EFFECT OF DENTAL PLAQUE EXTRACTS ON MAMMALIAN CELLS IN CULTURE

An *in vitro* investigation into chemical factors from dental plaque which may be responsible for chronic gingivitis in man

by

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DISCUSSION

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To grow long in the tooth is another way of saying that a person is getting older, and many people believe that recession of gums from the teeth (periodontal disease) is an exclusively age dependent process. Although this is partly true, it appears that bacterial accumulations on teeth (dental plaque), and not age, may be the principal aetiological factor. I am grateful to Professor G.C. Cowley, formerly Senior Lecturer in Oral Medicine at the Glasgow Dental Hospital and School, and now Professor of Preventive Dentistry at the University of Dundee, for indicating how important a role he believed dental plaque to play in the periodontal disease process. That belief was the inspiration of this thesis.

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SUMMARY

Chronic gingivitis is generally believed to result from irritation to cells at the gingival junction. This is brought about by the accumulation of micro-organisms on teeth. These micro-organisms and their products are known as dental or bacterial plaque and constitute a bacterial ecosystem whose appearance and consistency differentiate it from other kinds of deposits on teeth. The dental plaque contains a variety of substances which are potentially pathogenic. Presumably some of these are extractable, and may be toxic *in vitro*. The aims of the present investigation were to examine the effects of extractable material from dental plaque on the growth of mammalian cells in culture, and to characterise any material having a cytotoxic effect. In addition, it was necessary to characterise the composition of the extractable plaque material, and to compare it with saliva and with serum.

Dental plaque was collected from the exposed surfaces of teeth, extracted into a modified Earle's saline solution, and homogenised. The mixture was centrifuged and the supernatant fraction (the plaque extract) was sterilised by millipore filtration. Unstimulated saliva was collected by spitting into an ice cooled beaker and then centrifuged. The supernatant fraction was retained, and the sedimented fraction was extracted as described for plaque. The supernatant fraction was sterilised by millipore filtration but the sediment extract was too viscous to be sterilised in this way. All three preparations were fractionated by membrane or Sephadex ultrafiltration and iso-electric focusing in polyacrylamide gels, as well as being examined by immunoelectrophoretic techniques. The total solid extractable from 1 g wet plaque

was determined after lyophilising samples of plaque extract. The ultraviolet spectra, and the amounts of protein, hexose, and sialic acid in these preparations were also determined. Mammalian cell cultures were grown in Eagle's medium with Earle's saline, calf serum to 10%, and penicillin and streptomycin each to 100 units/ml. Cells were incubated in 5% CO_2 in air at 37°C for 6 to 12 h, and then treated with either saline, plaque extract, or the saliva supernatant fraction. The dishes were reincubated for a known period, and the cells either counted, or measured for incorporation of tritiated thymidine into DNA.

The results showed that per g wet weight, the plaque preparations contained similar amounts of extractable material, including material which was toxic to 4 different types of mammalian cells in culture. This toxic material caused a gradual loss of the internal contents of HeLa cells and prevented their growth, a linear dose response relationship being observed. Plaque was found to be a much richer source of toxic material than saliva supernatant fraction. The nature and amount of extractable material from plaque differed markedly from that in saliva and serum preparations. Plaque extracts did not appear to contain any salivary material, but did contain a number of serum proteins. The toxic material was relatively stable. Most of it was found to have a molecular weight greater than about 30,000 and possibly to form aggregates. The dimer aggregate appeared the most active. The toxic material was inactivated after treatment with 5 % trichloroacetic acid at 0°C for 30 min. Toxic material in PE appeared to inhibit some process involved in DNA synthesis within 1 h of treating the cells.

The results suggested that gingival fluid, and not saliva, is substrate for the dental plaque ecosystem. The gingival fluid, an inflammatory serum exudate whose flow rate corresponds to the severity of chronic gingivitis, is a much richer source of nutrients than saliva. The increased bacterial growth which occurs as plaque develops probably results from the presence of gingival fluid. This proliferation and development of dental plaque would increase the amount of material which may directly irritate cells at the gingival junction. Toxic material may thus establish and maintain the flow of gingival fluid, and cause the development and persistence of plaque at the gingival margin. The toxic material was not identified but did not appear to consist of endotoxins or proteases.

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INTRODUCTION:

1. PERIODONTAL DISEASE AND CHRONIC GINGIVITIS

(a) Pathology and epidemiology of periodontal disease

(i) Occurrence and prevalence

Periodontal disease is most frequently apparent as an inflammatory lesion of the tissues supporting the teeth in the jaws (MacPhee and Cowley, 1969). It appears particularly widespread among primates and domestic animals. Some form of the disease is nearly always observed in most human beings, although its severity varies considerably from community to community (Waerhaug, 1966). The disease causes discomfort and loss of teeth but is not normally a direct cause of death. In human beings over age 40, periodontal disease is the major reason for the extraction of teeth.

(ii) Clinical signs and symptoms

In its mildest form, periodontal disease occurs as inflammation of the gingivae, which become red and bleed easily (gingivitis). In the chronic condition, the attached and marginal gingivae may become hyperplastic and false pockets between the teeth and the gingivae may form.

More advanced forms of periodontal disease exhibit a loss of gingival and periodontal membrane collagen together with alveolar bone (periodontitis). This process is accompanied by the formation of true pockets between the teeth and gingivae and some degree of apical recession of the gingival margin.

No classification of the clinical states has been universally accepted. The division of the disease into gingivitis and periodontitis is determined by whether the gingival attachment to a tooth is at the amelo-cemental junction, or has receded apically as a result of recession or true pocketing (MacPhee and Cowley, 1969).

Chronic gingivitis and periodontitis are common lesions and are usually painless. However, sometimes the gingival margins become extremely red and tender and may ulcerate (acute gingivitis), or periodontal abscesses may develop (acute periodontitis). These may cause moderate or even severe pain within the mouth. The degree to which periodontitis has progressed apically need not be related to the severity (acuity) of the gingivitis. MacPhee and Cowley (1969) apply the term 'atrophic gingivitis' to recession where a clinically apparent gingivitis seems to be absent. This type of condition is probably classified better as recessive periodontitis. Once recession of the gingival margin has occurred, or once pocketing has formed, the teeth may become mobile and eventually exfoliate.

(iii) Epidemiology and controlled clinical studies

Although many factors have been proposed as being responsible for periodontal disease, a number of epidemiological studies (Lövdal et al., 1958; Greene and Vermillion, 1960; Russell, 1963; Sheiham, 1970) have strongly suggested that the disease severity is directly related to the age of the subject and his oral hygiene status (his mouth cleanliness). When these two factors are combined, less than 10% of the variance in the severity of the disease remains to be accounted for (Russell, 1963).

In the absence of adequate tooth cleaning methods, microorganisms in the mouth accumulate on teeth surfaces. A short term clinical study revealed that after abstaining from oral hygiene measures for up to three weeks, all young adult subjects with previously healthy gingivae accumulated bacterial deposits on all teeth surfaces and exhibited gingivitis. The causal role of the bacterial deposits was confirmed by the fact that the inflammation completely resolved within a few days following the removal of the deposits and the re-institution of effective oral hygiene procedures (Löe, et al., 1965).

As a result of these and other studies (Theilade, et al., 1966; Löe, et al., 1967; Jensen, et al., 1968), gingivitis is now generally accepted as being caused by bacteria which invariably proliferate on uncleaned teeth surfaces. These microbial deposits are both soft and hard (calcified) and are commonly known as dental plaque and dental calculus respectively (Dawes, et al., 1963) - see section 3. The soft bacterial deposits are also known as bacterial plaque (Löe, 1969)

Three year longitudinal studies on human beings have shown that attention to cleaning the teeth reduces the severity of gingival inflammation, the rate of alveolar bone loss, and the depth of pockets (Lövdal, et al., 1961; Suomi, et al., 1971). However, a progression of gingivitis to periodontitis has never been clearly demonstrated in man, although such a transition has been demonstrated in experimental animals (Saxe, et al., 1967).

The epidemiological and clinical studies indicate that gingivitis is normally caused by the accumulation of bacterial deposits on teeth and that periodontitis becomes more severe if sufficient time is allowed to elapse without proper oral hygiene procedures being implemented. Similarly, these studies indicate that gingivitis predominates in youth and that periodontitis predominates in old age. In the absence of the crucial experiments, it seems reasonable to believe at present that periodontitis is probably the consequence of prolonged gingivitis, and that both are caused by inadequate oral hygiene habits.

(b) Dental plaque and the disease process

(i) Aetiological factors

Gingivitis and periodontitis may be related to three groups of factors. (1) Environmental factors, (2) Predisposing factors, and (3) Host factors.

The oral microbiota arises from micro-organisms in the host's environment which becomes established in his mouth shortly after birth. Later, once teeth erupt, the oral flora

includes additional strains of bacteria (Cornick and Bowen, (1971). The oral microbiota is extremely complex and differentiates to form bacterial ecosystems in different regions of the mouth (Socransky and Manganiello, 1971). The formation of these ecosystems may be governed, amongst other things, by limitation of oxygen, (Ritz, 1967), dietary sugars (Carlsson, 1968), bacteriocin production (Kelstrup and Gibbons, 1969), and growth enhancement factors (Parker, 1970). The rate of onset and the severity of periodontal disease which the host exhibits may thus depend on the state of his oral flora and the dental plaque ecosystem which develops from it (environmental factors).

Burnett and Scherp (1968) have suggested that gingivitis and periodontitis mainly result from insufficient control by the host of plaque accumulation to his teeth. The dental plaque normally requires a direct application of force to be dislodged from a tooth surface. The area of tooth covered is limited by friction from the tongue, cheeks, and food (Carlsson, 1968). The mass formed appears related to the consistency of the diet (Krasse and Brill, 1960; Egelberg, 1965a, b), the location of the tooth (Carlsson and Egelberg, 1965), and the tooth-brushing habits of the individual (Koch and Lindhe, 1965). These factors control the ease with which plaque forms and the length of time it remains undisturbed (predisposing factors).

Although both gingivitis and periodontitis themselves increase the ease of plaque retention and accumulation, the health and genetic endowment of the host presumably influence

the rate at which the disease progresses and the way in which it manifests (host factor).

(ii) An experimental approach to the study of chronic gingivitis and periodontitis

The products of the micro-organisms adhering to teeth probably irritate the surrounding tissues. This thesis is concerned with discovering and investigating these toxic substances and relating them to the aetiology of chronic gingivitis and periodontitis. Although it is not proven that chronic gingivitis in time gives rise to chronic periodontitis, the evidence suggests that it does and that periodontitis may be considered as an extension of gingivitis into tissues lying deeper in the periodontium. However, the findings (RESULTS) were extrapolated to provide information which is relevant, at present, only to the aetiology of chronic gingivitis in man.

2. THE DISEASE PROCESS IN GINGIVITIS

(a) The dento-gingival junction

The oral epithelium surrounding a tooth is known as the epithelial attachment or epithelial cuff and is everted at the gingival margin to form a shallow gingival sulcus, also termed a crevice or pocket (Löe, 1967; Weiss and Neiders, 1970). The sulcus is less than 0.5 mm deep. New techniques have recently permitted the epithelial attachment to be examined intact under the electron microscope and the resulting evidence enabled it to be described in more detail than hitherto (Schroeder and Listgarten, 1971). This evidence provides the most reliable background to date against which the results of the present investigation can be evaluated.

The attachment between tooth and oral epithelium is termed a junctional epithelium, the alternative term, 'crevicular epithelium' being discarded. So also is the term 'crevice'. The term 'pocket' is re-defined to mean a pathologically deepened sulcus - see section 2c (Schroeder and Listgarten, 1971). The junctional epithelium extends occlusally 3 to 4 mm from the region of the amelo-cemental junction to the base of the gingival sulcus and also becomes thicker in this direction. It has no keratinised or granular cell layers, and is composed of basal and spinous cells only. It is surrounded by a basal lamina which appears to be continuous round its most apical extension. The portion of this lamina which is continuous with the oral epithelium, and which lies over the connective tissue of the gingiva, is termed the outer basal lamina. The portion which covers the primary enamel cuticle (see section 3a) extends coronally to the gingival sulcus and is termed the inner basal lamina.

The gingival sulcus lies coronal to the free surface of the junctional epithelium which forms its base. Its lateral walls are the enamel surface and the sulcular termination of the oral epithelium. The junctional epithelial surface consists of 'cell ends' and is an exception to the usual orientation of epithelial cells in relation to a free surface.

It is possible to obtain biopsies which show the junctional epithelium and other gingival tissue to be free of any inflammatory cells (Löe, 1967, 1968). However, the junctional epithelium has a high rate of cellular turnover (Skougaard, 1970; Schroeder and Listgarten, 1971) and frequently contains at least a few polymorphonuclear leukocytes. The rate of cell division is most rapid and the presence of polymorphonuclear leukocytes is most obvious in the coronal portions of the junctional epithelium; i.e. at and close to the base of the gingival sulcus. Invariably, polymorphonuclear leukocytes can be picked up from the sulcular orifice by briefly inserting a blunt plastic strip (Attström, 1970).

(b) The gingival fluid exudate

A fluid exudate from the sulcular orifice can be demonstrated (Brill and Krasse, 1958). Its flow rate correlates more closely with the severity of gingivitis (Mann, 1963; Löe and Holm-Pedersen, 1965) than with the depth of pocketing (severity of periodontitis) (Mann, 1963). Even in the absence of plaque, mild mechanical irritation evokes this fluid flow (Brill, 1959; Brill and Krasse, 1959; Oliver, et al., 1969). This exudate contains several serum proteins (Brill and Brönnestam, 1960) and a sodium to

potassium ratio which is lower than in normal serum (Krasse and Egelberg, 1962). It is thought to be an inflammatory exudate.

No evidence has yet indicated what effect the gingival fluid may have on periodontal disease. Its existence indicates that the gingival epithelium is permeable. Intravenously injected fluorescein has been collected on to paper strips applied to the sulcular orifice (Brill, 1962) and homologous serum proteins labelled with fluorescein isothiocyanate have been found passing through the junctional epithelium near the base of the sulcus (Cowley, 1964). Most material applied to the sulcular orifice does not seem to penetrate the gingival tissues as it is opposed by the flow of gingival fluid. The gingival fluid contains some of the antibacterial properties of serum, and it has been concluded that the gingival fluid may have a protective influence in disease and add to the antibacterial properties of saliva (Jenkins, 1966). On the other hand, the gingival fluid has no detectable bacteriocidal or bacteriostatic effect on the growth of oral bacteria in vitro (Collins and Gavin, 1961) and may indeed be the nutrient source for bacteria at the gingival margin (Weinstein and Mandel, 1964).

(c) Microscopic changes in gingivitis

Early inflammatory changes occur in the gingival connective tissue adjacent to both the external basal lamina of the junctional epithelium and the basal lamina of the oral epithelium (Schroeder, 1970a). Simultaneously, there is a loss of intercellular material (Thonard and Scherp,1962; Schultz-Haudt,

et al., 1963; Toto and Sicher, 1964) and an opening of intercellular spaces within the junctional epithelium (Thilander, 1968; Freedman, et al., 1968).

Schroeder and Listgarten (1971) speculated that these changes might well have been due to the very large number of leukocytes now observed, particularly in the coronal portions of the junctional epithelium. These leukocytes occupy intercellular spaces between the junctional epithelial cells and appear to separate them from each other and from the tooth surface. At the most severely affected area, the base of the sulcus, the junctional epithelial cells disintegrate and the demarcation between sulcular epithelium and junctional epithelium becomes more marked. The net result of these changes is a gradual increase in the depth of the sulcus (Schroeder and Listgarten, 1971). As the depth of the sulcus increases, the oral sulcular epithelium occupies a gradually increasing portion of the sulcular lining. This pathologically deepened sulcus is termed a pocket (Schroeder and Listgarten, 1971). The extension of oral sulcular epithelium which forms the pocket's lateral wall (the pocket epithelium) is frequently rather thin and is occasionally ulcerated. The base of the pocket may remain coronal to the amelo-cemental junction (gingivitis), or extend apically (periodontitis).

(d) Response of epithelium to injury

The unusual permeability of gingival epithelium in permitting the passage of serum and leukocytes into the oral cavity, together with the proliferation of oral epithelium associated with pocket formation, is evidence that the sulcular

epithelium is exposed to an increasing degree of irritation and injury as periodontal disease develops.

i) Epithelial injury

If epithelium is injured, a great number of mitoses appear in the adjacent uninjured basal and prickle cell layers (Pinkus, 1952; Hell and Cruickshank, 1963; Epstein and Sullivan, 1964). The nature of this increase in mitotic activity can be explained if epidermal cells are assumed to be continually synthesising growth inhibitors (chalones) which normally prevent a rapid rate of cell division. Epithelial injury is thought to decrease the chalone concentration in the affected area, causing nearby undamaged cells to divide (Bullough and Laurence, 1961; Bullough, 1962). Chalones appear to have been isolated from the epithelia of a number of different species (Bullough et al., 1967).

Damage to more than the outermost layers usually causes a mild inflammatory response in the underlying connective tissue as a result of its affecting the basal layer (Pincus, 1952; Bullough and Laurence, 1961). However, a connective tissue injury to within 0.1 mm of the overlying epithelial layer failed to induce any epithelial proliferation (Bullough and Laurence, 1961).

Epithelial cells tend to move around and beneath an injured area as the burst of mitotic activity gets under way (Winter, 1964a). This continues until the epithelium is restored to its full thickness over the injured area (Winter, 1964b). The cells move beneath a leukocytic layer underlying a scab which first protects the injured site. It appears, however, that the epithelial cells and not the leukocytes secrete an enzyme locally which breaks down the scab collagen

and thus facilitates their movement (Winter, 1964a,b).

ii) Role of collagenase in regenerating epithelium

A collagenolytic enzyme has been isolated from human skin fragments cultured in vitro (Eizen, et al., 1970).

The greatest degree of collagenase activity could be obtained when the connective tissue portion of the skin (dermis) was cultured separately, although a small amount of activity was always detected when epidermis was cultured alone. Freeze thawing and puromycin treatment blocked the appearance of this collagenase, which appeared to be synthesised de novo, not being detected until 24 to 48 h after incubation. When a ten-day-old human wound edge was cultured, however, collagenase activity seemed to be equally divided between the dermal and epidermal fragments. It appears that regenerating epithelium is able to synthesise its own collagenase.

This collagenase cleaves the collagen molecule at a specific site at neutral pH. Although solutions of mammalian collagen undergo progressive denaturation in acid at temperatures above 30°C, there is no evidence that intact collagen molecules are denatured in vivo by acid production. The initial step in collagen denaturation may therefore be carried out in vivo by this enzyme.

This collagenase has been discovered in many human and animal tissues including bone, gingiva, polymorphonuclear leukocytic granules, and rheumatoid synovium. The enzyme can only be directly extracted from polymorphonuclear leukocytes, the other tissues, including gingiva requiring to be cultured. The collagenase from gingiva appears to be

derived from both epithelium and connective tissue, although the relative contribution from each tissue has yet to be demonstrated (Fullmer, et al., 1969).

Together with inflammation, proliferation of the oral epithelium and its apical migration have long been apparent as signs of periodontal disease (Znamensky, 1902; Fish, 1935; Wilkinson, 1935; Aisenberg and Aisenberg, 1948). It is possible that the oral sulcular epithelium tries to reunite with the more apical portions of the junctional epithelium beneath a region of irritation, and that epithelial collagenase is secreted in this process (Orban, 1949; also see section 4a). In this way gingivitis may lead to destruction of the periodontal membrane fibres.

(e) Cause of gingivitis

As previously described, Schroeder and Listgarten (1971) regarded the disintegration of the junctional epithelial cells, which form the epithelial base of the gingival sulcus, as being due to the pressure of emigrating polymorphonuclear leukocytes. Schroeder (1970 a,b) observed that the largest accumulation of leukocytes was always found directly apical to the base of the sulcus - i.e. in the coronal part of the junctional epithelium. The volumetric gradient of these granulocytes seemed to him to reflect the increasing concentration of a chemotaxin towards the base of the sulcus. Such an attractant has been shown to be manufactured by a number of bacteria known to be present in plaque and has indeed been found in water soluble extracts of *in vivo* plaque (Temple, et al., 1970).

Although detectable amounts of material can diffuse from the orifice of a clinically non-inflamed sulcus into the underlying tissue (Egelberg, 1963; Fine, et al., 1969; McDougall, 1971), it seems doubtful that sufficient quantities of material such as bacterial leukotaxin can diffuse into the healthy tissues to set up the observed granulocytic emigration (Rizzo, 1970). Indeed, the postulated inward diffusion of leukotaxin has to be set against the established outward path due to the gingival fluid exudate flow (section 2b).

However, Spector and Willoughby (1968) have noted that disrupted tissue cells contain powerful leukocytic chemotaxins. The increasing granulocytic infiltration of the junctional epithelium may therefore reflect a chemotaxin production by those epithelial cells which have been irritated. Such endogenous chemotaxins may easily diffuse into the subepithelial capillary plexus and cause the emigration of granulocytes more effectively than bacterial leukotaxins. Endogenous leukotaxins do not appear to mediate the inflammatory response (Spector and Willoughby, 1968). This may explain why the severity of the leukocytic infiltration was not related to the area of inflamed connective tissue in the region (Schroeder, 1970b).

As outlined in section (2d), an area of inflammation only becomes manifest when the intra-epithelial damage is sufficiently severe. In gingivitis, therefore, a predominance of granulocytic emigration to inflammation probably reflects the severe irritation of the sulcular epithelium rather than of the underlying connective tissue.

(f) Plaque and gingivitis

The presence of the granulocytes in clinically non-inflamed sulci, and the high cell turnover rate of sulcular epithelium, suggest that minor irritation to the junctional epithelial lining of the gingival sulcus may be occurring, even in the absence of plaque. Although the sulcus is probably never sterile (Löe, 1967) it is not certain if this mild irritation is bacterially induced or not when plaque appears to be absent. Accordingly, it has been proposed that the gingival margin area may suffer from mechanical injury (Fish, 1935; Schroeder and Listgarten, 1971), possibly the impact of hard food particles on the gingival margin, or a shearing stress on the most coronal portions of the junctional epithelium during functional tooth movements.

If this is so, it is probable that from time to time such stresses may induce a slight gingival fluid exudate (section 2b). If micro-organisms are not repeatedly and thoroughly removed from the tooth and gingival margin, they proliferate and form the bacterial plaque (section 3c)
It is tempting to hypothesise that the exudation of gingival fluid is the reason why plaque so readily develops at the gingival margin of teeth, since the serum-like composition of this fluid almost certainly contains more nutrients than does saliva (section 2b; also see DISCUSSION c).

In adolescents, only the local extension of plaque into the sulcus, rather than its merely covering the orifice, was associated with a marked increase of leukocytic infiltration

into the junctional epithelium, the indication of incipient pocket formation, and a marked connective tissue inflammation (Schroeder, 1970a). Of these 3 parameters, only the area of connective tissue inflammation in gingival biopsies correlated well with the clinical assessment of gingivitis (Schroeder, 1970b).

The more plaque is removed, the less it will extend into the sulcus, In the absence of plaque, neither gingivitis nor periodontitis normally appears to occur.

(g) Chronic gingivitis - a definition

Chronic gingivitis is the response of the healthy gingivae to continuous contact with irritating substances in dental plaque. This response is composed of leukocytic emigration through the junctional epithelium, proliferation of the oral sulcular epithelium, and inflammation of the underlying connective tissue. These indicate damage to cells in the gingival epithelium and connective tissue respectively. In time, this may result in collagen loss and alveolar bone loss (periodontitis).

3. THE DENTAL PLAQUE

(a) Integuments of the teeth

In an early review, Stephan (1953) defined dental plaque as 'a general designation for all soft extraneous material adhering to teeth surfaces.' As this material is of developmental, salivary, bacterial, and dietary origin, Stephan's definition has to be modified. Dawes, et al., (1963) suggested the terms 'primary enamel cuticle' and 'reduced enamel epithelium' for the two developmental integuments; 'acquired pellicle' for the non-bacterial structure acquired after eruption; 'dental plaque' for the soft bacterial deposits; 'dental calculus' for the mineralised bacterial deposits; and 'food debris' for the dietary deposits but also including material removed from the teeth on rinsing.

The acquired pellicle gradually replaces the developmental layers once a tooth has become functional. Ultrastructurally, the acquired pellicle is a cell-free layer on the enamel surface and lies between it and the dental plaque (Meckel, 1965; Leach and Saxton, 1966). It may appear continuous with the inter-bacterial matrix of the dental plaque, or it may be entirely absent (Frank and Brendel, 1966). The pellicle appears to be principally formed from saliva but may also contain material from bacteria and serum (Dawes, 1968). The acquired pellicle adheres tightly to enamel and can only be removed by treating it with dilute mineral acid or with violent abraision (Armstrong, 1968). Presumably, it can sometimes be removed by the dental plaque bacteria.

The bacterial deposits, the dental plaque and dental calculus, account for the greatest mass of tooth integument. The dental plaque must be scrubbed to remove it adequately from

the tooth surface (Armstrong, 1968) - see sections 1b i, and 3d. The dental calculus is believed to arise from dental plaque if it accumulates too much calcium and phosphate ions from the gingival or salivary fluids. Dawes and his associates (Dawes, et al., 1963; Jenkins, 1965; Dawes, 1968) consistently describe dental plaque as being 'the soft concentrated mass.... which develops within a short time of refraining from toothbrushing.' This definition is slightly confusing as it could imply that no plaque can be detected if toothbrushing is maintained. Löe, et al., (1965) have shown that even if the oral hygiene is extremely good, some bacterial deposits are observed on teeth surfaces. These are mainly cocci and short rods (Löe, et al., 1965). Abolishing toothbrushing is a procedure for standardising the dental plaque deposit, and the reference to toothbrushing is probably better to be omitted from its definition.

In general, food particles do not seem to remain long on the teeth. One study has shown that soft food particles disappear from the mouth more than 5 minutes after eating (Caldwell, 1968). Only bacterial matter is thus normally removed by rinsing, or by irrigating the teeth with a water-jet. At present, there appears to be no sound evidence which indicates that the outer layers of dental plaque are composed of food debris. Accordingly, Egelberg (1970) has observed that dental plaque should include the material removed from the teeth on rinsing and that the term 'food debris' should thus denote only food particles. These are usually seen interdentally, and are readily identified clinically.

(b) Definition of dental plaque

In this thesis, dental plaque is defined as the soft deposit of micro-organisms which adheres to the intact surfaces of erupted teeth. The terms 'bacterial plaque' or 'plaque' are synonymous.

(c) Standardised plaque

Investigators have standardised their plaque material prior to collection by controlling the age of the subjects (Dawes and Jenkins, 1962; Löe, et al., 1965); the area of collection on the teeth and in the mouth (Poole and Gilmour, 1971); the time after eating and abstaining from oral hygiene (Silverman and Kleinberg, 1967); by adopting a rinsing and drying procedure (Soder and Frostell, 1966); or by inserting an artificial surface which can be removed with the plaque (Jenkins, 1965; Grøn, et al., 1969). Perhaps the best method has been to use subjects who have no gingivitis or periodontitis, and then to harvest the plaque after a fixed interval without any toothbrushing or other oral hygiene procedure (Löe, et al., 1965; Theilade et al., 1966).

(d) Bacterial development of dental plaque

Studies have shown that plaque develops in an orderly manner on a clean tooth. Its development takes two to six days to complete when toothbrushing is abolished. Initially, gram positive cocci and short rods predominate, but then the microbial flora becomes increasingly complex until within a week it is composed of cocci, rods, fusobacteria, filaments, vibrios, and spirochaetes, with gram negative species now predominating. (M^{c}Dougall, 1963a,b; Löe, et al., 1965; Slack and Bowden, 1965; Howell, et al., 1965; Theilade, et al., 1966; Ritz, 1967). Mature plaque appears to be composed of different bacterial colonies in an apparently random arrangement, and ultrastructurally it appears either filamentous or non-filamentous (Schroeder and De Boever, 1970). Mature plaque is generally believed to vary greatly from site to site in the mouth (M^{c}Hugh, 1970),

probably because of this random presence of different bacterial strains.

(e) Plaque and gingival health

When the composition of plaque from the gingival pockets of patients with periodontal disease was compared with that from the gingival sulci of patients without periodontal disease, little difference in the types or numbers of bacteria was found despite the fact that the former had much more plaque per tooth. Only spirochaetes were significantly increased (from zero to 1%) in the diseased group (Socransky, et al., 1963; Gibbons, et al., 1963). However, when the criteria for the absence of gingivitis were made very strict before tooth cleaning was abolished, major differences in the bacterial composition were observed as the plaque developed (Löe, et al., 1965). The inclusion of mature plaque from areas of mild gingivitis in the pooled material of the earlier studies, in which the two groups were separated arbitrarily by pocket depth, may have obscured the differences (Theilade, et al., 1966).

It seems that large differences in the bacterial composition of plaque can be demonstrated only when very strict clinical criteria are used to diagnose the absence of gingival inflammation. Only the gingival condition, and not the pocket depth, therefore seems to indicate the degree of plaque maturity. It is possible the pocket depth indicates the time the plaque has been left undisturbed (see section 1a iii). Most plaque found on teeth is thus mature, since very few people exhibit the criteria of perfect gingival health described by Löe.

(f) Specific factors in plaque possibly responsible for disease

A variety of proteolytic and other tissue destructive enzymes have been shown to be present in sterile extract filtrates of plaque from the gingival margin of patients with disease, and also to be produced by plaque bacteria (Schultz-Haudt, et al., 1954; Schultz-Haudt and Scherp, 1955; Schultz-Haudt and Scherp, 1956; Dewar, 1958; Hampp, et al., 1959; Soder and Frostell, 1966; Soder, 1969). In addition, a group of plaque micro-organisms was found to cause a transmissible abscess in guinea pig skin (Macdonald, et al., 1960; Macdonald, et al., 1963) and various micro-organisms have been described as causing a transmissible form of periodontal disease in rodents (Jordan and Keyes, 1964; Gibbons, et al., 1966; Dick, et al., 1968; Socransky, et al., 1970).

Gingivitis frequently resolves in the presence of local or systemic applications of antibiotics (Strock, 1944; Ostrander, 1953) and antibiotics or antiseptics prevent plaque formation (Löe, et al., 1967; Löe and Rindom Schiött, 1970). However a form of periodontal disease occurs in germ-free mice which is clearly not associated with the presence of local bacteria (Baer, et al., 1964; Baer and Fitzgerald, 1966).

Although both innate and bacterial factors appear to play a part in the development of periodontal disease in man (section 2f), direct evidence for this assumption is lacking. The bacterial factor appears to correlate most closely with the severity of periodontal disease in man. Whatever causes plaque to accumulate and develop at the gingival margin may also be associated with the production of toxic substances by its component micro-organisms.

4. INVESTIGATING THE DISEASE PROCESS

(a) Experimenting with the periodontal lesion

Recent experiments have shown that short term leukopenia in dogs causes a reduction in the severity of their gingival inflammation. This reduction is greatest during the 3 days of maximal leukopenia (Attström and Egelberg, 1971; Attström et al., 1971). The inflammatory process itself may therefore cause as much destruction as the irritants from bacterial plaque which provoke and maintain it. Irritating factors from plaque cannot be separated *in vivo* from the destructive factors due to the inflammatory response and the epithelial proliferation (see section 2d). As the former factors are responsible for the persistence of the latter, the most fundamental approach to the disease process is to study the plaque factors on cells *in vitro*, away from complexities of the whole host organism.

(b) *In vitro* techniques for studying the response of gingiva to plaque

There are three methods of keeping tissue such as skin or gingiva alive *in vitro* (Cruickshank, 1965). (1) Organ culture - the explant retains its normal structure for a few days. (2) Cell culture - the inter-relationship between cells is quite different from that *in vivo*. (3) Tissue culture - in which there is disorganised growth of cells from an explant.

(i) Organ cultures

When epithelium or epidermis is organ cultured, it behaves consistently in a manner analogous to that occurring during the initial stages of repair to a break in continuity

of the epithelium *in vivo*. The epithelium proliferates around the dermis until the culture is sealed from the medium and dies for lack of nourishment (Cruickshank, 1965). This process of encirclement has also been described in gingival explants (Powell, 1967).

Epithelial cells in organ culture divide maximally all the time, unlike such cells *in vivo* (Matoltsy and Sinesi, 1957; Hell and Cruickshank, 1963). Irritation of the culture results in a slowing of epithelial cell division, differentiation, and encirclement. Unfortunately, because these parameters normally vary widely from explant to explant (Cruickshank, 1965, Rosenoer and Jacobson, 1966), it is difficult to assess the effects of toxic or irritating material.

Only one study into the effect of dental plaque on gingival epithelium using organ culture has been reported (Powell, 1969a,b). and the study demonstrated the assessment difficulties just described above (e.g. the difference between control and treated cultures in one experiment was found to be as great as the difference between controls in another). It seems that another system may be more suitable than organ culture for studying the effect of plaque material on cells *in vitro*.

(ii) Preparation, nature, and use of cell cultures

Cell cultures are derived from unorganised tissue cultures, or primary cell cultures (Paul, 1970). These latter cultures are obtained by mechanically and/or enzymatically breaking up a tissue and incubating the fragments in tissue culture growth medium. The cells gradually adapt to their new environment

and grow out of tissue fragments. The experimental problems associated with organ culture conditions still apply.

