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THE EASE OF TRANSLOCATION OF *Salmonella enteritidis* THROUGH THE EGGSHELL WALL: AN IMMUNOCYTOCHEMICAL / ULTRASTRUCTURAL STUDY

By

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Thesis submitted for the degree of Doctor of Philosophy
in the
Faculty of Veterinary Medicine at the University of Glasgow

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In memoriam:

To my beloved godfather (Dindo Nelson), godmother (Dinda Lely) and grandfathers (Vô Pinheiro and Vô João), for all the inspiration and guidance.

Wherever you are now, you are always in my mind and will never be forgotten.

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Science and the scientists must always, in my opinion, target and serve the people and their needs. No veterinarian can renounce from the responsibility of improving the human food supply. Famine in the world, with today's available technology, is a shame which every one of us has the duty to fight against.

Life teaches us many things, and we tend to become more selective as time passes. I am happy to say that these years here in Glasgow were fruitful in every sense. It has been much more than simply an opportunity to expand my professional knowledge. In fact, it has been an extremely important lesson of life. It has been an honour to live in a country with such wonderful people, from whom I am proud to say, I will carry with me forever a sense of citizenship, solidarity and idealism. Thanks Scotland, my adopted land.

I modestly wish to pay homage here to most of the Brazilian people who, struggling every day for survival, have all my respect, admiration and belief that Brazil will be, some day, a socially balanced and just place to live, an objective to which I very much hope to be able to contribute to achieve. Let's never turn our backs and forget the poor and the oppressed, as we, in our positions, may be their only chance.

Finally, my loving thanks go to all my family, who have provided encouragement and support when it was most needed, especially to my father, Jurandyr, and to the person who walked and shared with me all the steps of this work, my wife Fernanda, to whom I dedicate this thesis.

Imagine
John Lennon

Imagine there's no heaven
It's easy if you try
No hell below us
Above us only sky
Imagine all the people
Living for today

Imagine there's no countries
It isn't hard to do
Nothing to kill or die for
And no religion too
Imagine all the people
Living life in peace

You may say I'm a dreamer
But I'm not the only one
I hope someday you'll join us
And the world will be as one

Imagine no possessions
I wonder if you can
No need for greed or hunger
A brotherhood of man
Imagine all the people
Sharing all the world

You may say I'm a dreamer
But I'm not the only one
I hope someday you'll join us
And the world will live as one

PÁTRIA MINHA
Vinícius de Moraes

A minha pátria é como se não fosse, é íntima
Doçura e vontade de chorar; uma criança dormindo
É minha pátria. Por isso, no exílio
Assistindo dormir meu filho
Choro de saudades de minha pátria

Se me perguntarem o que é a minha pátria, direi:
Não sei. De fato, não sei
Como, porque e quando a minha pátria
Mas sei que a minha pátria é a luz, o sal e a água
Que elaboram e liquefazem a minha mágoa
Em longas lágrimas amargas.

Vontade de beijar os olhos da minha pátria
De niná-la, de passar-lhe a mão pelos cabelos...
Vontade de mudar as cores do vestido (auriverde !) tão feias
De minha pátria, de minha pátria sem sapatos
E sem meias, pátria minha
Tão pobrinha !

Porque te amo tanto, pátria minha, eu que não tenho
Pátria, eu semente que nasci do vento
Eu que não vou e não venho, eu que permaneço
Em contato com a dor do tempo, eu elemento
De ligação entre a ação e o pensamento

Eu fio invisível no espaço de todo o adeus
Eu, o sem Deus !
Tenho-te no entanto em mim como um gemido
De flor; tenho-te como um amor morrido
A quem se jurou; tenho-te como uma fé
Sem dogma; tenho-te em tudo em que não me sinto a jeito
Nesta sala estrangeira com lareira
E sem pé direito.

Ah, pátria minha, lembra-me uma noite no Maine, Nova Inglaterra
Quando tudo passou a ser infinito e nada terra
E eu vi Alfa e Beta de Centauro escalarem o monte até o céu
Muitos me surpreenderam parado no campo sem lua
`A espera de ver surgir a Cruz do Sul
Que eu sabia, mas amanheceu...

Fonte de mel, bicho triste, pátria minha
Amada, idolatrada, salve, salve !
Que mais doce esperança acorrentada
O não poder dizer-te: aguarda...
Não tardo !

Quero rever-te, pátria minha, e para
Rever-te me esqueci de tudo
Fui cego, estropiado, surdo, mudo
Vi minha humilde morte cara a cara
Rasguei poemas, mulheres, horizontes
Fiquei simples, sem fontes.

Pátria minha... A minha pátria não é florão, nem ostenta
Lábaro não; a minha pátria é desolação
De caminhos, a minha pátria é terra sedenta
E praia branca; a minha pátria é o grande rio secular
Que bebe nuvem, come terra
E urina mar.

Mais do que a mais garrida a minha pátria tem
Uma quentura, um querer bem, um bem
Um *libertas quae seras tamen*
Que um dia traduzi num exame escrito:
"Liberta que serás também"
E repito !

Ponho no vento o ouvido e escuto a brisa
Que brinca com teus cabelos e te alisa
Pátria minha, e perfuma o teu chão...
Que vontade me vem de adormecer-me
Entre teus doces montes, pátria minha
Atento `a fome em tuas entranhas
E ao batuque em teu coração.

Não te direi o nome, pátria minha
Teu nome é pátria amada, é patriazinha
Não rima com mãe gentil
Vives em mim como uma filha, que é
Uma ilha de ternura, a Ilha
Brasil, talvez.

Agora chamarei a amiga cotovia
E pedirei que peça ao rouxinol do dia
Que peça ao sabiá
Pra levar-te presto este avigrama:
"Pátria minha, saudades de quem te ama...
Vinícius de Moraes".

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SUMMARY

1. This thesis provides, in the first instance, a comprehensive survey of the literature pertaining to *Salmonella* and the putative role of the defence mechanism inherent in the shell and its contents.

2. Evidence is presented to indicate that:

a) The cuticular layer of the shell is rarely present as an even covering at any stage in the laying year. So, its role as a first line of defence is questionable.

b) The shell membranes do inhibit bacterial transfer to some degree, even when they are structurally disrupted; however, if the challenge is great enough, then their function as effective barriers is reduced.

c) In the absence of the shell membranes, *Salmonella enteritidis* Phage type 4 does not move freely across the shell, but it is either facilitated or inhibited in its passage by structural variation in the true shell, particularly at the level of the mammillary layer. Statistical data support in most instances a significant and positive correlation between the presence of structural defects and bacterial transfer.

d) In a three tier battery system, a tier effect exists with respect to ease of translocation of microorganisms, with eggs from the top tier being more susceptible, *i. e.* structurally inferior.

e) The results confirm earlier work that shell quality declines with age, and extends this finding to show that this morphological deterioration is accompanied by a decreased resistance to bacterial movement.

f) Patent gas exchange pores, whilst obvious portals for bacterial ingress, are in this respect of secondary importance to structural defects within the shell.

3. Evidence is also provided to substantiate the assumption that birds, irrespective of strain, display diverse shell structural quality. One of the

strains evaluated (strain B) was structurally better than the other (strain A), at the beginning and middle of lay, and was also more capable of withstanding bacterial challenge in all three laying periods tested.

4. The housing system can influence shell quality; thus Barn and Battery eggs were structurally superior to their Range counterparts, at the end of lay.

5. The tagging of *Salmonella* with immunogold markers proved to be a valuable technique, which allowed a more precise localisation of the bacteria within the shell's ultrastructure, as viewed by the Scanning Electron Microscope (S.E.M.). This method gave support to other findings in this work, confirming that bacterial transfer was specifically encouraged by late fusion and alignment of the mammillae and pitting occurrences, with the cone layer probably implicated in the process of penetration *in vivo.*

6. Finally, a comprehensive review of the literature available on growth promoters and their action in animals is provided, and experimental results show that the use of the growth promoter virginiamycin in the feed at 30 ppm appeared to improve the structural quality of the eggshell, particularly at the end of lay.

CHAPTER 1

GENERAL LITERATURE REVIEW

1. GENERAL LITERATURE REVIEW

" Quot homines tot sententiae; suo quoque mos"
(So many men, so many opinions; each to his own taste)
Terence, in "Phormio"

1.1. INTRODUCTION

Eggs have been recognised as an important food from the time primitive men snatched them from the nests of wild birds. According to Romanoff and Romanoff (1949), compared with the hen's egg, no other single food of animal origin is eaten and relished by so many culturally diverse populations the world over and none is served in such a variety of ways. Its popularity is justified not only because it is so easily obtained, economically efficient and has so many uses in cookery, but also because it is almost unsurpassed in nutritive excellence.

Eggs also have a definite place in preventive medicine and are of therapeutic value in the treatment of many dietary deficiency diseases. The egg is not only excellent for body maintenance, but also promotes growth, lactation and reproduction, all of which make rigorous demands and are very likely to uncover deficiencies in a food.

1.2. THE HEN'S EGG

1.2.1. OVOGENESIS

The oviduct of the fowl consists of six regions, according to Simkiss (1968) and Solomon (1991). From the ovary to the cloaca they are: a) the infundibulum (or funnel), which receives the oocyte after it has been shed by the ovary; b) the magnum or albumen-secreting region; c) the isthmus, which forms the shell membranes; d) the tubular shell gland, where the calcification of the shell begins; e) the shell gland pouch (uterus), where the bulk of shell growth occurs and f) vagina.

Ovogenesis, *i.e.* yolk development, begins 10 to 12 days preceding ovulation, when the developed ovum is released from the follicular membrane (yolk sac) into the upper section of the oviduct (infundibulum). The yolk is then moved caudally by peristaltic movement, towards the magnum, where the albumen mass is secreted around the yolk (Romanoff and Romanoff, 1949; Solomon, 1991).

In the isthmus region of the oviduct, the tubular glands secrete the paired shell membranes. The mammillary cores, around which calcium (Ca) salts seed, are also of isthmian origin (Richardson, 1935). The very beginning of the calcification of the true shell occurs in the tubular shell gland, where the mammillary mantle is established. Subsequently, the egg enters the shell gland pouch, where it remains for about 20 hours, and during this time the cone, palisade, vertical crystal layer and cuticle are produced (Solomon, 1991).

1.2.2. EGGSHELL FORMATION, COMPOSITION AND FUNCTIONS

The egg is a remarkable natural package. Board (1977) has defined the egg as a limited universe to which the cuticle, true shell and shell membranes provide a mediating boundary to the bulk environment. According to Romanoff and Romanoff (1949), the shell of the bird's egg is a relatively smooth, hard, calcareous coat, attached to the outer of the two membranes. So firm is the attachment that shell and membrane can be separated only with difficulty.

The avian eggshell is composed chiefly of calcite, one of the three modifications of calcium carbonate ($CaCO_3$), with a little magnesium, phosphate and chloride, and traces of a few other elements (Tyler, 1950). The calcified component is carried in a sparse matrix of glycoprotein with a characteristic amino acid and carbohydrate content (Baker and Balch, 1962; Becking, 1975). Protein itself represents 4 % of the eggshell, with 1 % water and the inorganic elements being responsible for the other 95 % (Shenstone, 1968).

The eggshell can be divided in two portions: the organic and the calcified fractions (layers of crystallisation). The organic fraction consists of shell membranes, the mammillary cores, the shell matrix, and the cuticle. These will be examined in more depth, subsequently. Although these components constitute only a small fraction of the entire eggshell, their integrity is critical to its formation and strength. The calcified portion of the shell can be divided into the mammillary knob layer, the palisade layer, and the outer surface crystal layer (Sturkie, 1986) (figures 1 and 2).

"Spherulites" of calcite (Tyler, 1969) grow from fairly uniformly distributed centres of crystallisation (mammillary cores). In the centre of each core, there is a small mass of protein attached to the outer shell membrane (O.S.M.) (Simkiss and Taylor, 1971). From the mammillary cores, crystals of

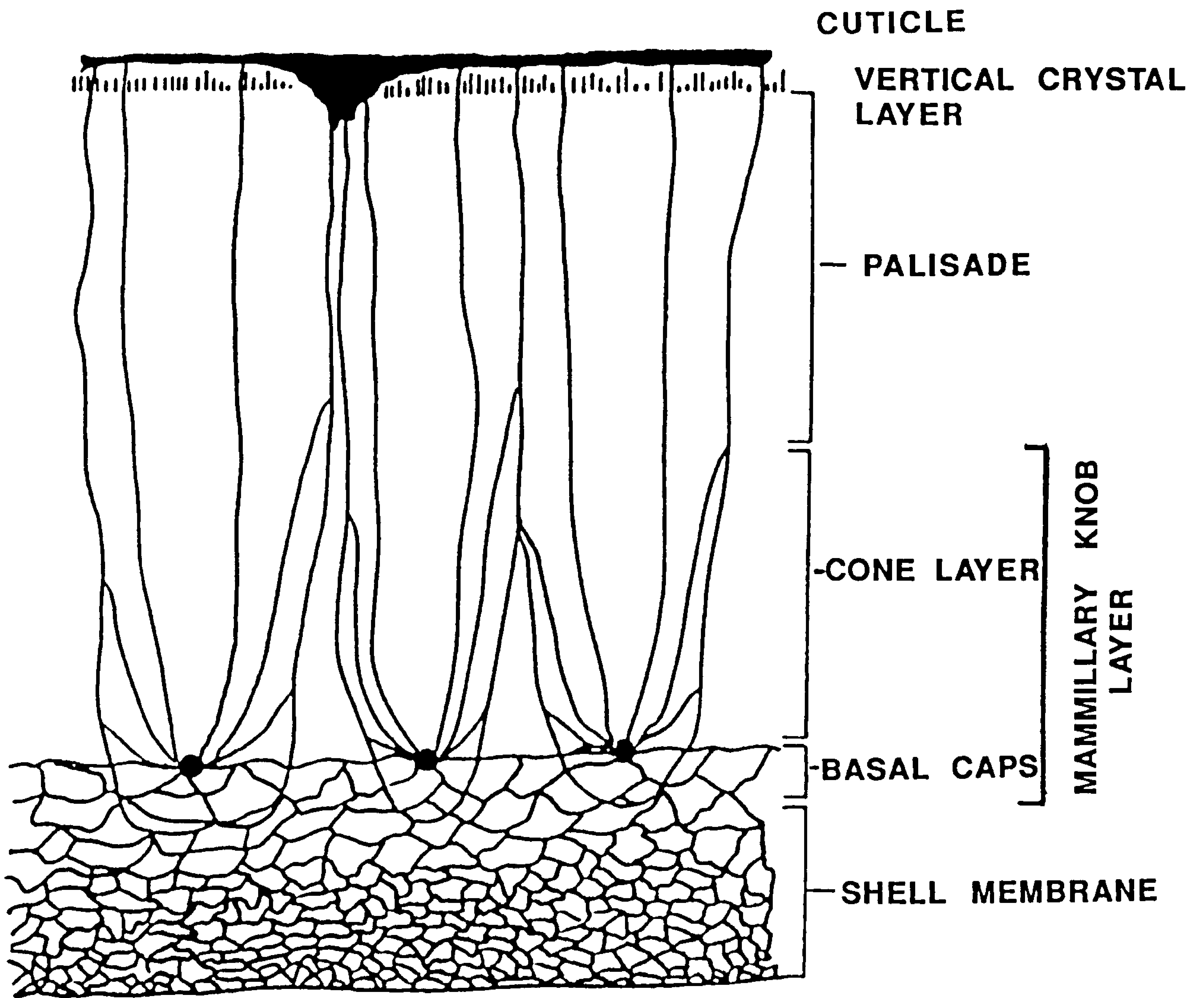


Figure 1: Diagrammatic representation of a cross-section through the hen's eggshell (modified from Parsons, 1982).

