in the rumen bacterium Butyrivibrio sp C_3 and differs from the Asp flavus one which is an exoenzyme (Hay et al., 1961; Krishnamurty et al., 1970).

Thus, the mode of rutin hydrolysis and metabolism (e.g. uptake and fate of the sugars) by the streptococcal isolates cannot be assessed here. This is not crucial though to the present investigation which was mainly concerned with the liberation of the genotoxic, quercetin from rutin. In the study by Cheng et al. (1969), some rutinolytic strains of Selenomonas and Butyrivibrio (other than Butyrivibrio sp C_3) were also found unable to attack the heterocyclic ring of the aglycon quercetin, which was consequently accumulated as a yellow precipitate in the cultures in rutin media, although the locale of their glycosidases was not determined.

A most interesting finding was the fact that this streptococcal rutinase unlike the one synthesized by Asp. flavus seems to be constitutively synthesized by the isolates as judged by the activity in cell-free extracts derived from the isolate grown in the absence of rutin (Table 2 and Fig. 16) and the hydrolysis of rutin, in cultures of the isolate, in the presence of excess of glucose (in broth and TYC) occurring at a rate even higher to that observed when the isolate was grown in limiting glucose concentration. Data also show (Table 3 and Fig. 17) that cell-free extracts obtained from the isolate which was grown in the presence of rutin possess higher rutinase activity than the extracts from the isolate grown in the absence of rutin. The

difference in activity between these two preparations was statistically significant ($p < 0.02$) and points to the fact that rutinase can be further induced by being synthesized at levels higher than that of its basal level in the presence of its substrate rutin in the growth media. This is not unusual for many constitutive enzymes to be partly inducible as in the case of the constitutive saccharase of some E. coli strains which can show up to 30-fold increase in activity when sucrose is added to the medium (Lamanna and Mallette, 1965). The assumption made here is that we are dealing with a single enzyme system in both preparations.

Rutinase was found to be active over the greater part of the physiological range of the "oral" pH with the optimum pH of 6.5, but its activity decreased precipitously at pH below 5.5 (Fig. 20); Asp. flavus rutinase, in contrast, has its optimum pH at 5.6. Although this streptococcal rutinase is inactive at low pH (i.e. 5.0 and 4.5) the loss in activity is not permanent and any autolytically liberated enzyme in acidic dental plaque could be reactivated at higher pH (Table 6).

Even though the effect of oxygen concentration on the enzyme synthesis was not thoroughly investigated (enzyme preparations were derived from cells grown anaerobically), the failure of the isolates to produce quercetin on rutin agar when incubated aerobically suggests that rutinase is practically synthesized under reduced oxygen tensions. Examination for rutinase activity in cell-free extracts from the isolates grown in well-aerated broth cultures would be appropriate

to complete the above findings on solid media. However, once synthesized rutinase retains its activity at ambient oxygen concentration.

Rutinase seems to be devoid of any dialysable co-factor(s) as dialysed preparations were found also active in carrying out the hydrolysis of rutin (pH studies). The characteristics of the rutinolytic enzyme system of the isolate and other microbes, are listed in Table 8.

While this study has demonstrated that the isolates can hydrolyse rutin *in vitro*, it cannot be stated with certainty that they perform this function *in vivo*, granting that the interactions among microorganisms in the natural setting are very complex and unpredictable (Meers, 1974; Ellwood *et al.*, 1980). One might expect though, that they would if substrate was available (constitutive rutinase), and since many common foodstuffs and beverages as well as smokeless tobacco contain rutin, this would not normally be a problem. The extent of rutin hydrolysis would then depend upon the period of time the substrate was retained in the mouth and its local concentration, the intra-oral enzyme activity and the prevailing local conditions (poor oral hygiene factor?). More specifically, this may include such variables as the intensive and excessive consumption of materials containing rutin and its levels in these materials, its prolonged retention in the areas of concentration (gingival-buccal fold, alveolar-lingual sulcus and floor of the mouth), its availability for hydrolysis (extraction efficiency of the oral fluids, presence of

TABLE 8

Some characteristics of the rutinolytic enzyme system present in the cell-free extracts of the streptococcal isolate and other microbes

Organism	Regulation of Synthesis	Synthesized	Locale	Optimum pH	Specificity
Isolate	constitutive[1]	anaerobically	cell-bound	6.5	specific
Asp. flavus[2]	inducible	aerobically	extracellular	5.6	specific
Butyrivibrio sp. C_3[3]	-	anaerobically	intracellular	-	-[4]

(1). Partly inducible.

(2). Hay, Westlake and Simpson, 1961.

(3). Cheng et. al., 1969; Krishnamurty et al., 1970.

(4). The organism is capable of growth on the flavonoid glycosides quercitrin, naringin and hesperidin as well as on rutin. Pure enzyme preparations were not studied.

lime), the number of rutin hydrolysing bacteria, the microbial interactions and other ecological factors (Switzer, 1977; Bowden, Ellwood and Hamilton, 1979). Reduced pO_2, sugar availability and the ambient intra-oral pH would favor such a reaction, assuming a glycosidase system with characteristics similar to those described for the streptococcal rutinase. In fact, the epidemiological and laboratory findings, as discussed in the Literature Review section, are consistent with the most favourable conditions for this reaction to occur.

Based on this compilation of supportive evidence, a working hypothesis is presented that genotoxic flavonoids are involved in the cancer of the upper alimentary tract and the oral microflora participates in the carcinogenic process through liberation of the active flavonoid aglycones in situ.