From the primary cell culture, or from disorganised tissue culture outgrowths, it is possible to obtain a continuously growing cell culture which has a limited period of survival (Hayflick, 1965). But occasionally, some cells in the culture may form more rapidly growing colonies and can be harvested and subcultured indefinitely. In this case, the culture is said to have become established (Paul, 1970). Epithelial cells sometimes become established quickly - after 3 weeks of culture, or 4 to 8 subcultures (Cruickshank, 1965). This procedure has been used to establish an epithelial-like cell line from normal adult human gingiva (Smulow and Glickman, 1966) and also from normal human skin (Wheeler, et al., 1957).

Established epithelial cells in culture are flat, polygonal, and have polyploid nuclei. The cells adhere well to a glass or plastic surface and grow rapidly to form a monolayer. After about four days of growth, the culture degenerates, possibly because nutrients do not penetrate the thick layer of cells which has developed. The cells have a distinctive appearance, but those derived from different epithelial sources cannot normally be distinguished from each other (Willmer, 1965). Established fibroblast cell cultures have similar general characteristics but may differ markedly from epithelial-like cells in their appearance and are able to move about on a glass or plastic surface (Willmer, 1965).

Cell cultures grow like micro-organisms and can be accurately quantified, since the amount of tissue is determined

by the number of cells in the culture. Cell culture techniques are now widely used in many different biological investigations, although the relationship of cells in culture to cells *in vivo* is not well defined. The growth conditions, intercellular relationships, and karyotypes in culture may all differ from those of cells *in vivo*. Accordingly, chemical agents which relate to metabolic processes associated with cell survival and growth are better suited for evaluation with cell cultures than those interfering with the more diverse functions of differentiated cells. These latter functions cannot be fully reproduced in cell culture (Rosenoer and Jacobson, 1966).

(c) *Cell cultures as a means for studying the irritating material from plaque*

The most obvious use of cell cultures is that they provide a means for evaluating the mode of action of a drug or toxin directly on isolated cells. Such studies can only be carried out effectively with a pure substance, otherwise the result is the net effect of different modes of action by different substances in the mixture.

As distinct from evaluating the mode of action, however, cell cultures are frequently used as a means of assessing or titrating for the presence of toxic material (Schindler, 1969; Solotorovsky and Johnson, 1970). This may be done by comparing treated and untreated cultures as regards cell morphology, staining with dyes only absorbed by damaged cells, the pH of the culture medium, the amount of protein or other cell substance, or the net uptake of radioactive precursor into cell

substance. Clearly the most direct parameter is to measure the number of cells in the culture as either the growth rate, or the growth yield after a fixed time interval.

The irritating matter in plaque may be regarded as being transposable from *in vivo* by exposing epithelial or connective tissue cell cultures to extracts of plaque material. If it is true that chronic gingivitis is caused by persistent cell injury *in vivo*, the effect of plaque material on cells in culture should be to prevent their growth and survival. Not only is this supposition likely (Schindler, 1969), but this approach is ideally suited to investigating the nature and mode of action of the irritating material in dental plaque.

In view of the complex composition of plaque, its extracts may contain a number of substances which have a toxic effect on cell cultures. Chemical characterisation and identification of these substances would seem to be a necessary prelude to detailed studies of their mode of action.

(d) Studies with plaque material using cell cultures

The effect of plaque on established epithelial and fibroblast cell lines has been investigated using cell cultures (Cobb and Brown, 1967). Plaque material was added to culture medium above monolayers of both epithelial (KB) and fibroblast (L929) cells and it was shown that the number of cells in the treated cultures decreased more than in controls. Some cultures were treated with solid particles, whilst others were treated with non-sterile material. These treatments would damage cells *in vitro* but not *in vivo*. Furthermore, the fact

that cells in the control cultures decreased in number with time indicates that all the cultures were unhealthy as the cells should normally increase in number with time.

The effect of plaque material on the growth of fibroblast cells (BHK 21) has been studied (Baboolal, et al., 1970). Plaque was extracted into physiological saline and centrifuged. The solid fraction was re-extracted with phenol and re-centrifuged. The supernatant fractions from both centrifugations were dialysed, the non-diffusible fractions lyophilised to dryness and added to the cells at a final concentration of 0.5 mg/ml in the culture medium. The diffusate was not investigated. Treated cultures showed abnormal and degenerative cellular changes which become more pronounced with time. Both plaque fractions inhibited growth by about 50%. The absolute increase in cell number that occurred in the untreated cultures was not reported, although such information would seem to be essential for a critical evaluation of the results. The cells were not attached to the dish when plaque material was added, so that any interference with attachment would prevent growth in vitro, whereas in vivo the cells are already attached. Finally, material which is diffusible through dialysis tubing is as likely as non-diffusible material to be toxic in vivo, and therefore a comprehensive study should consider both.

The review of published studies indicates that a thorough study into the effect of plaque material on cells in culture has not been carried out.

(e) Problems in the use of plaque

The general epidemiological finding - the more plaque, the more disease - implies that the more plaque collected and pooled, the more toxic material should be available for study. Most available plaque is already mature (Section 3e). Neither the nature nor the amount of solid material which is extractable from wet plaque has been established and plaque has frequently been assumed to contain material from saliva (Jenkins, 1966).

In line with investigations into the effect of extractable plaque material on cell cultures, more general investigations into the nature and distribution of extractable (unstandardised) plaque material from different individuals or groups of individuals would establish a baseline against which standardised plaque may eventually be compared and aid in determining the nature and distribution of any cytotoxic material found to be present.

5. AIMS OF THE INVESTIGATION AND SUMMARY OF PRINCIPAL FINDINGS

(a) To examine the effect of extractable material from dental plaque on the growth of mammalian cells in culture.

(b) To examine the composition of extractable material from dental plaque and to compare it with saliva and with serum.

(c) To attempt to characterise any growth inhibiting material from plaque by its chemical characteristics and its mode of action.

These aims were largely achieved. The results showed that per g wet weight, all plaque preparations contained similar amounts of material which was toxic to mammalian cells in culture. This material gradually lysed the internal contents of the cells and prevented their growth, a linear dose response relationship being observed. Plaque was found to be a much richer source of toxic material than saliva (RESULTS, section 1).

The nature and amount of extractable material from plaque differed markedly from that in saliva or serum preparations. Plaque appeared to contain no material from saliva, although some substances from serum, particularly proteins, were observed (RESULTS, section 2).

The toxic material was found to be relatively stable, and could be fractionated by various methods (RESULTS, sections 3 and 5). It was found that about 80% of the toxic material had a molecular weight greater than about 30,000 and possibly formed aggregates. The dimer aggregate appeared the most active. The toxic material was inactivated after treatment with 5% trichloroacetic acid at $0^{\circ}C$ for 30 min.

The toxic material in PE appeared to inhibit DNA synthesis in HeLa cells (RESULTS, section 4).

MATERIALS and METHODS

Investigative Procedure

No suitable assay procedure for examining cytotoxic material from plaque has been described. The investigative procedure therefore depended on the outcome of a primary investigation to establish what suitable cell culture assay might be used. This section, however, enumerates all the procedures, including those undertaken subsequent to the development of a cytotoxic assay.

(1) Plaque

(a) Plaque collection

Considerable amounts of plaque are essential for the preparation of an extract, its manipulation, and the setting up of an acceptable number of test and control cultures. As discussed above, unstandardised whole mouth plaque from different groups of donors was used (INTRODUCTION, 4e).

Using a plastic spatula, plaque was removed from all the exposed tooth surfaces from patients of widely differing ages attending the Glasgow Dental Hospital for treatment. Patients with a history of infectious hepatitis, or presenting with any form of oral ulceration were excluded. Patients with acute ulcerative gingivitis were also excluded except for about four initially. Most plaque came from patients with chronic gingivitis and was usually associated with chronic periodontitis. On average, each patient provided about 50 mg. of wet plaque, although the standard deviation of 16 whole mouth samples was 60% of this mean. Plaque was collected to just below the free gingival margin which was disturbed as little as possible.

The plaque was yellowish white and was discarded if contaminated with more than the odd streak of blood. If bleeding from the gingival margin was excessive, the mouth was rinsed with tap water and the area subsequently avoided. The mouth was not usually rinsed prior to plaque collection otherwise. Immediately on removal, the plaque was placed in polythene vials on ice. Within 2 h, the vials were frozen and stored at -20°C. Gram stained smears of the pooled material on two different occasions were found to exhibit all the normal plaque micro-organisms, but few polymorphs. The plaque was always used within 2 months of collection.

(b) Preparation of a Plaque Extract. PE.

The plaque from different donors was pooled and weighed. A volume of carrier saline (a solution containing 68.0 g/l NaCl w/v, 4.0 g/l KCl w/v, 2.0 g/l $MgSO_4.7H_2O$ w/v, and 3.93 g/l $CaCl_2$ w/v, and diluted 1 to 9 before use) was added to a known weight of wet plaque. (This carrier saline solution could be made into Earle's saline, and used for the cell culture medium by adding the appropriate components.) At 4°C, the mixture was homogenised. After centrifuging the mixture at 30,000 x g, the supernatant fluid, the plaque extract (PE), was filtered through a sterile 0.22 μ millipore filter to exclude bacteria. The extract was slightly acidic. The sedimented fraction of the plaque was discarded.

For the initial series of experiments, the plaque was pooled from at least 20 donors and was homogenised with either a Silverson (Silverson Machines Ltd., Chesham, Bucks.) or MSE homogeniser (Measuring & Scientific Equipment Ltd., London, S.W.1.). Subsequently, a Potter-Elvehjem homogeniser (Jencons, Scientific, Ltd., Hemel Hempstead, Herts.) was used (40 strokes of the rotating teflon pestle) to extract both unpooled plaque (single donors), and pooled plaque (80 to 100 donors per pool).

(2) Saliva

Unstimulated saliva was collected by spitting into a beaker in an ice bath and centrifuged at 30,000×g at 4°C immediately afterwards. The supernatant fraction (Saliva Supernatant, SS) was decanted and retained. The sedimented fraction, which is largely composed of micro-organisms (Burnett and Scherp, 1962), was extracted with carrier saline using a Silverson homogeniser as described for plaque and was designated the Sediment Extract, SE. Unlike PE, the SE was colourless, odourless, and was too viscous to be passed through a 0.22 μ millipore filter. Both SE and SS remained clear after standing for over 3 months in a refrigerator at 1°C.

(3) Water and extractable solid content of plaque

Wet plaque contains from 70 to 90% water (Dawes and Jenkins, 1962; Grøn, et al., 1969) and this observation was confirmed. The volume (ml) of water plus extractable solid in wet plaque can therefore be only slightly less than its wet

weight (g). The total solid in PE was determined by taking 0.5 or 1.0 ml samples in previously weighed test tubes and reweighing the tubes after lyophilising their contents to dryness for at least 6 h. By subtraction of the appropriate saline blank, the concentration of extracted plaque solid in PE (and hence in wet plaque) was found [see Table I, Results section (1b)].

(4) Dialysis

Visking tubing, 0.25 inch diameter, (Scientific Instrument Centre Ltd., London, W.C.1.) was boiled for 10 min in 5% w/v versene (B.D.H. Chemicals Ltd., Poole, Dorset, Analar Grade) in distilled water, washed, and then boiled in distilled water and washed again.

PE, SE and SS were dialysed 3 times for 8 h each time against 20 times their volume of distilled water. The diffusates (Low molecular weight fraction, L) were lyophilised to dryness, and redissolved in the same volume of distilled water as their respective non-diffusible fractions (High molecular weight Soluble fraction, HS). A precipitate formed in the non-diffusible PE fraction during dialysis (High molecular weight Insoluble fraction, HI) and was removed by centrifugation. It was found to redissolve partly when mixed with the same volume of carrier saline as the other fractions. Silverman and Kleinberg, (1967) have described a similar fraction after dialysis of plaque material extracted into NaOH. Kleinberg, et al., (1971) also observed a precipitate in the non-diffusible portion of a saliva supernatant fraction obtained after wax

stimulation. In the present experiments, a precipitate was not observed in this saliva fraction, possibly because unstimulated and not wax stimulated saliva was employed.

The three plaque fractions were sterilised by millipore filtration. A suitable volume of 10 x concentrated carrier saline was added to the non-diffusible fraction to replace that lost on dialysis, the other two fractions being diluted with the same volume of 1 x carrier saline.

(5) Ultrafiltration

(a) Amicon filtration

A known volume of sample was applied to an Amicon filter (Amicon Ltd., High Wycombe, Bucks,) at 4°C with 75 p.s.i. nitrogen. This filter restricts passage to solutes below a defined molecular weight, depending on the type employed. When the volume above the filter had been reduced 6-fold, the same volume of carrier saline was applied. After refiltering, the small volume above the filter was removed and, in the case of PE, diluted with carrier saline to the original volume applied. This fraction, excluded from passing through the filter, was cloudy and the precipitate was removed by centrifugation at 16,000 g at 4°C. The sediment was discarded. The supernatant fraction was millipore filtered and labelled the Amicon High molecular weight fraction, AH. The volume of the combined fractions was diluted with carrier saline to twice the volume of fraction AH and labelled the Amicon Low molecular weight fraction, AL. Different volumes of each fraction were added

to give the same concentration of plaque material as that found in assays containing the unfractionated extract. [see below, section (10b)]. The filter's efficiency was checked by observing that material in fraction AL absorbed much more strongly at 260 nm than material in fraction AH (RESULTS, 2b, 5a and 5c), and also that most protein remained in the excluded fraction (AH).

In the case of SS, the non-filterable fraction was used without dilution back to its original volume, the filtrate being combined and assayed for protein as a check on the filter's efficiency.

(b) Sephadex

A 22 x 0.9 cm column of G 10 or G 150 Sephadex was prepared according to the manufacturer's instructions (Pharmacia, GB., Ltd., London, W.11) and equilibrated overnight with carrier saline or distilled water. Fluid was passed through the columns at 9 ml per hour with a BTL Chromapump (Baird & Tatlock, London, Ltd., Chadwell Heath, Essex). Fractions (1.0 ml, G 150; 0.6 ml, G 10) were collected on a BTL Chromafrac (Baird & Tatlock), the whole apparatus being kept in a cold room at 4°C. Better fractionation was achieved subsequently by using a 82 x 1.5 cm G 150 Sephadex column and a 52 x 0.9 cm Sephadex G 10 column.

The G 150 column was standardised by passing through solutions of bovine haemoglobin (Sigma, London, Chemical Co., London, W.6), whale myoglobin (Sigma), bovine

chymotrypsinogen [Miles-Servac (PTY) Ltd., Berks. England], and human albumin (Hoechst Pharmaceuticals, Hounslow, Middlesex). About 5 to 10 mg of such protein was dissolved in either carrier saline, or 0.2M NaCl buffered at pH 8.1 with tris HCl(BDH). This latter solution caused the albumin to be eluted as monomers and dimers (Fischer, 1969). Different mixtures of the standards were applied to the column and eluted with either carrier saline or tris buffered saline. The elution of haemoglobin and myoglobin was followed by measuring their absorbance at 400 nm and the elution of other proteins was followed at 280 nm. Elution of dextran blue was followed at 600 nm.

The G 10 column was standardised by passing through standard solutions (2 mg/ml w/v) of deoxythymidine phosphate and adenosine (Koch Light Laboratories Ltd., Colnbrook, Bucks.); 5 µc (6-^{3}H) deoxythymidine (The Radiochemical Centre, Amersham, Bucks.); 10% w/v glycine and glucose respectively; and 30% w/v sodium chloride. The fractions were eluted with carrier saline or distilled water. Aliquots were assayed as described in section 9. Radioactivity was measured by assaying 0.1 ml aliquots in 10 ml NE-250 scintillant (Nuclear Enterprises Ltd., Sighthill, Edinburgh) using a Packard Tricarb Scintillation counter.

Unfractionated PE, and the diffusate from PE or SE were lyophilised to 4 - 10% the original volume of distilled water (50 - 100 mg extracted plaque material/ml) and 0.2 to 0.6 ml applied to either column after insoluble material had been removed by centrifugation at 4°C. The sample was applied with

a plastic syringe and catheter by dropping it through the eluting solution onto the gel surface. Aliquots were assayed as described below. In one experiment, 1 ml of PE prepared from 375 mg wet weight plaque per ml carrier saline was applied to the short column of G150 Sephadex without prior lyophilisation.

(6) Heat treatment

PE was heated in a boiling distilled water bath for 1 h. A white precipitate developed gradually and was subsequently removed by centrifugation and discarded.

(7) Ether extraction

PE was extracted twice at room temperature with 3 times its own volume of ether (BDH, analar grade). The aqueous layer was left for 2 h at room temperature to let some of the remaining ether evaporate and then stored overnight at 1°C. The ethereal layer was left to evaporate to dryness at room temperature for 20 h in a fume cupboard. The same volume of carrier saline as in the aqueous layer was added to the ether residue, and both fractions millipore filtered.

(8) Trichloroacetic Acid (TCA) extraction

At 0°C, PE was made 5% w/v TCA by the addition of a suitable volume of ice cold 50% w/v TCA (BDH, analar grade). The mixture was stirred on ice for 25 min. and centrifuged at 4°C at 30,000×g for 20 min. The greyish-white solid sediment was discarded. The supernatant fraction was extracted 5 times with ether as described above, until the pH of the aqueous layer was more than 4. The ethereal layers were discarded.

(9) Assays

(a) Ultraviolet absorbance estimation

This was scanned on an SP 800 spectrophotometer, or measured with an SP 500 spectrophotometer at a fixed wavelength. The absorbance of a solution was measured by using cells with a 1 cm light path.

(b) Protein estimation

This was measured as described by Lowry et al. (1951), using a 0.5 mg/ml chymotrypsinogen solution (Miles-Servac) spectroscopically estimated according to Wilcox et al. (1957) as standard.

(c) Carbohydrate estimation

This was measured as µg/ml glucose after treatment of the sample and of a glucose standard solution (70 µg/ml, w/v) with 5% phenol and 95% sulphuric acid (Dubois et al., 1956). Alternatively, carbohydrate was measured with the Orcinol Reagent using the method of Hartley and Jevons (1962).

(d) Amino acid estimation

The method of Moore and Stein (1954) was used to follow the elution of a glycine standard from a Sephadex G 10 column (Fig. 38).

(e) Sialic acid estimation

The procedure as detailed by Aminoff (1961), using thio-barbituric acid (BDH) was followed exactly. The preparations were assayed before and after hydrolysis (1 h at 80°C in 0.1 N H_2SO_4) to determine their bound sialic acid content.

(f) Sodium chloride estimation

To 1 ml aliquots of the test solution in labelled tubes, 0.1 ml of 257 mM silver nitrate was added and the tubes centrifuged 30 min at 800 g. The supernatant was carefully sucked out and the tubes dried overnight at 80°C. The tubes were weighed, thoroughly washed with water and rinsed with distilled water, again dried overnight at 80°C, and reweighed. The weight of precipitate obtained from 1 ml of standard NaCl solution was plotted against the NaCl concentration. (Fig. 39b; RESULTS, 5c).

(10) Cytotoxicity assays

(a) Selection of cell cultures

The best criterion for judging the health of a culture is its growth rate, which, like all criteria of health, is a relative measurement. Human cell cultures grow relatively slowly prior to establishment (Paul, 1970), the number of cells doubling about every 35 h. On the other hand, human established cell cultures grow faster and double about once every 24 h (INTRODUCTION, 4b). It was decided to work chiefly with HeLa cells, which are an epithelial-like established cell culture derived from a carcinoma of the human cervix (Gey, et al., 1952). These cells are therefore derived from human tissue whose structure is not too different from gingival epithelium. The extracted plaque material was thus applied to a controlled, quantifiable, fast growing, and easily obtained standard 'tissue'.

The effect of extracted plaque material on other cell cultures was also examined. These cultures were L 929 (Sanford, et al., 1948), a clone derived from mouse fibroblasts

(Earle, 1943); BHK 21 C13 (McPherson and Stoker, 1962) derived from a hamster kidney fibroblast; and cells derived from a primary foetal human lung fibroblast culture (4th passage on cell culture). The lung culture was prepared by and purchased from Bio-Cult Laboratories Ltd., Paisley. The other cell cultures were obtained by courtesy of the Department of Biochemistry, University of Glasgow, where all cultures were regularly checked for bacterial and mycoplasma infection. Growth of the cultures was sometimes assayed visually with a Leitz inverted lens microscope [E, Leitz (Instruments) Ltd., London W1N 8BB], but more often by measuring the cell number as described below.

(b) Preparation and treatment of cells

Cells were grown in Eagle's medium with Earle's saline, 10% (w/v) calf serum, and 100 units per ml of both penicillin and streptomycin.

Eagle's minimum medium amino acid solution (x 50), Eagle's minimum medium vitamin solution (x 100), calf serum, and penicillin - streptomycin solution (1,000 units per ml) were purchased from Flow Laboratories Ltd., Irvine, Ayrshire, or from Biocult Laboratories Ltd., Paisley. Eagle's minimum medium was enriched by including twice the minimal vitamin concentration. Equal volumes of the amino acid and vitamin solutions were diluted with solutions of glucose (BDH) and glutamine (Koch-Light Laboratories Ltd) until the medium was now only 10 times more concentrated than necessary. The pH was brought to 7.1 by adding approximately 1% v/v of 6N HCl, and the medium sterilised by passing it through a 0.22 μ millipore filter.

A suitable volume of the enriched Eagle's medium x10 was added to sterile Earle's salt solution from which sodium bicarbonate had been omitted. The pH was adjusted to 7.4 with about 1.5% v/v of 8.4% w/v $NaHCO_3$ solution, and the growth medium constituted by adding the calf serum and penicillin-streptomycin solution to the concentrations described above.

Stock cultures were grown at 36.5°C in a 5% CO_2 in air mixture in large flat bottomed tissue culture bottles obtained from Flow Laboratories Ltd. Aqueous trypsin solution was prepared containing 0.25% w/v trypsin (Difco Laboratories, East Molesey, Surrey), 0.3% w/v tri-sodium citrate, 0.6% w/v sodium chloride, and 1.5×10^{-5}% w/v phenol red. It was filtered through a Whatman's No.1 filter paper (H. Reeve Angel & Co. Ltd., London, E.C.4), and finally through a 0.22 μ millipore filter to sterilise it. Versene was dissolved in 0.1 M phosphate buffered saline (pH 7.1) to 0.2% w/v, and the solution autoclaved. The trypsin solution was diluted 1 to 4 with this versene solution immediately before use. The cells were harvested by washing them with the trypsin-versene solution, incubated 5 min at 37°C, and washed into suspension with EC_{10}. An aliquot of this suspension was suitably diluted with counting fluid (a solution of 0.7% w/v sodium chloride, 1.02% w/v citric acid, and 0.1% w/v mercuric chloride), and the number of cells counted with a Coulter counter Model D1 (Coulter Electronics Ltd., Dunstable, Beds., England).

Approximately 0.8×10^5 cells per ml were added to disposable plastic Petri dishes or trays which were purchased from Sterilin Ltd., Richmond, Surrey, or from Biocult Labor-

atories, Paisley. The cultures were left overnight in an incubator at 36.5°C and became attached to the base of the dish or tray. The incubator was ventilated with 5% CO_2 in air and kept at high humidity by bubbling the gases through distilled water. The following day, the dishes were randomised and the medium removed from not more than 20 at a time. Different volumes of plaque extract or other substance dissolved in carrier saline were added to the dishes and made to a constant final volume with carrier saline. Controls had carrier saline only. The other growth medium components were then added such that the carrier saline extract mixture contained in solution, all the EC_{10} components at the same concentration as in EC_{10} itself. The dishes were then re-incubated. The SS fraction was sterilised by millipore filtration as described for plaque. A concentrated solution of EC_{10} was diluted with this fraction and added to the cultures in Petri dishes. The medium was made to a constant final volume with EC_{10}. Control cultures only contained EC_{10}, and no fraction SS.

(c) Growth assay by cell number.

After a fixed time interval, the medium was removed. The cells were harvested in 1 ml trypsin-versene solution and counted with a Coulter Counter as described above.

Cells in culture grow exponentially to high cell densities. The growth was expressed relative to the cell number in control cultures after 96 h incubation (% Control cell number, %C in figs 3, et seq.). Controls increased 8 to 12 fold over 96 h, except for the foetal lung cells, which only tripled over 108 h.

(d) Growth assay by radioactive deoxythymidine uptake into DNA

Treated and control HeLa cells were incubated for a fixed time interval and then pulsed for 0 - 4.5 h with 5 or 10 μc [6-^{3}H] dThd tracer (20 curies/m mole; Radiochemical Centre), in medium made 0.0, 0.5, 1, 2, 5 or 10 x 10^{-6}M dThd. Incorporation of tracer into DNA was found by assaying cellular material which is insoluble in cold acid and ethanol and has DNase sensitvity (Adams, 1969a).

Following incubation with [^{3}H]dThd, the excess [^{3}H]dThd was removed by washing the HeLa cells with 3 x 3 ml cold Earle's saline balanced salt solution without detaching them from the dish. The radioactivity now soluble in ice cold 5% TCA was confirmed experimentally to be largely [^{3}H]dTTP, and this TCA soluble pool (AS pool) attains an equilibrium concentration about 10 min after incubation with [^{3}H]dThd (Adams, 1969a; Cleaver, 1967). Aliquots of the cold AS pool were removed into clean potassium free radioactivity counting vials, (Packard Instrument Ltd., Wembley, Middlesex). The cells were then washed three times with 2 or 3 ml ice cold TCA, once with ethanol, dried, dissolved in 0.3N NaOH, and an aliquot removed into vials as for the AS pool sample. The vials were incubated at 60°C until dry and 0.5 ml of 1.0 M hyamine in methanol (Nuclear Enterprises Ltd.) followed by 10 ml of toluene (scintillation grade) in which was dissolved 5 g/l PPO and 0.2 g/l dimethyl POPOP (Nuclear Enterprises Ltd.) was added prior to counting on a Packard Tricarb radioactive counter.

The results were expressed as counts/min (cpm) disintegrations per min (dpm), or p moles per 10^{5} cells. Cpm or dpm were converted into the percent dpm or cpm observed in control

cultures after the subtraction of blanks (control cultures exposed for less than 10 sec to tritiated thymidine).

In those experiments in which changes in the rate of DNA synthesis after 24 h PE treatment were measured, the number of cells in control cultures at 24 h was twice that at 0 h. The number of cells in treated cultures had either not increased, or had increased less than in control cultures. From a plot of growth to PE dose at 96 h (such as Fig 4; see RESULTS, section 1c) the y axis value from the data extrapolated to zero PE was taken as 100% control cell number instead of the growth of control cultures. The growth of treated cultures at 96 h was recalculated as a fraction of this extrapolated value. Considering the recalculated fraction as now being a fraction of the cell number in control cultures at 24 h, an estimate could be made of the growth in cultures after 24 h PE treatment.

(11) Iso-electric focusing in polyacrylamide gels

Iso-electric focusing is a technique which separates proteins as a function of their iso-electric point on a pH gradient. Proteins in a mixture become focused to form sharp bands at their iso-electric pH (Vesterberg and Svensson, 1966). The method has high resolving power and will fractionate proteins which differ in their iso-electric points by only 0.02 pH units. The method used was almost exactly as described by Beeley (1969). Iso-electric focusing was performed in 12 cm long 3 mm internal diameter glass tubes, marked 1 cm and 7 cm from the cathode end. The ampholine-acrylamide monomer solution consisted of a mixture of the following aqueous solutions:

1 ml 28% w/v acrylamide containing 0.735% v/v N,N' methylene bisacrylamide; 1 ml 40% sucrose; 0.1 ml 40% ampholine of pH range 3 - 10; 0.25 ml 1.6% w/v N,N,N'N' tetramethylethylene-diamine, and 0.5 ml 0.004% w/v riboflavin photopolymeriser catalyst. This solution was kept away from bright light until ready for polymerising. The monomer solution was mixed with 0.2 ml salt free protein sample containing 0.4 mg protein to a final volume of 0.5 ml.

With the cathode end downwards, the tube was filled to the 1 cm mark with 40% w/v sucrose, the sample monomer mixture added to the 7 cm mark, the tube topped up with distilled water, and the gel photopolymerised. After 1 h, the sucrose and water layers were removed and the tubes placed between two reservoirs. The anode reservoir was filled with 250 ml of 1.4% v/v orthophosphoric acid and the cathode with 2% v/v ethan-olamine. A potential of 100 V was applied to the gels for 3.5 h, and increased to 200 V for the subsequent 0.5 h to sharpen the bands. The gels were removed from the tubes by water pressure, marked at the cathode end, the ampholine removed, and the proteins precipitated by stirring the gels in 2 changes of TCA for a total of 36 h. The gels were stained for 1 h in 1% w/v Lissamine Green SF in 7% w/v acetic acid and destained with several changes of 7% acetic acid. The gels were stored indef-initely in 7% TCA at 4°C in test tubes. In addition to visual examination, the gels were scanned with a Joyce Loebl Chromoscan Mark II (Joyce Loebl & Co. Ltd., Princes Way, Team Valley, Gateshead 11).

Samples of PE and SE for analysis were dialysed as described above and the non-diffusible fractions concentrated by lyophilization. The SS fraction for analysis was reduced to about 5% its original volume by ultrafiltration through an Amicon UM 10 filter as described above (section 5a). Prior to mixing with the gel monomer, the protein content of each sample was estimated after any precipitate in the sample had been removed by centrifugation and discarded.

(12) Immuno-electrophoresis

This was performed using the Shandon Universal Electrophoresis Mark 2 Apparatus (Shandon Scientific Co. Ltd., London, N.W.10). The procedure as described by the manufacturer (Shandon Publications, Nos. SAE 01-366, and SAE 05-1068) was followed. At a suitable pH, proteins in a sample applied to a well in agar gel were separated electrophoretically. Standard antiserum was then added to a central trough and diffused from it to form precipitin arcs on contacting the antigen. Antigens are detected more easily if they are applied in a small volume after the sample has been suitably concentrated.

PE, SE, and SS salt free samples were prepared by dialysis or Amicon filtration as described for iso-electric focusing (section 11). The non-diffusible fractions were assayed for protein (section 9b), and lyophilised to dryness. Distilled water was added to bring the protein concentration to 50 mg/ml and 5 µl of each concentrated sample were applied to the well. Serum samples were applied to the well without prior dialysis or concentration.

The immunoelectrophoresis was performed in a 1% (w/v) gel of Oxoid Ionagar No. 2 (Oxoid Ltd., London S.W.1) in 0.1 M barbitone-acetate buffer (pH 8.6) diluted 1 in 4. A potential difference of 10 volts/cm was applied for 90 min. Antisera were added to the troughs in the gels and diffusion allowed to occur in a moist atmosphere for 24 - 28 h at room temperature. The gels were then washed in 2 changes of 0.85% (w/v) for 24 h followed by 2 changes of distilled water for 24 h. After drying, the gels were stained either in 0.5% (w/v) Amide Black 10B in methanol:glacial acetic acid (9:1, v/v) and mounted in Barlicot resin.

Rabbit anti-human serum and rabbit anti-human plasma albumin were obtained from Hyland Division, Baxter Laboratories, Thetford, Norfolk. Rabbit antisera to human plasma immunoglobulins A, and G, transferrin, ceruloplasmin, and haptoglobin, were purchased from Nordic Pharmaceuticals Division, Fraburg Ltd.,Furze Platt Road, Maidenhead, Berks. Rabbit anti-human mixed saliva serum was prepared by concentrating fraction SS protein to 10 mg/ml, diluting it 1:1 with Freunds complete adjuvant, and injecting it intramuscularly into Californian male rabbits. After repeating the injections weekly for 5 weeks, the rabbit serum was collected on the 6th week (F.J. McKean and J.A. Beeley, in preparation).

RESULTS

(1) EFFECT OF PLAQUE EXTRACTS ON MAMMALIAN CELLS IN VITRO

(a) Appearance and growth of normal and PE treated HeLa cells

Figs. 1 and 2 are phase contrast photomicrographs of normal and plaque extract treated HeLa cells after 96 h incubation. The untreated cells (fig. 1a) were closely packed. Many more cells appeared to be present in the normal culture as compared to the treated culture (fig. 1b), suggesting that treated cells had grown less well than untreated cells.

The untreated cells had dense nuclei and cytoplasm (fig 2a), whereas many PE treated cells appeared less refractive (fig 2b). The former were polygonal (fig 1a), whereas many of the latter were elongated, and some had threadlike intercellular connections (fig 1b). No giant multinucleate cells were seen (c.f. Baboolal, et al., 1970). Neither treated nor untreated cultures had many cells floating in the medium, although treating HeLa cells with high concentrations of saline did release many cells into the medium as well as prevent growth (section 1c). Extractable material from plaque did not seem to cause a loss of cell adhesiveness *in vitro* but rather to lyse the contents of the cells (fig 2b).