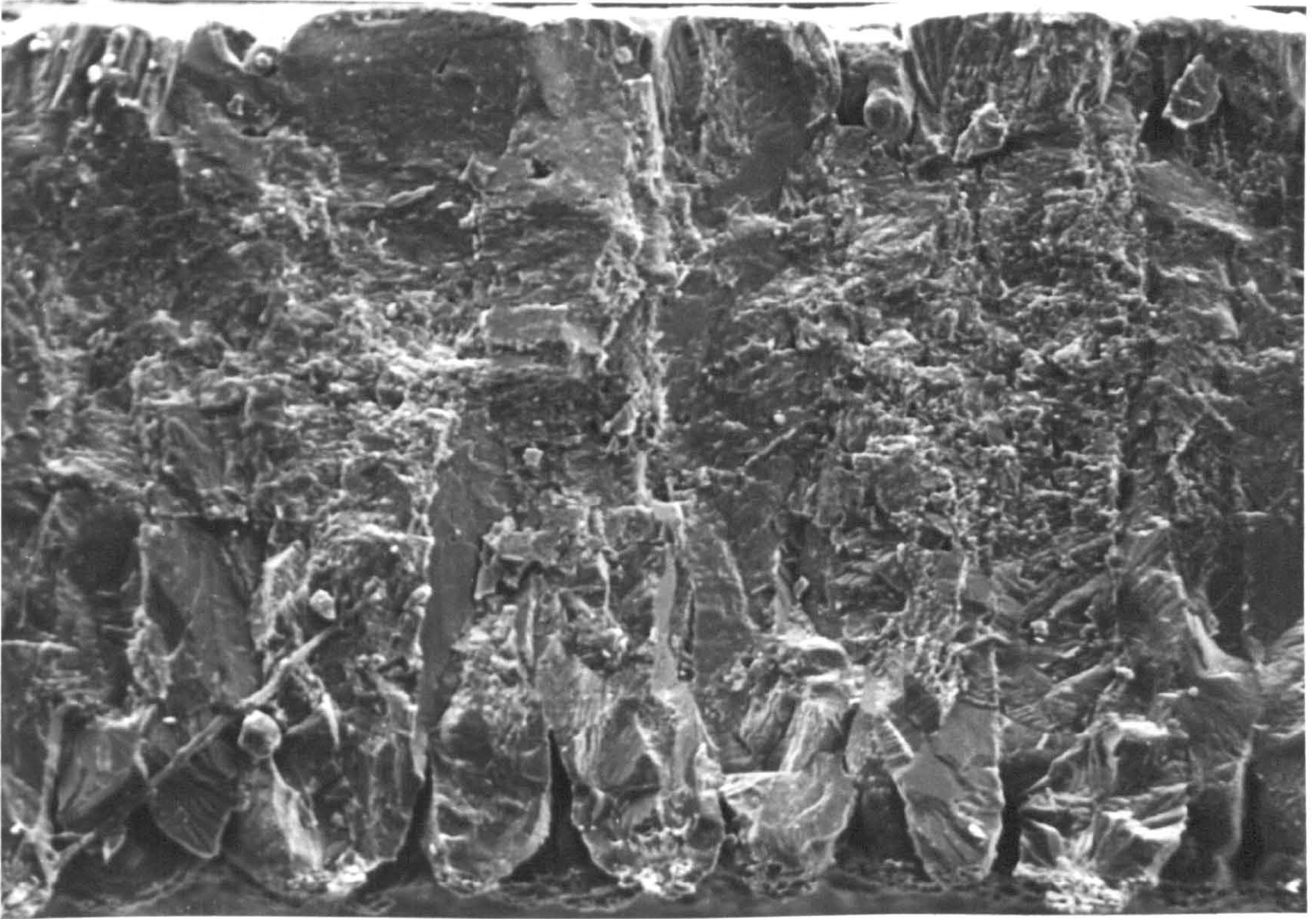


Figure 2: S.E.M. micrograph of a transverse section of fully formed shell (360 X).

calcite grow radially in all directions, to enclose the membrane fibres and thus firmly bind the calcified part of the shell to the membranes. Calcite crystals grow inward initially, being subsequently inhibited in this direction of growth by crystal poisons, possibly phosphate ions. These foreign ions act by either entering the crystal lattice in quantities sufficient to disrupt it, or by destroying the charged surfaces of the crystal, according to Simkiss (1964), Simkiss (1968) and Hodges (1974). Lateral crystal growth produces the basal caps (20 μm thickness) and growth outwards from the nucleation layer ultimately forms the cone layer.

Subsequent precipitation forms the palisade layer (crystal columns perpendicular to the shell surface), which accounts for about 2/3 of the thickness (total 300 μm) of the calcified shell, being responsible for its main strength (Becking, 1975; Hunton, 1987). The palisade layer is distinguished by its pit-like cavities (vesicular holes), which are 0.8 μm diameter, but within the mammillary base can be much larger, *c.* 9 μm (Hodges, 1974). It has a very compact upper part (with few vesicular holes). External to this, is a thin surface crystal layer (3-8 μm) with calcite vertically orientated . During and subsequent to the deposition of the latter, phosphate levels are increased, thus inducing the cessation of calcification (Simons, 1971).

The egg contents are as rapidly perishable as milk, yet the fragile shell, if in good condition, clean, undamaged and dry, will usually keep the egg edible for many months, even when stored at room temperature. Before spoilage or contamination with pathogens can take place, the microorganisms encounter several highly efficient barriers, nature's way of assuring that the propagation of the species will not be hindered by outside factors.

The shell therefore obviously provides the egg with its greatest natural protection against microorganisms from the environment. It has many contributions to make to the well-being of the embryo, thus, in addition to providing portals for the diffusion of respiratory gases, it contributes to the

conservation of water, and provides mechanical protection as well as scaffolding for the embryo (Board, 1977). These contributions are summarised in table 1.

Table 1 : Contributions of avian eggshell to the well-being of the embryo:

- Exchange of respiratory gases
- Prevention of asphyxiation
- Antimicrobial defense
- Mechanical protection
- Reservoir of Ca^{++}
- Camouflage

Source : Board (1980).

1.2.3. THE CUTICLE

The shells of all domesticated birds, hen, guinea fowl, turkey, goose and quail have a marked resistance to water, due to a natural protein-like film designated cuticle or bloom, which covers the outer surface of the shell and plugs to varying extents the pore canals. The cuticle is a product of the shell gland pouch (McCallion, 1953; Cooke and Balch, 1970).

The cuticle is a protein and carbohydrate complex and has a vesicular structure with irregular spaces between the vesicles of up to 0.5 x 2.8 µm diameter, particularly in the basal half (Simons and Wiertz, 1963), with brown eggs being reported to have denser structures and more granules (partially filling the vesicles) than white ones (Tyler, 1969). It has a thickness varying from 0.5 to 12.8 µm over the surface of the same egg (Simons, 1971), with an elemental composition of phosphorus, magnesium, chlorine, potassium, sodium and sulphur (Tullett *et al.*, 1976), and serves as a waterproofing agent and as a barrier to bacterial and fungal invasion (Baird *et al.*, 1980) with an effective protective life span of just a few hours succeeding oviposition (Vadehra *et al.*, 1970 a). Its surface is irregularly fissured, except when traversing the campanulate orifice of the pores where, presumably due to the stresses arising from drying, the fissures have a radial arrangement (Board, 1969). These cracks have been shown by Tullett *et al.* (1975) to provide channels for the diffusion of respiratory gases.

Sparks (1985) stated that some eggs are oviposited in a cuticle-less state. This is supported by Board and Halls (1973), who also found that 3.5 % of all brown eggs studied were cuticle-less, whilst 8.0 % were devoid of cuticle at one or other of the poles.

The reasons for the total or partial cuticle-less state are unknown (Board, 1975), although earlier, Alls *et al.* (1964), hypothesised that as the portion of the oviduct where the cuticle is applied exerts less pressure against the ends of the egg, then such areas will receive little or no cuticle at

all. The age of the bird was found by Bruce and Drysdale (1991) to influence the decline of cuticle deposition in eggs from older broiler breeder flocks, while Solomon and Watt (1990) showed that stress caused hens to lay eggs with patchy and abnormal cuticles.

As previously stated, the cuticle is believed to provide the egg with its first barrier to microbial penetration (Williams and Whittemore, 1967), and once it is removed, bacteria gain the ability to cross the shell (Tung *et al.*, 1979) and so, reduce the storage properties of the egg (Vadehra *et al.* , 1970a).

There are several factors, described in the literature, that may affect the protective function of the cuticle and, therefore, allow bacteria to penetrate the eggshell more easily, *viz.* :

a) the moist lustre present at oviposition disappears within 1 to 3 minutes, cannot be reproduced by subsequent wetting and is evidence of a change in the structure of the cuticle on freshly laid eggs (Sparks and Board, 1985).

b) it has been observed by Simons and Wiertz (1970) that during normal egg storage the cuticle shows thinning and shrinkage, either as a result of drying out or chemical change, with an increase in the number of cracks in the oval pore plaques of the cuticular surface.

c) the deposition of organic matter varies among breeds and among eggs laid by individual hens (North, 1978).

d) in spite of the cuticle being fairly resistant to water and detergents and to gentle rubbing with a cloth (Simons and Wiertz, 1966), it is less resistant to abrasives and chemicals like ethylenediamine tetra-acetic acid (E.D.T.A.) (Board and Fuller, 1974) and wiping eggs with sandpaper, wire wool or cheesecloth may cause them damage (Board, 1969), and even localised damage may permit bacterial entry through a few holes. Also fumigation with formaldehyde may lead to a reaction between the latter and the cuticle protein, resulting in its subsequent removal (Baker and Balch, 1962).

Increasing the eggs' storage temperature is recognised to accelerate the loss of cuticle (North, 1978). Board *et al.* (1979) reported the isolation of two species of *Pseudomonas* capable of digesting the cuticle. It is thought that these bacteria then allow yeasts to colonise the eggshell by utilising bacteria as a food source. Tung *et al.* (1979) also hypothesised that *Pseudomonas fluorescens* gained entry to the egg by digestion of the pore plug.

Sparks and Board (1985) affirmed that all the evidence to date has identified the cuticle as the most important barrier to water penetration and, by implication, bacterial penetration of the pores through the calcitic shell. In an earlier communication, Alls *et al.* (1964) found that cuticle removal increased bacterial contamination from 20 % to 60 %. According to Belyavin and Boorman (1980), the cuticle contributes to shell strength, although its influence may be limited to shell thickness.

1.2.4. THE PORES

According to Board (1977), in order to permit the diffusion of respiratory gases, the shell of an avian egg is 241-371 µm thick (Tyler, 1961a) and perforated with 7,000-17,000 funnel-shaped pores (Tyler, 1953 and Simkiss, 1968), the diameters of which are in the range 6-23 µm at the inner end and 15-65 µm at the mouth (Tyler, 1956 and Simkiss, 1967). In theory, each pore is partially filled with proteinaceous material, the cuticular plug. The latter serves to minimise water logging.

With respect to variation in density, the number of pores appears to be greatest at the equator or blunt pole of the shell (Romanoff and Romanoff, 1949). Their distribution is not random but tends towards uniformity and away from aggregation (Tyler, 1969).

Fujii (1974) stated that some shells contain a few malformed or "patent" pores, which have been acknowledged as major portals for the passage of antibiotics (Alls *et al.*, 1964) and ingress of bacteria (Board and Halls, 1973). Schmidt (1966) cited by Tullett (1975) and Sparks (1985) observed, during eggshell formation, that the incomplete fusion of the calcium spherites (cone tips) resulted in spaces remaining between some of them and that these spaces correspond to the origins of the pores.

In corroboration, Tullett (1975) found a positive linear correlation (r=0.918) between the number of mammillae per 0.25 mm^2 and the number of pores per cm^2 and hypothesised that if the mammillary density determines the pore density, then the packing of the mammillae may be expected to be important in terms of pore formation. The author also hypothesised that wider spacing of pores than would be predicted from an equivalent random distribution has been attributed to the fact that a pore cannot occur at any point on the eggshell, *e.g.* immediately above a site of crystallisation. The evidence therefore suggests that the formation of pores may be a physical result of the nature of mammillary growth, and a change in mammillary

density will produce a change in the number of pores within the eggshell.

Sharp (1937) indicated that the shell pores were the major breach for penetration of microorganisms into the egg, and Bryant and Sharp (1934) cited in Lorenz *et al.* (1952) stated that bacterial invasion normally occurs only through the large pores in the eggshell.

Attempts have been made by several workers to correlate eggshell porosity with bacterial penetration with varying results. Thus Fromm and Monroe (1960) are in support of a correlation, while Reinke and Baker (1966) refute these earlier findings. The fact that there are some pores which do not extend through the thickness of the shell but end abruptly at a variety of depths in the shell may contribute to these conflicting opinions (Bryant and Sharp, 1934 and Silyn-Roberts, 1983).

Vadehra *et al.* (1970b) stated that the blunt end of the egg, where porosity is at a maximum, has been shown to be the most vulnerable to infection by *Pseudomonas aeruginosa* and, correspondingly, regions of lesser porosity, *i.e.* the equatorial region and the narrow end of the egg were found to be less vulnerable to infection (Tryhnew *et al.*, 1973).

1.2.5. THE MEMBRANES

The paired shell membranes, which are formed in the upper 2/3 or granular region of the isthmus (Sparks, 1985), are *ca.* 70 μm thick (Simons and Wiertz, 1963) and held firmly together, except at the blunt end of the egg, where they separate to enclose the air space. The inner shell membrane (I.S.M.) lies immediately over the albumen, and the outer shell membrane (O.S.M.) is attached to the true shell (Mayes and Takeballi, 1983). Moran and Hale (1936) and Simons and Wiertz (1963) describe three layers of fibres in the O.S.M., while the I.S.M. has only two distinct layers.

Masshoff and Stolpmann (1961) cited in Simons and Wiertz (1963), showed that the egg membranes consist of a network of branched fibres (with pores of *ca.* 1 μm diameter, (Wolken, 1951; Bellairs and Boyde, 1969; Fujii and Tamura, 1970 and Tung and Richards, 1972)). According to Garibaldi and Stokes (1958) and Lifshitz *et al.* (1964), the O.S.M. shows interstices larger than bacterial dimensions.

The fibres are on average 0.8 to 1 μm thick, and each has a keratin core surrounded by a less electron-dense mucopolysaccharide mantle of *ca.* 0.5 μ (Romanoff and Romanoff, 1949). The I.S.M. is reported to be more porous than the O.S.M. (Hays and Sumbardo, 1927 cited in Mayes and Takeballi, 1983), which is surprising in view of the reputation of the former as a more effective barrier to translocation of bacteria (Vadehra and Baker, 1972).

Simons and Wiertz (1963) describe the fibres of the O.S.M. penetrating the inner part of the mammillary layer base (which consists of a single layer of prismatic bodies with their rounded bases pointing towards the membranes) and anchoring in its organic matrix. The first contact between membrane and mammillary layer is made by irregular protrusions at the extreme base of the mammillae, penetrating into the meshes of fibre network. The membrane fibres penetrate each mammilla up to a depth of *ca.* 20 μm,

i.e. about 1/5 of its height and so form the mammillary core (Tyler and Simkiss, 1959). By doing so, they provide a substrate for shell deposition, acting also as a bag enclosing the albumen and preventing it from clogging the pores of the shell (Bellairs and Boyde, 1969).

With respect to the contact between I.S.M and O.S.M., Simons and Wiertz (1963) describe a patchy and tenuous link between the two. The authors also maintain that the compactness of the organic matrix of the spongy layer is positively related to shell strength.

In relation to bacterial penetration, the shell membranes act as a filter (Haines and Moran, 1940 and Garibaldi and Stokes, 1958), being more impenetrable to bacteria than the shell. Lifshitz *et al.*, (1964) reported that the I.S.M. was the most effective barrier in preventing bacterial penetration of the egg contents, the shell ranked second and the O.S.M. was the least important. The fact that they are not arranged in a regular manner (with the distance between them and adjacent mammillae, measuring as little as 10 or as much as 150 µm (Bellairs and Boyde, 1969), may encourage bacteria to remain in these spaces and thus have access to water, in order to reproduce. Although acting as microbial filters, their resistance to penetration diminishes upon contact with microorganisms, according to Amin and Draughon (1990), and they are also affected by the presence of iron and the age of the egg (Hartung and Stadelman, 1963).

Membrane resistance is quickly breached when large bacterial inocula are used (Brooks, 1960; Hartung and Stadelman, 1962; Board, 1964 and Board *et al.*, 1968), especially when eggs are held at 37 °C (Board and Ayres, 1965), with the microorganisms having been recovered from the inner surface of the I.S.M. within minutes of the challenge (Williams *et al.*, 1968). Based on that observation, Board and Fuller (1974) commented that the membranes are capable of imposing only a temporary barrier to the inward movement of bacteria, and once they have passed through the shell membranes, the viscosity of the albumen ensures that they remain in a

clump.

With respect to the mode of bacterial penetration through the membranes, proteolysis has been implicated (Mayes and Takeballi, 1983), and Brown *et al.*, (1965) and Candlish (1972) have found zones of hydrolysis surrounding the bacteria located in the membranes, supporting the theory that enzymes (mucinase, polysaccharidase) are actively involved in the penetration process, perhaps aided by the effects of microbial growth on the membranes (Walden et al., 1956 and Garibaldi and Stokes, 1958). Board (1965) dismissed this hypothesis. Hartung and Stadelman (1963) considered the enzymatic digestion by *P. fluorescens* cells could possibly clear the albuminous substance (material present between the fibres) from the interstices, permitting early and rapid passage. Brown *et al.*, (1965) claimed the same can happen in a *P. aeruginosa* infection. Contrary evidence has also been produced, refuting the role of proteolytic enzymes (Wedral, 1971 and Vadehra and Baker, 1972).

Certain authors maintain that the shell membranes possess some kind of bactericidal activity. Korotkova (1957), cited in Board (1966), and Vadehra *et al.* (1972) detected lysozyme in the shell membranes and hypothesised it to play an important role in the defence of the developing embryo. This role has been categorically denied by Kraft *et al.*, (1958a) and, *sensu latu*, by Mayes and Takeballi (1983).

Growth of bacteria on the shell membranes *in situ* is restricted if iron (Fe^{++}) is unavailable or deficient (Brooks, 1960 and Board, 1964), and the antimicrobial properties of the albumen are believed to be primarily responsible for confining multiplication to the shell membranes. Multiplication occurs only when the yolk makes contact with the I.S.M. (Board, 1964 and Board and Ayres, 1965). It is more prone to happen in eggs held at room temperature (Board, 1964) or in aged eggs (Hartung and Stadelman, 1963).