However, many basic problems are still awaiting solution. The assessment of the carcinogenic potential of quercetin in oral epithelial tissues of experimental animals and the determination of rutinase and other flavonoid glycosidases levels in oral fluid and plaque homogenates ("oralase") of human subjects are most needed. Determination of the levels of quercetin (free and conjugated) in various products should then be followed if positive results for carcinogenicity are obtained. In fact, the necessity to assess conclusively the genotoxic potential of quercetin has been emphasized even by those investigators who found it negative in their bioassays (Saito et al., 1980; Sugimura, 1982) and recently the National Toxicology Program in the United States have included quercetin in the list of compounds to be

tested for their biohazard potential (Sugimura, 1982).

If quercetin proves to be definitely carcinogenic, its contribution to the genesis of human cancer would be plausible by virtue of its widespread occurrence (Tamura et al., 1980; Nagao et al., 1981). It should also be pointed out that a discovery of such a ubiquitous genotoxic material would not be unique nor shocking as other ubiquitously occurring agents such as radiation (ionizing, UV and particular) have long been known for their genotoxicity (Shellabarger, 1976; Rantanen, 1980; Rief, 1981). Their teleologic function in providing the source of variation (by a mutational action), according to the Darwinian scheme, for the evolution of species, cannot be overemphasized (Brown, 1980).

It must be pointed out that even if quercetin proves to be an oral carcinogen in animals, it should not be assumed, of course, that foodstuffs like vegetables and fruits are carcinogenic. Such thinking would be deceptively oversimplistic, not taking into account the existing data and the facts about carcinogenesis. Thus, there is the remarkable inherent resistance of the human body to environmental insults. Even the most potent known human carcinogens, such as the polycyclic aromatic hydrocarbons (e.g. the byproducts of tobacco combustion) require intensive and repeated contact before they produce tumors (Wynder and Hoffmann, 1967; Weisburger, Cohen and Wynder, 1977; Gluckman et al., 1980). Furthermore, the multifactorial nature of the carcinogenic process requires the participation of a diverse multitude of factors besides that of the carcinogen (initiator) such as promo-

tors and other cocarcinogens and modulators as well as a susceptible host (Sivak, 1979; Wattenberg, 1979; Miller et al., 1979; Slaga, 1980; Schmahl, 1980; Lynch, Lynch and Guirgis, 1980; Higginson, 1980). Thus, quercetin may be carcinogenic or cocarcinogenic (Saffiotti and Shubik, 1956; Sugimura, 1979; Vose, Coombs and Bhatt, 1981) only under certain circumstances related to its local concentration (dose), time and mode of exposure, the presence of other extraneous factors (chemicals, radiation and viruses) and the internal environment (susceptible epithelium and host response).

A more general question is addressed here on the possible role of the oral microflora in intra-oral carcinogenesis, through various mechanisms. How poor oral hygiene is related to oral cancer? Is the connecting string of the candida-speckled leukoplakia-malignancy triad an etiologic one (Banoczy, 1977; Mehta et al., 1981)? Could also tobacco (chewing, dipping and smoking) affect oral microbial metabolism of genotoxic and promoting agents, through its well known effects on the oral microbial ecology (e.g. increase in the rate of plaque accumulation and the incidence of periodontal disease) (Waerhaug, 1967; Colman et al., 1976; Arendorf and Walker, 1980; Bergstrom, 1981; Lindemeyer et al., 1981)? In fact, such questions have not received the experimental attention commensurate with their potential importance in the carcinogenic process, as presented by the epidemiological studies.

Clearly this is an area which deserves a respectable amount of exploration. I think the conditions are now propitious for the eluci-

dation of the biochemical-biological determinants of "poor oral hygiene" as related to intra-oral carcinogenesis (Smith, 1979; Preston-Martin et al., 1982). This interest is further timely in view of the recent advances in research related to microbes in cancer etiology in other parts of the body (Hicks et al., 1977; Hill, 1980; Murray et al., 1980; Goldin, 1980; Backus and Affronti, 1981; Cantwell and Kelso, 1981). Thus, hypotheses and methodologies are now available to the oral biologists for the investigation of the production, activation, modification or degradation of genotoxic agents and promotors of carcinogenesis by the oral microflora (Mandel et al., 1977; McCoy et al., 1977; Balish et al., 1977; McCoy et al., 1979; Rosenkranz, Karpinsky and McCoy, 1980; Wilkins et al., 1981; Goldin et al., 1981; Goldman, 1981; Wu and Wong, 1981; Woodruff et al., 1982). The thought of the involvement of the oral flora in a disease process other than that of caries and periodontal, namely intra-oral cancer, is truly intriguing. Activation of flavonoid glycosides could be but one example of its participation in the local carcinogenic process.

SUMMARY

Oral streptococci, isolated from the mouths of two healthy subjects, were found capable of hydrolysing innocuous rutin, a flavonoid glycoside, to its genotoxic aglycon quercetin, *in vitro*. The isolates were identified as strains of *Strep. milleri*. The glycosidase, rutinase, was studied in cell-free extracts derived from one of the isolates, grown anaerobically in batch cultures by the use of a bioassay, the Ames test, in which S-9 was replaced by the cell-free extracts. This streptococcal rutinase showed the following characteristics: (1) constitutive, (2) partly inducible, (3) cell-bound, (4) preference for rutin, and (5) most active at pH 6.5. Type culture collection strains of other oral streptococci (*Strep. salivarius* ATCC #25975 and *Strep. mutans* strain 6715-10) showed no capacity for rutin degradation. A hypothesis for a novel role of the oral microflora in a disease process other than that of caries and periodontal, namely intra-oral cancer, is presented. The possibility of a bacterial liberation of the genotoxic quercetin *in situ* could be but one example of its involvement in the local carcinogenic process.

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