This lysis was slow in appearing. The first stage was seen after a few hours treatment as droplets, possibly lysosomes or phagosomes, giving a spotty appearance to the outer cytoplasm of the most severely affected cells. Gradually these droplets became more extensive and merged together. Eventually, the whole cell became engulfed until only the plasma membrane seemed to have remained. Fig 2b shows these changes in a

1a

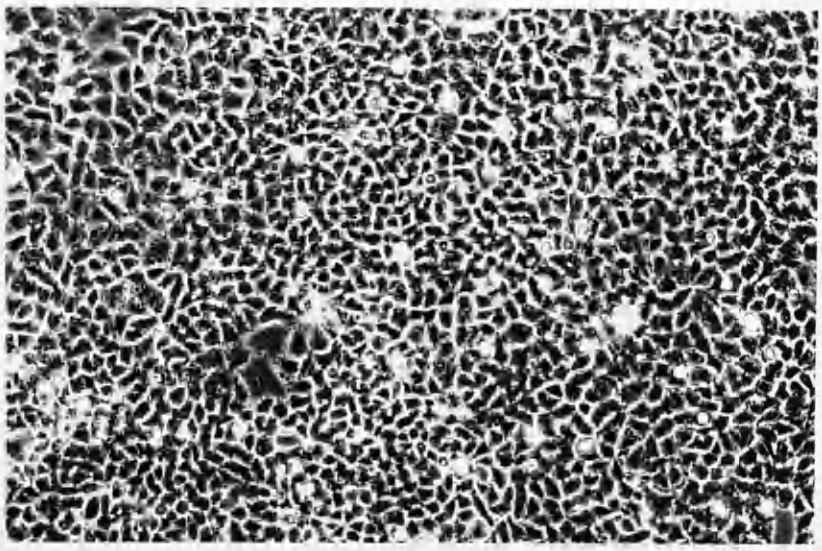


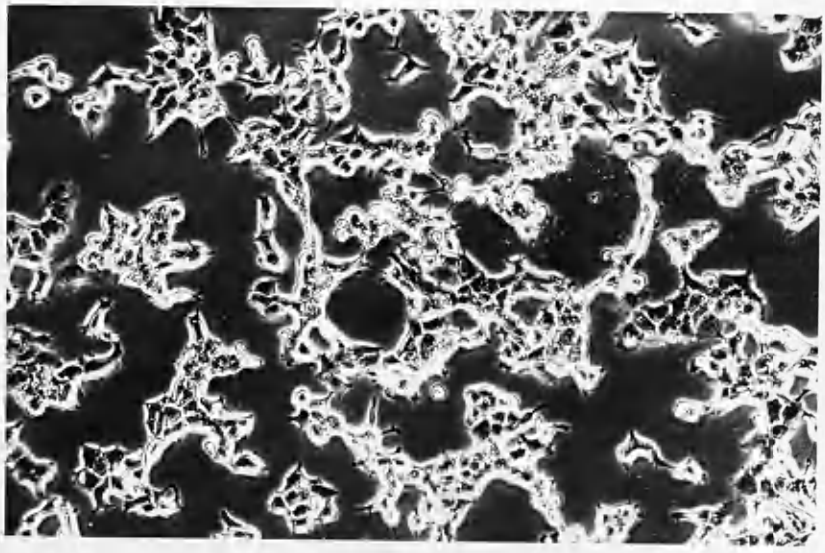
Fig. 1. Phase-contrast microscopic appearance of control and PE treated HeLa cells after 96 h incubation

350 mg wet plaque per ml of carrier saline was homogenised with a Potter homogeniser, centrifuged, and the supernatant fraction (PE) added at a dilution of 1 to 10 in growth medium.

Top: Control cells (treated with carrier saline instead of PE) x 100 (fig 1a).

Bottom: PE treated HeLa cells x 100 (fig 1b).

1b



2a

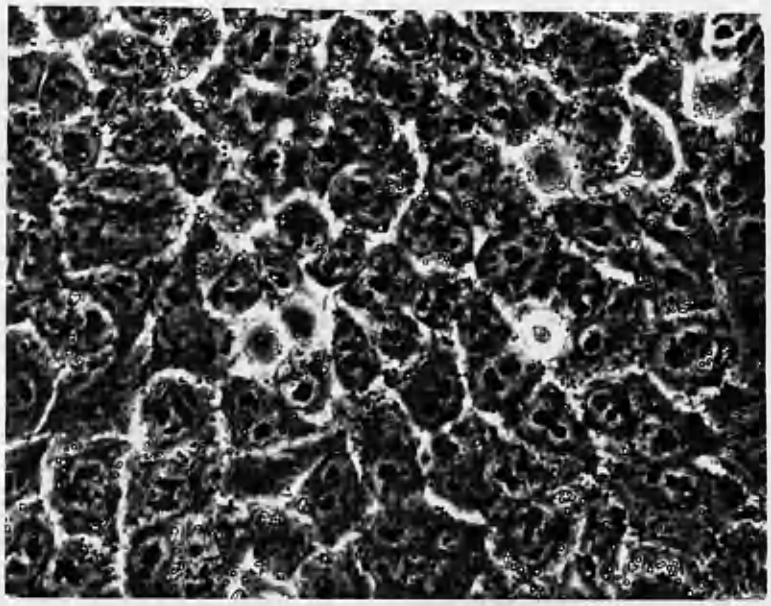


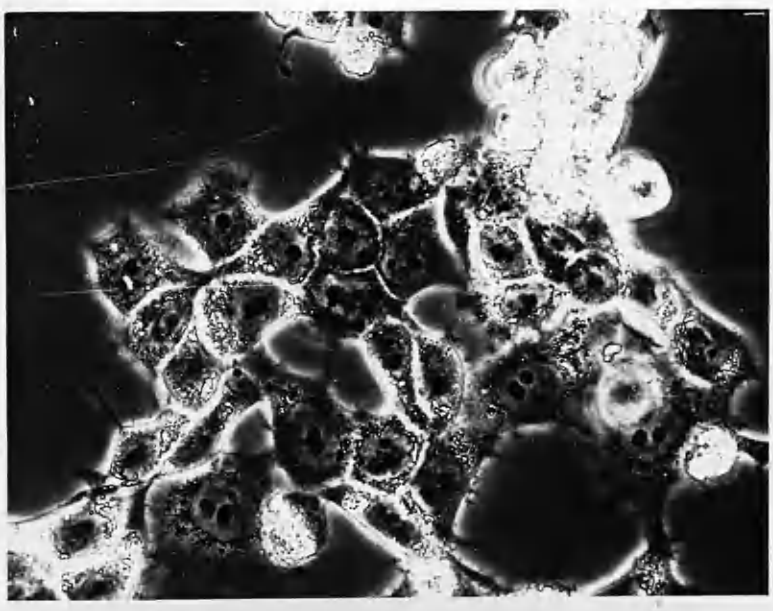
Fig. 2. High power phase-contrast microscopic appearance of control and PE treated HeLa cells after 96 h.

See legend to fig 1.

Top: Control cells x 400 (fig 2a)

Bottom: PE treated cells x 400 (fig 2b).

2b



culture treated with insufficient PE to completely stop cell growth. In such a culture, all the stages of lysis could be observed within 96 h treatment.

Fig. 3 is a plot of growth relative to control cultures at 96 h against time for both treated and untreated cultures. Cells were treated with two different amounts of extracted plaque material. The greater the PE dose, the greater was found to be the inhibition of the growth rate. The rate of growth in untreated cultures (but not in treated cultures) declined after 72 h. Up to this time, untreated cultures doubled every 25 h, 0.30 ml treated cultures every 36 h, and 0.45 ml treated cultures every 58 h.

(b) Measurement of the concentration of extractable material in plaque

The concentration of extractable material in wet plaque was calculated as described in table I. Wet plaque was found to have a mean concentration of 64.3 mg/ml extractable solid. Taking the dry weight as 20% of the wet weight, then 32% $\pm$ 6 (standard deviation) of the dry weight of plaque is extractable solid.

(c) Growth yield in the presence of added plaque material or saline

Two pools of plaque from two groups of 30 donors respectively were both extracted to a ratio of 60 mg wet plaque per ml carrier saline. Increasing amounts of these extracts were added to a large randomised group of HeLa cell cultures and incubated for 96 h. Fig 4 shows that the growth of HeLa cells relative to

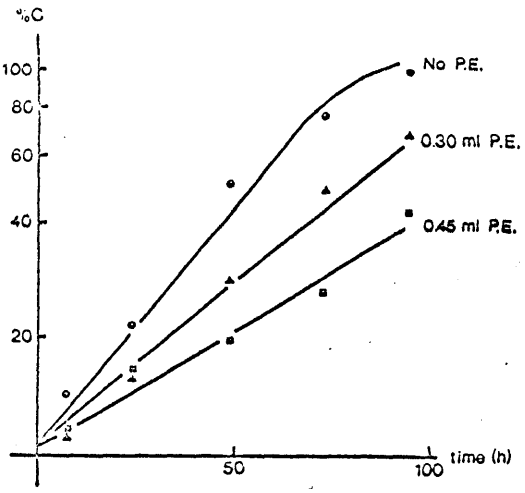


Fig. 3. Growth of treated and control HeLa cell cultures relative to time of incubation

Cells were grown in 3 ml EC_{10} in 5 cm Petri dishes. Control cultures contained 24.9×10^5 cells after 96 h. The number of cells in a culture after any length of incubation was expressed as a percentage of this number (%C), See Methods, 10c. Using a Silverson homogeniser 150 mg wet plaque was extracted per ml carrier saline and the cells treated (Methods, 10b) with carrier saline only (no PE) o—o, 0.30 ml PE ▲—▲, and 0.45 ml PE ■—■.

Table I

Determination of extractable solid concentration in plaque

(1) PE No.	(2) Wet weight plaque added per ml carrier saline	(3) Total solid in PE (mg/ml)	(4) Carrier saline in 1 ml PE (saline blank)	(5) Extracted solid (mg/ml)	(6) Dilution of plaque in PE	(7) Extractable plaque solid (mg/ml)
1	0.06	11.1	7.8	3.3	17.7	58.4
2	0.10	13.5	7.5	6.0	11.0	66.0
2a	0.10	15.0	7.5	7.5	11.0	82.5
3	0.15	14.6	7.2	7.4	7.7	57.0
3a	0.15	15.2	7.2	8.0	7.7	61.6
4	0.15	17.9	7.2	10.7	7.7	82.4
5	0.20	13.9	6.9	7.0	6.0	42.0
					mean	64.3

Notes:-

Col. 1. Assay performed in duplicate on extracts nos. 2 and 3.

Col. 3. Result after lyophilising PE.

Col. 4. Carrier saline in PE (saline blank) was calculated from its total salt concentration, 8.3 mg/ml. As 1 g wet weight plaque had a volume of 1.0 ml. the concentration of carrier saline in PE was $8.3/(1 + y)$ mg/ml where y is the value in column 2.

Col. 5. Col. 3 - Col. 4.

Col. 6. As y g plaque was diluted in $1 + y$ ml PE, the dilution was $(1 + y)/y$.

Col. 7. Col. 5 x Col. 6.

controls decreased as the concentration of plaque material in the culture medium increased. But increasing the concentration of plaque material in the medium could have increased the osmotic pressure and this increase, rather than an increasing amount of toxic material from plaque, might have accounted for the growth decrease observed. Hence in fig 4 is also plotted the growth yield against an increasing concentration of sodium chloride (additional to the 6 mg/ml normally present in the growth medium). This indicates the maximum possible inhibition of growth that osmotic pressure alone might cause.

The amounts of inorganic phosphate, sodium, and potassium ions extractable from wet plaque have been estimated as a percentage of its dry weight (Dawes and Jenkins, 1962). By assuming the phosphate is present as $CaHPO_4$, and sodium and potassium as NaCl, and KCl, respectively, these salts, which probably account for most of the osmotic pressure in PE, were calculated to account only for about 30% of the total extractable solid from plaque.

Growth was completely inhibited when 2.3 mg plaque material was added per ml of growth medium, whereas the osmotic equivalent of 8.5 mg excess sodium chloride was found (by extrapolation) to achieve the same effect. Thus extractable plaque material did not inhibit growth by an osmotic effect but by its containing toxic substances. Fig 4 also shows that control cultures grew less than cultures treated with 0.1 to 0.5 mg extracted plaque material. It appears that extractable plaque material includes both toxic substances, and nutrients which EC_{10} lacks.

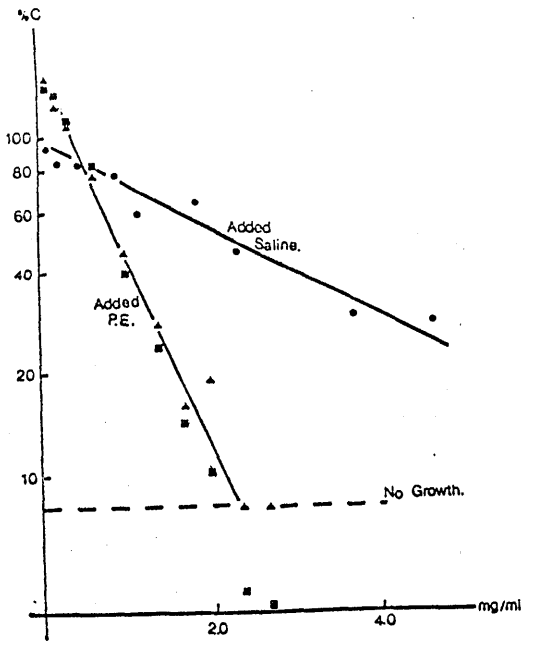


Fig. 4. Growth of HeLa cells relative to increasing amounts of PE or saline added in the culture medium after 96 h treatment

Cells were grown and treated as in Fig. 3. Control cultures (no PE) contained 20.1 x 10^5 cells after 96 h. The % control cell number (%C) was plotted against the amount of extracted plaque material or saline added in the EC_{10} (mg/ml). Plaque from donor group 1 extracted with MSE homogeniser ▲—▲ . Plaque from donor group 2 extracted with Silverson homogeniser ■—■ . Added saline ●—● .

The same concentration of added plaque material from two different groups of donors caused the same change in the cell growth yield. An inverse linear relationship between log growth and dose was observed. Such a relationship is not uncommon in this kind of experiment (Paul, 1970). Analysis showed that both sets of points from the PE treated cultures and the set of points from the saline treated cultures (excluding controls) had linear correlation coefficients greater than 0.9. The probability, P, that any of these regression lines was not straight was less than 1×10^{-4}. The toxic effect of plaque preparations from 19 different groups of patients was assayed 24 different times, some extracts being assayed more than once. The smallest group had about 20 donors and the largest 140 donors. The probability, P, that no linear correlation existed between growth and dose was less than 0.04 for all 24 assays and less than 0.01 in 19 of these estimations. (Each assay was from 3,4 or 5 different concentrations of plaque material in the growth medium). No growth occurred when 2.23 mg/ml $\pm$ 0.57 (standard deviation) extracted plaque material was present in the culture medium.

Table II shows that after plaque had been extracted with the MSE or Silverson homogeniser, similar concentrations of the extracted material in the culture medium prevented growth. After plaque had been extracted with the Potter pestle however, a slightly lower concentration of extracted material sufficed to prevent growth. Only the difference between the Potter and Silverson extracted plaque was statistically significant ($P < 0.05$; 't' test). As this difference between the means

Table II

Mean concentration of plaque material required to prevent growth[1]

Plaque extracted with MSE homogeniser (3 samples) mg/ml	Plaque extracted with Silverson homogeniser (19 samples) mg/ml	Plaque extracted with Potter homogeniser (2 samples) mg/ml
2.3 ± 0.29[2]	2.25 ± 0.62	1.80 ± 0.19

Notes:- (1) It was assumed that 1 g wet plaque contained 64.3 mg extractable solid, and occupied a volume of 1 ml.

(2) Standard deviation.

is small however, and as only 2 Potter preparations were assayed in this way, it can be argued that the statistics mean very little (see section 3).

Extrapolating the growth of treated cultures to zero mg/ml extracted plaque solid in the medium gave the number of cells in a culture containing only the nutrient from PE. Such a culture was estimated to contain 125% (94% to 166%) the cell number in control cultures (log % control cell number being 2.096 $\pm$ 0.125, standard deviation). In only 3 of the 19 different preparations was no increase relative to controls noted on extrapolating the data.

(d) Effect of PE on the growth of other cell cultures

As already stated, there is an inverse linear relationship between dose of plaque material and the log growth of HeLa cells (fig. 4). However, when the results obtained with L 929 fibroblasts are plotted in a similar manner, a straight line is not obtained (fig. 5a). At concentrations below 1.2 mg extracted plaque material per ml growth medium, L 929 cells were less affected by the toxic material than were HeLa cells, whereas at concentrations above 1.2 mg/ml neither cell line grew.

Fig. 5b shows that BHK 21 C 13 cells responded to increasing concentrations of plaque material like L 929 cells rather than like HeLa cells. The C 13 dose response curve appeared sigmoid. Low doses of extracted material markedly increased the growth yield relative to controls and the yield was also much greater than that of HeLa cells treated simultaneously with the same concentrations of the same extract. On the other hand, doses of more than 1.0 mg/ml caused slightly less growth than HeLa cells.

Fig. 5c shows that both human foetal lung cells and HeLa cells responded almost identically to the presence of plaque material in the medium.

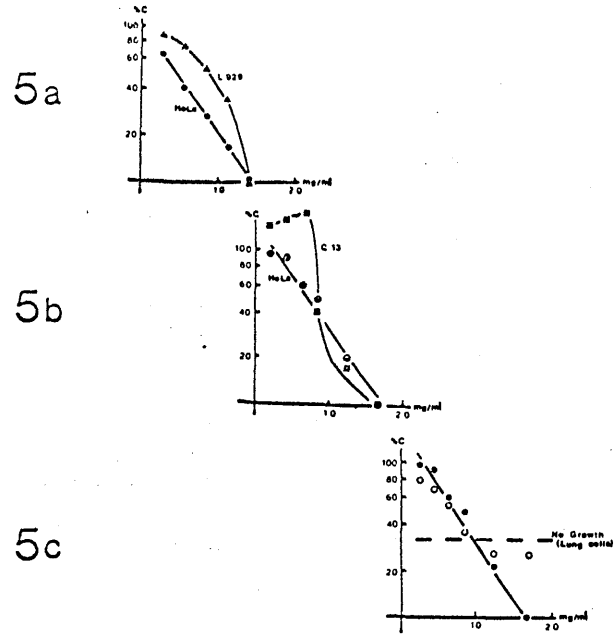


Fig. 5. Relative growth of different cell types in the presence of extracted plaque material

Cells increased more than 10-fold in number over the 96 h incubation period when plaque material was absent, except for human foetal lung cells which only increased 3-fold over 108 h. All cells were grown in 3 cm diameter Petri dishes in 1.5 ml EC_{10}. The x and y axes are as in Fig. 4.

Top: (Fig.5a) HeLa cells. 100% growth was 10.6 x 10^5 cells ●—●.
L 929 cells. 100% growth was 11.9 x 10^5 cells ▲—▲.

Middle: (Fig.5b) HeLa cells ●—●.
BHK 21 C 13 cells. 100% growth was 7.5 x 10^5 cells ■—■.

Bottom: (Fig.5c) HeLa cells ●—●.
Human foetal lung cells (4th passage after primary cell culture) 100% growth was 4.9 x 10^5 cells o—o.

More than 1 mg/ml extracted plaque material in the culture medium therefore inhibited the growth of different types of mammalian cells in culture, although smaller concentrations caused different growth responses in cells derived from different species.

(e) Growth inhibiting material in plaque and saliva

The results of the experiments described above demonstrate that the extractable plaque material is toxic to mammalian cells *in vitro* and therefore that substances in plaque may have a direct toxic effect on the adjacent gingival tissues. If so, the toxic substances may also diffuse from plaque into the saliva. Saliva should therefore inhibit the growth of HeLa cells.

Fig 6 is a plot of the growth of HeLa cells against the dilution of water soluble material extracted from plaque (PE) or in saliva supernatant fraction (SS) and present in the growth medium. Saliva supernatant fraction diluted less than 1 to 1.2 prevented growth, whereas even cultures treated with the extractable plaque material diluted 1 to 25 prevented growth. Cultures treated with the same dilution of saliva supernatant fraction increased 10 fold as in controls. Not until cultures were treated with more than a 1 in 100 dilution of extractable plaque material did their growth approach that of controls (fig 6). Substances in saliva thus inhibited the growth of HeLa cells, but plaque was richer in its content of toxic material. The toxic substances in plaque may therefore diffuse into saliva or into the gingival tissues.

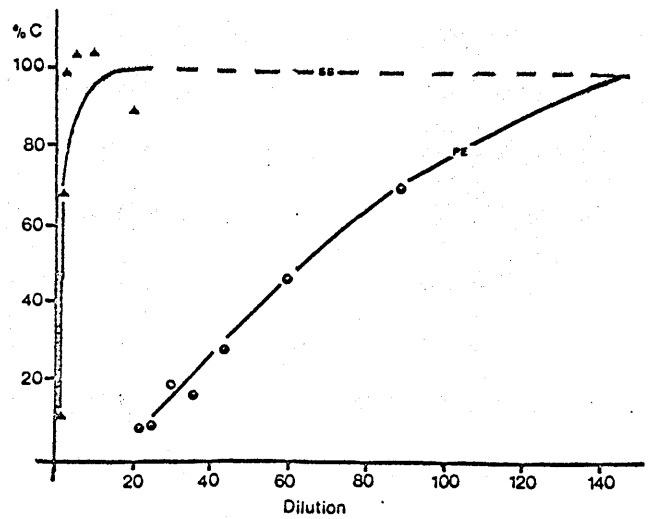


Fig. 6. Growth of HeLa cells treated with plaque and salivary material

The growth of HeLa cell cultures (%C) was plotted against the dilution of material extracted from plaque in the medium. This is data from the experiment in fig. 4 for plaque extracted with an MSE homogeniser o — o. Saliva supernatant fraction (SS) was millipore filtered, and used to dilute a concentrated solution of EC_{10}. The growth (%C) was plotted against the dilution of SS in the medium ▲ — ▲.

(f) Concentration of protein, carbohydrate, and 260 nm absorbing material in whole mouth plaque pools from different individuals, and groups of individuals

In the above experiments, plaque from a large number of different donors was pooled prior to its extraction and perhaps not all the plaque from one donor contained toxic material. Unfortunately, the amount of plaque from each donor was too little to extract and assay for toxic material with the available techniques, although the protein and carbohydrate content of such extracts was determined. In addition, plaque extracts all contained a large 260 nm absorbance peak and were assayed for their concentration of 260 nm absorbing material as well. Table III shows that the extractable fraction of plaque from different donors and from different groups of donors appeared to contain similar amounts of protein, carbohydrate, and 260 nm absorbing material per g wet weight. It is therefore reasonable to assume that all the individual donors' plaque samples contained similar amounts of toxic material per g wet weight.

(g) Summary and conclusions

On average, about 2 mg/ml extracted plaque material in the growth medium prevent any increase in cell number over 96 h treatment. This material lysed the cell contents *in situ*, without causing the cells to detach from the dish. This lysis *in vitro* is reminiscent of Schroeder's description (Introd. 2b), that the junctional epithelial cells at the base of the sulcus disintegrate during early inflammation *in vivo*.

Only cells from human tissue gave a good linear relationship between cell number after 96 h and the dose of plaque material.

Table III

Composition of extracted fraction of plaque from individual mouths, and from groups of individual mouths

Sample	Samples assayed	E_{260}[1] (Absorbance/ ml wet plaque)	Protein mg/ml wet plaque	Carbohydrate[2] mg/ml wet plaque
Grouped whole mouth plaque (Pooled plaque)	9 groups of 10 to 80 individuals	48.8 ± 7.7[3]	6.4 ± 1.6	6.9 ± 2.0[4]
Individual whole mouth plaque	16 individuals	57.9 ± 18.5	6.5 ± 1.7	5.4 ± 1.6[5]

Notes:- (1) See Methods, 9a

(2) Assayed by the method of Dubois, et al (1956)

(3) Standard deviation

(4) 5 groups

(5) 14 individuals

The effect of PE on the growth of mammalian cells *in vitro* could not be accounted for simply by the osmotic effect of its extractable material. Per ml volume, about 20 times more toxic material was extracted from plaque than was found in saliva supernatant.

No major differences in the amount of material extracted from plaque from different individuals were found relative to that from plaque pooled from different groups of individuals, and this implies that toxic material could well have been present in all the individual plaque samples.

The conclusion is that plaque micro-organisms seem to be a source of cytotoxic material. This material appears to be present in constant amounts per *g* wet weight of plaque, and apparently causes a slow disintegration or lysis of epithelial-like cells both *in vitro* and *in vivo*.

(2) COMPARATIVE STUDIES ON THE BIOCHEMICAL COMPOSITION OF EXTRACTED PLAQUE MATERIAL, SALIVA, AND SERUM.

(a) Concentration of protein, carbohydrate, and 260 nm absorbing material in the extractable fraction of plaque and in saliva.

The extractable fraction of plaque contained about 20 times more toxic material than was present in saliva (section 1e). Table IV shows that the extractable fraction of plaque had a greater concentration of protein, carbohydrate, and 260 nm absorbing material than had either the extractable fraction of the saliva sediment, or the saliva supernatant fluid. Similarly, the concentration of these extractable substances in the saliva sediment fraction, was greater than in the saliva supernatant fluid but less than was extractable from plaque.

The mean concentration of material in the plaque extract, and in both saliva preparations, was found to have a large variation. However, in all preparations the concentration of protein correlated well with the concentration of carbohydrate or 260 nm absorbing material ($P < 0.04$). This was particularly true for the saliva supernatant and extractable plaque fractions ($P < 0.007$). It therefore appears that different individuals have a large variation in the water content of plaque and saliva samples. This variation (as reflected by the standard deviation values in table IV) suggests that the water content of saliva may be almost twice as variable as that of plaque.

It is possible that the extractable plaque material absorbing at 260 nm came largely from the nucleic acids of

Table IV

Comparison of the extractable fractions of plaque and saliva sediment with each other, and with saliva supernatant fraction

	E_{260}[1] (absorbance/ml wet plaque)	Protein (mg/ml)	Carbohydrate (mg/ml)
Saliva supernatant fraction (n = 17)[2]	2.29 ± 61%[3]	1.13 ± 40%	0.21 ± 48%
[4]Saliva sediment (n = 3)	7.51 ± 27%	3.29 ± 32%	not assayed
[5]Plaque (n = 25)	54.6 ± 29%	6.38 ± 24%	5.80 ± 31%[6]

Notes:- (1) See Methods, 9a.

(2) n = number of samples assayed.

(3) Standard deviation as % of the mean.

(4) The dilution of extracted saliva sediment (Silverson homogeniser) was calculated as described below for plaque.

(5) The dilution of extracted plaque fraction (Potter homogeniser) was calculated from the wet weight plaque to saline ratio used in making the PE. 1 g. wet weight of plaque was assumed to contain 1 ml of water plus extractable solid (Methods 3 and Table I). The results are the means from all plaque samples assayed in Table III.

(6) n = 19.

necrotic bacteria (section 2b i, and fig. 7). Most 260 nm absorbing material appeared to be diffusible, and could have been ribonucleoside, or free nucleic acid bases (section 2b, ii). It also appeared that the 260 nm absorbance due to protein in PE was insignificant (fig 7). Assuming that the 260 nm absorbing material was composed of equimolar concentrations of the 4 most commonly found free bases and ribonucleosides, and taking their respective molar absorbances in acid (Calbiochem, 1964), as the pH of PE was about 5.5, it was calculated that the E_{260} of extractable plaque material corresponded to about 1.2 mg/ml free base plus ribonucleoside mixture. Hence, from table IV, the concentration of extractable protein plus carbohydrate plus 260 nm absorbing material in plaque was 13.4 mg/ml. The extractable inorganic salt concentration in plaque was 20 mg/ml (section 1c). Altogether, these substances account for about 50% of the total solid extractable from 1 g wet plaque (section 1b).

Table IV shows that the ratio of protein concentration to E_{260} was about 0.5 in both saliva preparations, but only about 0.1 in the extractable plaque fraction. Likewise, the ratio of protein to carbohydrate was 0.2 in the saliva supernatant fraction, but 1.0 in the plaque preparation. However, the ratio of carbohydrate concentration to E_{260} in the saliva supernatant was similar to that in the plaque preparation. But on the whole, both the quantity and relative proportion of extractable substances in plaque appeared different from those in the saliva preparations, and further investigation of the 260 nm absorbing material and protein from plaque and saliva was carried out (sections 2b and 2c).

(b) Ultraviolet absorbing substances

(i) Ultraviolet spectra

The results illustrated in figs 7a, 7b and 7c were typical findings after examination of 3 saliva sediment, 4 saliva supernatant, and 6 pooled plaque preparations. Each spectrum was distinct and reproducible. Most ultraviolet absorption was due to the nature and amount of diffusible material in each preparation.

Uric acid has an absorption peak at 285 nm in acid, and 292 nm in alkali (Dawson, et al., 1969). Uric acid has been shown to be a normal constituent of saliva (Hawkins, et al., 1963), and may therefore have been responsible for the spectral peak observed at about 285 nm in whole saliva, and 290 nm in its diffusible fraction.

The base content of nucleic acid material has a spectral absorption peak in the region of 260 nm. PE may therefore have contained nucleic acid material from autolysis of some bacteria in plaque *in vivo*. Most of this material appears to be in the form of low molecular weight derivatives.

(ii) Elution profile of diffusible material absorbing at 260 nm from PE and SE.

The diffusible fractions obtained by dialysis of PE and SE were concentrated and 0.15 ml of each was applied to a column of G 10 Sephadex. The column was eluted with distilled water.

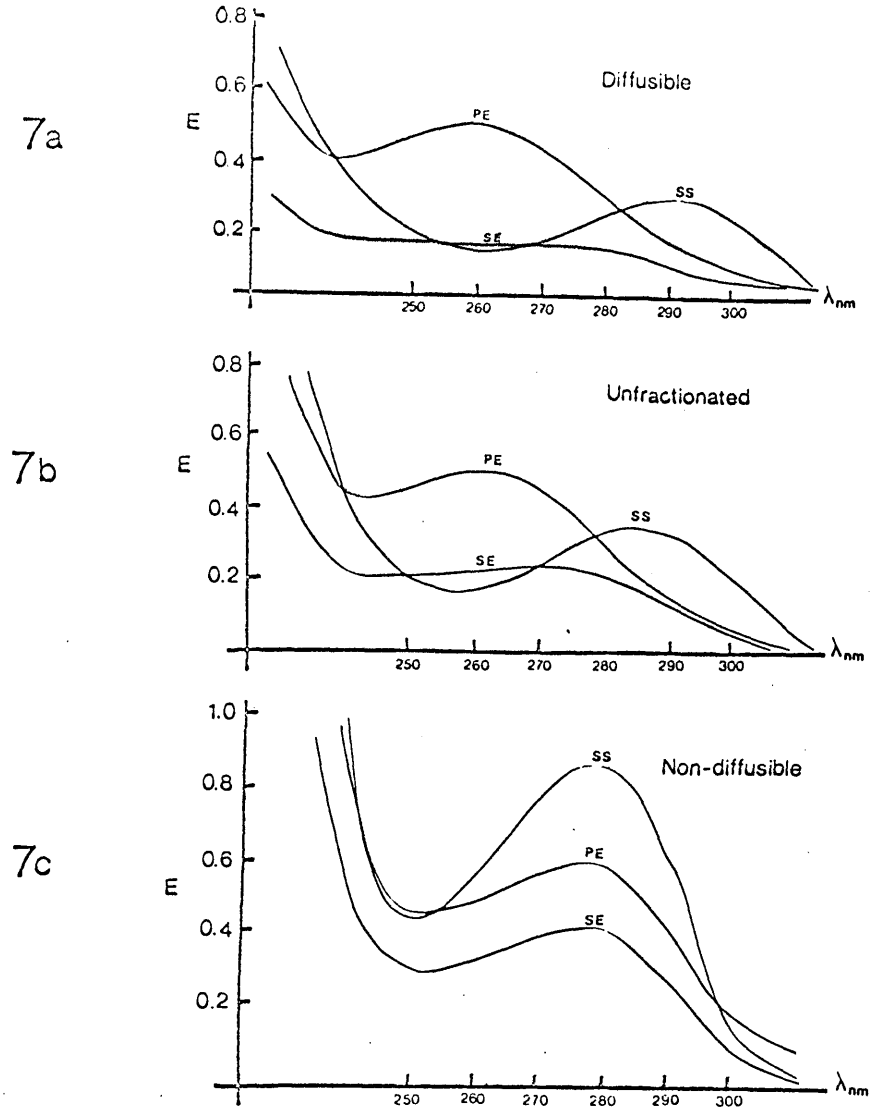


Fig. 7. Ultraviolet spectra of plaque extract (PE) saliva sediment extract (SE) and saliva supernatant fraction (SS)

Extinction (E) was plotted against wavelength in nanometers (λ nm). Plaque and saliva were obtained, and extracts prepared with a Silverson homogeniser (Methods, 1 and 2). 150 mg wet plaque or saliva sediment were homogenised per ml of carrier saline. The 3 preparations were dialysed against distilled water (Methods, 4).

Top: Diffusible fractions; diluted 1 in 10 with carrier saline.

Middle: Unfractionated preparations; diluted 1 in 10 with carrier saline.