Vadehra *et al.* (1969) showed that cracked eggs were no more inclined

to infection by *Salmonella* than were normal-shelled eggs, provided they were handled properly, but since the membranes are exposed in cracked eggs, the chlorine solutions used to wash the eggs – or even chelating agents such as E.D.T.A. or penicillamine (Vadehra and Baker, 1972) – will probably break the structure of the membranes, thus making them more permeable, and increasing spoilage.

1.2.6. CHEMICAL DEFENCES

The eggshell is equipped with a range of antimicrobial defence substances, which are summarised in table 2.

Despite these antimicrobial attributes, various *Salmonella* serotypes are able to remain viable in the albumen, which appears to have very little inhibitory effect on it after it has penetrated the shell (Adler, 1965), especially at temperatures of 20 °C and 30 °C.

The alkaline state of the albumen (pH 9.5) is an inimical factor which also accentuates the chelating potential of ovotransferrin (most of the common contaminants of rotten eggs were unaffected when inoculated in albumen adjusted to pH 6-8, but were killed at pH 9-10) (Tranter and Board, 1982; Board and Tranter, 1986). The physical restraint provided by the thick viscous nature of the albumen in retarding bacterial movement is also an important defence factor.

Brooks and Taylor (1955) and Shenstone (1968) state that the egg is a complex physicochemical system in which enzyme-mediated energy transfer and chemical transformations are limited, in the main, to the cells of the blastoderm. With eggs intended for human consumption, these activities are minimised by low-temperature storage. This also delays the rate of deterioration of the physico-chemical systems and loss of the structural integrity of the main components of an egg.

Component	Activity
Lysozyme	a) Hydrolysis of β (1-4) glycosidic bonds in bacterial cell wall peptidoglycan (acting specifically on the polymer n-acetyl glucosamine n-acetyl muramic acid, splitting the link between them). This occurs mainly with Gram + bacteria, because the Gram - does not have this substrate as a major constituent of their cell wall, or it is complexed or covered, and thus is unavailable for lysozyme detection. b) Flocculation of bacterial cells. c) Formation of oligosaccharides from bacterial cell wall tetrasaccharides by transglycosylation.
Ovotransferrin	- Chelation of Fe^{++}, Cu^{++}, Mn^{++}, Co^{++}, Cd^{++}, Zn^{++} and Ni^{++}.
Avidin	- Binding (chelation) of Biotin (or vit. H or one of the components of the B-complex), rendering it unavailable to bacteria that require it.
Ovoflavoprotein	- Binding (chelation) of Riboflavin (or vit. G or vit. B_2), rendering it unavailable to bacteria that require it.
Ovomucoid	- Inhibition of Trypsin.
Ovoinhibitor	- Inhibition of several proteases.

Table 2: Biological properties of some antimicrobial proteins of the albumen. (modified from Tranter and Board (1982) and Board and Tranter (1986)).

1.3. NATURAL FACTORS THAT AFFECT THE FUNCTIONS AND QUALITY OF THE EGGSHELL

The "perfect" shell as such does not exist, according to Solomon (1987). The dynamic process of calcification, which takes some 20 hours to complete, witnesses the formation of many aberrant crystal forms, both within and upon the shell and the magnitude and nature of these defects will exert considerable influence on the physical parameters of the shell. Many different tests have been devised to evaluate shell quality and are widely used by the industry. These include: candling, specific gravity, strength and non-destructive deformation. The ultrastructural evaluation technique used in this work is being increasingly used as a more accurate, if time consuming, means of determining shell quality.

Many factors influence the thickness of the eggshell, *e.g.* the season of the year, the heredity endowment of the hen, her nutrition and her physiological efficiency. Thinner shells are more susceptible to breakage than thicker ones, and Hunton (1987) concluded that the correlation between shell thickness and breakage was usually greater than the correlation between other estimates of strength and shell damage. Previously, Wells (1967) found that resistance to crushing and impact and egg specific gravity were significantly correlated with the percentage of cracked eggs, while Solomon (1985b) concluded that there is a significant correlation between the incidence of mammillary abnormalities and shell cracking.

There is a discrepancy among the studies of Simons (1971) and van Toledo *et al.*, (1982). The former observed that eggshells designated "superior quality" had a higher density of mammillary knobs than "poor quality" shells. The latter concluded that eggshells with low breaking strength had a higher density of mammillary knobs. King and Robinson (1972) suggested that the mammillary layer was generally thinner in the fractured surfaces of weak eggshells. Hunton (1989) claims that eggshell quality represents only half of the contribution to the cracking of an individual

eggshell, the size and nature of the insult to which the egg is exposed providing the other half of the explanation.

It has been suggested that an increased amount of water in the albumen will interfere with the rigidity of the template for shell deposition, resulting in the production of a structurally inferior shell (Solomon, 1985b and Tullett, 1987). Earlier, Tyler (1969) reported that wetting the shell with water makes it weaker, but the effect disappears if the shell is dried again. Water seems to hydrate the organic matrix of the shell, thereby reducing its strength.

In a survey of cracked eggs, it was found that there was a much higher proportion of structural abnormalities in the cone layer associated with the crack line than with other parts of the intact shell (Solomon, 1985a).

According to Sherwood (1958) and Gilbert (1971), several factors affect egg composition, *viz.* breed, age, position of the egg in a sequence, rate of lay, time of the year, ambient temperature, food quality and quantity, noise and disease. It is widely agreed that some strains of birds show consistently poorer shell quality than others, and also that age influences shell quality. Solomon (1987) and Tullett (1987) agreed that shell quality decreases with age, in terms of decreased resistance to cracking. More eggs had heavy spotted shells when produced by older hens, according to Bogdanov *et al.* (1989). This could be related to a decreasing ability to absorb calcium from the intestine and to mobilise skeletal calcium.

Ewing (1963) claimed that rate of lay or intensity of egg production may also be correlated with shell thickness, with high producers in a flock often producing the thinnest shells. Temperatures over 21.1 °C were shown to cause a decrease in eggshell quality and thickness, which can be exacerbated by high humidity (Petersen, 1965). It has been suggested that these temperature levels reduce the bird's blood Calcium level (Conrad, 1939; Warren and Schnepel, 1940 and Ewing, 1963). The latter author also suggests that with temperatures less than 21 °C, 3.75 % Ca is necessary to

keep eggshell quality at its best, while with temperatures over 21 ^{o}C, a Ca intake of 4.5 to 5 % is recommended.

Since Calcium is not stored in the shell gland (Simkiss and Taylor, 1971), it has to be supplied continuously during eggshell formation, and any reduction in Ca utilisation from either the feed or the skeleton will result in the production of eggs with inferior shell quality (Balnave, 1988).

On the other hand, the mobilisation of medullary bone is known to generate an increased concentration of phosphate in the blood (which is also known to be negatively correlated with eggshell quality). Calcium is stored in the skeleton as a Calcium phosphate salt, hydroxyapatite (Miles and Harms, 1982). This will be discussed in detail in chapter 6, but suffice it to say that there is evidence that eggs laid later in the day (with a greater proportion of shell formation occurring during daylight, when birds are consuming feed) have superior shell quality (Roland et al., 1973).

Balnave (1989) suggests that a bicarbonate deficiency could induce the production of poor quality eggshells. He observed that when hens were supplied with saline drinking water, they also produced poor quality eggshells, a feature which persisted even when the sodium chloride was removed from the water for an extended period.

In an earlier communication, Simons and Wiertz (1963) claim that although factors such as those related to shell thickness and to mineral content do show a high positive correlation with shell strength, the total of these factors cannot account for the full resistance of the shell against breakage.

The matrix may have a significant influence on shell strength, and Powrie (1977) observed positive correlations between the nitrogen content of the shell and shell strength, and the compactness of the organic matrix and shell strength (Simons and Wiertz, 1963).

Hughes and Black (1976) found that when birds were subjected to handling stress, the proportion of eggs with equatorial bulges increased. They suggested that, under stress, adrenaline is released, causing strong shell gland contractions that in turn break the egg. Solomon (1988b), in a similar way, indicated the importance of the effects of stress in causing eggshell abnormalities. Likewise, a change in stocking density was sufficient to cause structural changes in the eggshell for up to 30 days (Watt, 1989). Fear induced decreased shell thickness in chickens (Stiles and Dawson, 1961) and also an increased percentage of shell-less eggs (Hewitt, 1939).

There are obviously many external and internal factors which encourage the occurrence of poor quality shells, but irrespective of the etiology of these factors, they all have the same end result – they increase the vulnerability of the egg to bacterial translocation.

1.4. THE ROUTES AND FACTORS AFFECTING BACTERIAL TRANSLOCATION AND CONTAMINATION OF THE EGG

There are essentially two routes through which microorganisms may gain entry to the yolk and albumen. The first of these is congenital contamination, whereby microorganisms are incorporated into the egg contents during its formation in the oviduct. The second means of infection is extragenital contamination, in which microorganisms penetrate the integument of the egg once it has been laid (Drysdale, 1985) mainly as a result of faecal contact (Forsythe *et al.*, 1967).

1.4.1. CONGENITAL INFECTION

According to Hutton (1991), transovarian transmission, although possible, is very difficult to demonstrate experimentally, even in artificially infected hens (Nicholas and Andrew, 1991 and Baskerville *et al.*, 1992). Attempts to produce ovarian infection by artificial exposure were reported to be either inconclusive or negative (Snoeyenbos *et al.*, 1969; Shivaprasad *et al.*, 1990 and Bolder *et al.*, 1991), or even mixed (Gast and Beard, 1990a).

Nevertheless, it is possible that certain paratyphoid organisms such as *Salmonella enteritidis* and *S. typhimurium* have a greater capacity to invade the internal organs or to localise in some remote site within the body (Faddoul and Fellows, 1966), and although *Salmonella* serotypes other than *S. pullorum* and *S. gallinarum* have not been frequently isolated from the contents of clean uncracked eggs, possible transovarian contamination of eggs with *S. enteritidis* has been inferred from its recovery from the contents of intact eggs and the ovaries of laying hens (Barrow and Lovell, 1991), but only during a fairly brief period after exposure (Williams, 1984; Shivaprasad *et al.*, 1990 and Gast and Beard, 1990a,b,c).

S. enteritidis has been shown to be a very invasive pathogen, capable

of infecting not only the ovary and the egg contents as discussed above, but also the oviduct (Hopper and Mawer, 1988), the liver and spleen (Shivaprasad *et al.*, 1990 and Barrow and Lovell, 1991) and the alimentary tract of clinically normal laying hens in an infected flock (Lister, 1988).

St. Louis *et al.* (1988) suggested that hens colonised by *Salmonella* may contaminate eggs only intermittently, and that culturing of pooled eggs may be necessary to detect it. The explanation may be that, as with *S. pullorum* infection, the number of affected ovules in birds with *S. enteritidis* may vary, with normal ovules as well as infected occurring in the same ovary. Evidence in favour of the transovarian route of contamination has been provided by O'Brien (1988), Bygrave and Gallagher (1989), Cooper *et al.* (1989), Bradshaw *et al.* (1990) and Madden (1990).

The isolation of *Salmonella* from the egg contents however cannot be accepted as single proof of transovarian infection, because of the possibility of shell penetration by the pathogen (Snoeyenbos *et al.*, 1969). Isolation from ovarian tissue should also be treated with caution, as peritoneal infection might produce ovarian surface contamination. Another possibility of experimental error was conceded by Gast and Beard (1990b), who accepted that contamination of the egg contents might have happened during the 4-day holding period of the eggs, before sampling.

Barrow and Lovell (1991) orally infected chickens and demonstrated that the birds remained healthy throughout the experiment (asymptomatic, "healthy carriers"), although *S. enteritidis* was isolated frequently from the ovarian interstitial tissues, but not from the yolk separated from these tissues, nor from the oviduct or eggs, and the number of isolations from the caecal and cloacal swabs was much higher than from birds inoculated intravenously. Their final conclusion was that eggs were more likely to become contaminated during their passage through the cloaca than as a result of ovarian infection. Similarly, Baskerville *et al.* (1992) were unable to isolate *S. enteritidis* from the egg contents (despite a few of the shells being

positive) from experimentally infected hens.

The earlier concept of the oviduct as a sterile tube (Rettger, 1913 cited in Stokes *et al.*, 1956; Stuart and McNally, 1943; Tanner, 1944 cited in Miller and Crawford, 1953; Lorenz *et al.*, 1952; McNally, 1953; Brooks and Taylor, 1955 and Bryan, 1968) has now been refuted by Bruce and Drysdale (1986) and Sesma *et al.* (1987). Board (1977) has reported that occasionally the oviduct is thrown into violent antiperistalsis, with the result that stones, feathers, *etc.* and presumably microorganisms, are included in otherwise normal eggs, but this phenomenon is considered very unusual and of academic interest only.

Shivaprasad *et al.* (1990), based on their own experiments and on those of Timoney *et al.* (1989), suggested that the recently emerged epidemic clones of *S. enteritidis* have an enhanced ability to invade and survive in the bloodstream and infect organs such as the oviduct and peritoneum. Humphrey *et al.* (1991a) and Dolman and Board (1992) put forward the theory that during the passage of the egg through the oviduct, the albumen is seeded with a few cells of *S. enteritidis*, and these would remain dormant, even in eggs stored at room temperature, for 2-3 weeks. After this time, physical and chemical changes take place in the egg contents, with some nutrients leaking out from the yolk by alterations in the structure of the yolk membrane, and so favouring the multiplication of the bacteria. Alternatively, the inactivation of ovotransferrin in older albumen may allow Gram - bacteria access to Fe^{++} ions previously not available. Similarly, Shivaprasad *et al.* (1990) found that albumen was more frequently contaminated than yolk, and suggested that the source of the organism was the oviduct or peritoneal cavity.

Barrow and Lovell (1991) also isolated *S. enteritidis* from oviducal swabs but not from eggs removed from the oviduct, and from yolk and ovary tissue but not from the fully formed egg.

The importance of intestinal and/or systemic infection as a very first step in the mechanism of egg contamination should not be underestimated. Baxter-Jones (1991) suggested that *S. enteritidis* was more invasive than other serotypes, with the organism having the ability to leave the gastrointestinal tract and infect other tissues, particularly the ovary and the oviduct. Humphrey *et al.* (1989a) were, however, unable to find contaminated egg contents in systemically infected older birds (52 w. o.), and only four out of 375 eggs in the younger group (18 w. o.). They also believed *S. enteritidis* PT 4 may be retained in the viscera for long periods after infection, even with no specific antibodies being detected. Olesiuk *et al.* (1969) had previously reported similar results with *S. typhimurium.* The early work of Turnbull and Snoeyenbos (1974) reported that the ability of *S. enteritidis* to penetrate the intestinal mucosa fell rapidly with increasing age of birds, suggesting that older birds have a higher resistance.

Workers are certainly puzzled by the absolute unpredictability of *Salmonella* excretion and seroconversion in birds known to be infected. This has caused tremendous difficulties in diagnosing the disease, with serious consequences in terms of control and/or immunisation. Humphrey *et al.* (1989b) suggested that the production of contaminated eggs may be intermittent or, according to Gast and Beard (1990a), even only for a brief period following exposure, with serological and bacteriological tests detecting infection long after the hens had ceased to produce contaminated eggs, thereby adding the possibility of false positives, and so complicating the diagnostic situation even more.

To add one more factor to an already confused frame, Baker *et al.* (1980a) and Salvat *et al.* (1991) concluded from their experiments that not only can hens excrete *S. enteritidis* in their faeces without serological conversion or contaminated egg contents, but the opposite can also occur (Hassan *et al.*, 1990 and Humphrey *et al.*, 1991b). Such controversy probably explains why no guaranteed diagnostic test or control scheme is yet available to solve this problem.

Intermittent faecal shedding by birds, caused possibly by large amounts of bacterial intracellular multiplication stressing the host cell to such an extent that it bursts and releases intracellular organisms (Finlay and Falkow, 1989), means that some birds in some orally infected flocks could remain indefinitely infected and shed *Salmonella* when stressed at a later time (Shivaprasad *et al.*, 1990 and Bolder *et al.*, 1991). This could result in contamination of the egg contents or eggshells (Baker *et al.*, 1980a), for up to 21 days post-infection. A *S. thompson* infection was otherwise reported to become localised in the gallbladder of a bird which remained excreting it for at least 18 months.

Humphrey (1989) found that the production of infected eggs by experimentally infected hens was intermittent though clustered. His results also showed that on some days a significant proportion of eggs may have *S. enteritidis* in their contents.

Gast and Beard (1990b) conceded that extensive or persistent colonisation of the intestinal tract was not a prerequisite for the production of contaminated eggs. Humphrey (1991a) and Humphrey *et al.* (1991b) also claimed that there is no relationship between faecal carriage and the contamination of egg contents with *S. enteritidis*. Whether the congenital route is the source of infection is a matter of debate. Birds however are faecal shedders of *Salmonella* and as a consequence the shell must be regarded as a prime target.

1.4.2. ENVIRONMENTAL (EXTERNAL) CONTAMINATION

The main sources of microorganisms on the outside of eggs are the intestinal tract of the fowl (Stuart and McNally, 1943) and other sources in the environment (Rosser, 1942; Hinshaw and McNeil, 1948; Harry, 1963 and Board *et al.*, 1964), *e.g.* the nest box, dust, litter, feedstuffs, air, contaminated water, shipping and storage containers, human beings and other creatures (pigeons, fowls, wild birds, cats, dogs, lizards, and other wild or domesticated animals on the farm, plus cockroaches, fleas, beetles, and flies) (Buxton and Gordon, 1947 and Nagaraja *et al.*, 1991). Under today's commercial conditions of rearing chickens, however, it seems unlikely that animals other than rodents would have an important role in the contamination process, although in the south cone of South America, pigeons and other birds have been specifically implicated in transmitting *Salmonella* infections to chickens.

According to Romanoff and Romanoff (1949), the egg immediately becomes contaminated in the nest, acquiring a profuse and heterogeneous microflora, with numbers of bacteria varying widely, from 10^2 to 10^7 /shell, depending on the amount of faeces, dust or soil present (Board, 1969). As the cuticle is moist at this stage, microbial invasion of the shell could conceivably occur. Sixteen bacterial genera have been recovered from the shell and are listed in the table 3. Organisms having simple nutritional requirements and, like Gram + bacteria, because of their tolerance to dry conditions, dominate the flora on eggshells. The Gram -, on the other hand, are the principal contaminants of rotten eggs. In any case, the ultimate determinant of viability will be the growth rate (Board and Tranter, 1986).

In a survey carried out by Solowey *et al.* (1946), 2 % of clean eggs and 16 % of dirty eggs were found to contain *Salmonella* spp. on the surface or in the pores of the shell. This is in accordance with the findings of Radkowski (1990), while Humphrey *et al.* (1991a) isolated *S. enteritidis* in the contents of only 0.6 % of a more than 5,000 egg survey and Baker *et al.* (1980b) found none, even on eggs positive on shell culture.

Table 3 : Type of microorganisms present on the shell of the hen's egg :

Type of organism:	**Frequency of occurrence:** [a]
Streptococcus	1
Staphylococcus	2
Micrococcus	3
Sarcina	1
Serratia	2
Bacillus	2
Pseudomonas	2
Achromobacter	2
Alcaligenes	2
Flavobacterium	2
Cytophaga	2
Escherichia	2
Aerobacter	2
Aeromonas	1
Proteus	1
Arthrobacter	2

[a] Present : 1 = Occasionally
2 = On most eggs but in small numbers
3 = Always present in large numbers

Source : Adapted from Board (1977).

According to Drysdale (1985), the immature cuticle of a newly-laid egg is moist and does not provide an effective barrier to the entry of microorganisms, and the presence of moisture on the egg is known to enhance microbial penetration. Due to this favourable condition, it is most likely to occur at the point of lay when, according to Baxter-Jones (1991), microorganisms may become entrapped within the glycoprotein cuticle. Eggs with a poor cuticle were found by Bruce and Drysdale (1991) to have a significantly higher incidence of bacterial contamination (40 %) compared with those with a medium or good quality cuticle (26 %).

The presence of water or any form of humidity enhances bacterial penetration through the eggshell, according to Rievel (1939), Williams *et al.* (1968), Board and Halls (1973), Board and Fuller (1974), Sparks and Board (1985) and Padrón (1990). The latter author claims that water is not essential for microbial penetration to occur. Earlier works by Gillespie and Scott (1950) and Board *et al.* (1964) suggested that bacterial multiplication would not occur in shells under normal conditions of storage. Under such circumstance, water is a limiting factor. Lifshitz (1963) demonstrated that the cuticle and shell material, with their content of neutral sugars and hexosamines, are capable of supplying bacteria with nutrients for growth. The studies of Williams *et al.* (1968) and Padrón (1990) proved that almost immediate penetration of open shell pore areas is possible under ideal moisture and temperature conditions. The presence of faecal material tended to enhance the penetration process (Vadehra *et al.*, 1969 and Humphrey *et al.*, 1989c), probably by reducing surface tension, or by contributing with some chemical (*e.g.* iron), helping to break the egg's natural defences (Bruce and Drysdale, 1991). In view of their origin, *i.e.* the cloaca, complete freedom from faecal smears will be impossible, and so with this adherent contamination and with the drop in temperature from 40 - 42 ^{o}C to ambient, the egg is most vulnerable, because this reduction in temperature causes the air sac to contract (Board, 1966), and to pull water and bacteria through the shell and against the I.S.M..

When exposing eggs experimentally to an inoculum containing five different microorganisms including *S. enteritidis*, Dolman and Board (1991) found the latter dominated the infection of eggs incubated at 37 °C, but not those stored at 4 °C, 15 °C and 20 °C. This question of storage temperature has received a good deal of attention, and is considered to be the determining factor affecting the growth response and growth rate of *Salmonella*, above 16 °C (Bradshaw *et al.*, 1990) proportioning a rapid amplification of even low numbers of cells to levels high enough to cause human infection (up to 10^6-fold, when held at 21 or 27 °C for more than 20 days) (Kim *et al.*, 1989 and Humphrey *et al.*, 1991a).

Eggs were definitely found to be safer if stored at 4 °C. Clay and Board (1991) observed that no albumen contamination occurred at this temperature for 30 days (although the organisms remained viable). Humphrey *et al.* (1989d) proved the same in relation to growth in the yolk. The fact that the viability was still present at 4 °C was however considered to be detrimental by Baker (1990). A storage temperature of 8 °C is permitted within the E.C..

There is a critical relative humidity (R.H.) near 97 %, above which growth, and below which death of microorganisms takes place (Simmons *et al.*, 1970). In corroboration, Christian and Scott (1953) and Christian (1955) suggested *Salmonella* requires a minimal water activity for growth of 0.93 to 0.97, while Drysdale (1985) found 97 % R.H. enhanced penetration of *Salmonella* and subsequent contamination of eggs.

In an earlier communication, Stokes *et al.* (1956) claimed that *Salmonella* organisms failed to penetrate the membranes at 1 °C and died during 6 months of storage at this temperature. If the egg is removed within the first month and placed in an ambient temperature of 35 °C, the organisms will overcome their previous repressed growth characteristics due to the low temperature and will develop. These observations have been corroborated by Board (1964) and Brown *et al.* (1970) who maintain that the position in

which the egg is stored can influence the ease of egg spoilage. They recommended eggs must be stored with the blunt end up, because in so doing, the yolk does not migrate and so contact with the membrane is avoided. This is in total disagreement with the later work of Clay and Board (1991), who found that in eggs stored with the air cell uppermost, gross contamination developed, regardless of the size of the inoculum, which was associated with the deterioration and decay of shell structure.

Porosity is not positively correlated with bacterial penetration, according to Wright and Frank (1956), Reinke and Baker (1966) and Bruce and Drysdale (1991). The latter suggested that some other variable characteristics other than porosity influence bacterial contamination. According to Board (personal communication), the egg is more prone to infection near the air space not because of the increased porosity at that point, but because the yolk gets closer to the air space as the egg ages.

There is a great danger of eggs that, even in the absence of a culminating infection of the contents, may still harbour bacteria at or near the shell membranes, which can allow contamination to occur when eggs are broken (Baxter-Jones, 1991) for food preparation or even for hatching (Schalm, 1937 and Borland, 1975).

In terms of elimination of microorganisms, it has been claimed by Forsythe *et al.* (1953) and Bierer *et al.* (1961a,b) that a good detergent will physically remove up to 92 % of the bacteria on the shell surface, but according to Gillespie *et al.* (1950), Sauter *et al.* (1962) and Sauter (1966), this does not guarantee freedom from spoilage during the storage of treated eggs. Winter *et al.* (1952 and 1955) cited in Board (1969) estimated that under farm conditions, the presence of bactericides in wash water achieves nothing more than a reduction in the level of the contamination of the shell.

As regards the influence of metal ions on penetration, Garibaldi and Bayne (1962) maintained that eggs washed in water containing 4.8 ppm of

Fe^{++} showed 6.2 % spoilage by *Pseudomonas* during storage, whereas of those eggs washed with water containing 0.2 ppm of Fe^{++}, only 0.8 % spoilage was detected. Sauter and Petersen (1969) extended this effect to any metal ions present in the water.

With regard to pH affecting penetration, Sauter *et al.* (1979) found maximum penetration rates of 42 % at pH 7.5 for *Salmonella typhimurium*, 22 % at pH 8.5 for *S. st. paul* and 34 % at pH 7.0 for *S. derby*, and that *Salmonella* sp. penetration rates were significantly less at pH 5.0 than at higher pH values and that there was an increased penetration from pH 5.5 to pH 7.0 for all serotypes tested.

In terms of time required for penetration, Williams *et al.* (1968) found that at 37.2 ^{o}C (99 ^{o}F), only 6 minutes elapsed before penetration of *S. typhimurium* to the inner surface of the I.S.M. could be detected, and it can also happen in a matter of minutes at room temperature (*ca.* 24 ^{o}C or 75 ^{o}F). During the first 30 minutes of incubation at 99 ^{o}F after shell exposure to *S. typhimurium*, it was observed by the same authors that 12.3 % of eggs showed penetration through cuticle and shell, while only 0.95 % showed penetration through cuticle, shell and O.S.M. and only 0.44 % demonstrated penetration through cuticle, shell and both shell membranes.

Stokes *et al.* (1956) and Williams *et al.* (1968) claimed that the percentage of total eggs penetrated does not appear to be as dependent on the number of organisms used in exposure tests as on the relative susceptibility of the shell surface to penetration, reinforcing the importance of egg shell quality in restraining bacterial penetration.

Sauter and Petersen (1974) maintain that penetration is dependent more on shell structure than on the *Salmonella* organism population to which eggs are exposed. These views have been refuted by Rosser (1942), Hartung and Stadelman (1963), Brown *et al.* (1966) and Board and Fuller

(1974), who maintain that the heavier the inoculum, the greater the opportunity for bacteria to penetrate imperfectly sealed pores. Shell thickness was not found to influence penetrability, but was dependent rather on the extent of structural defects in the shell (Kraft *et al.*, 1958; Williams *et al.*, 1968; Williams and Dillard, 1969; Vadehra *et al.*, 1970; Sauter and Petersen, 1974; Smeltzer *et al.*, 1979; Stuart, 1981 and Bruce and Drysdale, 1991).

Egg spoilage follows a number of well documented steps (Gillespie and Scott, 1950), *q.v.*: 1) microbial penetration of the shell; 2) growth on, and possible digestion of, the shell membranes and 3) infection of the albumen. Under normal commercial conditions, according to Board (1966), bacteria have to penetrate at least to the mammillary layer before they are in a position to grow and infect the albumen. In spite of this *sine qua non* condition above, Williams *et al.* (1968) found that motility was not required for *Salmonella* to penetrate through the outer shell structures but motility, however, made a marked difference in both the number of eggs penetrated and the speed and degree of penetration, as logic indicates. Stokes *et al.* (1956) proved that non-motile strains like *S. pullorum* and *S. gallinarum* could also penetrate the egg as readily as motile *Salmonella* strains, perhaps due to proteolytic enzyme activity (Williams *et al.*, 1968). Tung *et al.* (1979) showed, by Scanning Electron Microscope (S.E.M.) studies, microorganisms filling an apparent channel in the mammillary knob, with the bacteria appearing to have created the channel in the organic matter by themselves.

Several workers have demonstrated (Tyler, 1961b; Williams *et al.*, 1968 and Vadehra *et al.*, 1970b) that shell thickness does not significantly influence the number of eggs penetrated by *S. typhimurium*, or by *Pseudomonas* (Lorenz *et al.*, 1952), putting more emphasis on the structure of the shell surface, its pore system and the shell membranes, the age of the egg, the influence of genetic line variations on the specific characteristics and quality of the outer structures. A contrary opinion has been expressed by

Orel (1959), March (1969) , *i.e.* the thicker the shell structure, the less the possibility of contamination.

Eggs of excellent shell quality (specific gravity of 1.090) were found by Wright and Frank (1956) and Sauter and Petersen (1974) to be considerably more resistant to penetration by various *Salmonellae* than were eggs of lower shell quality. Average rates of penetration approximately doubled with each reduction of 0.010 in the specific gravity of the eggs. In their trials, eggs of low shell quality were penetrated 14.3 % of the times by *S. orienburg* and 82.1 % by *S. typhimurium*, with the corresponding figures for good shell quality being 3.8 % and 21.5 %. The earlier work of Sauter and Petersen (1969) reported the incidence of *P. fluorescens* spoilage for eggs of high, medium and low levels of quality (specific gravity of 1.085, 1.077 and 1.070 respectively) averaged 6.3 %, 19.4 % and 29.1 % over 8 weeks of storage.

Fracturing of the shell (Brown *et al.*, 1966; Humphrey, 1989 and Humphrey *et al.*, 1989c) increases an egg's susceptibility to microbial degradation (up to 1,000 times, according to March (1969)), and unwashed cracked eggs appear to be less prone to spoilage than washed, cracked eggs when exposed to infection (Vadehra *et al.*, 1969). Even though, bacterial multiplication would still be dependent upon factors such as specific types of bacteria and the age of the egg (Board *et al.*, 1964).

Brown eggs also appear to be more resistant to spoilage than white eggs (Alford *et al.*, 1950 and Trussel *et al.*, 1955a,b), perhaps due to the protective quality afforded by the pigment/cuticle relationship in the brown variety.

The incidence of shell contamination was considered to be much higher than that of *Salmonella*-contaminated contents by a myriad of workers (Stokes *et al.*, 1956; Mundt and Tugwell, 1958; Harry, 1963; Mellor and Banwart, 1965; Forsythe *et al.*, 1967; Bryan, 1968; Knowles, 1971; Smith, 1971; Borland, 1975; Silva and Hipólito, 1978; Stuart, 1981; Wilding and

Baxter-Jones, 1985; Board and Tranter, 1986; Cox *et al.*, 1990; Barrow and Lovell, 1991; Baxter-Jones, 1991; Bolder *et al.*, 1991; Denton, 1991 and Nagaraja *et al.*, 1991), which are in support of the extra-genital contamination theory.

1.5. *Salmonella* and SALMONELLOSIS: GENERAL ASPECTS

1.5.1. CHARACTERISATION

Salmonellae are some of the most widely distributed organisms in nature, affecting on a worldwide scale practically all warm- and cold-blooded animals. There are *ca.* 2,100 serotypes

, according to Snoeyenbos and Williams (1991)); 100 of them affect chickens and turkeys (Adler, 1965), which constitute the largest single reservoir of *Salmonellae* organisms existing in nature (Snoeyenbos and Williams, 1991). They are gram-negative stained facultative bacilli belonging to the family *Enterobacteriaceae*, measuring 2 to 5 µm length and 0.7 to 1.5 µm width (Krieg and Holt, 1984) or 1 to 3 µm x 0.4 to 0.6 µm (Williams, 1984 and Nagaraja *et al.*, 1991) depending on the subspecies. With the exception of *S. pullorum* and *S. gallinarum*, all strains normally possess peritrichous flagella and are actively motile. Capsule formation has sometimes been observed particularly in mucoid strains (Buxton and Fraser, 1977), although this has since been denied by Jordan (1990). Spore formation does not occur (Buxton and Fraser, 1977).

. The classification of these organisms by antigenic analysis is based upon the original work of Kaufmann and White, and uses both somatic (O) and flagellar (H) antigens (Ag). Their use is based upon the fact that each Ag possesses its own genetically determined specificity (Buchanan and Gibson, 1974).

The division into serotypes is done in the following manner, according to Morales-Diaz (1989): those organisms with a particular "O" Ag in common are collected into an "O" group (A, B, C and so on) and arranged alphabetically by "H" Ag within the group. The "H" flagellar Ag is subdivided into: specific phase or phase 1 (designated with small letters) and group phase or phase 2 (designated by Arabic numerals). Thus, the complete

identification of *Salmonella enteritidis* is: *Salmonella* serotype *enteritidis* group D1, with an antigenic formula of:

1, 9, 12	:	g, m	:	1, 7
⇓		⇓		⇓
"O" Ag		Phase 1 "H" Ag		Phase 2 "H" Ag

Serotypes can be further subdivided, into biotypes (which present different sugar patterns and are genetically determined), and phage types (or sub-types, based on the sensitivity of cultures to a series of selected bacteriophages – viruses which infect bacteria – at appropriate dilutions, shown by the production of zones of clearing on agar plates previously seeded with the test organism, which are indicative of cell lysis and susceptibility). They can also be classified according to their resistance to transfer factors such as antibiotics or bacteriocins.

1.5.2. PREVALENCE, EPIDEMIOLOGY, PUBLIC HEALTH AND ECONOMIC ASPECTS

Many members of the *Salmonella* group cause "food poisoning" and are particularly insidious because their presence in food is usually undetectable since they produce little or no change in its odour or appearance (Romanoff and Romanoff, 1949 and Vadehra and Baker, 1973), so that an egg containing millions of organisms could easily pass inspection and since the initial degree of shell contamination does not need to be very large, probably less than 100 *Salmonella* cells for infection to occur, the real possibility exists that a single eggshell, heavily infected with *Salmonella*, could contaminate large batches of sound eggs, when broken out to produce liquid, frozen or dried whole egg, albumen or yolk (Stokes *et al.*, 1956 and Williams *et al.*, 1968).