Bottom: Non-diffusible fractions; not diluted.

Fig 8 shows that the 260 nm absorbance pattern from each extract was distinctive. The PE peaks were numbered as indicated. The excluded peak (V_o), was fraction 12, and the ratio of V_e (eluted volume) to V_o for the peaks 2, 3, and 4, corresponded to the ratios obtained from similar peaks after the same fraction of PE had been eluted with carrier saline (section 5c, table XI), although, in that event, the relative heights of peaks 2 and 3 were reversed (c.f. section 5c, figs 34 or 35).

Plaque material in peaks 2,3, and 4, absorbed maximally at 260 to 270 nm, 258 nm, and 250 nm respectively (fig 9,top).Peak 1 had no absorption maximum and is not illustrated. TMP and adenosine standard solutions were excluded relative to peak 2, whereas thymidine was eluted in peak 2 (section 5c). These observations suggest that few nucleoside derivatives in these preparations can have been phosphorylated. Ultraviolet scans of material in peaks a and b from saliva sediment showed that these had no absorbance maxima and are not illustrated. Peaks c and d absorbed maximally at 260 nm and 290 nm respectively (fig 9, bottom). Peak c was eluted in the same position as plaque peak 2 whereas peak d did not coincide with the position of any plaque peak. From its spectrum and V_e, peak d could have contained uric acid.

It was concluded that diffusible ultraviolet absorbing material extracted from plaque was mostly different from that extracted from the saliva sediment. Material which might have been salivary uric acid was detected in the sediment extract but not in the plaque extract.

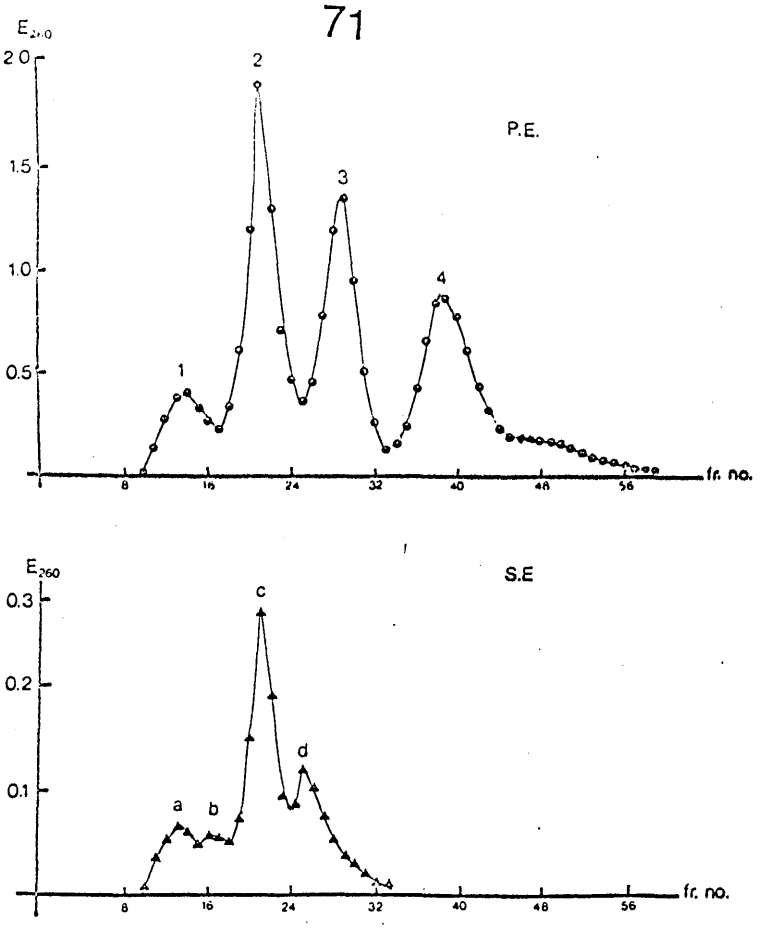


Fig. 8. Fractionation of the 260 nm absorbing material in the diffusible fractions of plaque and saliva sediment extracts

Absorbance at 260 nm (E_{260}) was plotted against fraction number. (Each fraction was diluted 1 to 1.7 with carrier saline prior to reading its absorbance at 260 nm). 3.0 ml of PE, prepared by homogenising 350 mg wet plaque per ml carrier saline with a Potter homogeniser, was lyophilised, and redissolved in 0.15 ml distilled water, applied to a 22 x 0.9 cm Sephadex G 10 column, and eluted with distilled water. The diffusible fraction of SE, prepared as described for Fig. 7, was lyophilised to dryness and redissolved to 1/70 of its original volume of water. The mixture was centrifuged, and the sediment discarded. 0.15 ml of the supernatant fraction was added to the same G 10 column, and eluted as for plaque.

Top: Plot of E_{260} against fraction number for PE diffusate (Fig. 8a) o—o.

Bottom: Plot of E_{260} against fraction number for SE diffusate (Fig. 8b) ▲—▲.

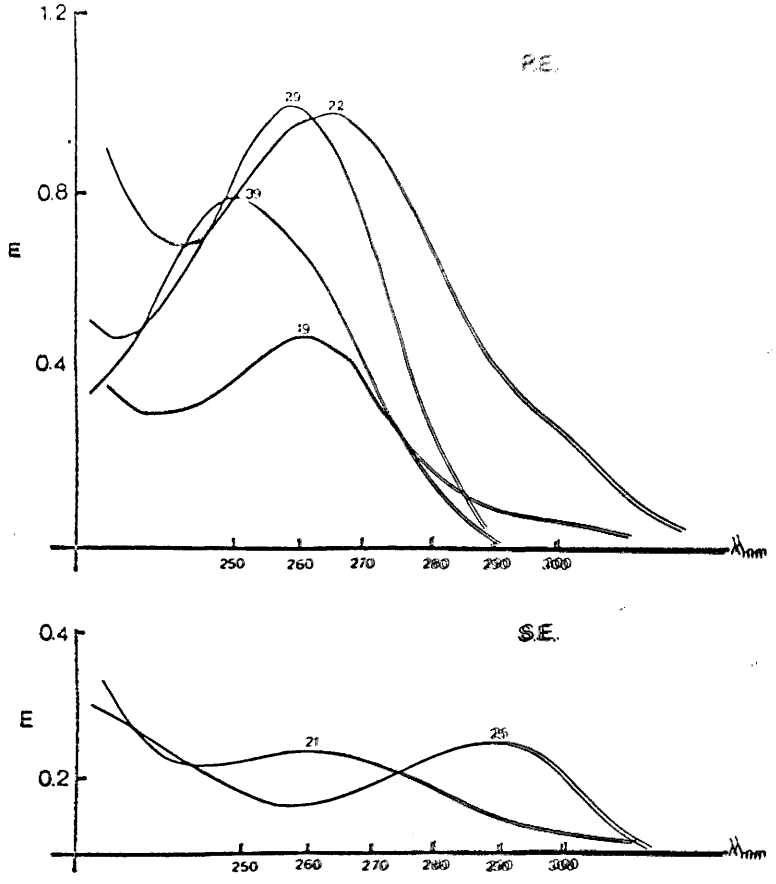


Fig. 9. Ultraviolet spectra of fractions from peaks in Fig. 8

Extinction (E) was plotted against wavelength in nanometers (λ).

Top: SE diffusate. Fractions nos. 19 and 22 from peak 2 in Fig. 8a.

Fraction no. 29 from peak 3 in Fig. 8a.

Fraction no. 39 from peak 4 in Fig. 8a.

Bottom: SE diffusate. Fraction no. 21 from peak c in Fig. 8b.

Fraction no. 25 from peak d in Fig. 8b.

(c) Proteins

The spectra of the non-diffusible fractions from all 3 preparations had absorption peaks around 280 nm (fig 7). This suggests that the major constituent of these fractions was protein. The plaque absorbance peak (275 nm) was slightly lower than the absorption maximum from the saliva preparations (279 nm). It seems that non-diffusible material which absorbed in the ultra-violet and was extracted from plaque may have differed from that obtained from saliva.

(i) Iso-electric pH separation of proteins on polyacrylimide gel

Fig 10a shows the appearance of lissamine green stained proteins from plaque extract (PE), saliva sediment extract (SE), saliva supernatant fraction (SS), and serum, after iso-electric focusing on polyacrylamide gel in a gradient of pH 3 to 10. Three of 6 major protein bands in SS correspond to bands in SE, and similarities in the position of minor bands are also apparent. The protein pattern from fraction SS after iso-electric focusing on polyacrylamide has been described in detail (Beeley, 1969). By contrast, PE has only a single major band which is always observed when this preparation is examined (4 different plaque pools).

Fig 10b is a densitometric tracing of all the lissamine green stained gels (omitting the overloaded PE gel) and shows more clearly the similarity in protein composition of the two saliva preparations. The tracings also show that only one major band is present in the plaque preparation. This band appears not to coincide with bands in either saliva preparation. The

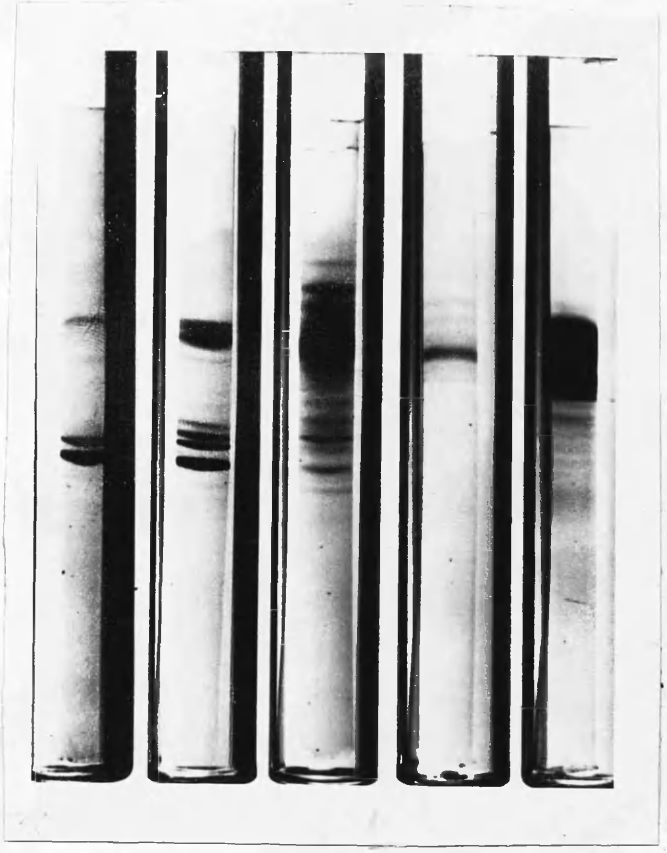


Fig. 10a.

Photographs of lissamine green stained gels.

(left to right: SE, SS, PE, PE 1/10, serum).

Fig. 10. Pattern of proteins in plaque, saliva, and serum preparations after iso-electric focusing on polyacrylamide gels over pH range 3 - 10

Plaque extract, PE. Saliva supernatant fraction, SS. Saliva sediment extract, SE. PE 1/10 is 0.04 mg extracted plaque protein instead of 0.4 mg. (Methods, 11).

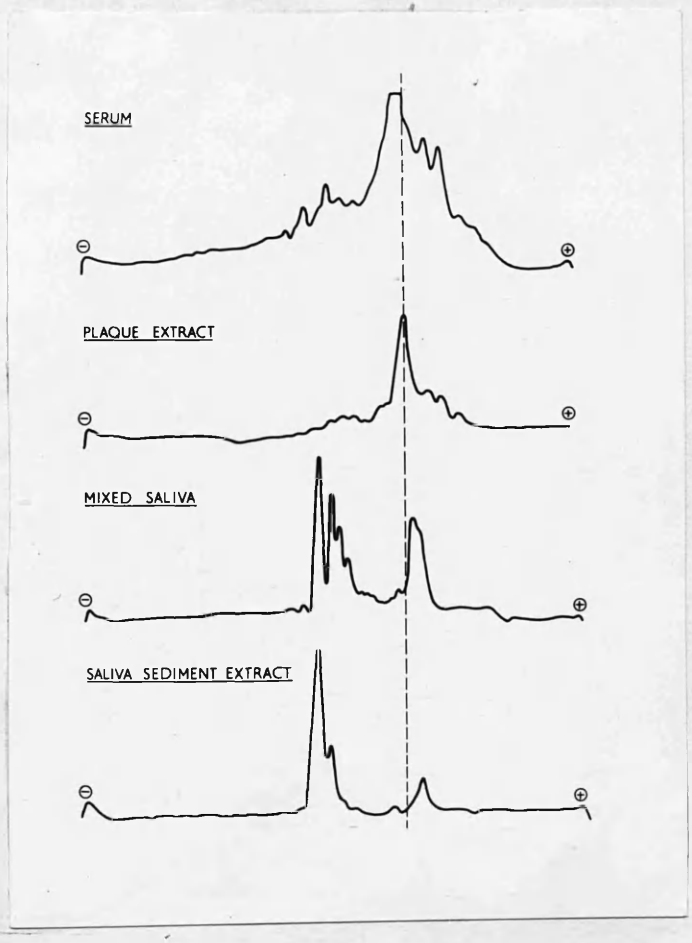


Fig. 10b.

Densitometric tracings of 4 of the gels pictured in Fig. 10a, only the more dilute PE treated gel being recorded.

protein pattern from serum is distinctive, and the heavily stained bands observed correspond to its albumin fraction (Dale and Latner, 1968). It is possible that the major plaque protein is also serum albumin.

(ii) Immuno-electrophoretic separation of proteins

Fig 11a shows immuno-electrophoretic slides of each of the 4 preparations using rabbit antiserum to human normal serum (anti-HNS) in the central trough to detect the proteins as precipitin arcs after electrophoresis of the sample. A number of proteins were detected in human normal serum (serum) by this technique. Serum proteins were also detected in the plaque preparation, and these were identified as including albumin, IgA, IgG, and transferrin. Ceruloplasmin and haptoglobin appeared to be absent. Albumin, transferrin, and IgG have been shown to be constituents of the gingival fluid exudate (Brill and Brönnestam, 1960). No serum proteins were found in the saliva sediment preparation. The saliva supernatant fraction contained two precipitin arcs, of which the heavily stained one was identified as albumin, and the weak one as IgA.

Fig 11b shows the same preparations examined by using rabbit anti-serum to saliva supernatant fraction (anti-SS) instead of anti-HNS to detect protein antigens. Only 5 antigens were clearly detected in the saliva supernatant preparation, of which 2 were possibly in the sediment preparation, but none in the plaque preparation.

Figs 11a and 11b show that the saliva supernatant fraction contained material in the albumin region which did not produce

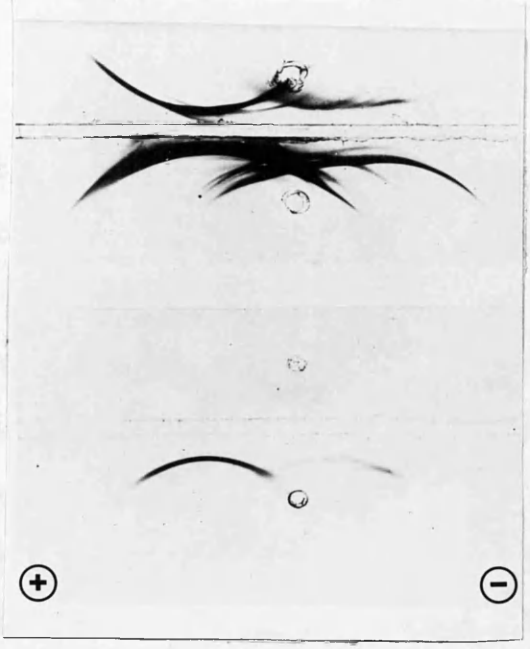


Fig. 11a.
Immunoelectrophoretic patterns with anti-HNS in troughs.
From top to bottom wells contain PE, serum, SE, and SS.

Fig. 11. Immunoelectrophoretic pattern of precipitin arcs produced by plaque, saliva, and serum preparations to rabbit antisera to human normal serum and human saliva supernatant fraction respectively

Rabbit anti-human normal serum, Anti-HNS. Rabbit anti-human saliva supernatant fraction, Anti-SS. Plaque extract, PE. Saliva supernatant fraction, SS. Saliva sediment extract, SE. (See Methods, 12).

Top: Immunoelectrophoretic patterns with Anti-HNS in the trough.

Bottom: Immunoelectrophoretic patterns with Anti-SS in the trough.

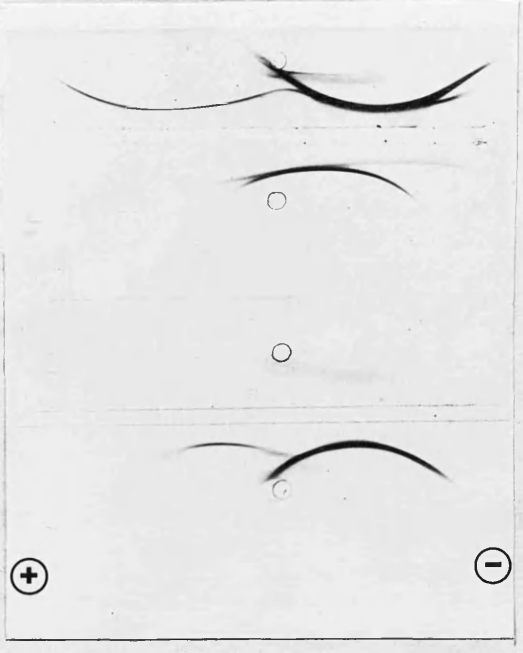


Fig. 11b.
Immunoelectrophoretic patterns with anti-SS in troughs.
From top to bottom wells contain SS, serum, PE and SE.

precipitin arcs against anti-SS, only against anti-HNS. This observation (from 3 different SS preparations, and two different anti-SS preparations) led to the finding that although saliva contained albumin, the arc in the albumin region between SS and anti-SS did not in fact result from albumin (F.J. McKean, and J.A. Beeley, In preparation). Therefore the absence of an arc in the albumin region when anti-SS is run against serum or PE, is due to the absence of anti-albumin in anti-SS. Why the albumin in SS is not antigenic is unknown.

Fig 11b shows that when serum was run against anti-SS, arcs in the IgA and IgG regions were observed. Although anti-SS did not contain anti-albumin, it was found to contain anti-IgA (F.J. McKean and J.A. Beeley, Personal communication). This observation may therefore account for the arc in the IgA region against serum in fig 11b. However, although PE was found to contain IgA when run against both anti-HNS and anti-IgA, no arc was observed when the same PE preparation was run against anti-SS. The reason for this is not known.

Despite these problems, figs 11a and 11b quite strongly suggest that the extractable plaque proteins came largely from serum, and not saliva.

(iii) Bound sialic acid content

Perhaps the most characteristic property of saliva, its viscous frothy appearance, is caused by its glycoprotein (mucin) content. Glycoproteins consist of a folded polypeptide chain to which short carbohydrate side-chains are attached. These side-chains may be branched, and frequently terminate in a

sialic acid residue, giving the molecule a strong negative charge at physiological pH. Sialic acid is responsible for the specific characteristics of glycoproteins (Gottschalk, 1966). The terminal sialic acid residues can be hydrolysed from the protein by a specific enzyme, neuraminidase, which has been shown to be present in saliva (Perlitsh and Glickman, 1966a, b). Cleavage of sialic acid from glycoproteins in saliva causes them to precipitate (Leach, 1963), probably because of the loss of their hydrophilic charged surfaces.

PE, SE, SS and serum preparations were therefore examined for the presence of sialic acid in their non-diffusible fractions before and after acid hydrolysis. The difference (table V) was a measure of the amount of sialic acid which was attached to glycoproteins in each preparation. Table V shows that the sialic acid content per mg protein from each of the 4 preparations was quite distinct. The bound sialic acid in SS was considerably greater than that of serum, although the estimated values in the SS samples were extremely variable, apparently due to the partial liberation of the bound sialic acid in some of the SS samples during preparation. Taking the protein content of saliva as 1.13 mg/ml (table IV), the value for bound sialic acid, 51.2 µg/ml, was similar to that of Perlitsh and Glickman (1967), who recorded a value of 54.7 µg/ml $\pm$ 21.8. The protein content of the serum samples was found to be 57.0 mg/ml $\pm$ 5.1, somewhat less than that recorded by Fawcett and Wynn (1956), which was 70 mg/ml. However, Fawcett and Wynn employed the Micro-kjeldal protein assay, and not the Lowry procedure. The Micro-kjeldal assay probably gives the larger values, since it measures the total

Table V

Protein bound Sialic Acid

Sample	ug/mg protein S.D.	Number of samples
Serum	10.0 ± 2.6	9
Saliva supernatant fraction (SS)	45.3 ± 22.4	8
Saliva sediment extract (SE)	147 ± 45.	8
Plaque extract (PE)	0.1 ± 0.3	6

nitrogen in a sample and not specifically protein. On the other hand, the bound sialic acid content of serum, 0.57 mg/ml ± 0.17, was close to the value recorded by Winzler (1958), which was 0.60 mg/ml ± 0.04.

The PE contained virtually no bound sialic acid although an abnormal colour was produced with the thiobarbiturate reagent. The sediment extract contained by far the most sialic acid per mg protein, and therefore may contain either the most glycoprotein or a glycoprotein containing an unusually large number of attached sialic acid residues.

(iv) Bound and free hexose

In addition to glycoproteins, some saliva and serum proteins have short hexose chains covalently linked to amino acid residues in the polypeptide chain, e.g. salivary amylase (Kauffman, et al., 1970). In addition, non-diffusible sugars may be present as polysaccharides and mucopolysaccharides, especially in the plaque and saliva sediment preparations.

Bound hexose and total sugars per mg protein were compared in each of the 4 preparations by assaying for hexose by the orcinol method after dialysis, and by the phenol sulphuric acid method before dialysis. The former assay was employed on different samples from the latter. As the amount of pentose in mammalian or bacterial tissues is virtually negligible compared to the amount of hexose, it is assumed that results obtained by using the non-specific phenol-sulphuric acid reagent may be comparable with results obtained from the orcinol reagent.

Most bound hexose per mg protein was found in the viscous sediment extract. But more was present in the plaque preparation

than in the saliva supernatant preparation, or in the serum preparation (table VI). In SS, 81% of the total hexose was bound, whereas in plaque only 44% of the total hexose was bound. In serum, 1.6% of the total hexose was calculated to be bound (table VI) due to its high glucose content.

Sephadex gel filtration confirmed that considerable amounts of free monosaccharide were present in PE (section 5c). Three PE preparations from 200 to 400 mg wet plaque per ml saline were therefore tested with Clinistix (Ames Company, Slough, Bucks.), a reagent which specifically detects glucose. Definite positive results were obtained within 15 sec of treatment with this reagent, whereas negative results were obtained from 3 samples of unstimulated saliva from subjects with chronic gingivitis.

It was concluded that plaque had a characteristic non-diffusible bound hexose content which was greater than that of serum, and that plaque contained free glucose. which was absent from saliva.

(d) Summary and conclusions

The results showed that the quantity and relative proportion of substances extractable from plaque largely differed from that in the saliva preparations.

Ultraviolet absorbance and Sephadex filtration studies showed that material with an absorbance peak corresponding to uric acid was found in both saliva preparations, but not in the plaque preparation.

Iso-electric focusing of proteins showed that the pattern from PE was distinct from that in both saliva preparations,

Table VI

Non-diffusible and total hexose

Sample	[1]Non-diffusible hexose ug/mg protein		[2]Total hexose (ug/mg protein)	Mean non-diffusible hexose as % of total
Serum	25.1 ± 2.2[3]	(8)[4]	1604[5]	1.6%
Saliva supernatant fraction (SS)	145 ± 29	(8)	180 ± 60 (17)	81%
Saliva sediment fraction (SE)	788 ± 124	(8)	not assayed	not assayed
Plaque extract (PE)	392 ± 127	(5)	891 ± 258 (19)	44%

Notes:-

(1) Estimated by the method of Hartley and Jevons, (1962).

(2) Estimated by the method of Dubois, et al., (1956).

(3) Standard deviation.

(4) Numbers in brackets indicate number of different samples assayed.

(5) Assumed that 0.9 mg/ml glucose (Long, 1961) was dissolved in the 8 serum samples containing a mean of 0.57 mg/ml protein.

which partially resembled each other. The iso-electric focusing pattern from PE was also distinct from that of serum. No antigenic similarity between proteins in plaque and those of the saliva supernatant fraction which were antigenic in the rabbit were detected by immuno-electrophoresis. Similar studies showed that more serum proteins were present in PE than in the saliva supernatant fraction. The extracted saliva sediment contained no serum proteins.

Serum and the plaque and saliva preparations were all found to have distinctive amounts of non-diffusible sialic acid and hexose per mg protein. Comparison of diffusible with non-diffusible hexose indicated that a considerable quantity of monosaccharide was present in the PE, and that plaque contained free glucose which was absent from saliva. Any glucose in plaque, therefore, was probably derived from serum.

The results suggest that gingival fluid and not saliva may be the substrate on which most dental plaque micro-organisms grow and develop. This could explain why such large differences were observed in the nature and quantity of material in the plaque and saliva preparations. (see DISCUSSION, c)

(3) PRELIMINARY FRACTIONATION OF CYTOTOXIC MATERIAL

Toxic material in PE was characterised (this section and section 5), and an attempt to examine its mode of action on the cell cycle was made (section 4). HeLa cells were used in these experiments since their linear dose response curve permitted a simple quantitative assay to be used.

(a) Dialysis and UM 2 Amicon filter treatment of Silverson extracted plaque.

In the experiments shown in figs 12, 13 and 14, the growth yield of HeLa cells was determined after treating them with increasing amounts of the various fractions from PE. The growth yield obtained after treatment with the unfractionated PE is also shown. The abscissa on each graph indicates the amount of unfractionated PE from which the fractions were prepared. The amount of toxic material in each fraction is proportional to the slope of the line.

Fig 12 shows that after dialysis of PE (Methods, 4) each of the three fractions, HS (High molecular weight Soluble fraction), HI (High molecular weight Insoluble fraction), and L (Low molecular weight fraction), caused little or no decrease in cell number. At that concentration of unfractionated plaque material in the medium where growth was completely

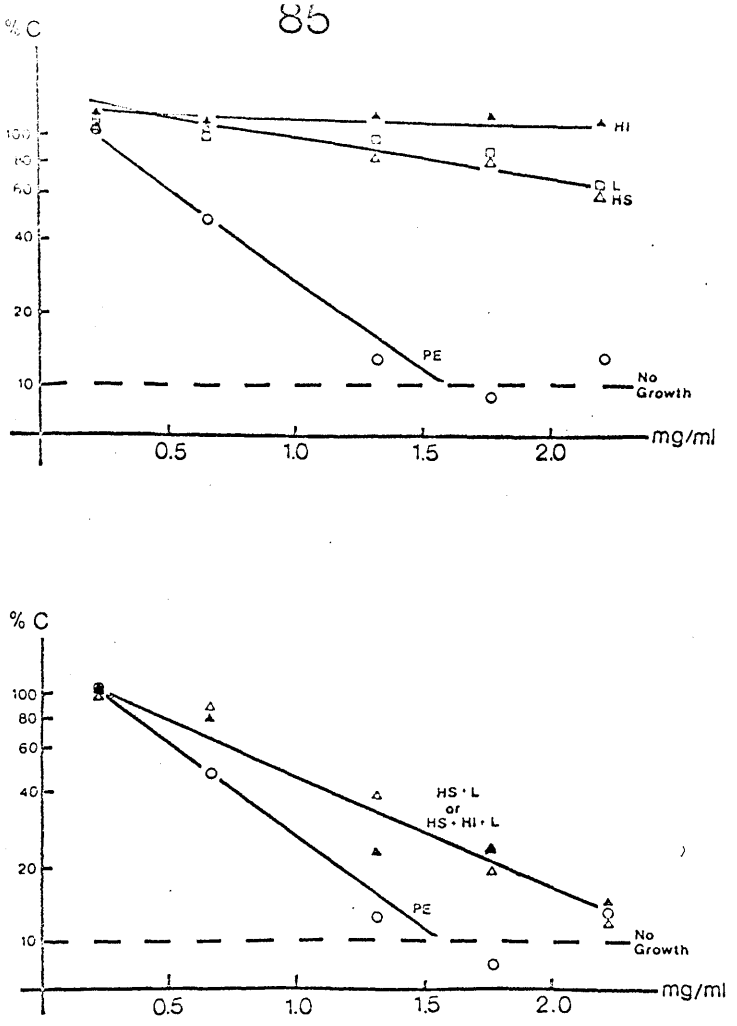


Fig. 12. Relative growth of cells treated with increasing amounts of PE or its fractions after dialysis

Cells were grown in 2 ml medium in 3 cm Petri dishes. Control cultures contained 9.9×10^5 cells after 96 h incubation. The results were plotted as in fig. 4. (Also see text, Results, 3a). Plaque was homogenised with a Silverson.

Top: Unfractionated PE o—o. Fraction L □—□.
Fraction HS △—△. Fraction HI ▲—▲.

Bottom: Unfractionated PE o—o.
Recombined PE (HS + HI + L) △—△.
Recombined fractions (HS + L) ▲—▲.

inhibited, its fractions HS and L permitted cells to grow virtually as well or better than controls. However, combining all 3 fractions, or combining fractions HS and L only, caused inhibition of growth intermediate between that of the separate fractions and the unfractionated PE. Material in fraction HI appeared to be completely non-toxic. From fig 12, the effect of toxic material in the recombined fractions was calculated to be more than twice that of the fractions separately, but still half that of the unfractionated extract. Half of the total amount of toxic material in the PE had therefore been lost irreversibly as a result of the dialysis procedure. The same experiment using PE from 2 other groups of plaque donors gave similar results.

After Amicon UM 2 filter treatment, the results were plotted as in fig 13. From fig 13 the toxic effect of each fraction separately was calculated to be 20% that of unfractionated material, but recombining the 2 fractions indicated that as much toxic material had been present as in the unfractionated extract. Although some toxic material in a Silverson extract had therefore been inactivated after Amicon filtration just as after dialysis, its activity was more completely restored on adding back the diffusible fraction after dialysis (see also table VII).

(b) <u>Amicon UM 20 filtration, and preliminary fractionation with G 150 Sephadex (Plaque extracted after homogenising it with a Potter homogeniser</u>

As distinct from the behaviour of toxic material in a

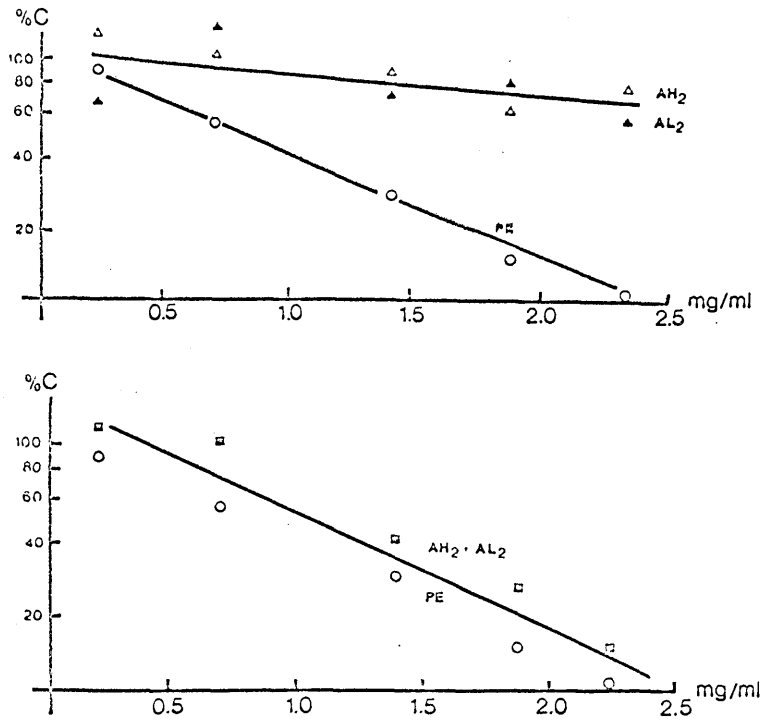


Fig. 13. Relative growth of cells treated with increasing amounts of PE or its fractions after Amicon UM 2 filtration

Cells were grown in 1.8 ml medium in 3 cm Petri dishes. Control cultures increased 8-fold in number to 7.1×10^5 cells after 96 h incubation. The results were plotted as in fig. 12. Plaque was homogenised with a Silverson.

Top: Unfractionated PE ○—○. Fraction AH_2 △—△.
Fraction AL_2 ▲—▲.

Bottom: Unfractionated PE ○—○.
Recombined PE (AH_2 + AL_2) □—□.

Silverson extract of plaque, filtration of a Potter extract of plaque through a 20,000 dalton cut-off membrane (UM 20E) showed that toxic substances in the excluded material accounted for 65% of the toxic activity in the unfractionated extract (fig.14). On the other hand, the toxic effect of material which had passed through the filter was 29% that in the unfractionated extract. This was a similar percentage to that obtained from the similar fraction of a Silverson extract. The separated high molecular weight material from a Potter extract therefore appeared to have retained its toxic activity, and little increase in activity was observed when the 2 fractions were recombined (fig. 14, table VII).