The incidence of "naturally infected" eggs is actually low, being less than one egg in 10,000, even in the so-called "high incidence" areas (Hunton, 1991), while Baxter-Jones (1991) found an egg transmission rate of

1 in 1,000 or 1 in 2,000. Duguid and North (1991) calculated from the results of a MAFF investigation in 1989, which showed a contamination rate of 1 in 15,000 eggs that with the British public eating on average 150 eggs per year (200 according to Chapman *et al.*, 1988), the chance of a person eating an infected egg was 1 in 100 per year. In a population of nearly 60 million, about 600,000 would eat an infected egg each year, but in most cases too few bacteria would be present in the egg to constitute an infective dose. The problem the authors overlooked was the fact that many of those infected could be in the "high risk" group (children, the elderly, pregnant women, immunocompromised), and for them this infection could have very serious consequences, given that "very few" bacteria can multiply to billions in a couple of days, depending on the conditions of storage (10 *S. enteritidis* PT 4 cells inoculated in an egg yolk multiplied to 10×10^8 within 48 hours at room temperature (Anonymous, 1989)). The symptoms of the disease in humans include vomiting and diarrhoea, and if not treated, severe dehydration and in extreme cases, death.

With respect to the number of organisms necessary to infect humans, McCullough and Eisele (1951) reported that *S. bareilly* and *S. newport* produced severe disease in human volunteers with 125,000 and 152,000 organisms respectively, although there are reports suggesting that only 17 organisms are enough, and even only 1 organism has been said to be sufficient (Blaser and Newman, 1982).

Food products containing raw eggs as well as poultry meats have been cited as sources of the organisms causing salmonellosis in man (Edwards, 1958; McCroan *et al.*, 1963 and Steele and Galton, 1966). In the U.K., case-control studies showed a significant association between infection with *S. enteritidis* phage type 4 (PT 4) and recent egg consumption (Coyle *et al.*, 1988), continuing to be a problem at the end of 1991 in humans (PHLS, 1992) and also in animals (Anonymous, 1992a). Similarly, epidemiological studies in the U.S.A. have determined that uncracked grade "A" shell eggs have been a major source of *S. enteritidis* in recent human outbreaks (St.

Louis *et al.*, 1988).

In Spain, most outbreaks of salmonellosis occurring recently have been transmitted by hen's eggs, and *S. enteritidis* is the causative agent in most of them (Perales and Audicana, 1989). The percentage of *Salmonella* isolates from egg-based foods in Italy has increased from 0.2 % in the period 1978-1980 to 8 % between 1984-86 (Fantasia and Filetici, 1989). The continuing association between eggs and outbreaks of *S. enteritidis* infections in the U.S.A. is confirmed by a M.M.W.R. (1988) report.

From January 1985 through October 1989, 189 *S. enteritidis* outbreaks in the U.S. caused 6,604 illnesses and 43 deaths, with contaminated eggs being the cause of nearly half of them, according to Blumenthal (1990). In Britain alone, the number of confirmed *S. enteritidis* human cases between 1982 and 1987 increased six-fold in England and Wales, with a similar increase observed in Scotland (Anonymous, 1988), while the number of cases reported for January through July 1988 (4,424) was more than double the number (2,000) for the same period in 1987 (Blumenthal, 1990), with eggs cited as the commonest source of *S. enteritidis* infection from food vehicles in the same period (Anonymous, 1989).

The phenomenon was not restricted geographically. In Brazil, where figures are available, the no. of *S. enteritidis* isolates increased 1,000 % over the total isolations of *Salmonella* in humans, from 1979 to 1987 (Rodrigue *et al.*, 1990). Similar increases in human infections are noted for: Hungary (László *et al.*, 1985); Canada, France, Ireland, Italy, Norway, Spain, Sweden, Yugoslavia and Zaire (Tauxe, 1988 cited in Shivaprasad *et al.*, 1990); Austria, Finland, Holland, Portugal and former West Germany (Anonymous, 1989). Some data regarding isolations of *S. enteritidis* PT 4 from food are shown in table 4.

Poultry infection has been reported to have also increased in many countries in the same period: former West Germany (Anonymous, 1989),

Table 4 : Isolations of *S. enteritidis* PT 4 from food in 1987:

FOOD:	No. of isolations referred for typing:
Raw liquid egg	103
Chicken	77
Unspecified poultry	29
Sea food	5
Carcass meat	3
Sausage meat	2
Meat pie	1
Dairy products	1
Total	221

Source: Public Health Laboratory Service (PHLS) / Division of Enteric Pathogens (DEP). Published in the first report of the Agricultural Committee of the House of Commons (1989).

Argentina (Eiguer *et al.*, 1990); France, the Middle East and The U.S.A. (O'Brien, 1990); Portugal (Machado and Bernardo, 1990) and Italy (Fantasia *et al.*, 1991). Similarly, Van de Giessen *et al.* (1989), cited in Bolder *et al.* (1991) reported that nearly 80 % of faecal samples taken from layer flocks in Holland were *Salmonella* positive, with 26 % being *S. enteritidis* isolates, while Poppe *et al.* (1991) found 52.9 % of the commercial layer flocks tested in Canada to be environmentally contaminated with *Salmonella*.

In the 1988 issue, fact and fiction were difficult to distinguish. Emotive statements such as "...*S. enteritidis* infection is epidemic in the U.K." (Coyle *et al.*, 1988) were unsubstantiated in subsequent discussion by the same authors. Irrespective of this lack of substance, these workers were reluctant to let go of a "good" story and so whilst merely suggesting that only one egg in a million is likely to be contaminated, they felt it necessary to conclude that all eggs, including intact clean eggs, should be regarded as possible sources of infection.

Another rather inconsistent communication was published by Telzak *et al.* (1990). In their work, all individually cultured eggs implicated with a nosocomial outbreak of *S. enteritidis* were found to be negative, in addition to the positive pooled batches of eggs not presenting the 54-Kilobase (Kb) plasmid found in all clinical isolates, food samples and one hen's ovary from a flock allegedly involved in the case. Irrespective of these findings, the authors implicated the eggs as the causative vehicle in the death of 9 patients with confirmed salmonellosis. They claim the plasmid DNA was lost because of plasmid instability.

The prevalence of human salmonellosis (other than *S. typhi*) in the U.S.A. and Canada alone is estimated to reach 3.7 million cases (with 40 deaths) per year, with an economic cost /market impact /lost productivity of up to US$ 4.8 billion a year (cost per case: US$ 1,350), according to Todd (1989). The medical expenditure alone was estimated as US$ 1.2 billion per year (Gangarosa, 1978). In the former West Germany, this cost was

estimated as £ 87 million per year (Krug, 1985). In an 8 month survey of the U.K. national costs of human salmonellosis, Sockett and Roberts (1991) calculated the total public sector costs as nearly £ 400,000, with the family costs to £ 95,000; the lost production due to illness £ 507,000 and the general total cost up to £ 1 million. The same authors also stated that, based on 23,000 cases reported in 1988 alone, the general costs could well exceed £ 16.3 million.

The economic implications of the *Salmonella* issue have been widely publicised, and despite the total financial loss to the poultry industry being difficult to measure, the losses due to low hatchability alone were estimated to cost the British poultry industry more than £ 1 million per year (Board, 1969), while losses incurred as a result of *Salmonella* infections in cattle, pigs and poultry in Germany and poultry in Canada were, respectively, £ 40 million and £ 1.65 million per year (Sockett, 1991). In the U.S. there was a loss of about US$ 77 million annually (Marsh, 1976). Public money is also spent to tackle the problem; thus the British government made available £ 7 million to support the egg industry, following the *Salmonella*-eggs crisis (Sockett, 1991).

O'Brien (1990) suggested that the current structure of the poultry breeding sector, dominated worldwide by a small number of companies helps to maintain a situation where unrecognised vertical transmission of *Salmonella* from grandparent stocks may be a reality, since flocks remain asymptomatic and demonstrate no appreciable variation in egg output.

An eradication programme would cost more than it could be justified by benefits derived from such eradication, according to Purchase (1979), with US$ 12.68 spent to generate US$ 1 of benefit (Finn and Mehr, 1977). Although probably unpractical, efficient control measures could be employed, as previously used in Sweden (Todd, 1980).

1.5.3. SALMONELLOSIS IN BIRDS

1.5.3.1. General

S. enteritidis PT 4 is highly virulent for both young chickens (Barrow, 1991) and older (30-40 weeks of age) birds (Fowler and Mead, 1992) and one of the major components of virulence is believed to be its invasiveness following oral inoculation (Hinton *et al.*, 1989 and Barrow, 1991). The fact that *S. enteritidis* shares surface Ag with the avian-adapted *S. pullorum* may partly account for the successful adaptation of the PT 4 strain to produce chronic invasive infection (Rampling *et al.*, 1989). This unusual invasiveness has strikingly arisen in Europe and the U.S.A. independently and simultaneously in phage types 4 and 8 (Timoney *et al.*, 1989).

It is acknowledged that stress-producing circumstances such as poor environment, overcrowding and other concomitant diseases (Borland, 1975), transportation, drastic changes in ambient temperature, heavy parasitic burden (D'Aoust, 1989) and the onset of egg production (Fowler and Mead, 1992) will contribute to the development of a systemic *Salmonella* infection. Humphrey *et al.* (1991c) reported that older specific pathogen free (SPF) birds (52 weeks old) were much more susceptible to a challenge with *S. enteritidis* PT 4, showing a severe response and prolonged faecal carriage (contrasting with the younger (20 w.o.) SPF birds, which appeared unaffected). The authors suggested that the effect was related to immunosuppression due to the fatigue associated with intensive laying and Ca depletion. In the younger birds, oestrogen could have helped in providing protection.

In terms of infection, although *Salmonellae* may enter the body through the pharynx, respiratory tract (Baskerville *et al.*, 1992), or the conjunctiva, the organisms usually gain entrance to the host by the oral route and are deposited in the intestine where they invade the enterocytes (Wray and

Sojka, 1977), having a predilection to establish a chronic infection in the caeca and crop (Nagaraja *et al.*, 1991).

Following adherence to the target cell, strains that produce diarrhoea multiply, secrete a toxin and invade the cell. *Salmonellae* within the cell secrete another cytotoxin, causing the cell to die and slough off. The severity of the disease depends upon the number of target cells involved. The signs are: progressive somnolence, eyes closed, wings drooping, ruffled feathers, marked anorexia, increased water consumption; profuse diarrhoea with pasting of the vent and evidence of cellular death (blood, debris and inflammatory cells), abdominal discomfort, blindness and conjunctivitis (Hirsch, 1990 and Nagaraja *et al.*, 1991). *Salmonellae* that are phagocytosed are not easily destroyed, because in the non-immune animal, the lysosomal contents of the macrophage will not readily affect it within the phagosome (Hirsch, 1990). Its ability to survive and multiply inside phagocytes is critical to the outcome of the infection, as in the intracellular location, the bacterium is protected from injurious substances such as antibiotics, antibodies and complements (Collins and Campbell, 1982).

Organisms like *Salmonella* and *Escherichia* may also produce a marked bacteraemia during the acute stage of the infection, particularly in young animals. After ingestion of these organisms, a few will penetrate the wall of the intestinal tract and be filtered off into the local lymph nodes with some entering the blood stream (primary bacteraemia) and becoming distributed in the organs of the body. A secondary and more severe bacteraemia may prove fatal (Buxton and Fraser, 1977).

Salmonella infected birds had a mortality which was highest in the first two weeks after hatching, with infection rarely causing mortality in birds more than 1 month old (Nagaraja *et al.*, 1991). Histopathology demonstrated that infected birds had areas of intestinal mucosa devoid of microvilli (probably causing the interference with nutrient absorption) (Bayer *et al.*, 1977).

Salmonella organisms have become remarkable in their ability (especially *S. enteritidis*) to cause infection, with an unpredictable disease outcome. It is possible that *S. enteritidis* has the same ability as *E. coli* (Finlay and Falkow, 1989), to be able to express two pili, which are serologically identical, yet possessing different binding specificities, which allow them, by genetically varying the minor tip protein adhesin, to gain the ability to bind to alternative receptors. Nolan *et al.* (1991) have addressed this hypothesis by isolating *Salmonella* serotypes from sick birds which were characterised by the production of pili and significantly greater invasiveness, because by doing so, *S. enteritidis* could adapt to infect ovary and gastrointestinal tract. This complete adaptation may have taken some years to be achieved, but now the invasiveness is high and the outcome rather puzzling. Also, microbes in general can avoid host immune responses by varying surface antigens (Meyer and Haas, 1988 and Seifert and So, 1988), and this could mean remaining inactive for some time within the host, changing some surface Ag in order to avoid Ab recognition, and then multiplying again. This mechanism would explain the phenomena of intermittent shedding and also the inability (either partially, not reproducing the real pathogenesis, or completely, with no infection happening at all) of many researchers to reproduce the natural course of infection by experimental procedures. An explanation of why laboratory-grown cultures do not establish themselves as effectively and easily as when it is inoculated mixed and grown in faecal contents, can be given by the fact that, when an "invader" organism enters an established flora, it will be significantly more successful when it has been growing in an environment which closely resembles that in which it is about to enter (Freter, 1974). Previous growth of the invader in a different environment (laboratory media) will result in a prolonged lag growth phase in the new environment, with most of its population being eliminated during this lag phase.

1.5.3.2. Treatment and development of immunity

The administration of antibacterial drugs to infected animals is designed to destroy organisms in the intestine as well as in the tissues. The intracellular location of the bacteria is a formidable barrier to antibacterial drugs, and there is dependence on host defence to destroy or contain such organisms (Clarke and Gyles, 1986). This mechanism could explain partially the intermittent shedding, by the irregular burst of cells (liberating Ag) and the multiplication of these and their expulsion by peristalsis.

Antibiotics frequently used to challenge *Salmonella* include ampicillin, chloramphenicol, kanamycin, tetracycline and trimethoprim-sulphamethoxazole (D'Aoust, 1989). Other recommendations exist, such as furazolidone (0.04 % in the feed for treatment and 0.01 % for prophylaxis) (Jordan, 1990), injectable gentamycin and spectinomycin (Nagaraja *et al.*, 1991), or lincomycin and streptomycin (Hirsch, 1990). The feed can also be treated by the inclusion of organic acids, to reduce contamination (Humphrey, 1991b).

Opinions vary, thus Knivett and Tucker (1972) claim that neither vaccination with live *Salmonella* cultures administered in water nor furazolidone therapy is adequately effective, once the pathogen is established in the intestines of a chicken. On the other hand, it must be always kept in mind that antibiotic treatment will almost certainly increase faecal shedding and also prolong the carrier state of birds.

Regarding the development of immunity, there are two levels of immunological protection. The first is humoral, in which Immunoglobulins (Ig) secreted by plasma cells opsonize target Ag and thus facilitate phagocytosis. Binding of IgG or IgM to cellular Ag can also activate the classic complement system, a series of serum proteins and enzymic reactions that leads to

bacterial lysis through cell membrane damage, attraction of phagocytic cells to the infected site, or coating the cell Ag with a complement (C3b) fragment to encourage phagocytosis (D'Aoust, 1989). Paratyphoid *Salmonella* serotypes, such as *S. enteritidis*, generally elicit weaker humoral Ab responses (Williams and Whittemore, 1975 and 1979).

The second level is the cell-mediated immunity, where two types of lymphocytes provide protection. Cytotoxic T lymphocytes (T killer cells) attach to and lyse viable Ag such as bacteria and viruses without undergoing self-destruction (D'Aoust, 1989). This type of immunity is of considerable importance in protection against Salmonellosis (Habasha, 1981 and Lindberg and Robertsson, 1983, both cited in Clarke and Gyles, 1986). Transfer of sensitised T cells confers protection, whereas transfer of macrophages, B cells and hyperimmune sera are not protective in the absence of immune T cells. The persistence of the vaccinating bacilli within the liver and spleen is also essential if a cell-mediated type of immunity is to be developed. When the systemic stimulus has been eliminated, immunity rapidly declines as seen from the increasing severity of later oral challenge infections (Collins and Carter, 1974). Despite that, neither cellular nor humoral mechanisms have been demonstrated by Bradshaw *et al.* (1990) to inactivate *Salmonella* in the yolks of unfertilised eggs.

Intestinal immunity is, on the other hand, an important protective mechanism that translates into a localised IgA-dependent response. Bacterial Ag activate B lymphocytes located in discrete aggregations of lymphoid tissues (Peyer's patches) in the intestinal submucosa. The activation ultimately results in the appearance of secretory IgA (s-IgA) plasma cells in the lamina propria, preventing adhesion and subsequent invasion of pathogens (Freter, 1974) and also immobilises cells and their toxins at the mucosal surface, thereby facilitating proteolytic inactivation (Abraham and Beachey, 1985; Kilian *et al.*, 1988 and D'Aoust, 1989). It seems increasingly apparent that immune protection against enteropathogenic bacteria depends largely on the mucosal immune system, which can best be aroused by oral

vaccination (Hone and Hackett, 1989). Thus, it is the s-IgA levels found within the intestinal contents, rather than the circulating IgG and IgM Ab titres that constitute the more accurate index of immunity to enteric disease (Fubara and Freter, 1973).