The results of a preliminary experiment with Sephadex G 150 are shown in figs. 15 and 16. To a 22 x 0.9 cm column was added 17.5 mg of Potter extracted plaque material in 1 ml. Fig. 15 is a plot of 260 nm absorbance, 280 nm absorbance, and protein concentration in each eluted fraction against fraction number. The first peak of material (fractions no. 10 and less) contained protein and material absorbing more at 280 nm than at 260 nm. The second peak of material (fractions no. 11 and over) contained a second protein peak as well as larger amounts of 260 nm absorbing material.

Fig. 16 shows the distribution of cytotoxic material in relation to the 260 nm and 280 nm absorbing fractions. Two peaks of growth inhibition were observed. From the relative area of the graph covered by each peak in fig 16, the relative amount of toxic material in each was calculated

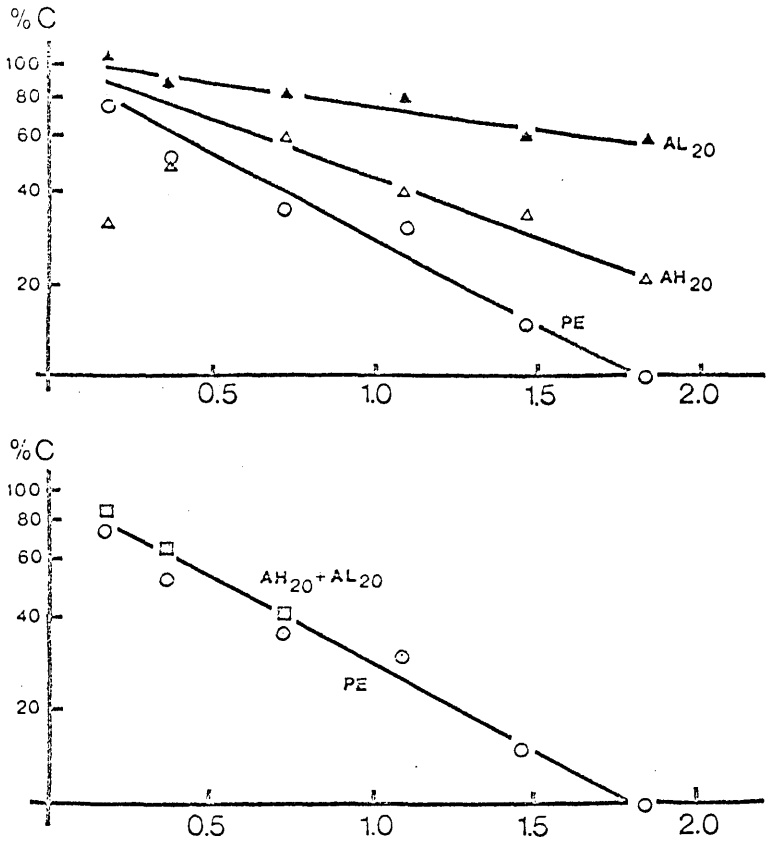


Fig. 14 Relative growth of cells treated with increasing amounts of PE or its fractions after Amicon UM 20E filtration

Cells were grown in 0.3 cm diameter cups in trays in 0.4 ml medium. Control cultures increased 12-fold in number to 22.7×10^4 cells after 96 h incubation. The results were plotted as in figs. 12 and 13. Plaque was homogenised with a Potter homogeniser.

Top: Unfractionated PE o—o. Fraction AH_{20} △—△.
Fraction AL_{20} ▲—▲.

Bottom: Unfractionated PE o—o.
Recombined fractions $AH_{20} + AL_{20}$ □—□.

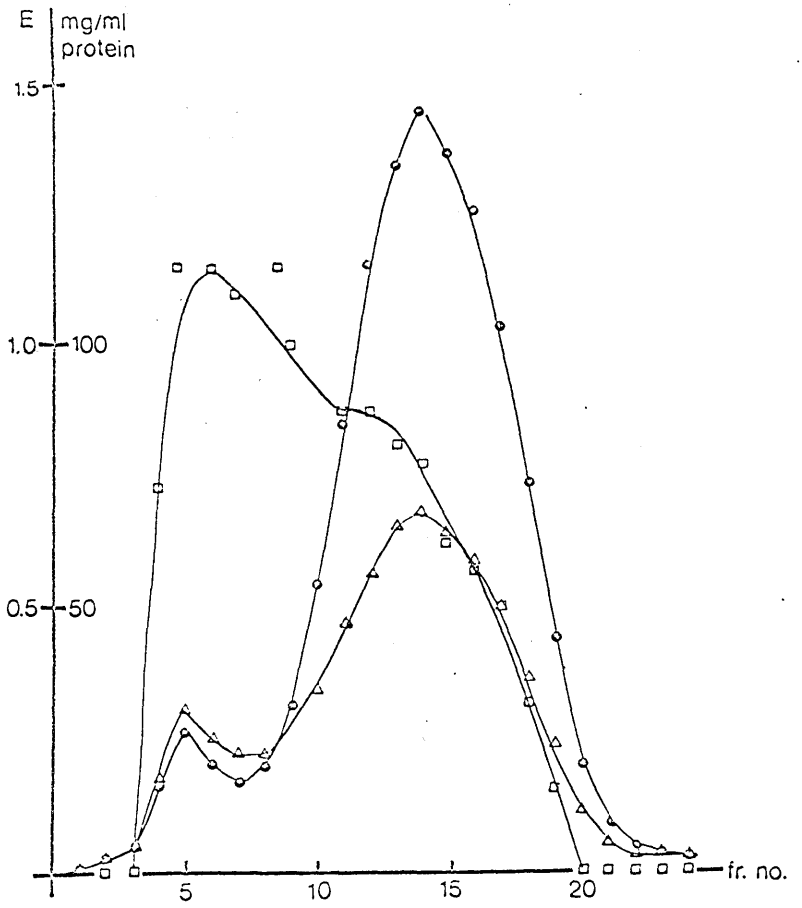


Fig. 15. Elution pattern of material in PE after preliminary fractionation on Sephadex G 150

17.5 mg extracted plaque material (Potter) was applied in 1.0 ml to a 22 cm x 0.9 cm (diameter) G 150 Sephadex column and eluted with carrier saline in 1.0 ml fractions. Ultraviolet extinction at 260 nm and 280 nm, and protein concentration, were plotted against fraction number (fr. no.).

Extinction (E) at 260 nm o—o.

Extinction (E) at 280 nm Δ—Δ.

Protein concentration □—□.

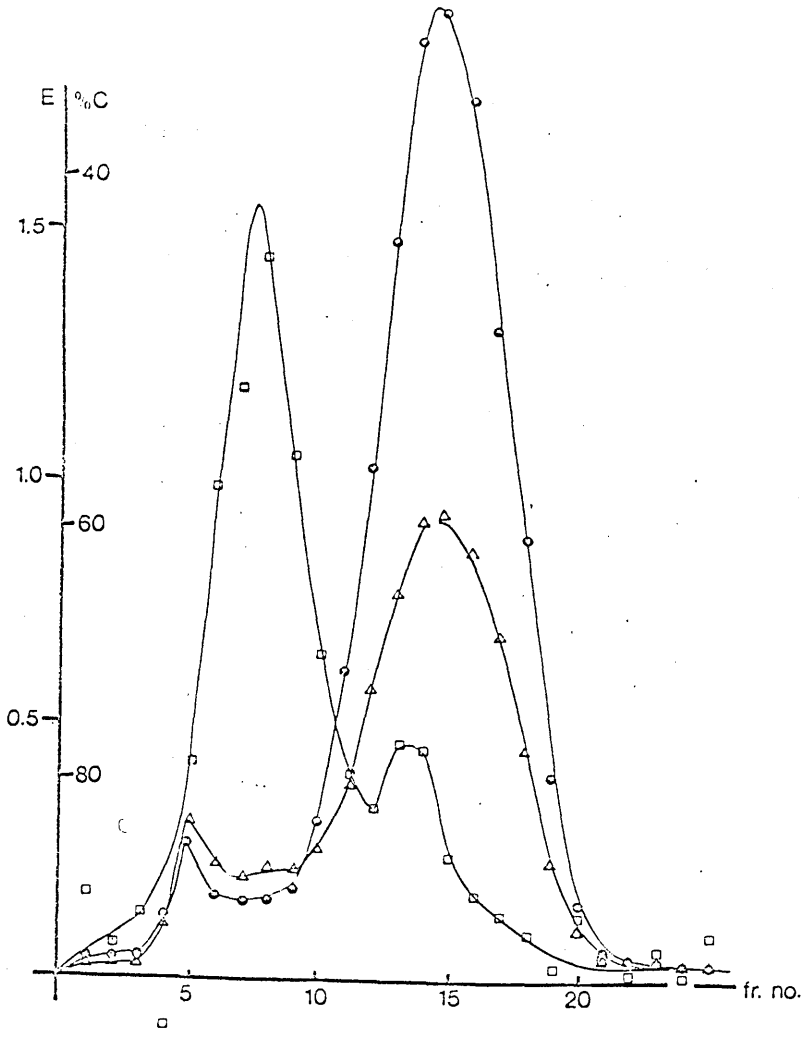


Fig. 16. Elution pattern of toxic material in PE after preliminary fractionation on Sephadex G 150

The experiment was identical to that in Fig. 15 except that growth as the % cell number in control cultures (%C) was plotted against fraction number (fr. no.). Protein was not assayed.

The cells were grown in 2.5 cm diameter Petri dishes in 1.6 ml EC_{10} containing a 1 to 2.5 dilution of each fraction. Control cultures, grew 10-fold over 96 h to 13.7 x 10^5 cells.

Extinction (E) at 260 nm o—o.

Extinction (E) at 280 nm △—△.

% control cell number (%C) □—□.

and is shown in table VII. As the retarded fractions on Sephadex G 150 contain substances whose molecular weights are less than 5,000 daltons, two peaks of toxic material were expected in view of the previous results.

The recovery of toxic material from the Sephadex column was 93%. This was calculated indirectly by dividing the 'total growth' recovered from the column by the 'total growth' added to it. The former can be found from fig 16, since some growth occurred when all the fractions were assayed. From fig 16, virtually all the 17.5 mg extracted plaque material added to the gel was recovered in fractions 5 to 18 inclusive. The growth (log % control cell number after treatment with each of these 14 fractions) was totalled to give the 'total growth' = 26.0. As the plaque material in each fraction had been diluted 1 in 2.5, the average amount of material in each of the 14 assays was 17.5/14 x 1/2.5 = 0.50 mg/ml.

The relationship between growth (as log % control cell number) and the mg/ml extracted plaque material in the medium was found from an aliquot of this PE prior to fractionation. The linear relationship fitted the equation $y = 2.027 - 0.621a$ ($P < 2 \times 10^{-3}$) where y is growth (log %C), and 'a' is mg/ml extracted plaque material. Substituting 0.50 mg/ml for 'a', and multiplying by 14, the 'total growth' added to the column was 24.2.

The recovery of growth from the column was therefore 26.0/24.2 = 107.4%, i.e. 7.4% more growth was recovered from the column than was added to it indicating that recovery of the growth inhibiting material was 92.6%. High molecular weight toxic material in the Potter extract was therefore inactivated

as little after Sephadex G 150 fractionation as after UM 20E filtration (see table VII).

(c) Growth inhibition by high and low molecular weight fractions of extracted plaque material

In table VII is summarised the percentage of toxic material found in the separate and recombined PE fractions (unfractionated PE = 100%). After dialysis, UM 2 filtration, and Sephadex G 150 filtration, the low molecular weight fractions accounted in each case for about one fifth of the toxic effect of the whole extract. After Amicon UM 20E fractionation, the low molecular weight fraction accounted for only a further 9% of toxic effect. These results suggest that toxic material in these 4 fractions of PE may be similar, and have a molecular weight of less than 2,000 daltons (see section 5c).

Table VII shows that Silverson extracted material in the high molecular weight fractions accounted for about 20% the toxic effect of unfractionated PE whereas Potter extracted material in the high molecular weight fractions accounted for about 70% the toxic effect of unfractionated PE. The total toxic effect of the Silverson extracted plaque fractions was markedly increased towards that of the unfractionated extract on recombining the fractions whereas the total toxic effect of the Potter extracted plaque fractions was already close to that of the unfractionated extract, even without recombining the fractions.

These results can be explained by assuming that the high molecular weight toxic factors in plaque were relatively stable

Table VII

Growth inhibition expressed as a percentage of that of whole PE[1] produced by the material in the high and low molecular weight PE fractions

	Silverson extract		Potter extract	
Fraction	Dialysis	Amicon UM 2 membrane	Amicon UM 20E membrane	Sephadex G 150
Low molecular weight	19%	20%	29%	19%
High molecular weight	19%	20%	65%	74%
Total recovery (separate)	38%	40%	94%	93%
Total recovery (recombined)	51%	100%	100%	-

Note:- (1) Method of calculating these values from Figs 12 - 16 is given in Results, sections 3a and 3b.

after Potter extraction, but not after Silverson extraction (possibly because the latter was more vigorous and took longer); and also by assuming that a low molecular weight factor(s) reactivated the Silverson extracted high molecular weight material. Taking all the results in table VII together, it is therefore concluded that toxic material in the diffusible fraction of PE may be distinct from that in the high molecular weight fraction, that toxic material in this latter fraction may lose and regain activity depending on the presence or absence of a diffusible factor(s), and that much toxic material in the high molecular weight fraction is inactivated more permanently after exposure to distilled water (after dialysis).

(d) Effect of heated PE on cell growth

Plaque was extracted with a Silverson and a sample of the PE was heated in boiling water for 1 h. Fig. 17 shows that some toxic material had been destroyed by heating. However, a repeat of this experiment with plaque from a different group of donors indicated the opposite, that the heat treatment had increased the toxic effect of PE. Further investigation into the effect of heating toxic material in PE appears necessary, as no proper conclusion can be reached from the present evidence.

(e) Summary and conclusions

Four different crude fractionation procedures with 2 different methods of plaque preparation gave results which indicated that toxic material consisted of at least 2 compounds;

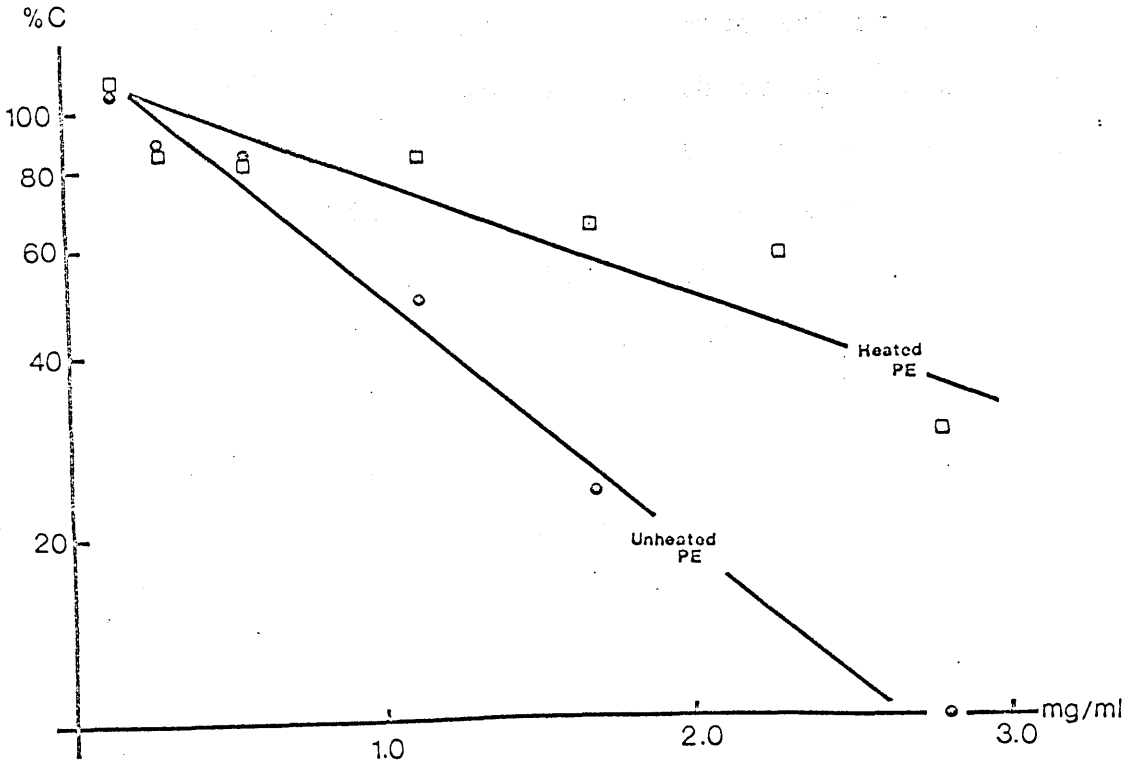


Fig. 17. Inhibition of HeLa cell growth when treated with heated and unheated PE

An aliquot of PE (Silverson) was heated for 1 h (Methods 6). The results were plotted as in fig. 4 (also see Results 3a). Cells were grown in 5 cm Petri dishes, and control cultures increased 10-fold over 96 h from 2.2 x 10^5 cells per dish.

Heated □—□. Unheated ○—○.

diffusible material of molecular weight less than 2,000 daltons, and non-diffusible material of molecular weight greater than 20,000 daltons. Toxic material in the former fraction accounted for about one fifth of the total toxic effect despite differences in the method of extraction and fractionation. On the other hand, toxic material in the latter fraction was stable after Potter extraction but appeared to lose and regain its activity after Silverson extraction of plaque, depending on the presence or absence of a diffusible factor(s). Exposure of Silverson PE to distilled water during separation of the high molecular weight fraction from the low molecular weight fraction inactivated irreversibly much toxic material in the former fraction.

Heating PE for 1 h in boiling water gave inconclusive results.

It was apparent that a Potter extract was more suitable than a Silverson extract of plaque for use in more detailed fractionation of the high molecular weight material on Sephadex G 150.

(4) EFFECT OF PE ON THYMIDINE UPTAKE INTO HELA CELLS, AND ON THEIR RATE OF DNA SYNTHESIS

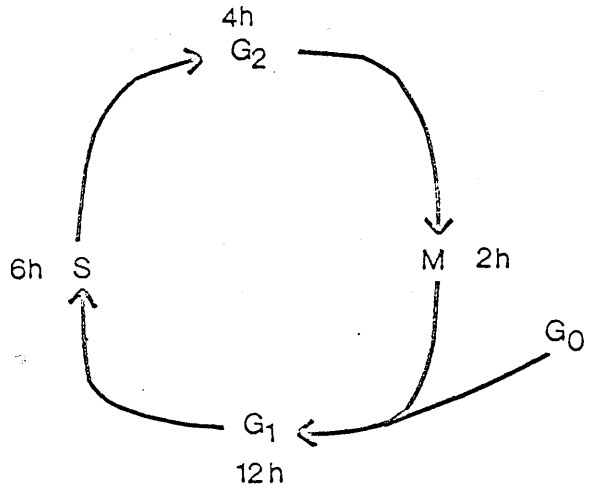
It would clearly be useful to have some means of assaying the effect of toxic material on HeLa cells other than the reduction in cell number in treated cultures. Such an assay could usefully corroborate some results of the investigation as well as perhaps provide some evidence as to the mode of action of the toxic material in PE, which may of course be the net effect of different modes of action by different substances.

(a) Inhibition of thymidine uptake into DNA at different times after PE treatment

The number of cells in a cell culture will increase until a lack of nutrients or some other factor limits their growth. The process whereby the cells divide is cyclical and it is now well established that DNA synthesis occupies only a part of this cycle. The cell cycle is shown diagrammatically in fig 18 (Quastler, 1963). For different cells, each stage of the cycle takes a specific time and the time required by HeLa cells has been indicated in fig. 18.

Preceding mitosis (M), synthesis of new DNA (S) occurs in the nucleus. The DNA precursor pool, extractable with 5% trichloroacetic acid and known as the acid soluble pool (AS pool), includes deoxythymidine triphosphate (dTTP) which is formed within the cells from ribonucleotides (Davidson, 1969). However, extracellular thymidine in the medium is taken up into the cell AS pool and is phosphorylated, mostly to its triphosphate, in the process (Cleaver, 1967). These endogenous

Fig 18. The Cell Growth Cycle.



G_1 post-mitotic pre-synthetic gap.

G_2 post-synthetic premitotic gap.

G_0 fertile cells not actually proliferating.

S period of DNA synthesis.

M period of mitosis.

and exogenous sources of dTTP are supplementary. Unsynchronised cultures of growing cells incorporate dTTP into DNA at a constant average rate under standard conditions (fig 19).

If too much toxic material is added to the culture, DNA synthesis is completely abolished, and no [^{3}H]deoxythymidine, [^{3}H]dThd, is incorprated into DNA. In cells treated with tritiated thymidine, the radioactivity incorporated into DNA per cell over a fixed time interval measures the apparent rate of DNA synthesis. If toxic material in PE inhibits cell growth as a result of affecting the cell cycle at a point other than S phase, its effect on DNA synthesis would not be immediate - i.e. at least 18 h would elapse before maximal inhibition of the rate of DNA synthesis is attained.

Fig 20 shows the effect of PE (1 mg/ml) on the incorporation of radioactive thymidine into DNA. Almost maximal inhibition was obtained within 1 h following the treatment although slightly greater inhibition was observed by 24 h. Material in PE therefore seemed to inhibit the apparent rate of DNA synthesis almost immediately, i.e. in S. phase. However, demonstrating this immediate effect on DNA synthesis does not exclude the possibility that other parts of cell metabolism are affected earlier or simultaneously.

(b) Thymidine uptake at 24 h and growth at 96 h.

Cultures were treated with PE to give the concentrations shown in fig. 21, and the effect on DNA synthesis (measured by [^{3}H]dThd incorporation during a 1 h pulse after 24 h PE treatment) compared with growth yield at 96 h. The thymidine uptake curve

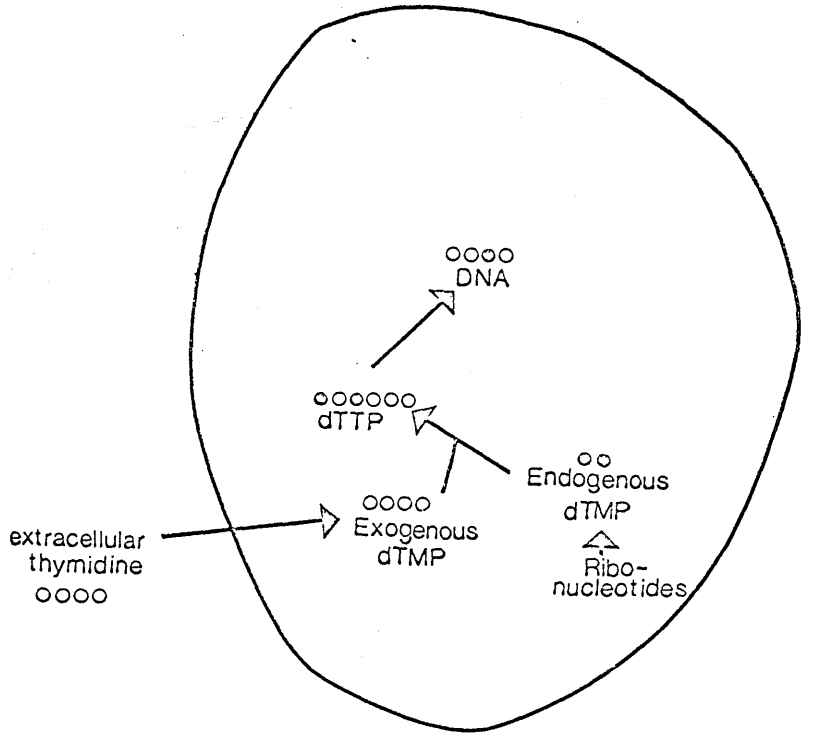


Fig. 19. Diagram illustrating the source of exogenous and endogenous thymidylate and their incorporation into DNA

Pyrimidine monophosphates (ribonucleotides) give rise to endogenous dTMP which is phosphorylated to dTTP. Extracellular thymidine is incorporated into the cell as exogenous dTMP which is also phosphorylated to dTTP, and competes with the endogenously synthesised dTTP for incorporation into DNA.

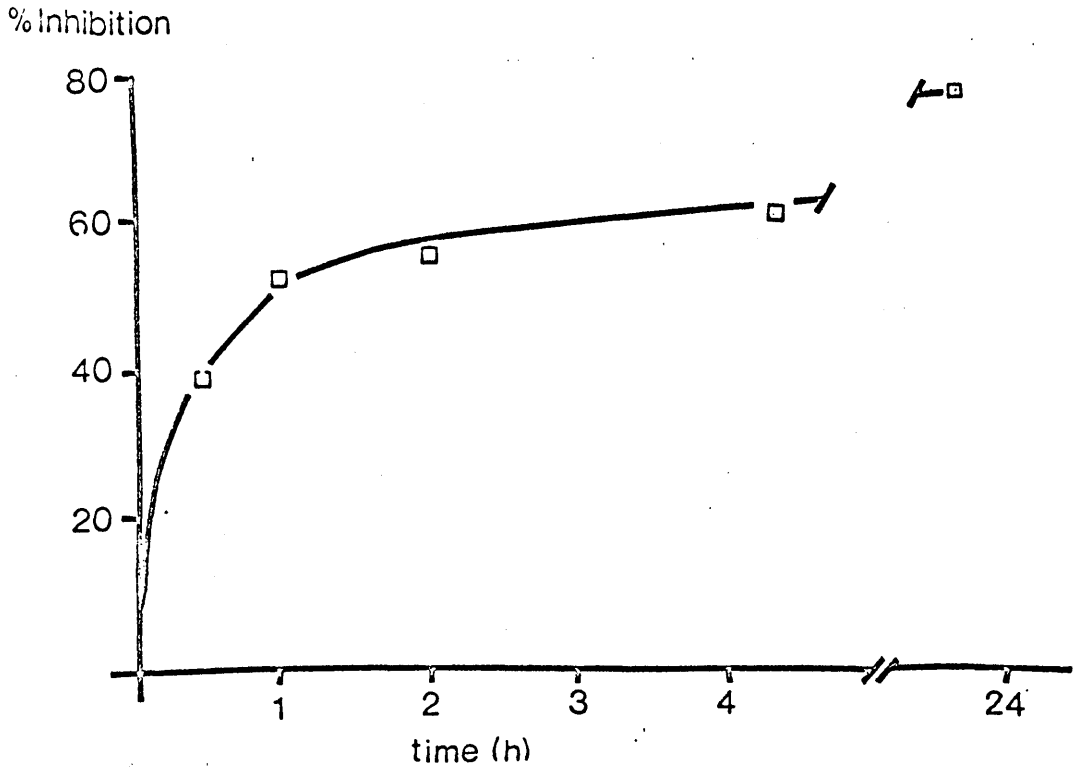


Fig. 20. Inhibition of thymidine uptake into DNA of PE treated cultures relative to time

2.0×10^5 cells in 3 ml EC_{10} in 5 cm Petri dishes were treated with 1 mg/ml extracted plaque material (MSE homogenised plaque), and [6-^{3}H]dThd, 10 µc at 10^{-5}M. Duplicate control and treated cultures were harvested at 0.5, 1.0, 2.0 and 4.5 h (Methods, 10d). The incorporation of [^{3}H]-dThd was estimated as cpm (Methods 10d), and increased from 0.16 to 2.50×10^3 cpm in control cultures. The % inhibition was plotted against time of harvesting.

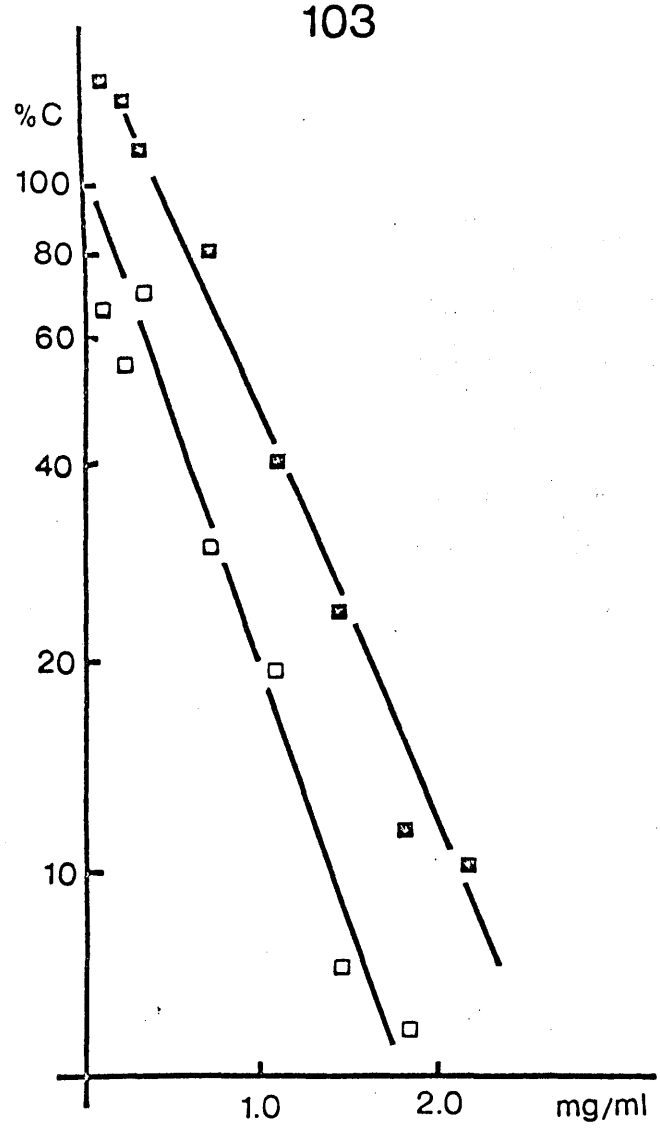


Fig. 21. Relationship of the apparent rate of DNA synthesis to growth in cultures treated with increasing amounts of PE.

1.6 x 10^5 cells were grown in 5 cm Petri dishes, treated with increasing amounts of extracted plaque material, and harvested after 24 h for [6-^{3}H]dThd uptake into DNA (1 h pulse of 10 uc at 10^{-5}M dThd), and after 96 h for relative growth. The latter result was previously shown in Fig. 4 for Silverson homogenised plaque. The apparent rate of DNA synthesis was calculated as the dThd uptake/ 10^5 cells/h (Methods, 10d). The dThd uptake was measured as a fraction of dpm in control cultures.

Cell number assay at 96 h ▫—▫.
100% = 20.1 x 10^5 cells.

Thymidine uptake assay at 24 h ▫—▫.
100% = 37.1 x 10^3 dpm

was displaced to the left, possibly because at 24 h control cultures were growing faster than any treated culture. It was only after 72 h that the growth of control cultures slowed down relative to PE treated cultures (fig 3, section 1a). Drawing the dThd incorporation curve through the 100% point in fig 21, it was observed that the dThd uptake curve appeared slightly steeper than the cell number curve, suggesting that thymidine uptake may have been slightly more inhibited than the cell growth by material in PE.

(c) Effect of the diffusible fraction of PE on cell growth and DNA synthesis

The experiment described in fig 12 was repeated to test the effect of dialysed PE on DNA synthesis at 24 h. It appears from fig 22 that the effect of fraction L on the inhibition of DNA synthesis is greater than its effect on cell growth. This may be the result of diffusible material from PE reducing uptake of [^{3}H]dThd into DNA without reducing the true rate of DNA synthesis. The diffusible fraction of PE probably contains a large amount of thymidine (fig 38; section 5c), and this would have the effect of reducing the specific activity of the tritiated thymidine and so reducing the incorporation of radioactivity into DNA. That little difference was seen between the two assay methods when whole PE was used (fig 21), is probably because the effect of cold thymidine is minor compared with the effect of the toxic material (see section 4d).

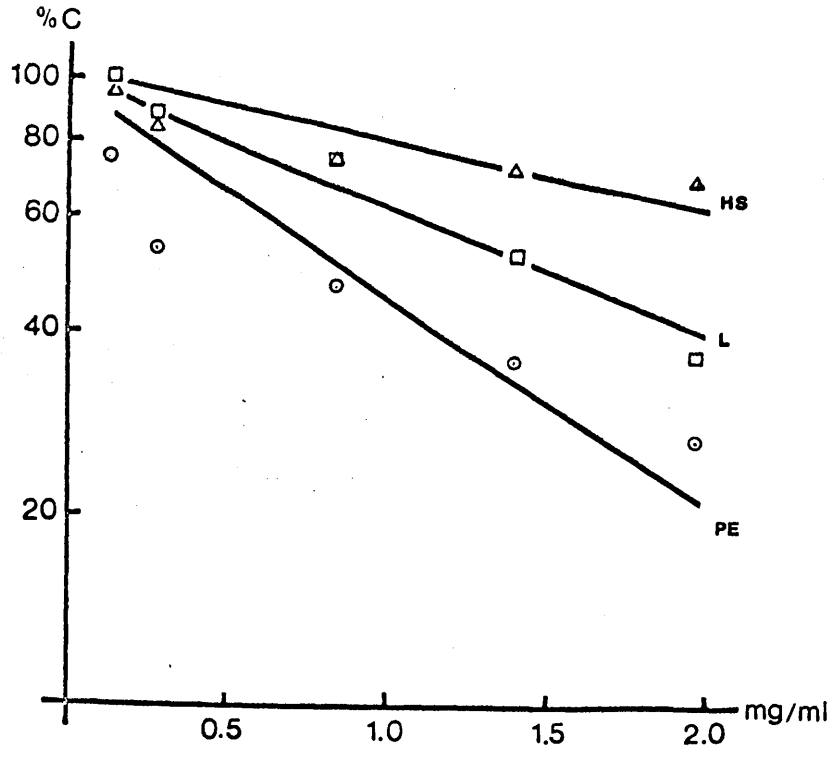


Fig. 22 Effect of 24 h treatment of cells with PE fractions after dialysis when measured by thymidine uptake into DNA.

1.8×10^5 cells in 5 cm diameter Petri dishes were grown in EC_{10} containing unfractionated PE, or its subfractions L, or HS respectively (Methods, 4). Cells were harvested after 24 h by measuring [^{3}H]dThd uptake into DNA as described in figs. 20 and 21, and in methods 10d. (A duplicate set of cultures were harvested after 96 h, when, unlike the PE treated cultures, those treated with fractions HS or L were found to have grown like controls.)

Unfractionated PE (Silverson) o — o.

Non-diffusible fraction, HS Δ — Δ.

Diffusible fraction (L) □ — □.

(d) Determination of the true rate of DNA synthesis, and of the amount of thymidine in the AS pool in cells treated with PE or its dialysed fractions

By measuring the uptake of thymidine into DNA at different specific activities, it was possible to extrapolate the results to indicate both the true rate of DNA synthesis in treated and untreated cultures, and the respective endogenous dTTP pool sizes (Adams, 1969b). An increased dTTP pool size in PE treated cultures indicates the amount of thymidine contributed by PE in such cultures.

(i) Rationale

Fig 23 shows that the amount of radioactive thymidine incorporated into DNA increased with increasing concentration of thymidine, and then decreased. As the dThd concentration in the medium increases, more and more exogenous dTTP is incorporated into the AS pool, and high levels of dTTP prevent DNA synthesis by feedback inhibition. Fig 23 therefore indicates that the exogenous dThd concentration must not increase beyond about 10^{-5}M if the rate of DNA synthesis is to remain unaffected by the presence of exogenous dTTP in the AS pool.

Fig. 24 shows that exogenous thymidine incorporated into the AS pool and DNA increased in a non-linear fashion as the external thymidine concentration was raised from 0.5 to 10 x 10^{-6}M. This incorporation into DNA is the result of two factors; (1) the addition of increasing amounts of exogenous dTTP to the endogenous dTTP, and (2) feedback inhibition of thymidine uptake into the AS pool by increasing concentrations

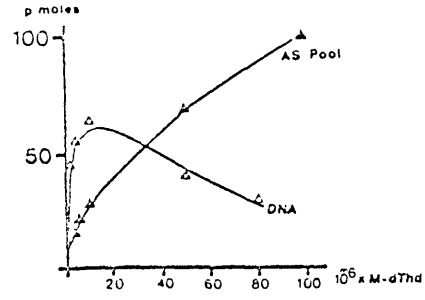


Fig. 23. Relationship of p moles dTTP in AS pool and DNA to the thymidine concentration in the medium

5 x 10^5 HeLa cells in 5 cm diameter Petri dishes were incubated for 24 h, and then pulsed for 1 h with 10 µc [^{3}H]dThd tracer in 3 ml EC_{10} made from 2 to 100 x 10^{-6}M thymidine. Incorporation of thymidine into the AS pool and DNA (Methods, 10d) was plotted against the thymidine concentration in the medium.

p moles dTTP in AS pool ▲—▲. p moles dTTP in DNA △—△.

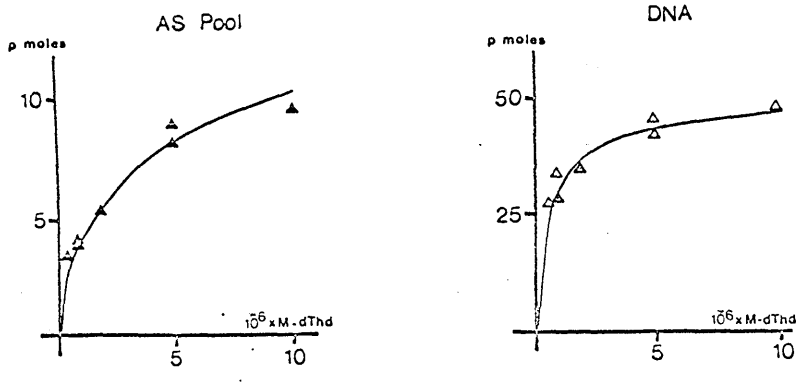


Fig. 24. Relationship of p moles dTTP in AS pool and DNA to those thymidine concentrations in the growth medium at which its incorporation into DNA approaches maximum

1.7 x 10^5 Cells in 5 cm Petri dishes grew to 3.6 x 10^5 cells over 24 h, and were then pulsed with [^{3}H]dThd for 1 h as in Fig. 23, except that the 3 ml EC_{10} was made from 0.5 to 10 x 10^{-6}M thymidine. The results were plotted as in Fig. 23.

Left: p moles dTTP in AS pool

Right: p moles dTTP in DNA

of dTTP. The latter factor was eliminated when the incorporation of exogenous dTTP in DNA (y) was plotted against the amount of exogenous dTTP in the AS pool (x), as in fig 25a This relationship depends on the amount of exogenous dTTP, as a fraction of the total dTTP (exogenous + endogenous) which is incorporated into DNA (fig 18); i.e. $x/(x + a) = y/b$.....(1)

x = amount of exogenous dTTP in the AS pool (p moles/AS pool volume).

y = amount (p moles) exogenous dTTP incorporated into DNA/h.

a = amount of endogenous dTTP in the AS pool (p moles/AS pool volume).

b = amount (p moles) of exogenous dTTP which would be incorporated into DNA if its AS pool concentration were so high that any DNA made from endogenous dTTP would be negligible.

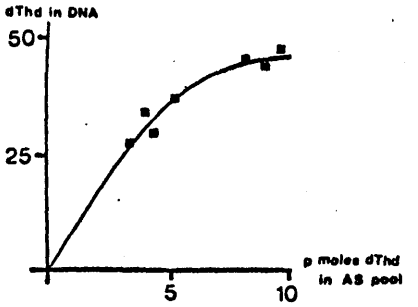
From equation (1),

$1/y = a/b.1/x + 1/b$ .....(2).

As x and y were measured, their double reciprocal relationship (equation 2) should be linear if both a and b are constant for a given number of cells. This is found to be so (fig 25b). Extrapolating to $1/x = 0$ gives a value for $1/b$, the true rate of DNA synthesis. Extrapolating to $1/y = 0$ indicates the value of x which is equal to a; i.e. that amount of endogenous dTTP which is normally present in the AS pool.

This approach (Adams, 1969b) has several limitations. It assumes that exogenous dTTP in the AS pool at the external concentrations employed has no effect on the true rate of DNA

25a



25b

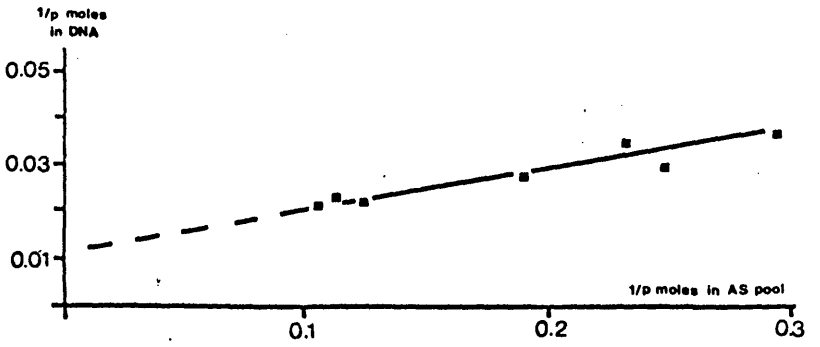


Fig. 25. Relationship of p moles exogenous dTTP incorporated into DNA to the p moles exogenous dTTP in the AS pool of 3.6×10^5 cells

This is the data in fig. 24 replotted.

Top: p moles dTTP in DNA against p moles dTTP in AS pool.

Bottom: 1/p moles dTTP in DNA against 1/p moles dTTP in AS pool.

synthesis, nor on the rate of synthesis of thymidylate by endogenous routes. It also assumes that dTTP from external or internal thymidine is in direct equilibrium. Finally, this approach can only be used when cells are making DNA.

(ii) Experimental

Cells were treated for 24 h with unfractionated PE, the three dialysis fractions separately, the three fractions recombined, and the carrier saline only (controls), and then re-incubated for 1 h with tritiated thymidine at concentrations of between 0.5 and 10 x 10^{-6} M. Fig 26 shows the results of double reciprocal plots of thymidine incorporation into DNA and the AS pool. The probability, P, that each line was not straight was less than 0.01. From these lines, the rate of DNA synthesis and the concentration of dTTP in the AS pool were determined from the y and x intercepts respectively. These values, divided by the number of cells in the differently treated cultures (table VIII), gave the true rate of DNA synthesis (V) and the endogenous dTTP concentration per 10^5 cells.

Table VIII shows that relative to controls the rate of DNA synthesis was only markedly reduced when cells had been treated with unfractionated PE or the recombined fractions. Material in the recombined extract reduced DNA synthesis more than material in the three separate fractions but less than in the unfractionated extract. This effect resembles that on growth (section 3a).

Only cells in S phase incorporate dTTP into DNA, and only 25% of HeLa cells in an asynchronous culture are in S phase at

Fig. 26. Estimation of endogenous AS pool size and rate of DNA synthesis in cultures treated with no PE, unfractionated PE, each of the 3 PE dialysis fractions separately, and all 3 fractions recombined

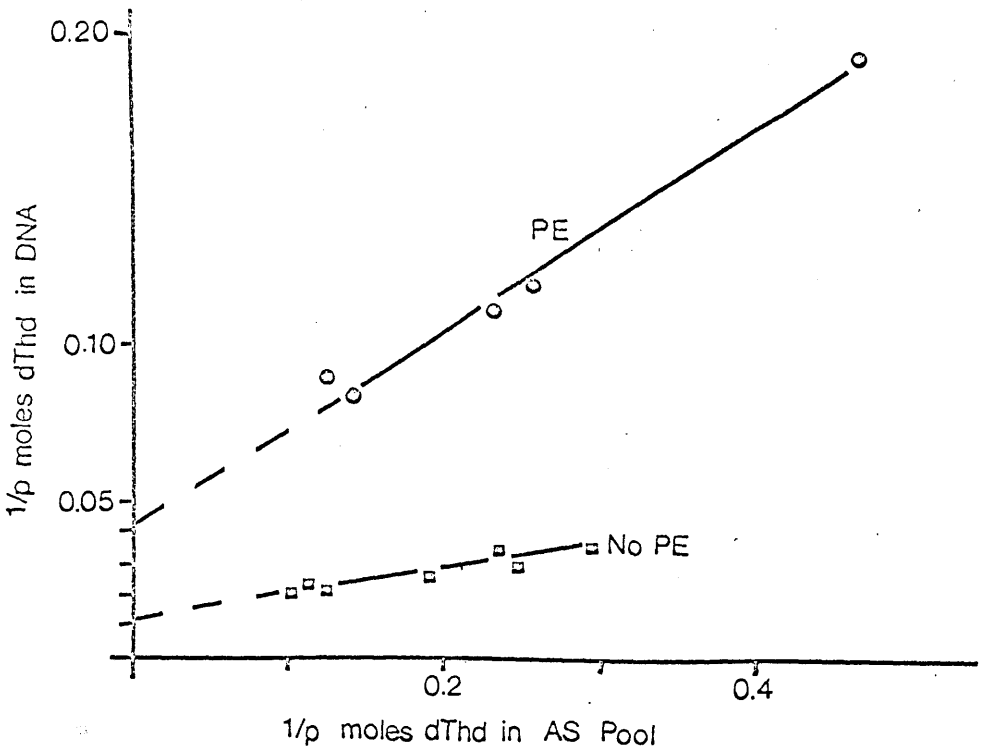
Remainder of the experiment shown in Fig. 24, the amount of plaque material added to treated cultures being shown in Table VIII. The results were plotted as a double reciprocal plot as in Fig. 25. The 'true' rate of DNA synthesis and the AS pool size were obtained by extrapolation of the best straight line through each set of points to the y and x axes respectively.

Top: Unfractionated PE o—o.

No PE (carrier saline only) ▫—▫.

Bottom: Recombined PE ⊙—⊙. Fraction L o—o.

Fraction HS △—△. Fraction HI ▲—▲.


0.20
0.10
0.05
1/p moles dThd in DNA
PE
No PE
0.2
0.4
1/p moles dThd in AS Pool

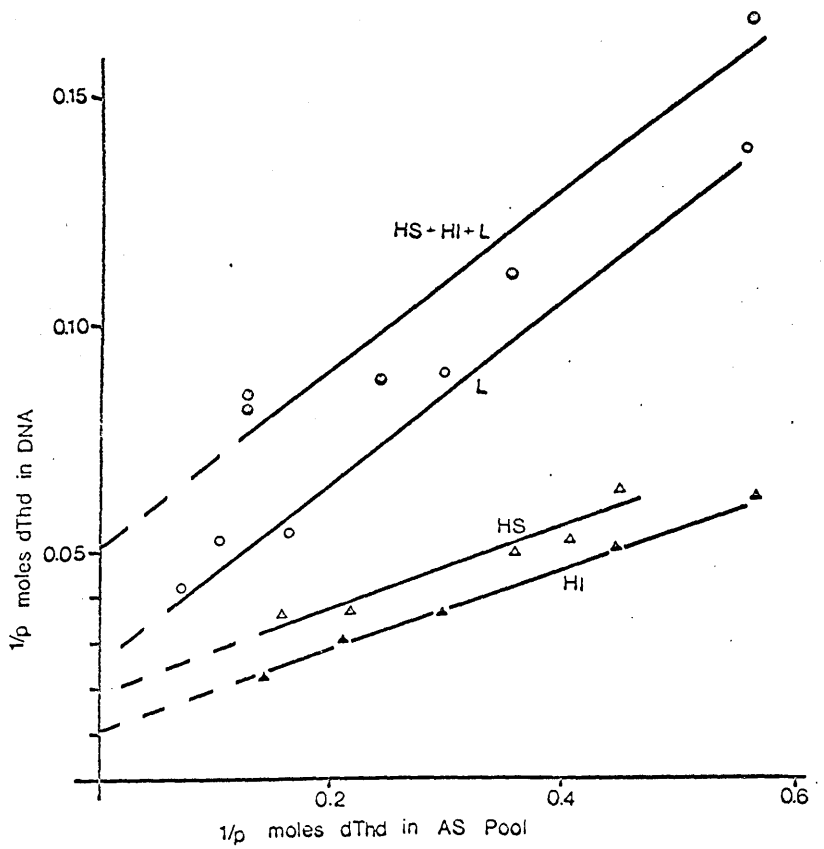

0.15
0.10
0.05
1/p moles dThd in DNA
HS+HI+L
L
HS
HI
0.2
0.4
0.6
1/p moles dThd in AS Pool

Table VIII

The true rate of DNA synthesis (V), and the concentration of dTTP in the AS pool of HeLa cells after treatment with PE and its sub-fractions after dialysis

Treatment	[1] Cells/dish x 10^5	[2] V p moles/10^5 cells/h.	V as % control	[2] dTTP in AS pool/10^5 cells	Increase in AS pool %	Conc. of plaque material in medium
Controls	3.6	21	100%	1.60	0%	0.0
Fraction HI	3.6	28	133%	2.50	56%	2.0
Fraction HS	2.2	24	114%	2.33	33%	2.0
Fraction L	2.2	18	86%	3.46	116%	2.0
Recombined fractions	1.7	12	57%	2.28	42%	2.0
Unfractionated PE at 1.5 mg/ml	1.8	13	62%	2.08	31.%	1.5
Unfractionated PE at 2.0 mg/ml	1.7	0	-	0.0	-	2.0

Note: (1) Controls contained 1.7 and 3.6 x 10^5 cells at 0 h and 24 h respectively. The growth of treated culture at 24 h was calculated from the appropriate results in fig 12, taking 3.6 x 10^5 cells as 100% growth (see Methods 10d).

(2) Obtained by extrapolation to y and x axes in fig. 26, and dividing by the appropriate number of cells/dish.

any one moment (fig 18). From table VIII, the rate of DNA synthesis from dTTP in control cultures was calculated to be 0.84×10^{-9} moles/10^6 cells in S phase/h. Cells treated with fractions HS and HI incorporated dTTP into DNA slightly faster, at about 1×10^{-9} moles/10^6 cells in S phase/h. The rate of incorporation of dTTP into DNA in L 929 cells was found to be 2×10^{-9} moles/10^6 cells in S phase/h (Adams, 1969a). This difference in rate could have been due to differences in the mean generation time between the two experiments.

Table VIII also shows that the concentration of endogenous dTTP in the AS pool (1.6 p moles/10^5 cells) was increased in all the PE treated cultures. This indicates that dThd was present in the PE. From Table VIII, treatment of cells with the diffusible fraction of PE was calculated to result in 6.8 additional p moles being present in the AS pool of 3.6×10^5 cells. This was found to correspond to 3.5×10^{-6}M dThd in the medium (fig 23a). Similarly from Table VIII, treatment of cells with the non-diffusible fractions corresponded to only 0.4×10^{-6}M dThd in the medium (fig 23a).

There was 9 times more thymidine in medium treated with the diffusible fraction than in medium treated with either of the non-diffusible fractions. Thymidine detected in the non-diffusible fractions could have been retained due to incomplete separation during dialysis, or as a result of DNase action on DNA oligonucleotides, both enzyme and substrate having possibly been extracted from plaque in addition to thymidine (see sections 2d and 5a). It was calculated that thymidine in the diffusible fraction accounted for about 2% of all the 260 nm absorbing material extracted from plaque (section 2a, table IV).

Synthesis of exogenous and endogenous dTTP is closely linked to that of DNA (Adams, 1969b). Hence in cultures treated with the unfractionated or recombined PE, the dTTP concentration in the AS pool increased less than in cultures treated with the diffusible PE fraction alone (Table VIII).

Thymidine in the medium of cells treated with PE decreased the specific activity of radioactive thymidine added to measure the apparent rate of DNA synthesis in the experiments shown in figs 20, 21 and 22. At the specific activity used for these measurements the decrease was calculated to be 30% when the amount of toxic material in PE was sufficient to stop cell growth completely. Inhibition of tritiated thymidine uptake in these experiments was therefore caused largely by true inhibition of DNA synthesis.

(c) Summary and conclusions

The results indicate that toxic material in PE may have inhibited DNA synthesis by acting directly on S phase of the cell cycle.

Tests on each of the 3 fractions obtained by dialysis of a Silverson PE showed that alone none had much effect on the true rate of DNA synthesis, although when recombined the rate was markedly reduced. However, the same concentration of unfractionated PE prevented DNA synthesis altogether. This is the same result as was obtained when the effect on growth was tested (section 3a).

Under the conditions of assay, thymidine in PE reduced uptake of tritiated thymidine into DNA by not more than 30% of the total reduction in uptake brought about by toxic material in PE.

(5) ANALYTICAL FRACTIONATION OF CYTOTOXIC MATERIAL ON SEPHADEX.

(a) High molecular weight fraction.

The material in PE was applied to a 82 x 1.5 cm column of Sephadex G 150 and the eluate collected in 1.0 ml fractions. The 4 preparations examined are listed in Table IX where the total amount of extracted plaque material applied to the column and assayed in the effluent fractions is indicated for each experiment.

Fig 27 shows that the eluted fractions had 3 peaks of ultraviolet absorbing material. The excluded peak absorbed at 260 nm and 280 nm equally, the partially excluded peak absorbed more at 280 nm than at 260 nm, and the retarded peak absorbed much more at 260 nm than at 280 nm. This last peak was also much more intense than the others (c.f. figs 15 and 16; section 3b). On the same plot is shown the fractionation of protein and growth inhibiting material in the excluded and partially excluded fractions. The fractions numbered 110 to 150, which were retarded and absorbed strongly at 260 nm, were pooled and fractionated on Sephadex G 10 (Methods, 5b) - the results being given separately below (section 5c). The protein was eluted from Sephadex G 150 as 2 broad peaks, one excluded and the other partially excluded from the gel. The maximum protein peak coincided with the ultraviolet peak which was partially excluded, and absorbed maximally at 280 nm as in figs. 15 and 16. The toxic material fractionated into 4 peaks as indicated by the degree of growth inhibition in the eluted fractions.

Fig 28 shows the elution pattern of the toxic and other material in the extracted fraction of the plaque pooled from a different group of donors. The totally

Table IX

Total extracted material applied to G 150 Sephadex

Preparation	Wet weight plaque/ml carrier saline (g).	Extracted plaque solid mg/ml	Applied on gel after lyophilisation to 0.5 ml (mg)	Dilution of eluted fractions in cytotoxicity assay
Potter (fig 27)	0.350	16.6	74.7	1 in 1.5
Potter (fig 28)	0.327	15.8	94.8	1 in 1.3
Potter (fig 29)	0.400	18.0	108.0	1 in 1.3
Silverson (fig 30)	0.150	8.4	157.7	1 in 2.0

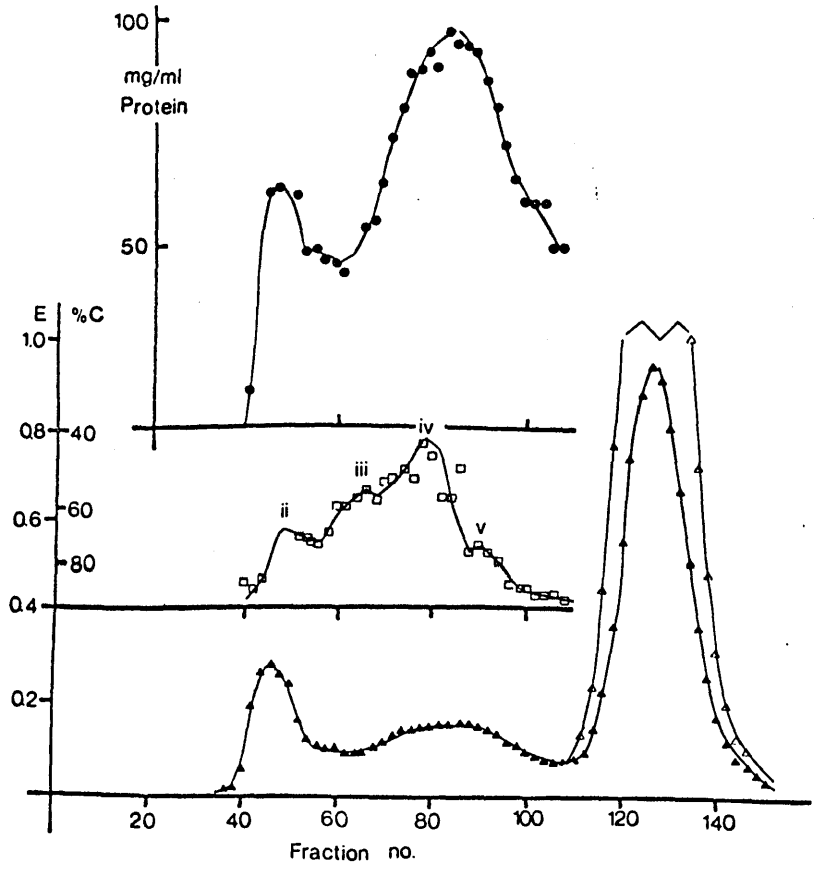


Fig. 27. Fractionation of protein, toxic material, and ultraviolet absorbing material in PE using Sephadex G 150

Plaque was extracted with a Potter homogeniser. 75mg extracted material was applied to the 82 x 1.5 cm G 150 Sephadex column in 0.5 ml (see Methods, 5b).

Top: Protein concentration (mg/ml) against fraction number (Methods, 9b) ●—●.

Middle: Growth (%C) against fraction number (Methods, 10c and Table X) □—□.

Bottom: Absorbance (E) against fraction number (Methods, 9a) E_{280} ▲—▲. E_{260} △—△.

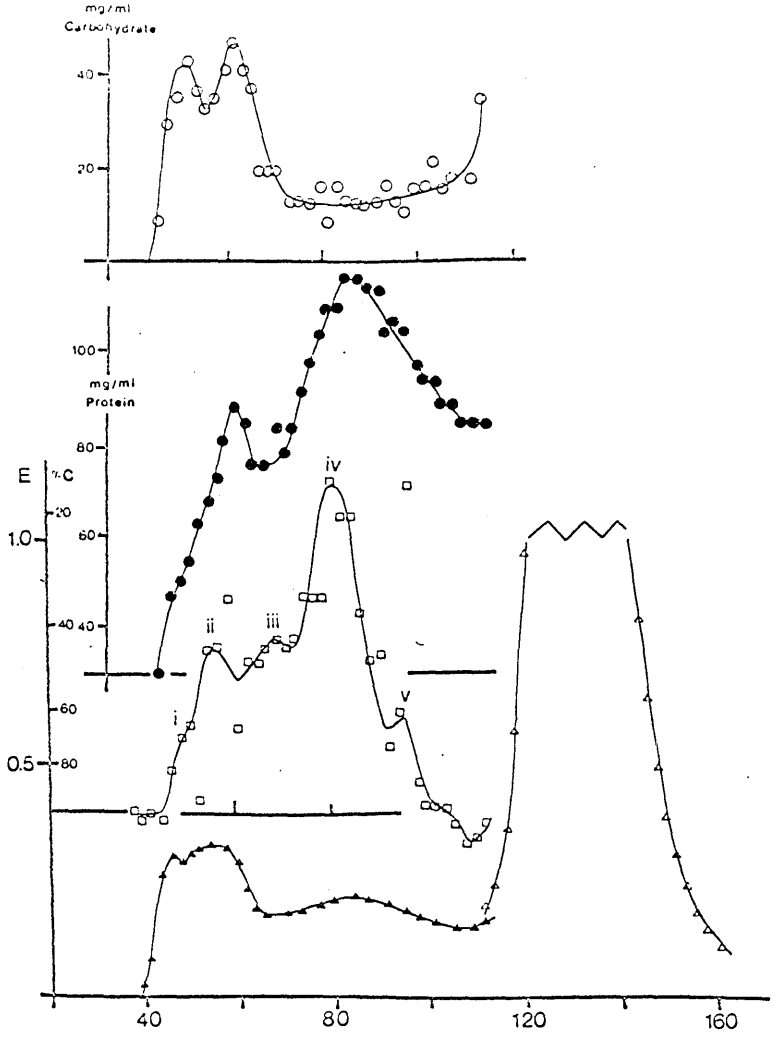


Fig. 28. Fractionation of toxic and other material in PE using Sephadex G 150

Extracted plaque material (95 mg; Potter homogeniser) was applied in 0.5 ml to the 82 x 1.5 cm G 150 Sephadex column (Methods, 5b).

1st Top: Carbohydrate (mg/ml) against fraction number (Phenol reagent, Methods, 9c) o—o.

2nd Top: Protein concentration (mg/ml) against fraction number (Methods, 9b) ●—●.

3rd Top: Growth (%C) against fraction number (Methods, 10c, and Table X) □—□.

Bottom: Absorbance (E) against fraction (Methods, 9a).

E_{280} ▲—▲. E_{260} △—△.

excluded fractions separated much better than in the previous experiment, and much of the protein, toxic, and ultraviolet absorbing material that was apparently in the excluded fractions in fig 27 were composed of 2 peaks quite close together. Nevertheless, a pattern very similar to that in fig 27 was found for these three classes of material in the eluted fractions. The carbohydrate in each fraction was eluted as 3 peaks, 2 close to the excluded volume, and the indication of a third in the retarded volume. Intermediate fractions all had a low concentration (not zero) of carbohydrate. The second carbohydrate peak coincided with the first large protein peak (fraction no. 58).

The fraction of PE excluded from an Amicon UM 20 membrane was subsequently applied to the Sephadex G 150 column. Fig 29 shows the ultraviolet absorbing and growth inhibiting peaks in the eluate. These peaks were found in almost identical positions to those from the extracted plaque material from different groups of donors in the previous experiments (figs 27 and 28). The excluded ultraviolet absorbing peak was greater than previously observed, and did not split as in fig 28. The middle 280 nm absorbing peak (fraction no. 80) corresponded in position to the protein peaks in figs 28 and 29. This peak seemed to be larger and more closely related to the largest peak of toxic effect than in the previous experiments. The small growth inhibiting peak (peak v) observed at fraction 92 in figs 27 and 28, was absent. Some material which had an absorbance peak at 260 nm had apparently not passed through the UM 20 membrane, and was still found in the retarded fractions on elution from G 150.

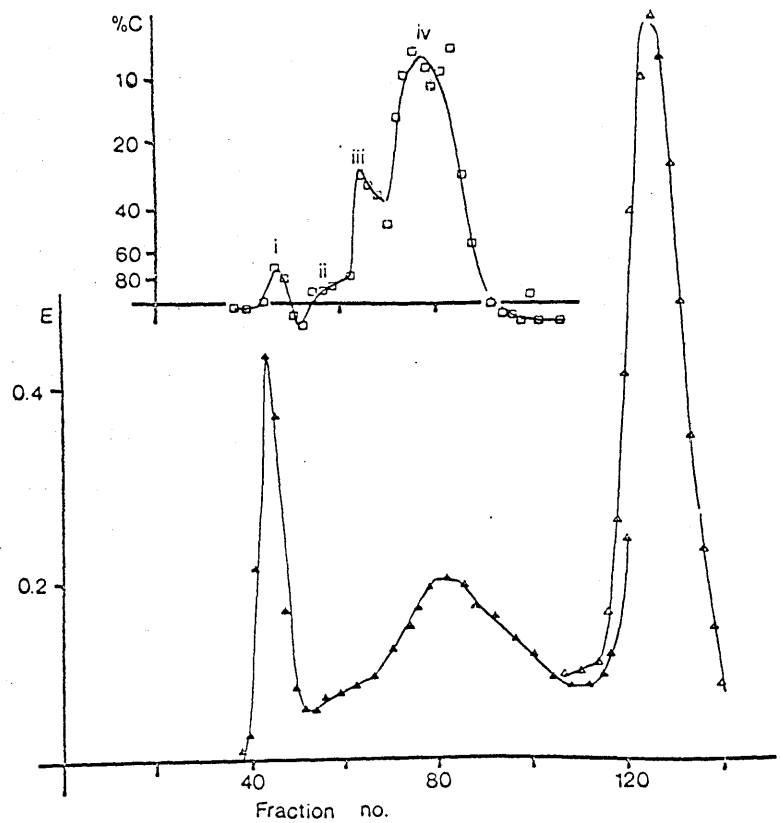


Fig. 29. Fractionation on G 150 Sephadex of the toxic and ultraviolet absorbing material in the PE fraction excluded from the Amicon UM 20E filter.

PE was fractionated with the UM 20E Amicon filter (see legend to fig. 14). The non-filterable material was applied in 0.5 ml to the 82 x 1.5 cm G 150 column (Methods, 5b). The equivalent of 108 mg unfractionated plaque material was applied.

Top: Growth (%C) against fraction number (Methods, 10c; Table X) □—□.

Bottom: Absorbance (E) against fraction number

E_{260} △—△. E_{280} ▲—▲.

This 260 nm absorbing material may have been produced by the action of nuclease on nucleic acid fragments in the unfilterable fractions (c.f. section 4d; table VIII). However, the amount of 260 nm absorbance in the retarded fractions was much less than the amount found after whole PE had been applied to the column (c.f. figs 27 and 28).

After extraction of plaque with a Silverson homogeniser (fig 30), protein and ultraviolet absorbing material were eluted in positions similar to those in the previous experiments. Toxic material eluted somewhat differently, however, and showed only a small peak iv. This peak had been the main growth inhibiting fraction in previous experiments after plaque had been extracted with the Potter pestle (figs 27, 28 and 29).

The elution volume (V_e) of chemically similar classes of solutes from a Sephadex column, is related to their molecular weight. For any length of column, the ratio V_e/V_o (V_o = column's void volume) is proportional to log molecular weight, especially if the column is long and $V_o < V_e < V_t$ (V_t = column's total volume), (Andrews, 1964; 1965).

A standard curve for the elution of various proteins, dextran blue, and glucose from this column was constructed (fig. 31). Assuming the toxic substances were protein, V_e/V_o for each eluted toxic peak was evaluated and log molecular weight read off the graph. The results are shown in Table X. It was found that toxic material could be interpreted as being aggregates of a substance whose molecular weight was about 30,000. The major peak of activity therefore appeared to be the dimer (M.W. approx 60,000).