Pathogenic *Salmonellae* seem able to cross the intact mucosal barrier, even in actively immunised individuals. Then, once they are taken up by the phagocytic cells within the lamina propria, the rapidly multiplying bacilli tend to be protected from the bactericidal action of the circulating Ig (Collins and Carter, 1972). As only a small number of *Salmonellae* need to reach the bloodstream to bring about an infection, the host defence system is required to prevent virtually all of the *Salmonellae* from crossing the intact intestinal mucosa and entering the lymphatic drainage system (Collins and Carter, 1974). The adherence to the target cell is prevented by Ab specific for surface structures of *Salmonella*, possibly fimbriae (Hirsch, 1990).

As far as maternal antibody is concerned, antibodies produced by the hen are transferred to the egg, where the Immunoglobulins are partitioned: higher levels of IgG in the yolk and higher levels of IgA and IgM in the white (Rose *et al.*, 1974), with the chick embryo receiving IgA and IgM antibodies by swallowing the albumen. Since the major function of secretory IgA appears to be Ag exclusion, this passive protection might conceivably inhibit bacteria colonisation of the chick during the first day of life, when it is most vulnerable. The deposition of IgA in the white of the egg, which was demonstrated by Schiemann and Montgomery (1991), following an oral booster vaccination, would suggest that the avian intestine and oviduct are part of such a common mucosal immune system.

Finally, Humphrey *et al.* (1991b) found a clear relationship between the birds' antibody (IgG and IgM) levels and its generation speed and the size of the infective dose. They also found a close relationship between the latter and the duration of faecal excretion, but no parallels could be drawn between faecal excretion and Ab levels.

1.5.3.3. Serology screening and control

Higher serological titers will reflect that infection has occurred some time in the past, even though the birds might not be excreting *Salmonella* organisms at the time of sampling (Barrow and Lovell, 1991).

Some authors recommend that the ELISA test can be used to detect antibodies to *S. enteritidis*, even in the yolk of hens that have been naturally exposed to infection (Nicholas and Andrews, 1991), while others like Chart *et al.* (1990a) claim that the whole serum agglutination test is the most appropriate test for screening chicken sera for Ab to the "O" antigens of *S. enteritidis*, rather than the ELISA alone, which they found inconclusive and therefore unreliable. It is done as a flock test and not as an individual bird test. There is a great deal of inconsistency regarding the application of these serological methods for the detection of Ab against *S. enteritidis*. The latter were effective experimentally (Gast and Beard, 1990d), but unreliable in naturally infected flocks (Williams and Whittemore, 1979; Cooper *et al.*, 1989 and Chart *et al.*, 1990b), where the presence of Ab, although suggesting that the flock had been infected, did not necessarily indicate current infection.

Regarding the isolation of the microorganism, *Salmonella* can be isolated directly from the liver, gallbladder or yolk sac, but the intestines and especially the caecal contents are more prone to reveal the presence of infection (Jordan, 1990). Cloacal swabs would be a feasible way of detection, but the intermittent shedding factor makes it an unreliable instrument, subject to false-negative results. According to Blood *et al.* (1983), animals should not be considered free of infection until 3 successive attempts to isolate the organism every 14 days have failed. The same applies conversely to egg screening. Morris (1990) claims that even in a bird which is infected with *S. enteritidis*, only about 1 in 200 eggs will be contaminated. The recent finding of a 16 Kb antigen specific for *S. enteritidis* by the Central Veterinary Laboratory (CVL) (Weybridge), allowed the development of a

monoclonal Ab directed against the Ag, and the application in a test kit for the detection of *S. enteritidis* commercialised under the name of "Sefex" (Dr. D. Taylor, personal communication).

In terms of general control, a high ammonia content present in old litter may help to control *Salmonella* contamination, according to Turnbull and Snoeyenbos (1973). Bryan (1968) advocated that the misconception that the so called "ubiquitous" organisms cannot be controlled must be replaced with a feeling of determination to apply the known principles of control (limitation of contamination, inhibition of growth and destruction of organisms).

"Draconian" control measures such as those introduced by the Zoonoses Order 1989, recommending the slaughter of flocks found to contain any infected birds were considered by Duguid and North (1991) as being out of line with practice in most other developed countries, and that it was damaging the British egg industry, particularly the small producers. *Salmonella*, according to Snoeyenbos *et al.* (1970) can recur even after animals were slaughtered and thorough cleansing and disinfection was applied. Similarly, Wilding and Baxter-Jones (1985) stated that it would not be feasible to prevent egg-borne *Salmonella* if the continual inputs into the system are to continue occurring.

1.5.3.3.1. Vaccines

Vaccines developed for controlling host specific serotypes tend to have little effect in reducing faecal excretion of host non-specific strains in poultry, but may control systemic infection of *S. enteritidis* PT 4 (Mead and Barrow, 1990), by inducing the production of IgM and IgG systemically, and not only IgA locally. It is considered by Barrow (1989) to be essential that the vaccine strains are easily differentiated from field strains. Suitable genetic markers can be used, and the strains should not induce the production of Ab which will be detected in an agglutination test.

Barrow *et al.* (1991), working on vaccination against *S. enteritidis* using a mutant strain, found no conclusive results for its use in layers or broilers, but opened the possibility of it being applied to breeding flocks in order to reduce vertical transmission to layers and broilers, as breeder flocks are early critical points for preventing *Salmonella* entry into the integrated poultry operation. Inactivated *Salmonella* vaccines were found to sacrifice a considerable part of immunising potency, because they produce a humoral response, whereas live vaccines activate both the humoral and cellular immune systems (D'Aoust, 1989), inducing significant specific and non-specific cell-mediated immune responses, which may account for their ability to induce a cross protective response (Lumsden *et al.*, 1991).

On the other hand, Timms *et al.* (1990) suggested that subcutaneous inoculation at three or at 3 and 6 weeks old with an inactivated oil adjuvant *S. enteritidis* PT 4 vaccine protected chickens against a massive experimental challenge at 5 or 8 w.o.. A possible problem in the establishment of sound protection by vaccination could be that lipopolysaccharide (LPS) may not be well recognised by the IgA plasma cell-generating system, or that there is a poor expression of IgA-inducing epitopes on somatic antigens (Hassan *et al.*, 1991). These facts, coupled with the previously recorded importance of the mucosal IgA protection mechanism would partly explain the inconsistency in the results reported regarding vaccine protection against *S. enteritidis*. It could even be that bacteria grown *in vitro* would not provide full expression of the antigenicity as they would otherwise do *in vivo*. Some flagellar Ag are believed not to fully express themselves in *in vitro* cultures (M. Peel, pers. comm.).

1.5.3.3.2. Competitive exclusion

The establishment of disease-producing organisms in the intestinal environment may be inhibited by other microbial species constituting the normal flora. Constant microbial competition and antagonism have to be overcome by pathogenic organisms before they can establish themselves and produce clinical disease (Buxton and Fraser, 1977). This fact, together with the evidence that: a) natural transfer of microbes from the hen to the chick is broken in modern poultry industry (and thus the opportunity for the chicks to get a versatile intestinal flora at the earliest possible age is lost (Nurmi, 1985)), and b) newly hatched chicks can be contaminated by a single cell of *Salmonella* (Pivnick and Nurmi, 1982 cited in Nurmi *et al.*, 1984) form the basis of the competitive exclusion mechanism, or the "Nurmi Concept". By establishing an adult flora at the earliest opportunity, the treatment overcomes the high susceptibility of the young bird to *Salmonella* colonisation and provides a degree of protection that is usually available only to the adult individual (Mead and Impey, 1986).

The protective effect is not significantly influenced by breed, strain or sex of the bird, but depends upon the introduction of viable bacteria, and not bacteria-free filtrates, and it appears that activation of the immune system is not required, although stress can reduce its effect (Mead and Barrow, 1990). The mechanism of protection is poorly understood, according to Barrow (1989), but there are suggestions that the normal flora competes with the *Salmonella* organisms for sites of attachment within the caeca (Snoeyenbos *et al.*, 1979). Other explanations include the inhibitory effect of bacterial metabolites such as hydrogen sulphide and volatile fatty acids and the low redox potential generated by these organisms. *E. coli* seems to be a major competitor to *Salmonella* in newly hatched chicks, and as the former is known to be one of the first microorganisms to colonise the chicks' gut, it will probably be able to exclude *Salmonella* simply by establishing itself first, and

blocking the essential space for *Salmonella* (Baba *et al.*, 1991).

The use of competitive exclusion has been attributed not only to the prevention of *Salmonella* colonisation (Fukata *et al.*, 1991), but also an increased growth rate in the treated chicks (Nurmi, 1985). The latter author cited others (Seuna and Nurmi, 1979 and Seuna *et al.*, 1980) who reported that the application of intestinal microbes can be combined with antibiotic treatment without negative effects, while Humbert *et al.* (1991) found even a synergistic effect between virginiamycin and bacitracin administration and competitive exclusion treatment. They believed the antibiotics possibly promoted the most protective bacteria, which had been administered as part of the competitive exclusion treatment. In contrast, Snoeyenbos *et al.* (1979) and Bailey *et al.* (1988) reported a decrease in the protective effect of competitive exclusion due to antibiotic administration.

This concept should not be treated as a panacea, as in reality tests made under field conditions have not always been successful (Nurmi, 1985) and also there have been reports of late contamination by *S. enteritidis* (30 to 40 weeks of age) of some broiler breeder flocks, each of which had been given a commercial normal adult chicken intestinal microflora at day old and which had been previously constantly negative for *S. enteritidis* PT 4 (Fowler and Mead, 1992). The same authors proposed, as an explanation, that a cryptic systemic infection could flare in some birds with residual low level infection that may remain after competitive exclusion treatment after day old.

It is certain that upon ingestion, the enteric organism is summarily exposed to higher temperatures, extremes of pH, different available nutrients, high concentration of bile salts, digestive enzymes, *etc.*. Some pathogens have learned to overcome the strong waves of peristalsis within the small bowel and to establish themselves in this niche, despite the presence of competitive bacterial populations (Finlay and Falkow, 1989).

1.6. AIMS OF THE WORK

The general objectives of the present work are:

I) to define and develop a microbiological technique which makes possible the study of bacterial penetration through the hen's eggshell, *in vitro*, and also permits subsequent examination of the shell with the Scanning Electron Microscope (S.E.M.);

II) to assess the efficacy of the eggshell with/without its membranes to restrain bacterial penetration, both qualitatively and quantitatively;

III) to analyse eggshell quality, using an ultrastructural evaluation technique developed for S.E.M. studies, with regard to eggs at beginning, middle and end of lay plus selected poor quality eggs, eggs from two different strains of birds, eggs from birds housed in barn, on range or in batteries and eggs from birds subjected to two different feeding regimes, one of them incorporating the growth promoter Virginiamycin;

IV) to correlate bacterial penetration through the eggshell wall with shell structural conditions *sensu latu* and *sensu strictu*, as influenced by age, strain of the bird, housing system and the addition of an antibiotic in the feed and

V) to develop an immunocytochemical technique which makes possible the close monitoring of bacterial translocation through the eggshell by using an immunogold marker, which allows precise localisation of the bacterial cell when examined with the S.E.M. in backscattered electron mode.

CHAPTER 2

EGGSHELL QUALITY AND BACTERIAL PENETRATION AS AFFECTED BY THE AGE OF THE BIRD (AGE EFFECT)

2. EGGSHELL QUALITY AND BACTERIAL PENETRATION AS AFFECTED BY THE AGE OF THE BIRD (AGE EFFECT)

2.1. INTRODUCTION

An estimate made in the late fifties placed the annual U.S. losses due to broken and cracked eggs at about 250 million dozen eggs (5 to 7 % of the total production at that time), which, priced at US$ 0.40 a dozen, would be worth about US$ 100 million (Ewing, 1963). Losses to the U.K. industry due to shell faults amount to at least £ 8 million a year, according to Belyavin *et al.* (1987). At present, about 7 % of eggs are downgraded in commercial packing stations, and over 90 % of these have shell faults. It is estimated that worldwide (but excluding the former U.S.S.R., South America and Asia, except Japan) losses from eggshell breakage alone exceed US$ 600 million (Hunton, 1987).

A great part of these losses can be attributed to structural defects within and on the surface of the shell, which weaken its capacity to withstand the various injuries to which it is submitted during the industrial process.

The ability to produce good quality shells can deteriorate suddenly in some birds (Belyavin, 1979 cited in Belyavin *et al.*, 1987), while others show a gradual decline in quality with age. The decline associated with time occurs even in birds of superior shell quality production (Boorman, 1985). Shell thickness also declines with age (Jull, 1953; Izat *et al.*, 1985; Belyavin *et al.*, 1987 and Belyavin *et al.*, 1991), and the longer the bird produces eggs, the thinner the eggs become. This means that with today's laying strains, bred for long periods of profitable production, the problem of shell quality may appear before it is economically advisable to cull the flock. Partial restoration of eggshell quality can be achieved by moulting, either natural or forced (Ewing, 1963; Strong, 1989; Watt, 1989 and Bain, 1990). Various

authors reviewed by Hunton (1987) showed that following a moult, several indicators of eggshell quality were improved, *e.g.* the lower the shell breaking strength prior to the laying pause, the greater the recovery after the moult period.

Although increases in calcium intake can be beneficial in improving shell quality, high intakes will not prevent the decline in quality associated with age (it has been speculated earlier by Petersen (1965) that this decline is caused by a reduction in the Ca retention capacity, as the birds become older).

Eggshell quality in general was found to become poorer as the birds age (Petersen, 1965; Bruce and Johnson, 1978; Strong, 1989; Watt, 1989; Bain, 1990; Harms *et al.*, 1990 and Belyavin *et al.*, 1991), with eggshell contamination being suggested by Bruce and Drysdale (1991) to increase accordingly. Cuticle deposition has also been shown to decline with laying flock age (Anonymous, 1991).

To the outsider, the battery house is assumed to provide a uniform environment with respect to temperature, humidity, lighting, *etc.*. According to Sparks (1991), the tiered systems lend itself to the production of eggs of variable quality. According to his findings, eggs from the top tier were inferior. The following experiments were designed not only to demonstrate the effect of age on shell structure, but to give some insight into the relationship between the position of the bird in a system and the integrity of the shell therein produced.

2.2. MATERIALS AND METHODS

2.2.1. GENERAL

The eggs used in this trial were collected at random from a strain of commercial brown egg layers (here and thereafter referred to as strain A), housed in a three tier battery system, fed a commercial layers diet and located in a controlled environment house at the West of Scotland Agricultural College (W.S.A.C.) (Auchincruive, Ayrshire, Scotland). Since this work was from an already ongoing experiment using commercial strains, we were requested by the Company to identify them by code letters. Eggs were collected from the beginning (birds 24 weeks old) and middle (46 w.o.) of lay. Because of schedule difficulties at W.S.A.C., eggs from the end (69 w.o.) of lay and a group of visually poor quality ("bad") eggs were obtained from a similar strain held under similar conditions from Harper Adams Poultry Research Unit (Edgmond, Newport, Shropshire, England).

A total of 106 eggs were used (32 from each age group and 10 from the "bad" group). The eggs from the Harper Adams site were collected by the farm manager with no regard to their position within the battery system. Nevertheless, it was considered of interest to make some statement concerning tier effects and shell structure, and so the work was carried out using only those eggs of known provenance from the beginning and middle of lay. The eggs from beginning and middle of lay were distributed: 50 % from the middle, 25 % from the top and 25 % from the bottom positions in the battery.

Salmonella enteritidis PT 4 (figure 3) was chosen as the test organism partly because of the recently reported high incidence of *Salmonella* infections in the United Kingdom and partly because it differs from the normal microflora of eggs and can, therefore, be isolated and distinguished from other contaminant microorganisms.

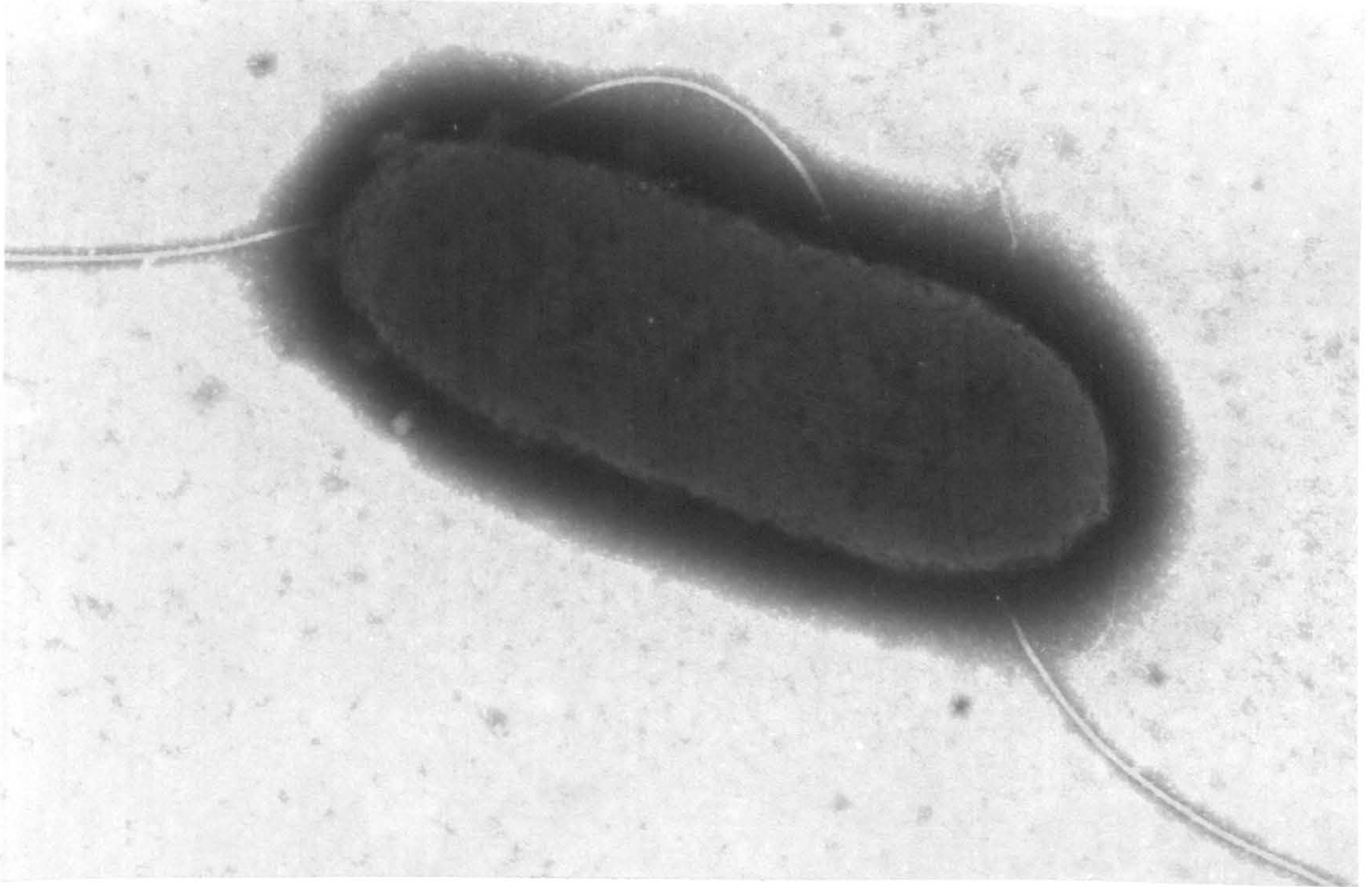


Figure 3: Transmission micrograph of *Salmonella enteritidis* PT 4 (50,000 X).

2.2.2. *S. enteritidis* PT 4 TECHNICAL PROCEDURES

Salmonella enteritidis phage type (PT) 4 was isolated from chickens and identified by the National Collection of Type Cultures (NCTC). The samples were lyophilized and stock cultures were kept in a Nutrient agar (Oxoid Ltd., Basingstoke, Hampshire, England) tube and on Dorset's egg slopes at 4 °C, from which one wire loop was used to transfer the organisms to a bottle containing 25 ml. of Nutrient Broth no. 2 (Oxoid Ltd.). The bottle was incubated in a rotatory auto-shaker (140 strokes/minute) at 37 °C for 24 hours. After that, and based on previous trials, serial 10-fold dilutions using 0.8 % (w/v) sterile saline (NaCl Analytical grade, Formachem Ltd., Strathaven, Scotland) were made, until a dilution of 10^{-6} was achieved, giving a final concentration of *ca.* 3 x 10^3 colony-forming units (C.F.U.)/ml., which means that the count of viable *S. enteritidis* cells was adjusted to provide a contaminating dose of *ca.* 30 C.F.U. per piece of shell. The term colony-forming unit was preferred to cells in describing the units from which colonies developed.

With respect to plate counting, colony numbers were determined by spreading 90 mm plates with 0.1 ml. of the appropriate dilutions of the bacteria. Colonies were counted after incubating the plates at 37 °C for 24 hours. Each plating of the 10^{-6} test dilution was done in triplicate at the beginning and end of the shells' manipulation period, in order to evaluate any change in bacterial numbers during this time. Variations in cell counts between replicates were usually very small, so the average concentration of viable microorganisms in the challenge suspension was 3 x 10^3 C.F.U./ml.. Plates were also made from the dilutions 10^{-4}, 10^{-5}, 10^{-7} and 10^{-8}, as a back-up to check the accuracy of the dilution procedure. All viable colonies were counted using a Colony Counter (Gallenkamp Ltd., England).

The identity of *Salmonella*-like colonies that formed on 0.8 % Brilliant Green (BG) agar (Oxoid Ltd.) following incubation was confirmed by

morphology, Gram-staining and serology procedures, by sub-culturing a colony on to another BG plate, from which typical, fresh 24 hour colonies were then checked serologically by slide agglutination test using *Salmonella* polyvalent somatic ("O") agglutinating serum (groups A-G) (Wellcome Diagnostics, Dartford, England) and subsequently other two tests using *Salmonella* somatic group D1 (Ag 9) (Wellcome) and *Salmonella* polyvalent flagellar ("H") agglutinating serum (phase 1 and phase 2 antigens) (Wellcome) and also biochemically using the API-20 E System (API System SA, La Balme, Les Grottes, France). In all cases, testing of the colonies recovered confirmed them to be the same as the organism inoculated on the shell. Pieces of shell taken from the same eggs were incubated on BG at 37 ^{o}C for 24 h. as controls. No growth of microorganisms was detected.

The concentration of the BG agar was modified from the 1.2 % (w/v) recommended to 0.8 % to provide an agar soft enough in which to insert the shell, but at the same time firm enough to prevent colonies of *Salmonella* merging due to the medium being too sloppy.

Similarly, the trials indicated that the concentration of C.F.U./ml. number was the most appropriate dilution of *Salmonella* broth to add to the shell in order to permit bacterial penetration without colony merging, while being heavy enough to give every opportunity for penetration under conditions used.

Before the definitive experimental procedure was settled, preliminary trials were carried out to test:

a) the efficiency of the plasma etching procedure with regard to contamination.

b) the effectiveness of Silicone High Vacuum grease (Edwards) as a sealing barrier on the shell to *Salmonella* broth.

c) the most suitable period to leave the contaminated shell on the BG agar.

d) the most appropriate incubation time of the agar plates for

colonies to develop.

e) the resistance of silicone grease to the high temperature of the autoclave.

f) the effectiveness of plasma etching procedure to remove remains of BG agar on the under surface of the shells that were exposed with no membranes attached.

g) the effect of autoclaving on shell ultra-structure.

Only after these questions had been solved and the necessary adjustments made, was the definitive experimental protocol carried out.

2.2.3. TREATMENT OF EGGSHELLS

A piece (*ca.* 1.5 cm^2) of shell was cut from the blunt end of each egg using a dental drill (NM 3000, Nouvag, Switzerland). The egg contents were then poured out and discarded, and the inside of the shell was rinsed twice with distilled water, in order to remove the albumen adhering to the membranes. Small areas of shell of *ca.* 1 cm^2 were then cut from the equatorial region of the egg.

A quarter of the samples were left with both the O.S.M. and the I.S.M. intact, and a quarter had the I.S.M. removed by peeling it away from the O.S.M., manually. The other half were plasma etched for 4 hours to remove all traces of membrane fibres, as described by Reid (1983). This technique uses low-temperature plasma treatment, during which specimens are surrounded by an atmosphere of oxygen gas at 133.3 Pascals, which is then made reactive (ionised) by the application of radio frequency power of 100 watts. Using this method, the reaction between the plasma and the carbon in the samples removes the organic component of any remaining membrane fibres by volatilisation, while leaving the crystalline material intact. The resultant membrane ash was removed from the shell samples by lightly applying a pressurised aerosol (Kenair Clean Air Duster, Kenro, Swindon, England). The plasma etching equipment used was the Nanotech Plasmaprep 100 Plasma chemistry unit.

Pieces of shell used for control purposes were selected from the same eggs in test, and all were handled in an identical manner.

2.2.4. POSITIONING THE EGGSHELL ON THE AGAR PLATE

The pieces of shell (cuticle side uppermost) were carefully placed on the surface of the 0.8 % BG agar in a 50 mm shallow form, single vent Petri dish (Sterilin Ltd., Hounslow, England), eliminating any air bubbles beneath, and ensuring that the cuticle was exposed above the level of the agar.

All plates containing the shells were then placed in an incubator and held at 28 °C for 15 minutes, in order to eliminate any moisture present on the cuticular surface, which could adversely influence the sealing process carried out subsequently. After drying, a ring of silicone high vacuum grease was added by means of a dental elastomer syringe (Espe Gmbh, Germany) attached to a fine nozzle.

At this point, 0.01 ml. (10 µl) drop of the *S. enteritidis* broth (at 28 °C ± 1 °C), diluted to 10^{-6} in 0.8 % sterile saline (see section 2.2.2.) containing approximately 30 C.F.U. was placed on the surface of the shell, inside the grease ring. The bacteria were then allowed to penetrate the eggshell for 20 minutes at room temperature (23 °C ± 1 °C), after which the shells were carefully removed from the agar and autoclaved for 30 minutes at 121 °C and 103.4 KPascals pressure, in order to kill any *S. enteritidis* still present in the shell. Control uninoculated shells were subjected to the same treatments. All plates were incubated at 37 °C for 24 hours, after which colonies were counted, in order to determine the number of C.F.U. exposed and the number of units that penetrated the shell.

After autoclaving, shells were drilled to remove the grease ring and those with no membranes attached were put for 2 hours in the plasma etching unit, to remove any remaining agar on the mammillary layer.

All shells were affixed to aluminium stubs with the outer surface

uppermost, in the first instance, with double-sided sellotape, to permit cuticle examination. Subsequently, the shells were detached and mounted inner surface uppermost, now fixed with conductive silver paint (Agar Scientific Co., Stansted, Essex, England).

All samples were coated with gold-paladium in an Emscope Sputter Coater SC 500 (Ashford, Kent, England) and then examined in a Phillips 501 B Scanning Electron Microscope (S.E.M.) with an accelerating voltage of 15 Kv. Mammillary counts (after Reid, 1984) were taken at three randomly chosen areas per sample at a magnification of 160x and at a working distance of 13 mm. Mammillary layer morphology was assessed using the criteria described by Reid (1984) and later modified by Watt (1985 and 1989), in which thirteen structural features were assessed per shell, according to the score system illustrated in figure 4. Pore counts at the egg equator were made on samples previously inoculated and prepared for S.E.M. and transformed to express counts per unit area (10 mm^2). These were the values used in the statistical analysis. Other countings on the cuticular surface of pieces of shell taken from adjacent areas and also on the mammillary side of these shells were used to proportionally confirm pore numbers.

Data from the untreated control eggshells will not be presented in the results because these shells were consistently negative for *Salmonella* and other bacteria in all sampling tests. This fact virtually eliminates the possibility that shells used in this experiment were previously (naturally) infected with *Salmonella* or any other bacteria, and permits interpretation of the results entirely on the basis of the experimentally introduced *Salmonella enteritidis* PT 4.

There was a small but not significant variation in the colony counts of culture broths used in exposing eggs, over the different groups. These were compensated in the analysis and interpretation of results by using a percentage of penetration as a value to estimate the degree of bacterial

penetration through the eggshell, thus providing a figure that can be interpreted independently of the number of C.F.U. exposed.

						SCORE:
MAMM. DENSITY:	area 1	area 2	area 3		mean:	
					s.d.	
CONFLUENCE:	none(3)	isol.(4)	mod.(6)	ext.(1)		
CAPS:	G (1)	G-(3)	P+(6)	P(8)	P-(10)	
EARLY FUSION:	ext.(1)	mod.(2)	isol.(4)			
LATE FUSION:	ext.(6)	mod.(3)	isol.(1)			
MAMM. ALIGNMENT	none (1)	isol.(2)	mod.(4)	ext.(7)		
TYPE B's	none (1)	isol.(2)	mod.(5)	ext.(8)		
PITTED	none (1)	dep.(5)	eros.(7)	hole(12)		
ARAGONITE	none (1)	isol.(2)	mod.(5)			
TYPE A's	none (1)	isol. (2)				
CUBICS	none (1)	isol. (2)	mod.(5)			
CUFFING	none (5)	isol. (4)	mod. (1)			
CHANGED MEMBRANE	none (1)	isol. (4)	mod. (8)	ext. (14)		
					TOTAL SCORE:	

Figure 4 : Procedure for quantifying shell quality by S.E.M.. Numbers in brackets represent the score ascribed to each individual characteristic.

2.3. RESULTS AND DISCUSSION

2.3.1. GENERAL

The values attributed here to each of the shell variables relate to the influence of the latter on shell performance. They are neither absolute values nor were arbitrarily chosen, but were derived as a result of the collaborative input from both a statistician and the experience of other researchers who have worked in the field of eggshell quality for several years.

However, at this time, it is accepted that modifications of the score system still have to be made to take account of the influence of new structural information in a more diversified approach, as for example eggs with more than one pitted area, the influence of structure on restraining microbial penetration, *etc.*.

The mean values for each structural characteristic, percentage of bacterial penetration and number of pores were calculated according to the period of lay and, where appropriate, position in the battery system. In tables 7, 8, 13, and 14, the mean values with standard deviation (S.D.) or standard error of the mean (S.E.) are presented for each particular characteristic, but for statistical purposes, the whole set of values for each character were tested, in order to give a consistent indication of the significance of the results. The percentage values (proportions) were all transformed using an arcsin or square root transformation (where appropriate) before statistical analysis, following the recommendations of Steel and Torrie (1980). As the results were similar for transformed and untransformed data, the untransformed percentages are reported for ease of data interpretation.

Analysis of variance (ANOVA), Student's t-test, Chi-squared tests and correlation coefficient and regression analysis when appropriate, were then

carried out on these data to determine if inter- or intra-group significant differences in the incidence of some or all of these structural traits and microbiological phenomena could be identified.

For easier reading, the tables and graphs are placed in numerical order at the end of each results' section. The figures, however, approximate to their reference in the text to permit easier and more effective binding of the thesis.

2.3.2. THE CUTICLE

In terms of ultrastructural assessment of the cuticle, results found in this experiment showed that it was very rarely even, with most (> 98 %) of the shells examined being classified as having abnormal (patchy or absent) cuticular layers (table 5) (figure 5).

It has obvious implications in the eggshell's capacity to resist microbial penetration, as suggested by Alls *et al.* (1964), who found cuticle removal increased bacterial contamination from 20 % to 60 %.

Board and Halls (1973) and Sparks (1985) have subsequently reported cuticle-less shells, but the extreme situation is not necessary, since even localised damage to the cuticle may permit bacteria to enter more easily (Board, 1969 and Board and Fuller, 1974).

According to Simons and Wiertz (1963), the normal cuticle (figure 6) has irregular open spaces of up to 0.5 x 2.8 μm, with neighbouring ones possibly diffusing to produce larger spaces, which facilitate bacteria such as *Salmonella* (0.7 to 1.5 μm width) (figure 3) to penetrate even an apparently "perfect" cuticle (figure 7).

Drysdale (1985) claimed that the "immature" cuticle of a newly-laid egg does not provide an effective barrier to the entry of microorganisms, as it is moist and moisture enhances microbial penetration. Since the cuticle is deposited immediately preceding oviposition, this puts emphasis on the need to have a clean nest and a clean environment to avoid problems at this critical point.