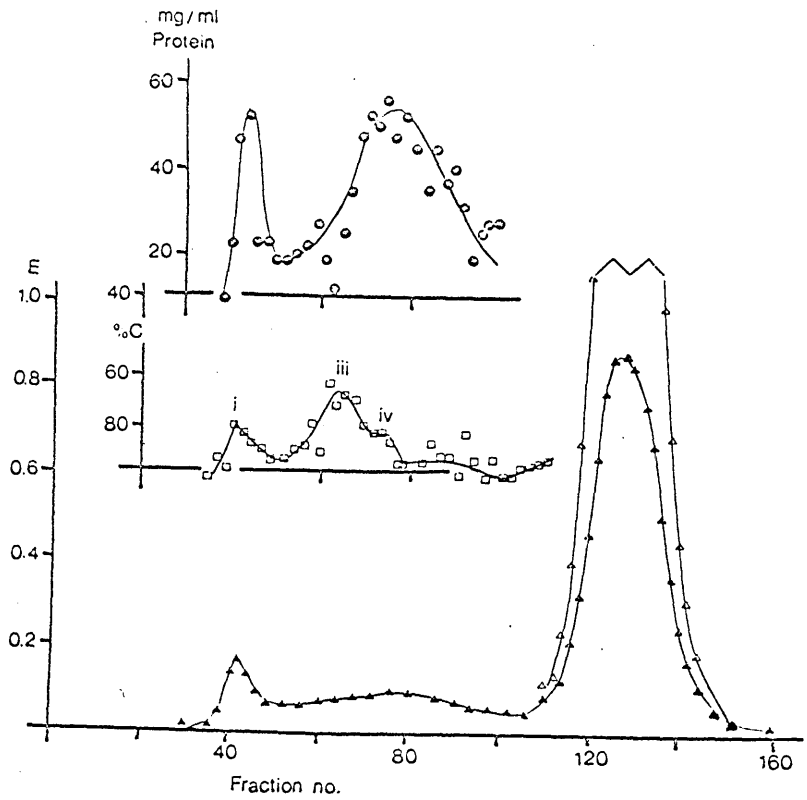


Fig. 30. Fractionation of Silverson extracted plaque on Sephadex G 150

158 mg extracted plaque material was applied to the 82 x 1.5 cm G 150 column.

Top: Protein concentration (mg/ml) against fraction number (Methods 9b) o—o.

Middle: Growth (%C) against fraction number (Methods, 10c and Table X) □—□.

Bottom: Absorbance (E) against fraction number
E_{280} ▲—▲. E_{260} △—△.

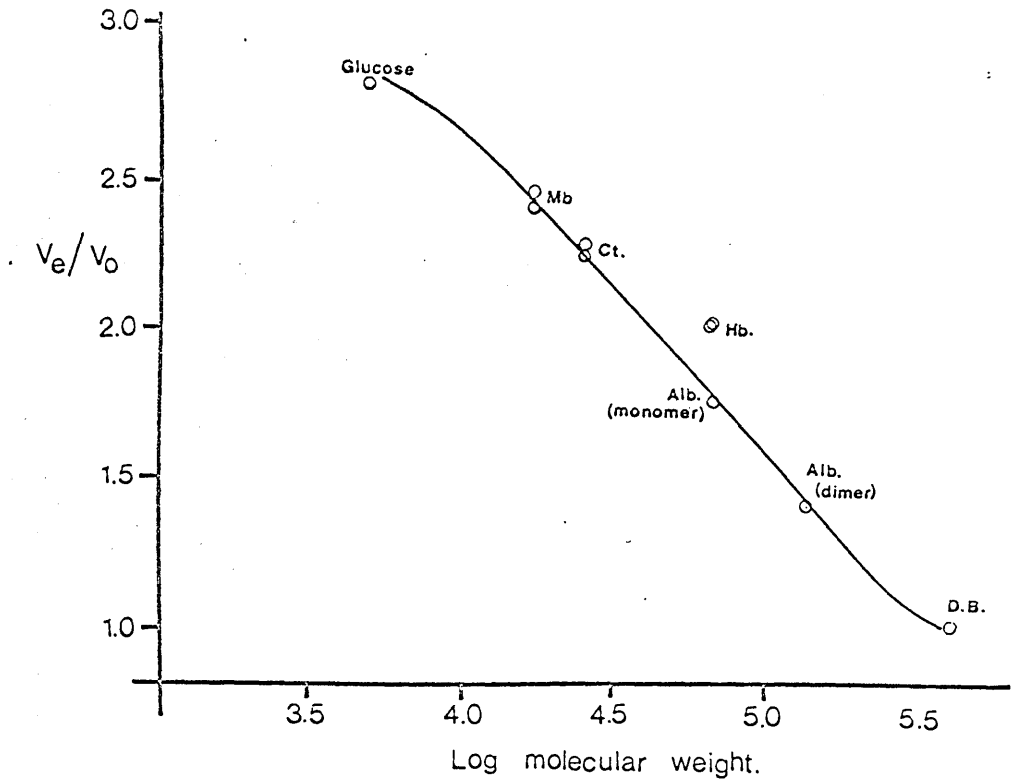


Fig. 31. Standard elution curve for various substances from Sephadex G 150

Plot of ratio of elution volume (V_e) to excluded volume (V_o) against log molecular weight of standard substance. (See Methods, 5b).

Mb. myoglobin; Ct, chymotrypsinogen; Hb, haemoglobin; Alb, serum albumin; D.B. dextran blue.

Table X

Calculation of molecular weights of toxic material eluted from Sephadex G 150 in the partially excluded fractions

Peaks No.	[1]Elution Vol. (V_e; ml).				[2]Range of molecular weights	A possible interpretation
Figs. No.	27	28	29	30	10^{-3} k	
i	-	45	46	42	234 - 281	240 (8)
ii	48	55	58	-	151 - 174	180 (6)
iii	66	66	65	64	105 - 115	120 (4)
iv	78	79	78	74	58 - 72	60 (2)
v	90	94	-	-	29 - 35	30 (1)

Notes:- (1) Excluded volume, V_o = 43 ml

(2) Obtained from fig 31, and V_e/V_o

These observations support the previous result (section 3) which suggested that the high molecular weight factor may be activated by a diffusible factor and form a toxic aggregate which disintegrates on fractionation if plaque is extracted with a Silverson homogeniser but not with a Potter pestle.

(b) Effect of treating PE with trichloroacetic acid at 0°C.

Trichloroacetic acid (TCA) is frequently used at 0°C to selectively precipitate proteins from a complex solution. As the most frequently encountered biologically active substances with molecular weights of the order observed in section 5a are proteins, the PE was treated with TCA and extracted with ether. The water layer was concentrated to 0.4 ml, and applied to the G 150 Sephadex. The non-retarded fractions were assayed for E_{260}, E_{280}, and toxic material. The results (fig 32) showed that neither growth inhibition nor material absorbing at 280 nm were observed.

However, toxic material may have been extracted into the ether layer in the course of TCA treatment. PE was therefore extracted with ether, and both layers assayed. Fig 33 is a plot of growth yield against the concentration in the medium of extracted plaque material, its ether soluble fraction, and its water soluble fraction. No growth inhibition was observed in cultures treated with the ether soluble fraction. The growth inhibition in cultures treated with the water soluble fraction was the same as in the presence of the unfractionated material (fig 33), or indeed if the 2 fractions were recombined (not shown).

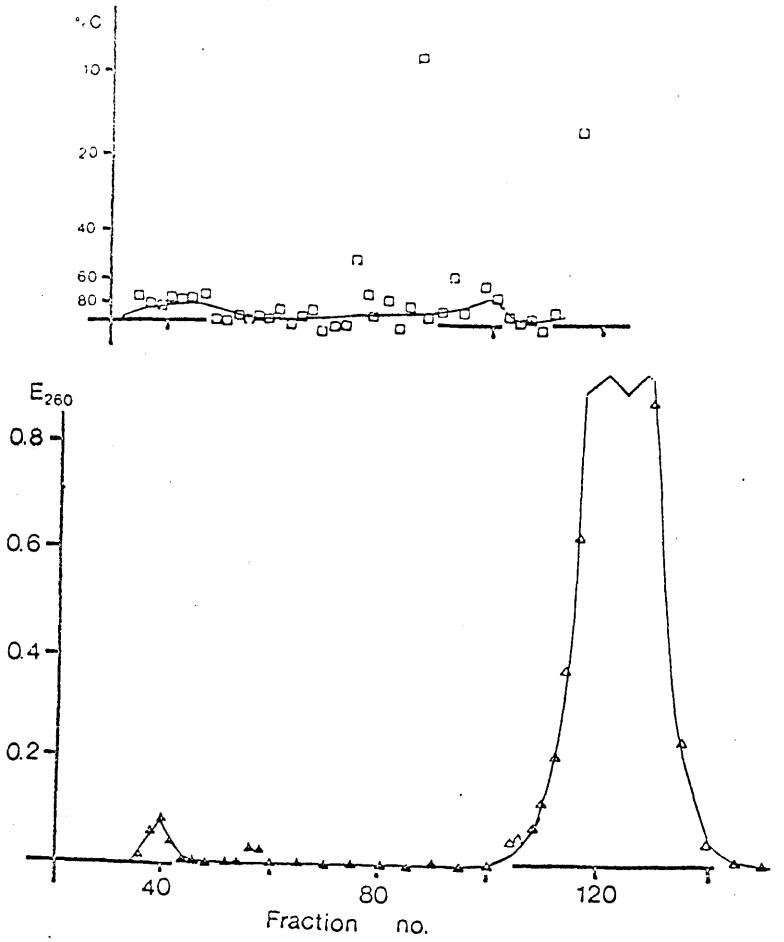


Fig. 32. Elution from Sephadex G 150 of extracted plaque material after treatment with trichloroacetic acid.

PE (Potter) was treated with TCA (Methods, 8), and the equivalent of 90 mg unfractionated plaque material in 0.5 ml was applied to the 82 x 1.5 cm Sephadex G 150 column (Methods, 5b).

Top: Growth (%C) against fraction number (Methods, 9b). The fractions were diluted 1 to 1.3 □ — □.

Bottom: Absorbance (E) against fraction number

E_{280} ▲ — ▲. E_{260} △ — △.

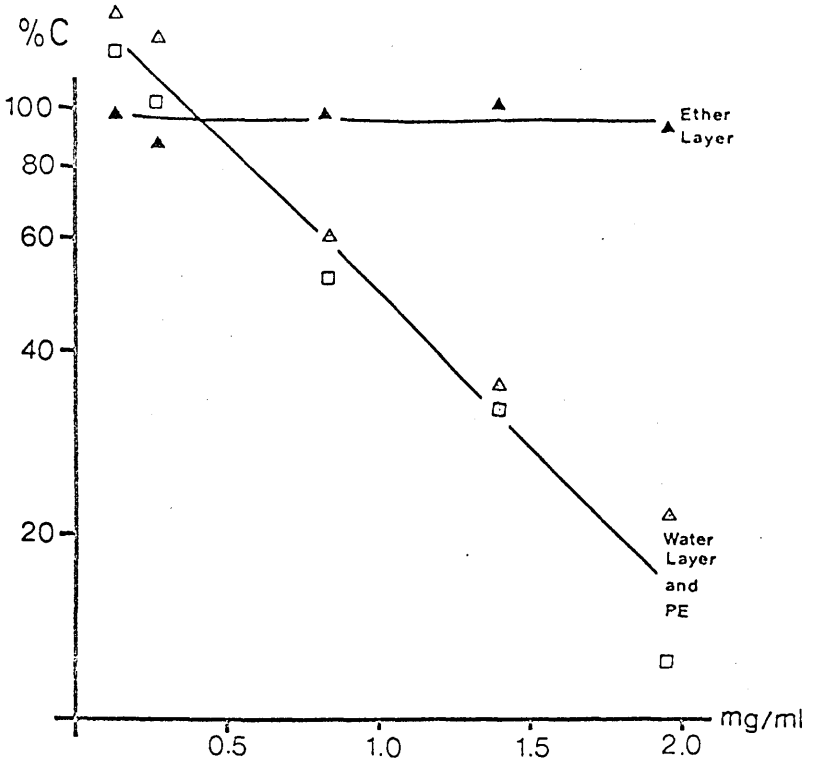


Fig. 33. Relative growth of cells treated with the ether soluble and insoluble fractions of PE

PE (Silverson) was extracted with ether (Methods, 7), and material in both layers was incorporated into EC_{10} (Methods, 10b). Cells grew in 5 cm Petri dishes in 3 ml medium. At 96 h, cells had grown 10-fold to 15.3×10^5 cells per dish. The growth (%C) was plotted against the equivalent amount of unfractionated plaque material added in the EC_{10}. (See legend to fig 12).

Ether soluble layer added ▲ — ▲.

Water soluble layer added △ — △.

Unfractionated PE added □ — □.

It was concluded that the toxic material was not soluble in ether, and therefore that toxic material and protein had been removed by TCA treatment. As toxic activity may have been destroyed by TCA treatment other than by its precipitation of protein, this result does not prove the toxic material to be protein.

(c) Low molecular weight fraction

The fractions retarded on G 150 Sephadex ($E_{260} > E_{280}$) were pooled, reconcentrated to about twice the volume applied to the G 150 column, and centrifuged to remove insoluble material. The solution (0.8 ml) was applied to a 22 x 0.9 cm G 10 Sephadex column.

Fig. 34 is a plot of E_{260} and growth inhibition against fraction number. Five peaks of E_{260}, and 2 peaks of growth inhibition were observed. Since the total volume of this column was 14 ml, much of the 260 nm absorbing material would appear to have had some affinity for the gel dextran, since its elution volumes were excessively retarded.

The diffusate of the PE against distilled water was applied to the same G 10 column (Methods, 5b). Fig. 35 is a plot of E_{260}, protein concentration, and growth inhibition in the eluted fractions. The positions of both the growth inhibiting peaks and the 260 nm absorbing peaks were very similar to those in Fig. 34. Toxic material which diffused into distilled water after extraction from plaque with a Silverson appeared to have been eluted from the gel in a similar position to toxic material which had been obtained after extraction with a Potter pestle, and was retarded on G 150 Sephadex.

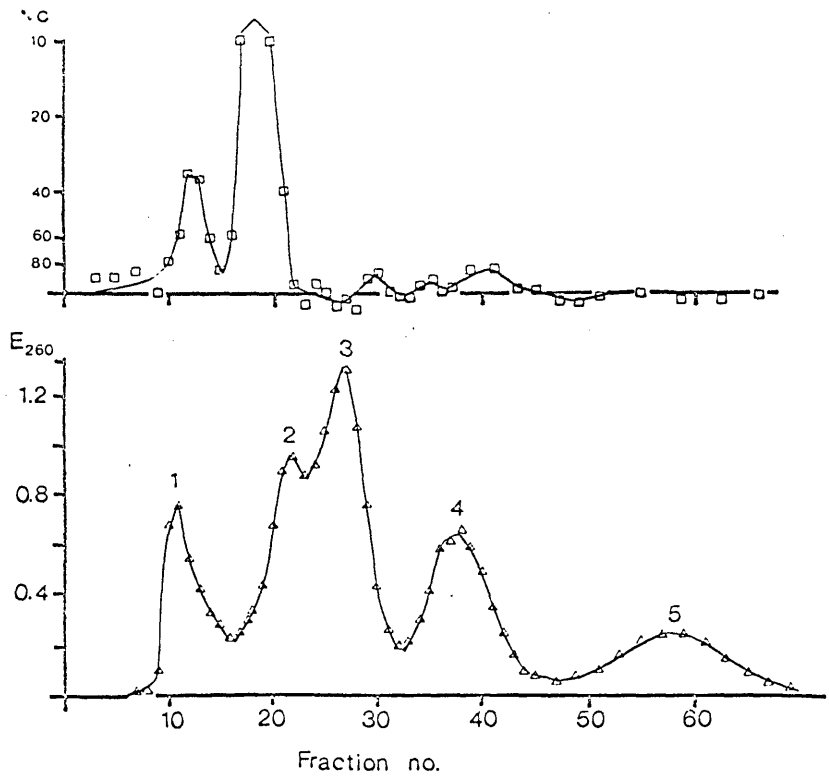


Fig. 34. Elution from G 10 Sephadex of PE fractions retarded on G 150 Sephadex in experiment shown on Fig. 27

The fractions retarded on G 150 Sephadex (fig. 27) were pooled, reconcentrated to about twice the volume applied to the G 150 column, and centrifuged to remove insoluble material. The supernatant fraction (0.8 ml) was applied to a 22 x 0.9 cm G 10 Sephadex column.

Top: Growth (%C) against fraction number - Methods, 10c. Each fraction was diluted 1 to 2 in EC_{10} □—□.

Bottom: Absorbance (E_{260}) against fraction number (1 to 1.7 dilution of each fraction) △—△.

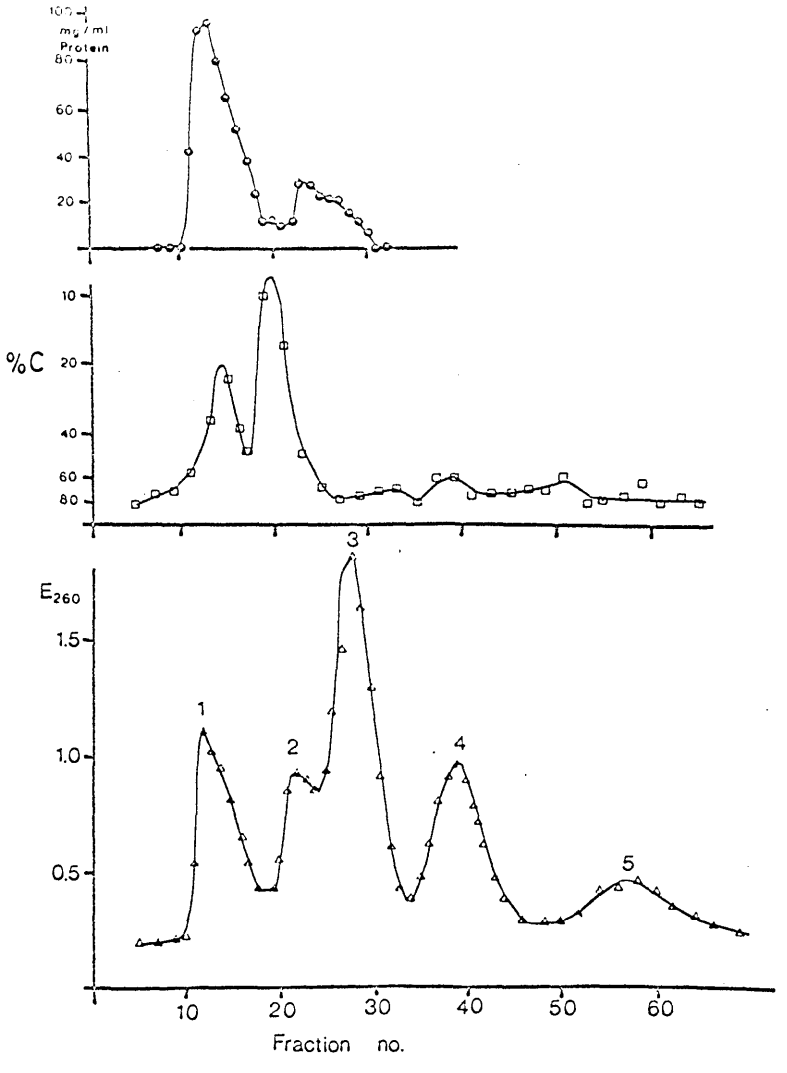


Fig. 35. Fractionation of PE diffusate from dialysis using G 10 Sephadex

Plaque was extracted with a Silverson, and the PE dialysed. The diffusate was reduced to 5% its usual volume (Methods,4), and 0.6 ml applied to the 22 x 0.9 cm G 10 Sephadex column.

Top: Protein (mg/ml) against fraction number. o—o.

Middle: Growth (%C) against fraction number - Methods, 10c). Each fraction was diluted 1 to 2 in EC_{10} □—□.

Bottom: Absorbance (E_{260}) against fraction number (1 to 1.7 dilution of each fraction) Δ—Δ.

In section 4, it was observed that dThd was extracted from plaque and interfered slightly with radioactive dThd uptake into HeLa cells. This dThd was assayed by its inhibiting uptake of high specific activity radioactive dThd in the growth medium of HeLa cells after 24 h treatment with the eluted fractions. The results (fig 36) indicated that two peaks of thymidine uptake inhibition were observed. The first peak was small and coincided with the first growth inhibition peak. The second peak was very large and extended well beyond the second growth inhibition peak.

The experiment was then repeated, collecting aliquots in 0.35 ml fractions instead of 0.6 ml. Each fraction was assayed for growth inhibition and radioactive dThd uptake inhibition. The results (fig 37) showed two peaks of growth inhibition as before, although the first peak was barely detectable, and no dThd uptake inhibition corresponded with it. Inhibition of dThd uptake did correspond to the second sharp growth inhibition peak, although a closely adjacent and yet larger peak of dThd inhibition corresponded to fractions eluted beyond the sharp growth inhibition peak where growth was apparently normal. This last peak can only have been caused by dThd reducing the specific activity of the radioactive tracer in growth medium containing those fractions.

The G 10 Sephadex was standardised, and the standard elution curve is shown in fig 38. It was observed that nucleosides were retarded (large V_e/V_o) relative to the other standards, and a separate curve has been drawn. Table XI indicates the elution volume and the mean V_e/V_o for the various peaks. The

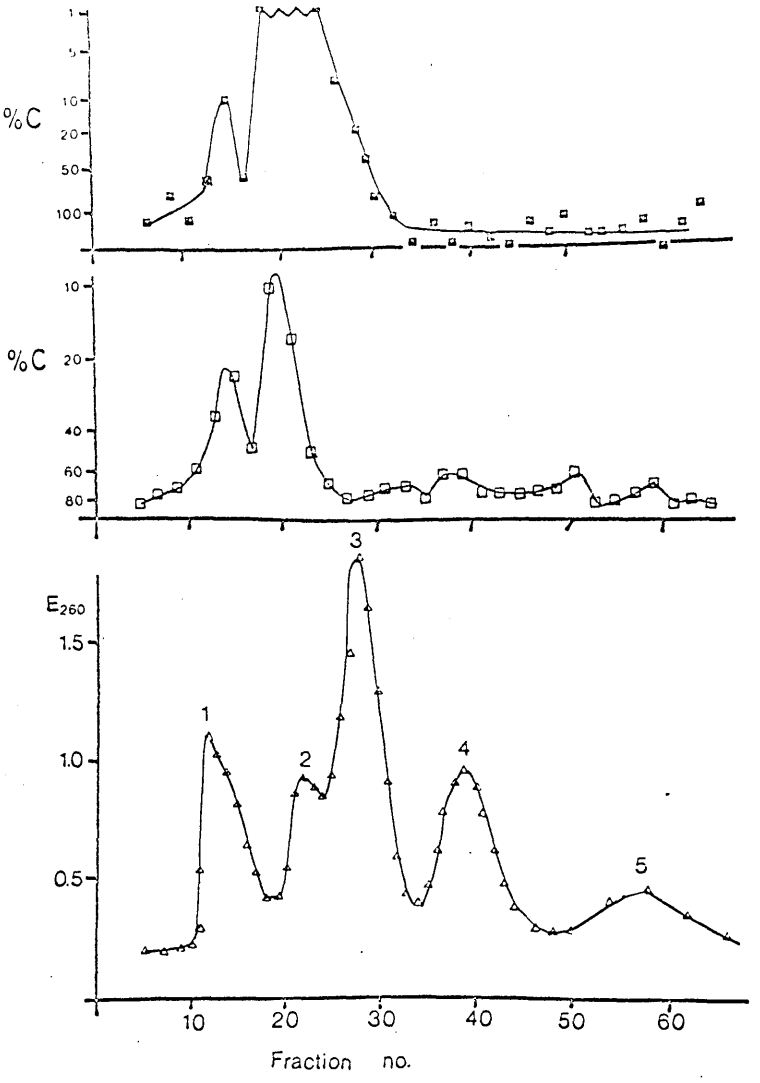


Fig. 36. A comparison of growth and thymidine uptake assays on fractions from PE diffusate after Sephadex G 10 fractionation

Same experiment as shown in Fig. 35 except that uptake of 5 µc [^{3}H]dThd (20 curies/mmole) over 1 h after 24 h treatment was measured as well as growth (Methods,10c and d).

Top: Relative uptake of [^{3}H]dThd into DNA (%C) - Methods 10d - against fraction number. 100% was 31.9 x 10^3 cpm; 1 to 2 dilution of each fraction in EC_{10} □—□.

Middle: Growth (%C) against fraction number - Methods, 10c. Each fraction was diluted 1 to 2 in EC_{10} □—□.

Bottom: Absorbance (E_{260}) against fraction number (1 to 1.7 dilution of each fraction) △—△.

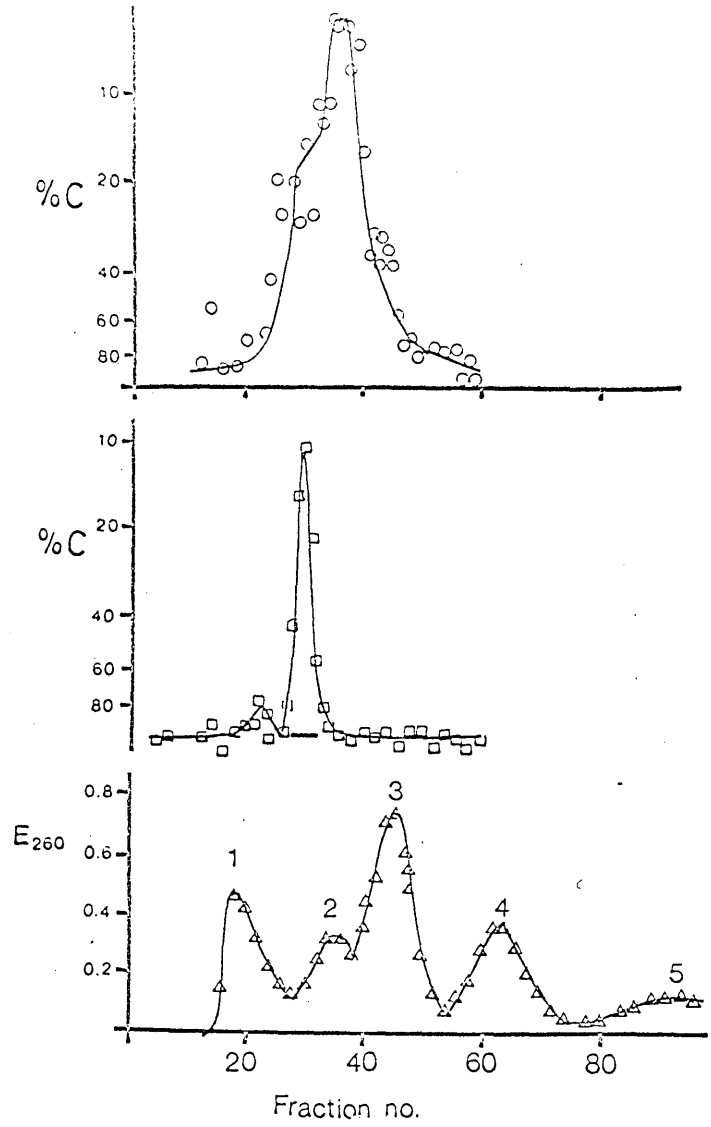


Fig. 37. Position of maximum inhibition of thymidine uptake in fractions from diffusible PE material eluted on Sephadex G 10

Similar experiment to that shown in Figs. 35 and 36, except that 0.4 ml diffusate (see legend to Fig. 35) was applied to the column, and the fractions were collected in 0.35 ml and not 0.6 ml aliquots.

Top: Relative uptake of [^{3}H]dThd into DNA (%C) against fraction number - Methods, 10d. (100% was 3.9×10^3 cpm; 1 to 9.5 dilutions) o — o.

Middle: Growth (%C) against fraction number - Methods, 10c. (1 to 4.8 dilutions in EC_{10}) □ — □.

Bottom: Absorbance (E_{260}) against fraction number (1 to 2.8 dilutions) Δ — Δ.

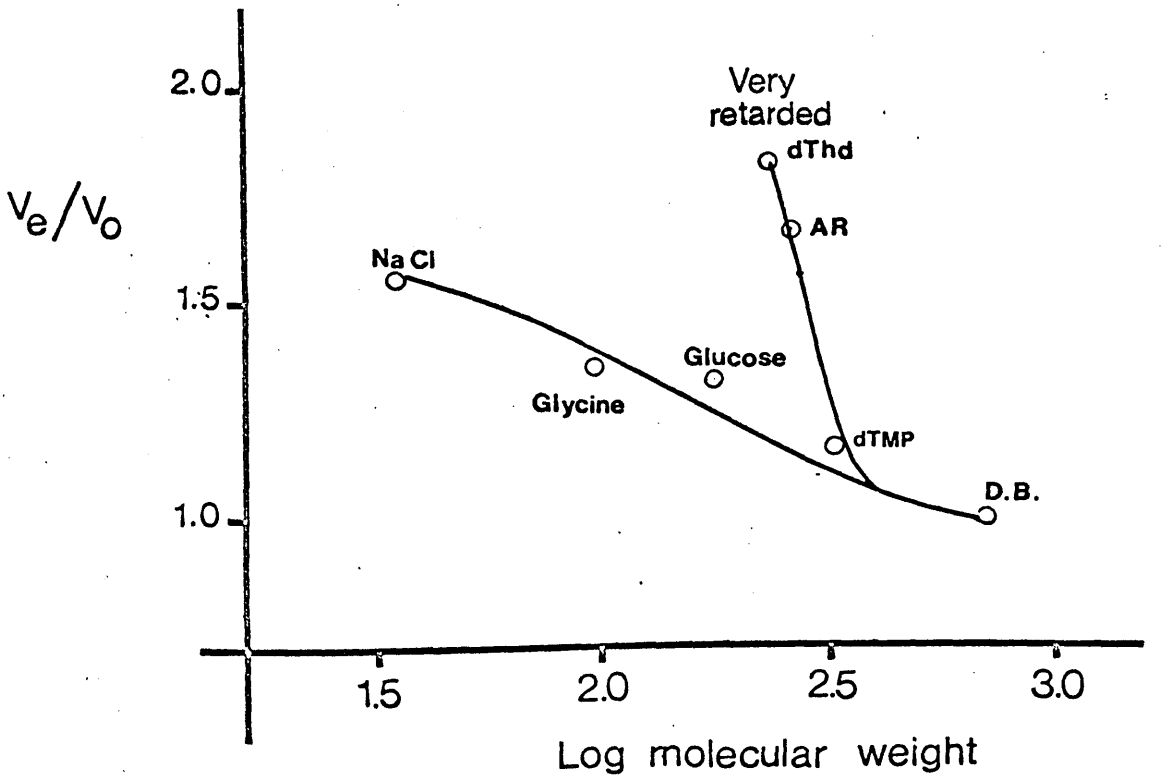


Fig. 38. Standard elution curve for various substances from Sephadex G 10

Plot of ratio of elution volume (V_e) to excluded volume (V_o) against log molecular weight of standard substance. (See Methods 5b).

D.B., dextran blue; AR, adenosine ribonucleoside; dThd, deoxythymidine ribonucleoside; dTMP, deoxythymidine monophosphate; NaCl, sodium chloride.

molecular weights of material in the severely retarded 260 nm absorbing peaks were not estimated as the elution volumes of suitable standards (free nucleic acid bases) were not determined. Table XI indicates that thymidine was a part of the material absorbing at 260 nm in peak 2. That other nucleic acid derivatives are also present in this peak is indicated in figs 8 and 9a (section 2).

When 0.4 ml of 30% w/v sodium chloride solution was applied to the 22 x 0.9 cm column, a peak of growth inhibiting material was eluted with distilled water at exactly the same place on the column as the second toxic peak. Using 0.25 M $AgNO_3$ to assay each eluted fraction, it was observed that the maximal weight of precipitate coincided with this peak (fig 39a). From the weight of precipitate obtained from treating material pooled from the second growth inhibiting peak (6 different experiments), and a plot of weight of precipitate per assay against the NaCl concentration (fig 39b), this toxic peak was found to be 400 mM saline.

Of this, 100 mM would be the eluting carrier saline, leaving 300 mM presumably due to excess saline. Table XII is the growth yield at different concentrations of excess saline (mg/ml) added to growth medium with this pooled fraction. By regression analysis, it was found that no growth occurred if 8.7 mg excess saline was added per ml growth medium in this way. Since 8.5 mg excess saline per ml medium prevented growth (fig. 3), it was concluded that the second growth inhibiting peak had

Table XI

Mean V_e/V_o ratios for material in PE eluted from Sephadex G 10

Substance	Fraction No. (0.6 ml aliquots) fig 34	fig 35	fig 37	fig 40	fig 41	Mean V_e/V_o	[1]Molecular weight	Probable identity
(a) 260 nm absorbing								
Peak No.1 (V_o)	11	12	18	21	12	1.00	-	-
Peak No.1a (V_e)	-	-	-	25	-	1.19	347	-
Peak No.2 (V_e)	22	22	34	41	22	1.90	234	thymidine
Peak No.3 (V_e)	27	28	45	53	26	2.41	-	-
Peak No.4 (V_e)	38	39	63	81	37	3.43	-	-
Peak No.5 (V_e)	58	57	83	-	58	4.89	-	-
(b) dThd uptake assay (fig 37)	-	-	35	-	-	1.94	230	thymidine
(c) dThd standard						1.82	240	thymidine
(d) Growth inhibiting								
Peak No. 1 (V_e)	13	15	22	26	-	1.21	224	Diffusible toxin
Peak No. 2 (V_e)	19	19	29	-	20	1.64	-	Retarded salt
(e) NaCl standard						1.56	-	Retarded salt

Note:- (1) Estimated from fig 38 and mean V_e/V_o

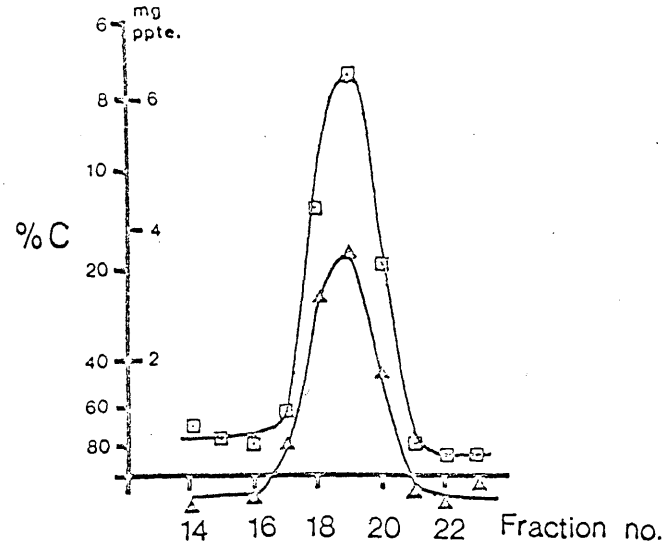


Fig.39a. Elution of growth inhibiting and silver nitrate precipitating material from Sephadex G 10 after application of sodium chloride standard solution

0.4 ml of 30% NaCl was applied to the 22 x 0.9 cm G 10 column, and eluted in 0.6 ml fractions. The fractions were diluted to 1.0 ml, and 0.6 ml of the diluted fractions were used in 1 ml EC_{10} to measure growth after 96 h (%C) - Methods, 10d □—□. 0.1 ml of the diluted fractions was added to 1 ml 0.25 M $AgNO_3$, and the weight of precipitate determined (Methods, 9f)▲—▲.

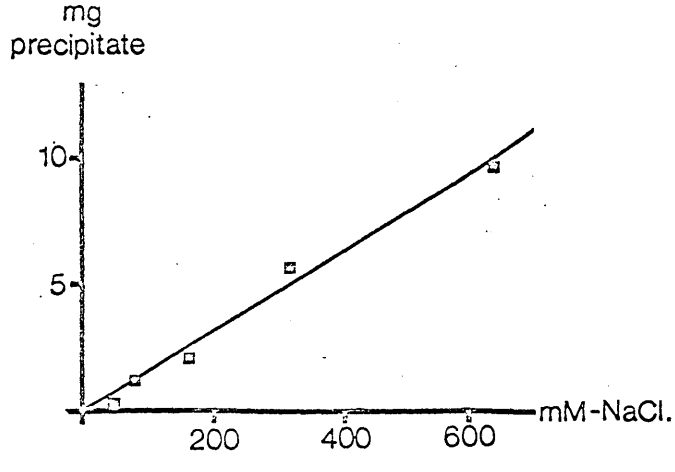


Fig.39b. Standard curve of weight of precipitate with 0.25M silver nitrate against the milli-molar concentration of sodium chloride

See Method, 9f.

Table XII

Growth yield at different concentrations of saline added with the second toxic peak from Sephadex G 10

Conc. saline (mg/ml)	Growth yield[1]
0.00	2.000
0.05	1.955
0.10	1.940
0.20	1.936
0.48	1.905
0.93	1.848

Note:- (1) Growth yield expressed as log % cell number in control cultures. Growth was from 1.1 to 13.2 x 10^5 cells per dish, and 13.2 x 10^5 = 100% growth

been produced only by the high saline concentration in which the G 150 retarded (or diffusible) fractions had been applied to the G 10 column (figs. 34 and 35).

Only the first growth inhibiting peak was therefore due to low molecular weight material in the extract which was toxic. Because of its V_e, this material is probably not excessively retained in the gel, and its molecular weight has therefore been estimated as about 224.

In another experiment, growth inhibition in the G 150 Sephadex pooled retarded fractions was assayed in duplicate by incorporation of 5 µc [H^3] dThd into DNA, as a 1 h pulse after 24 h treatment with each fraction. Only the mean estimation is recorded. Fig. 40 shows the concentration of protein, carbohydrate, 260 nm absorbing, and growth inhibiting material in the first eluted fractions. A single excluded protein peak was observed (c.f. fig. 35), although a long tail suggests that peptides and amino acids may also have been present. The carbohydrate was present as 2 peaks, the major peak having the same V_e as a glucose standard solution subsequently run through the same column. Most of the carbohydrate in this G 150 retarded fraction therefore appears to have been monosaccharide which may have arisen from hydrolysis of extracted polysaccharide. Since serum proteins were in the extract, however, some of the monosaccharide could be serum glucose (see section 2c, iv). The V_e for each 260 nm absorbing peak was as indicated in table XI.

In Fig. 40, the first peak of radioactive thymidine uptake inhibition corresponded to the first peak of growth inhibition in figs. 34 and 35, and therefore was due to toxic material in the extract.

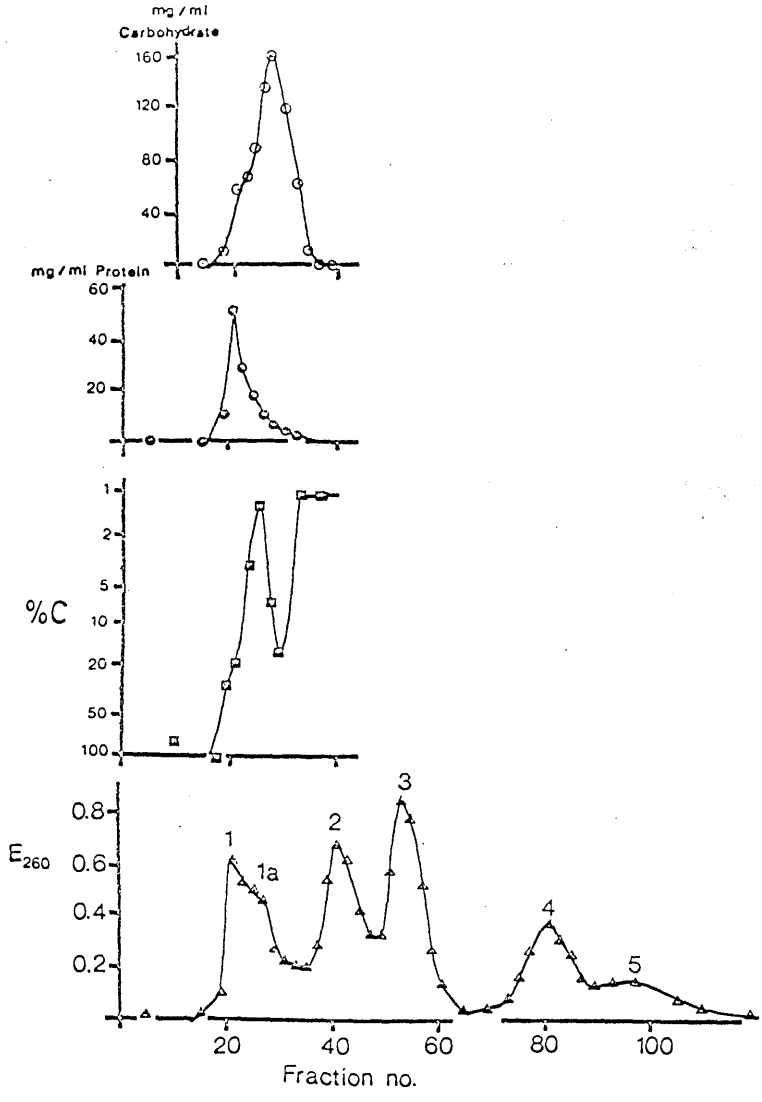


Fig. 40. Elution from Sephadex G 10 of toxic and other material from PE in retarded fractions after Sephadex G 150 treatment in experiment shown in Fig. 28

See legend to Fig. 34, replacing 'Fig. 27' with 'Fig. 28'.

1st. Top: Carbohydrate (mg/ml) against fraction number (1 to 1.7 dilutions) - Phenol reagent, Methods, 9c. o—o .

2nd Top: Protein (mg/ml) against fraction number (1 to 1.7 dilutions) ⊙—⊙ .

3rd Top: Uptake of 5 uc [^{3}H]dThd tracer (20 cm/mole) into DNA after 24 h treatment (%C) - Methods, 10d. (Fractions diluted 1 to 1.3 in EC_{10}) ▫—▫

Bottom: Absorbance (E_{260}) against fraction number (1 to 1.7 dilutions) △—△.

(d) Effect of trichloroacetic acid on PE growth inhibiting material retarded on G 150 Sephadex after fractionation on G 10 Sephadex

The 260 nm absorbing fractions from PE after TCA treatment, ether extraction, and G 150 Sephadex fractionation (fig. 32), were pooled, reconcentrated, and applied to the Sephadex G 10 column. The results are shown as plots of E_{260} and growth inhibition against fraction number (fig. 41).

The ultraviolet elution peak 1 (excluded peak) contained much less material than peak 1 from the equivalent PE fractions without prior TCA treatment (figs. 34 and 35). Similarly, less 260 nm absorbing material appeared in peak 2 relative to peak 3 after TCA treatment (fig. 41, bottom graph) than in Potter extracted plaque without TCA treatment (figs. 8a, 34 and 40). It appears that the TCA treatment could have hydrolysed some of the material appearing in peak 2, and caused it to appear in peak 3.

Fig. 41, top graph, shows that only one growth inhibiting peak was eluted from the G 10 column after TCA treatment. As this peak corresponded to the position of the saline peak (figs. 34 and 35), it was concluded that no toxic material had been present. As the TCA treated PE separated on G 150 Sephadex contained no toxic material either (section 5b), it was concluded that all the toxic material in PE had been lost or destroyed by the TCA treatment.

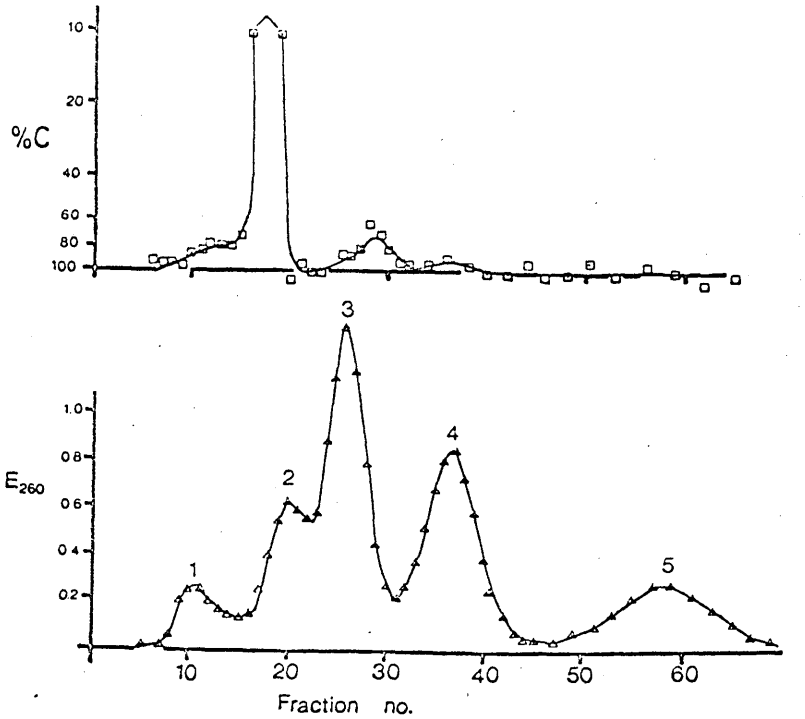


Fig. 41. Sephadex G 10 fractionation of material in pooled retarded fractions after Sephadex G 150 separation of TCA treated PE from experiment shown in Fig.32

PE (Potter) was treated with TCA (Methods, 8), and the equivalent of 90 mg extracted plaque material was applied to the 82 x 1.5 cm Sephadex G 150 column (Methods, 5b). The retarded fractions (Fig. 32) were pooled, reconcentrated to about twice the volume applied to the G 150 column, and centrifuged to remove insoluble material. The supernatant fraction (0.8 ml) was applied to a 22 x 0.9 cm G 10 Sephadex column.

Top: Growth (%C) against fraction number (1 to 2 dilutions of each fraction in EC_{10}) - Methods, 10c □ — □.

Bottom: Absorbance (E_{260}) against fraction number (1 to 1.7 dilutions) △ — △ .

(e) Summary and conclusions

The results of these experiments appear to confirm and amplify those in section 3. The toxic material retarded on Sephadex G 150 appeared to be the minor toxic component in the PE, and was eluted from Sephadex G 10 as a single peak. Assuming it was not abnormally retarded like the nucleic acid bases on Sephadex G 10, it appeared to have a molecular weight of 224. It was not present after treatment of PE with trichloroacetic acid.

A second and larger peak of toxic material eluted from Sephadex G 10 was shown to be an artifact caused by unavoidably high saline concentrations in the applied sample.

One of the 260 nm absorbing peaks eluted from Sephadex G 10 contained thymidine, confirming the evidence in section 4.

All the other toxic material appeared to have a molecular weight of at least 30,000. Its elution profile suggested that it might have been eluted as aggregates corresponding to a dimer, tetramer, hexamer, and octamer. The most active aggregates appeared to be dimer and tetramer.

Silverson extracted plaque contained much less toxic material, and in particular, much less of the active dimer after Sephadex G 150 fractionation. The relatively violent agitation that occurred during Silverson extraction of plaque may have altered the structure of this toxic material, and caused it to become relatively inactive once the diffusible factors simultaneously extracted from plaque had been removed. This idea is consistent with all the relevant observations in sections 3 and 5. Nevertheless, the high molecular weight toxic material could be altogether more complex than this interpretation suggests.

The most toxic fractions (peak iv) contained the most protein, and both toxic activity and protein were removed after TCA treatment. These associations could be fortuitous, and the nature of the high molecular weight material has yet to be determined. Peaks of toxic activity did not seem to correspond to peaks of 260 nm absorbing material or carbohydrate on either Sephadex G 10, or Sephadex G 150.

The elution pattern of toxic and other material from Sephadex G 10 and G 150 appeared to be reproducible from the PE from at least 4 groups of donors, and despite variation in the precise way in which the plaque had been prepared prior to Sephadex treatment. This corroborates the results in section 2 which indicate that plaque from different groups of patients contains similar kinds of extractable material.

DISCUSSION

(a) Source of biochemical material extracted from plaque

It has previously been calculated that micro-organisms and interbacterial matrix polysaccharide constitute 50% and 10% of the dry weight of plaque respectively (Critchley, et al., 1967). From this investigation, about 32% of the dry weight of plaque was found to be solid which was extractable with a Silverson homogeniser. The extracted material was apparently derived from serum, from cell metabolism, and from disrupted bacterial cells. About half the number of micro-organisms in plaque cannot grow *in vitro* (Socransky, et al., 1963; Poole and Gilmour, 1971), probably because some plaque bacteria have such complex growth and nutritional requirements that their conditions of culture *in vitro* are not known and because others are not viable. The latter autolyse in the mouth and are probably disrupted by the extraction procedure. The presence of nucleic acid material in the plaque extract suggests that autolysing bacteria are a significant proportion of the total microbial content.

(b) Plaque and salivary fluids

Saliva is reported to contain a range of from 0.4 to 0.7% solids (Jenkins, 1966). However, this large variation (from 0.4 mg/ml to 0.7 mg/ml) is due only to a very small variation in its water content, from 99.6% to 99.3%. Similar considerations apply to plaque. This explains the findings (RESULTS, 2a), that the variation in the mean concentration of the extractable solids in plaque, or of those dissolved in saliva,

Table XIII

Mean percentages of cultivable organisms in the adult oral cavity

Predominant Genera	Crevice area	Dental plaque	Tongue	Saliva
Gram positive facultative cocci	28.8	28.2	44.8	46.2
Gram positive anaerobic cocci	7.4	12.6	4.2	13.0
Gram negative facultative cocci	0.4	0.4	3.4	1.2
Gram negative anaerobic cocci	10.7	6.4	16.0	15.9
Gram positive facultative rods	15.3	23.8	13.0	11.8
Gram positive anaerobic rods	20.2	18.4	8.2	4.8
Gram negative facultative rods	1.2	not detected	3.2	2.3
Gram negative anaerobic rods	16.1	10.4	8.2	4.8
Spirochaetes	1.0	not detected	not detected	not detected

Note:- (1) Adapted from Socransky and Manganiello, (1971).

was due mainly to a variation in the water content of different samples. Hence the water content of saliva, and the extent to which it washes into plaque, seem to be important in determining the variation in the concentration of solids in both. Therefore, despite the marked variation in the microbial content of pooled unstandardised plaque (Poole and Gilmour, 1971), the variation in the composition of its extractable fluid fraction appears to be due more to differences in the wetness of the plaque than to differences in its microbial composition.

It was also found that constant amounts of total soluble solid, toxic material, nucleic acid material, protein, and carbohydrate, respectively, were extracted per g wet weight of plaque (RESULTS, 1) and were distinct from those per ml saliva (RESULTS, 1e and 2a). In addition, further studies disclosed that much of the material in saliva was qualitatively distinct from that in plaque (RESULTS, 2). The saliva does not appear to be a 'natural' plaque extract. Although small amounts of material may have come from plaque and vice versa, plaque and saliva appear to consist of quite different fluids.

(c) Plaque and salivary ecosystems

Table XIII has been adapted from Socransky and Manganiello (1971). It shows the mean percentages of the major cultivable species of bacteria in plaque and saliva. Plaque is divided into gingival crevice area plaque, and 'dental plaque', restricting the latter term to the plaque which does not lie at the gingival margin although there is no obvious point where it can be distinguished from the plaque which does.

Indeed, the number and types of cultivable bacteria from either division seem much the same (table XIII). Likewise, the bacterial flora adhering to the tongue is similar to the flora dispersed in saliva in clumps, the saliva sediment. In this case, despite the presence of an obvious dividing line (attachment to the tongue) both tongue and salivary ecosystems appear to be very similar. In fact, the data in table XIII could have been interpreted as being from two, and not four different ecosystems; namely the dental plaque ecosystem which adheres to the teeth, and the salivary ecosystem which does not adhere to the teeth.

Only the salivary ecosystem can be found in the absence of teeth. The two ecosystems apparently differentiate only when teeth are present and are left uncleaned (INTRODUCTION, 1b and 3d). If salivary fluid is the substrate for the bacteria in both ecosystems, it is difficult to understand why such a distinct dental plaque ecosystem invariably develops. Hence, it must be concluded that salivary fluid is almost certainly not the growth medium for the dental plaque ecosystem. This probably explains why the composition of salivary fluid was found to be quite different from that of the extractable plaque fluid.

If the plaque samples had been sufficiently contaminated with blood and saliva, the extractable plaque fluid would be expected to include a certain amount of both salivary and serumnal material. Despite almost no rinsing procedure being employed to prevent salivary contamination of the plaque samples on collection (METHODS, 1a), no salivary protein was

clearly detected in the extractable plaque fluid (RESULTS, 2c). Furthermore, many serumnal proteins, including some relatively common ones (ceruloplasmin and haptoglobin), were also not detected in the extractable plaque fluid (RESULTS, 2c) although trace amounts of whole blood had certainly contaminated a few of the plaque samples.

Virtually all the plaque was collected from patients who had gingivitis or periodontitis (METHODS, 1a). Such donors exude gingival fluid, which is probably an inflammatory exudate and contains serum proteins (INTRODUCTION, 2b). It seems reasonable that this fluid is at least as likely to be a source of the serum proteins and free glucose in the plaque fluid as whole blood contamination. When this assumption is considered in addition to the previous conclusion, that the salivary fluid is almost certainly not the substrate for the dental plaque ecosystem, the results indicate that the gingival fluid seems likely to be the substrate for the dental plaque ecosystem (INTRODUCTION, 2f). A reconsideration of Socransky and Manganiello's original interpretation of table XIII now suggests that their four different ecosystems may be considered to have different amounts of gingival or salivary fluids as their nutrient. The ecosystem from the gingival crevice area possibly grows exclusively on gingival fluid, the 'dental plaque' ecosystem possibly grows on gingival fluid plus traces of salivary fluid, the salivary ecosystem possibly grows on salivary fluid plus traces of gingival fluid, and the tongue ecosystem possibly grows on salivary fluid alone.

Oral bacteria have been shown to grow on gingival fluid *in vitro* apparently as well as on blood agar plates (Collins and Gavin, 1961). The possible antibacterial properties of gingival fluid may therefore be insufficient to prevent bacterial growth *in vivo*, but may explain why some bacteria from plaque seem unable to grow *in vitro*, and also, perhaps, why autolytic bacterial debris were found in the extractable plaque fluid.

Leach (1963) observed that as a precipitate developed in whole saliva at 37°C, sialic acid was hydrolysed from salivary protein. He showed that the precipitate was probably caused by neuraminidase cleavage of terminal sialic acid residues from salivary glycoproteins. By assuming that a similar reaction occurred in the mouth *in vivo*, he suggested that this salivary protein formed an early matrix for plaque development. He supported his suggestion with evidence that plaque contained no sialic acid.

The results (2c, iii) confirmed his observation but not his interpretation. If glycoprotein in saliva is being precipitated *in vivo* by the action of neuraminidase, protein in the salivary sediment ought to have contained less sialic acid per mg than protein in the salivary supernatant fraction. In fact the opposite result was obtained. The salivary sediment appeared to contain intact glycoproteins, or sialic acid rich glycoproteins; and not glycoproteins containing less bound sialic acid. Furthermore, proteins extracted from the salivary sediment were antigenically related to those in the salivary

supernatant fluid but were not detected in the extractable plaque fluid.

However, the precipitated salivary proteins may not be extractable either from plaque or the salivary sediment, may have become antigenically different from the unprecipitated protein in the salivary supernatant fluid, or both. Indeed, there is no simple way of disproving Leach's hypothesis since the origin of precipitated protein may be difficult to ascertain.

In any case, Leach's hypothesis does not explain table XIII; the different interrelationships of the cultivable bacterial species in the different oral ecosystems.

(d) Source of toxic material, and its relationship to periodontal disease.

The extractable plaque fluid was found to contain substances which were toxic to mammalian cells in culture and were present in similar amounts per g wet weight of plaque. Obviously it is not necessary that the same substances which affect one cell type in culture should either affect other cell types in culture or the cells *in vivo* of the gingival sulcus or pocket. However, even the last extrapolation is perfectly justified (INTRODUCTION, 4c), although many more investigations remain to be carried out into the nature and mode of action of the toxic material, how it comes to be found in the dental plaque ecosystem, and whether it really causes persistent inflammation of both the gingival and underlying tissues of the periodontium.

The salivary fluid also contained substances which were toxic to HeLa cells, but were about 20 times less concentrated than in the extracted plaque fluid (RESULTS, 1). Assuming that these toxic substances were the same in both the plaque and salivary fluids, it seems possible that the toxic material is synthesised mainly in the dental plaque ecosystem and can diffuse either into the gingival epithelium or into saliva.

Diffusion of toxic substances into the epithelial lining of the gingival sulcus, and especially into the unusually orientated junctional epithelial cells which form a part of this lining (INTRODUCTION, 2a), may possibly account, in part, for the close clinical association between the accumulation of plaque and the onset of gingivitis (INTRODUCTION, 1a iii).

(e) Nature and mode of action of the cytotoxic material

The toxic substances in the extractable plaque fluid were characterised by their preventing the growth of HeLa cells (RESULTS, 3 and 5). The linear log growth to dose relationship which HeLa cells exhibited enabled a relatively thorough characterisation of the toxic material to be obtained. However, it may well be desirable to repeat certain experiments using a different cell culture.

Only some of the toxic substances in the extractable plaque fluid were diffusible, fully retarded on Sephadex G 150, and apparently eluted as a single peak from Sephadex G 10. These had a molecular weight of about 225. However, many of the toxic substances were apparently non-diffusible. Their

elution in the incompletely retarded fractions with Sephadex G 150 suggested that they might either be an aggregating material, or toxic substances of a much smaller molecular size which were adhering to it. The aggregating material appeared to have a monomeric molecular weight of about 30,000 daltons.

The possibility that the toxic material is an aggregating protein or is adherent to one needs investigating by treatment of the plaque extract with a protease. Treatments of the plaque extract with heat, ammonium sulphate, urea, or thiol reagents, together with further analysis of the fractions after Amicon or Sephadex ultrafiltration, may elucidate further properties of the non-diffusible toxic substances.

The toxic substances were found to prevent growth and to cause a gradual disintegration of the HeLa cells within 96 hours (RESULTS, 1). On the other hand. DNA synthesis appeared to be mostly inhibited within only 1 to 2 hours' treatment with these substances. The effect of plaque extracts on other areas of cell metabolism such as RNA, protein, and cell wall synthesis, has yet to be investigated.

(f) Possible identity of the toxic material

At present, there seems little point in speculating what the in vitro acting toxic substances might be, although it seems relevant to point out that at present they appear to be neither bacterial endotoxins, nor bacterial proteases. These are present in the extractable plaque fluid (Simon, et al, 1969; Soder, 1969), and both have frequently been described as being important to the action of plaque on the

gingival tissues. Possibly because bacterial endotoxins have no direct toxic effect on most mammalian tissues (Weissman and Thomas, 1964), they do not affect the growth of mammalian epithelial-like or fibroblast cells in culture (Mesrobeanu, et al., 1960; Bergman and Weibull, 1969). Similarly, the elution pattern of proteases from plaque on Sephadex G 200 which is reported by Soder (1969), appears different from that of the toxic material on Sephadex G 150 reported here (RESULTS, 5a).

(g) Implications of these results for theories on the pathogenesis of chronic gingivitis and periodontitis.

In view of the above discussion, the results can be considered to provide evidence for the classical theory concerning the pathogenesis of periodontal disease; namely, that trauma irritates the gingival margin, and lowers its resistance to attack by oral bacteria (Fish, 1935).

The salivary ecosystem does not appear to irritate any of the oral epithelia which are in contact with it. On the other hand, leukocytes are detectable at the sulcular orifice when plaque is apparently absent and when the gingiva show almost no other signs of inflammation (INTRODUCTION, 2a and 2b). There is no reason why micro-organisms in the absence of plaque (i.e. in the salivary ecosystem) should be pathogenic here but not elsewhere in the mouth. Mild trauma to the sulcular epithelium may therefore occur intermittently as a result of its structure and function (INTRODUCTION, 2f). Such irritation may cause a brief flow of gingival fluid

from time to time in the absence of plaque (INTRODUCTION, 2b).

Those organisms from the salivary ecosystem which happen to lie at the gingival margin would then be washed with gingival fluid and proliferate to form the undeveloped plaque if not properly controlled by oral hygiene measures (DISCUSSION, c). Among the salivary organisms comprising this plaque, are some which now grow better, and produce more toxic material (DISCUSSION, d). The plaque extends into the sulcus and the toxic substances irritate the adjacent gingival tissues, particularly those cells of the junctional epithelium which lie at the base of the gingival sulcus. The irritation causes an increasing granuloytic infiltration of the epithelium and sooner or later the development of foci of inflammation in the adjacent connective tissue (INTRODUCTION, 2e). The connective tissue inflammation increases the flow of gingival fluid (INTRODUCTION, 2b). Although this fluid may have antibacterial properties and tend to wash out the sulcus, its nutrients are probably the major factor for the full development of the dental plaque ecosystem (DISCUSSION, c) and the lesion becomes clinically manifest as gingivitis.

Pocket formation ensues (INTRODUCTION, 2c) and protects the plaque accumulations in an area where they can be continuously washed with gingival fluid. Simultaneously or earlier, regions of the plaque may calcify owing to absorption of calcium and phosphate ions from either the gingival or salivary fluids (Chacker, 1968). This calcification also protects the uncalcified plaque which lies in a gingival sulcus or pocket.

This hypothesis proposes that physiological trauma at the gingival margin causes an intermittent flow of gingival fluid. This makes the adjacent tooth surface readily colonised by bacteria. The resulting plaque accumulations produce cytotoxic substances. This irritates the adjacent soft tissues, and increases both the frequency of gingival fluid exudation and its rate of flow (INTRODUCTION, 2b). The susceptibility of the host's cells to the toxic substances and the composition and flow rate of his gingival fluid in relation to his health and genetic endowment largely constitute the host factors. The bacteria which are able to produce the toxic substances and which have established within his dental plaque ecosystem largely constitute the environmental factors. The ease and length of time with which the plaque is not disturbed at the gingival margin allows the action of the toxic factors to continue and largely constitute the predisposing factors. These specific explanations of the aetiological factors periodontal disease (INTRODUCTION 1b i) are thus derived from the findings described in the RESULTS section.

(h) Possibility for preventing the development of chronic gingivitis and periodontitis.

It has already been shown that administration of antibiotics and antiseptics prevents the induction of gingivitis (INTRODUCTION, 3f). However, such measures are unsatisfactory for various reasons and at present only thorough and extensive oral hygiene measures can be recommended as being the best means of at least controlling the disease process.

Unfortunately, this method is only applicable to a highly motivated section of the population.

This investigation has shown that another preventive approach which is equally direct might now also be possible. Toxic material in plaque appears to result from the greater proliferation and development of oral bacteria in the presence of gingival fluid. By continuing investigations into the chemical nature of the toxic material and how it comes to be synthesised by bacteria in the dental plaque ecosystem, it might eventually become possible to prevent its synthesis or toxic activity by using a more generally applicable preventive measure than oral hygiene. In this way plaque development and gingivitis would be controlled, and probably also periodontitis.

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SUMMARY

Chronic gingivitis is generally believed to result from irritation to cells at the gingival junction. This is brought about by the accumulation of micro-organisms on teeth. These micro-organisms and their products are known as dental or bacterial plaque and constitute a bacterial ecosystem whose appearance and consistency differentiate it from other kinds of deposits on teeth. The dental plaque contains a variety of substances which are potentially pathogenic. Presumably some of these are extractable, and may be toxic *in vitro*. The aims of the present investigation were to examine the effects of extractable material from dental plaque on the growth of mammalian cells in culture, and to characterise any material having a cytotoxic effect. In addition, it was necessary to characterise the composition of the extractable plaque material, and to compare it with saliva and with serum.

Dental plaque was collected from the exposed surfaces of teeth, extracted into a modified Earle's saline solution, and homogenised. The mixture was centrifuged and the supernatant fraction (the plaque extract) was sterilised by millipore filtration. Unstimulated saliva was collected by spitting into an ice cooled beaker and then centrifuged. The supernatant fraction was retained, and the sedimented fraction was extracted as described for plaque. The supernatant fraction was sterilised by millipore filtration but the sediment extract was too viscous to be sterilised in this way. All three preparations were fractionated by membrane or Sephadex ultrafiltration and iso-electric focusing in polyacrylamide gels, as well as being examined by immunoelectrophoretic techniques. The total solid extractable from 1 g wet plaque

was determined after lyophilising samples of plaque extract. The ultraviolet spectra, and the amounts of protein, hexose, and sialic acid in these preparations were also determined. Mammalian cell cultures were grown in Eagle's medium with Earle's saline, calf serum to 10%, and penicillin and streptomycin each to 100 units/ml. Cells were incubated in 5% CO_2 in air at 37°C for 6 to 12 h, and then treated with either saline, plaque extract, or the saliva supernatant fraction. The dishes were reincubated for a known period, and the cells either counted, or measured for incorporation of tritiated thymidine into DNA.

The results showed that per g wet weight, the plaque preparations contained similar amounts of extractable material, including material which was toxic to 4 different types of mammalian cells in culture. This toxic material caused a gradual loss of the internal contents of HeLa cells and prevented their growth, a linear dose response relationship being observed. Plaque was found to be a much richer source of toxic material than saliva supernatant fraction. The nature and amount of extractable material from plaque differed markedly from that in saliva and serum preparations. Plaque extracts did not appear to contain any salivary material, but did contain a number of serum proteins. The toxic material was relatively stable. Most of it was found to have a molecular weight greater than about 30,000 and possibly to form aggregates. The dimer aggregate appeared the most active. The toxic material was inactivated after treatment with 5 % trichloroacetic acid at 0°C for 30 min. Toxic material in PE appeared to inhibit some process involved in DNA synthesis within 1 h of treating the cells.

The results suggested that gingival fluid, and not saliva, is substrate for the dental plaque ecosystem. The gingival fluid, an inflammatory serum exudate whose flow rate corresponds to the severity of chronic gingivitis, is a much richer source of nutrients than saliva. The increased bacterial growth which occurs as plaque develops probably results from the presence of gingival fluid. This proliferation and development of dental plaque would increase the amount of material which may directly irritate cells at the gingival junction. Toxic material may thus establish and maintain the flow of gingival fluid, and cause the development and persistence of plaque at the gingival margin. The toxic material was not identified but did not appear to consist of endotoxins or proteases.