In view of the imperfect nature of this outermost layer of all eggs examined, it is difficult to make assessment of its role as a barrier. In terms of the causes for its uneven distribution, there are two possibilities: a) stress, as

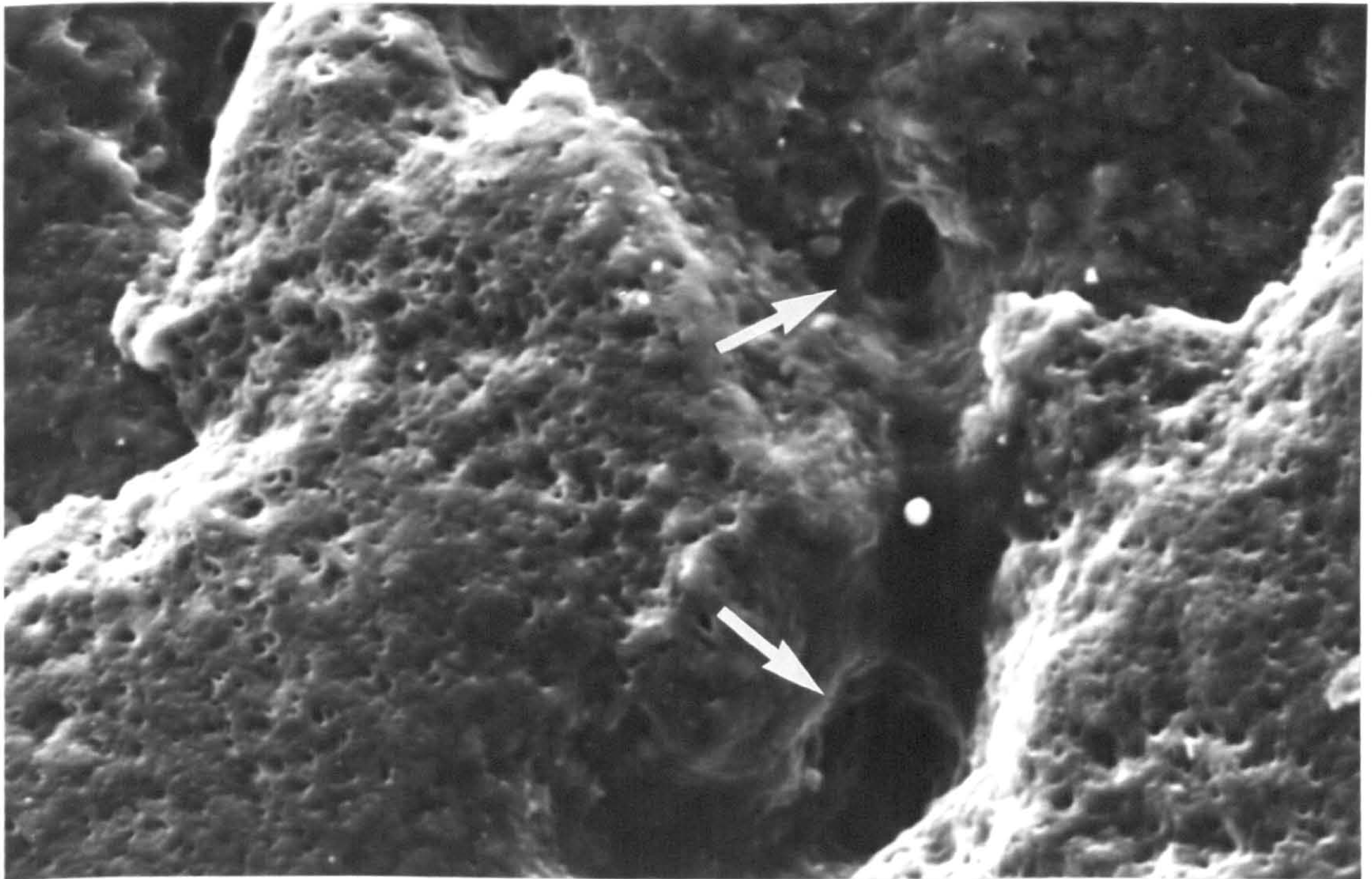


Figure 5: Patchy cuticular covering with external pore openings (arrow) (2,813 X).

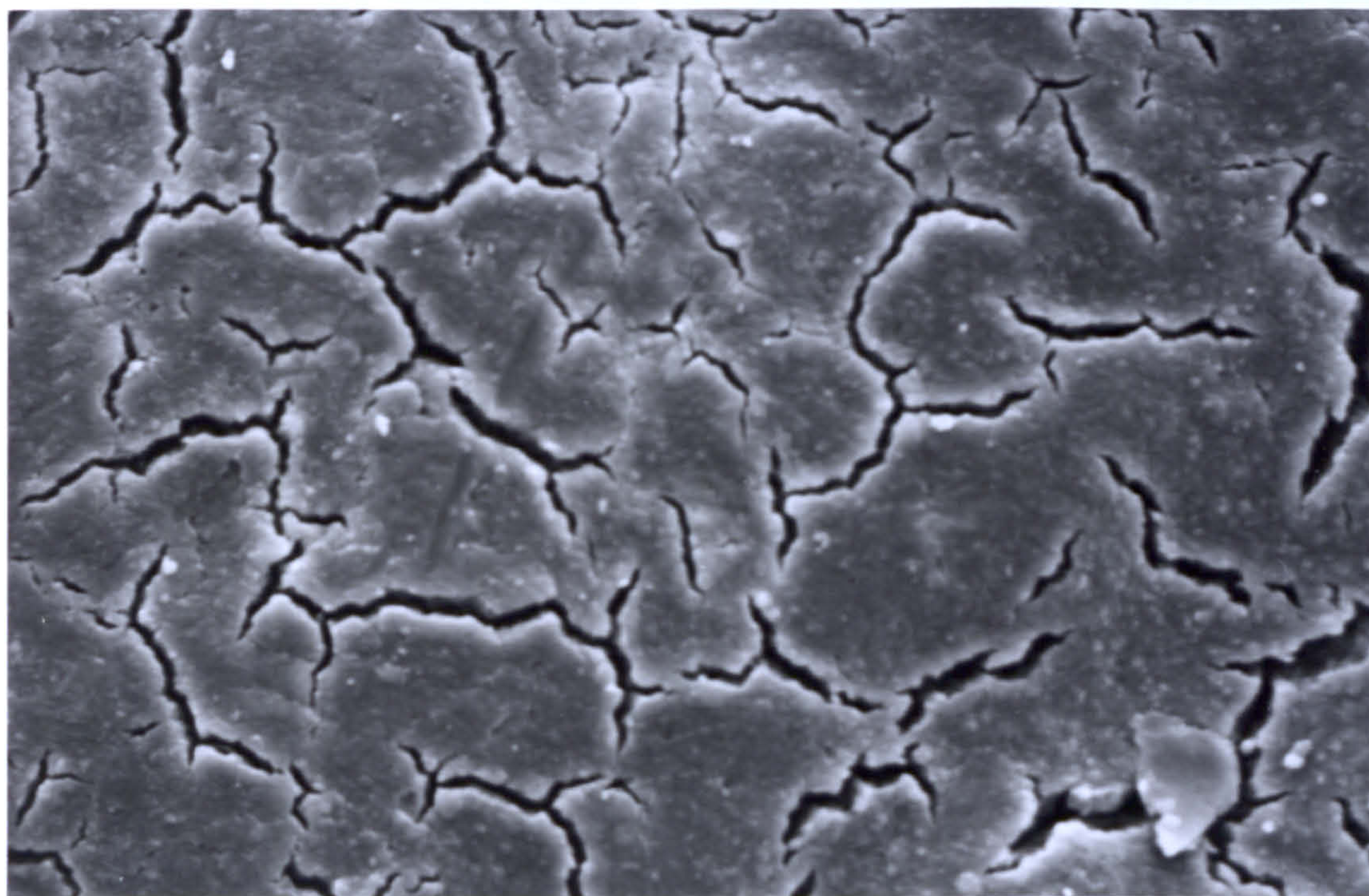


Figure 6: S.E.M. appearance of a normal cuticle with its characteristic cracked appearance (1,440 X).

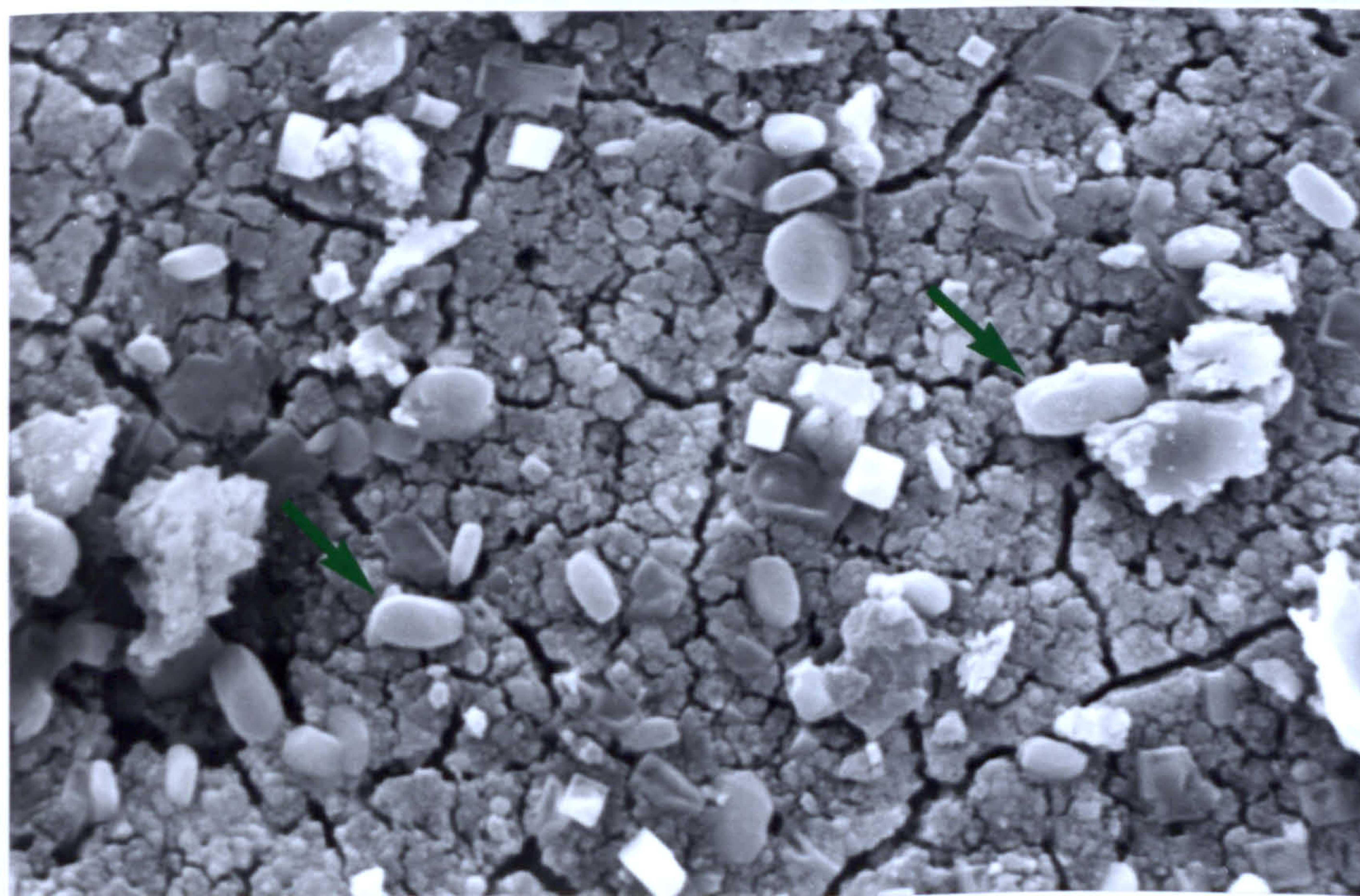


Figure 7: Bacteria (arrow) attached to the cuticular surface of the eggshell. The cubic crystals are salts from the suspending medium (5,625 X).

suggested by Watt (1989) or b) digestion, by microbial action during storage in a humid atmosphere (Board *et al.*, 1979).

Stress can produce long term effects on birds. Solomon and Watt (1990) observed that stressing birds by overcrowding causes egg retention and a variety of surface abnormalities. The same authors showed elsewhere (Watt and Solomon, 1988) that when a soft-shelled egg was present in the tubular shell gland, stress resulted in the termination of calcium transfer. They also found that subsequent eggs were characterised by various shell defects, including a patchy cuticular layer. Even two weeks later, despite appearing normal macroscopically, eggs still had defective mammillary layers and cuticular coverings. In the present situation, it is difficult to specify a particular "stressor" as the causative agent in the observed cuticular irregularities. It is possible that the "patchy" cuticle under today's intensive system of rearing is now the "norm".

	BEGINNING OF LAY	MIDDLE OF LAY	END OF LAY	"BAD" GROUP
NORMAL	1 (1.6 %)	0 (0 %)	1 (1.6 %)	0 (0 %)
ABNORMAL	63 (98.4 %)	64 (100 %)	63 (98.4 %)	15 (100 %)
TOTAL	64 (100 %)	64 (100 %)	64 (100 %)	15 (100 %)

Table 5: S.E.M. evaluation of cuticular surface. Cuticle classified as abnormal if it was patchy or absent, and as normal if it was complete.

2.3.3. THE PORES

The diameters of the pores – 9 to 35 µm (Romanoff and Romanoff, 1949 and Tyler, 1956) – indicate that they are large enough to permit bacteria to penetrate the egg (figure 8), with the occurrence of "patent" pores being acknowledged as major portals to the ingress of bacteria (Board and Halls, 1973). The thickness of the shell is not related to porosity, except in cases of abnormally thin shells, which are more porous, according to Tyler (1969). The same author suggested that some water loss can occur through parts of the shell other than the pores. Becking (1975) suggested that vesicular holes may be involved with gas exchange, supporting the hypothesis that at least part of the bacterial movement through the eggshell may occur through the vesicular holes. It is feasible that bacteria could penetrate through a non-patent pore, and spread via the vesicular holes, which are present in large numbers within the palisade layer, and remain dormant there until an insult or other phenomena puts them in contact with the albumen or any other water source (*e.g.* by an unproper egg washing procedure).

Pore numbers showed a highly significant ($P < 0.001$) increase with respect to bird age and egg quality (tables 13 and 14). However, according to Kraft *et al.* (1958b); Hartung and Stadelman (1963) and Reinke and Baker (1966), a poor correlation exists between porosity and spoilage.

The present results corroborate these earlier findings and support the concept proposed by Sparks (1985) that bacterial penetration is not pore dependent. Patent pores connecting freely with the external and internal environment of the egg are difficult to demonstrate (figure 9). They do, of course, exist, but they only represent a fraction of the total. In comparison, general egg quality indicators were more significant and stronger as parameters of the relationship between bacterial penetration and shell structure.

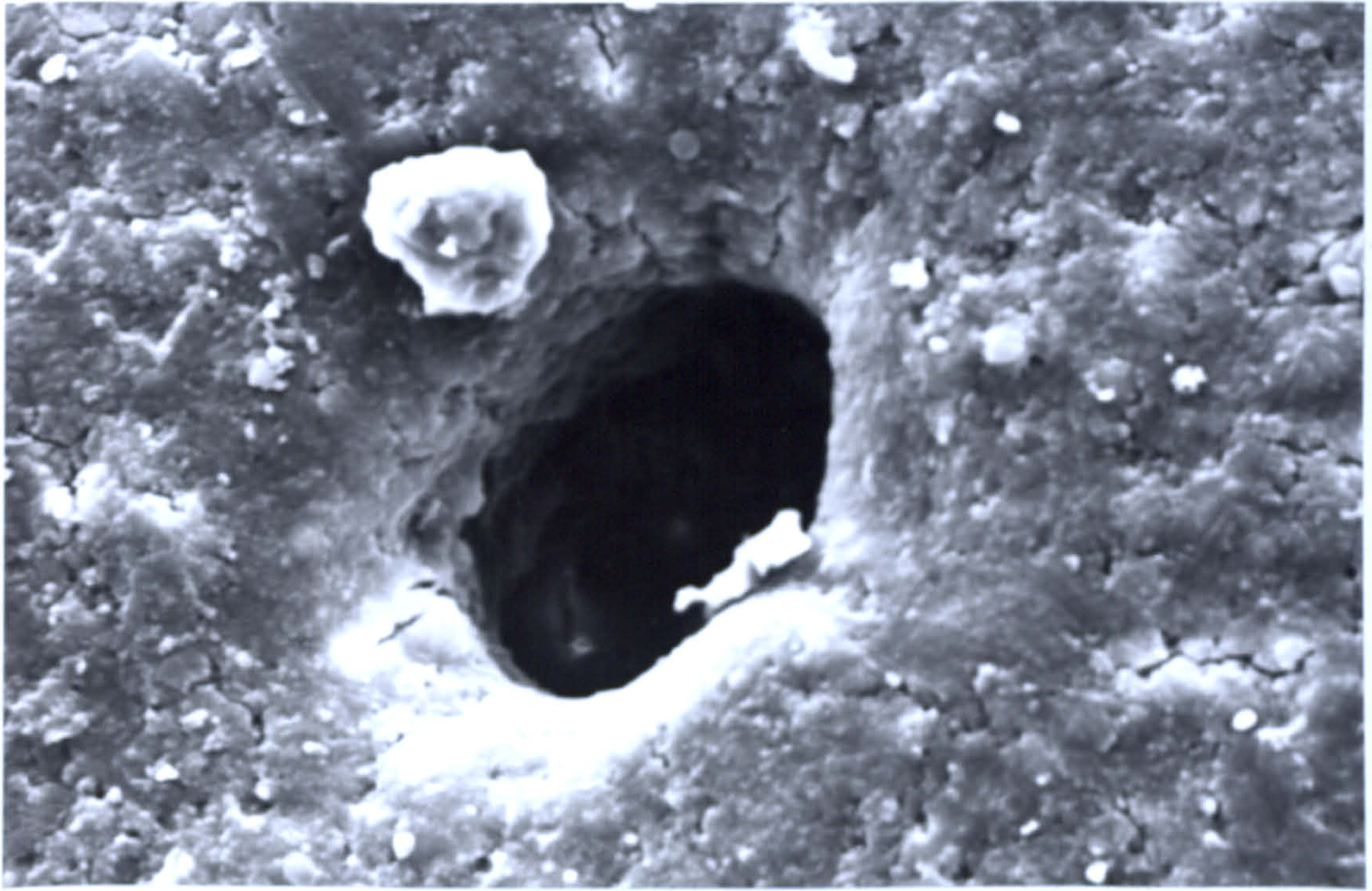


Figure 8: Unplugged gas exchange pore (2,813 X).

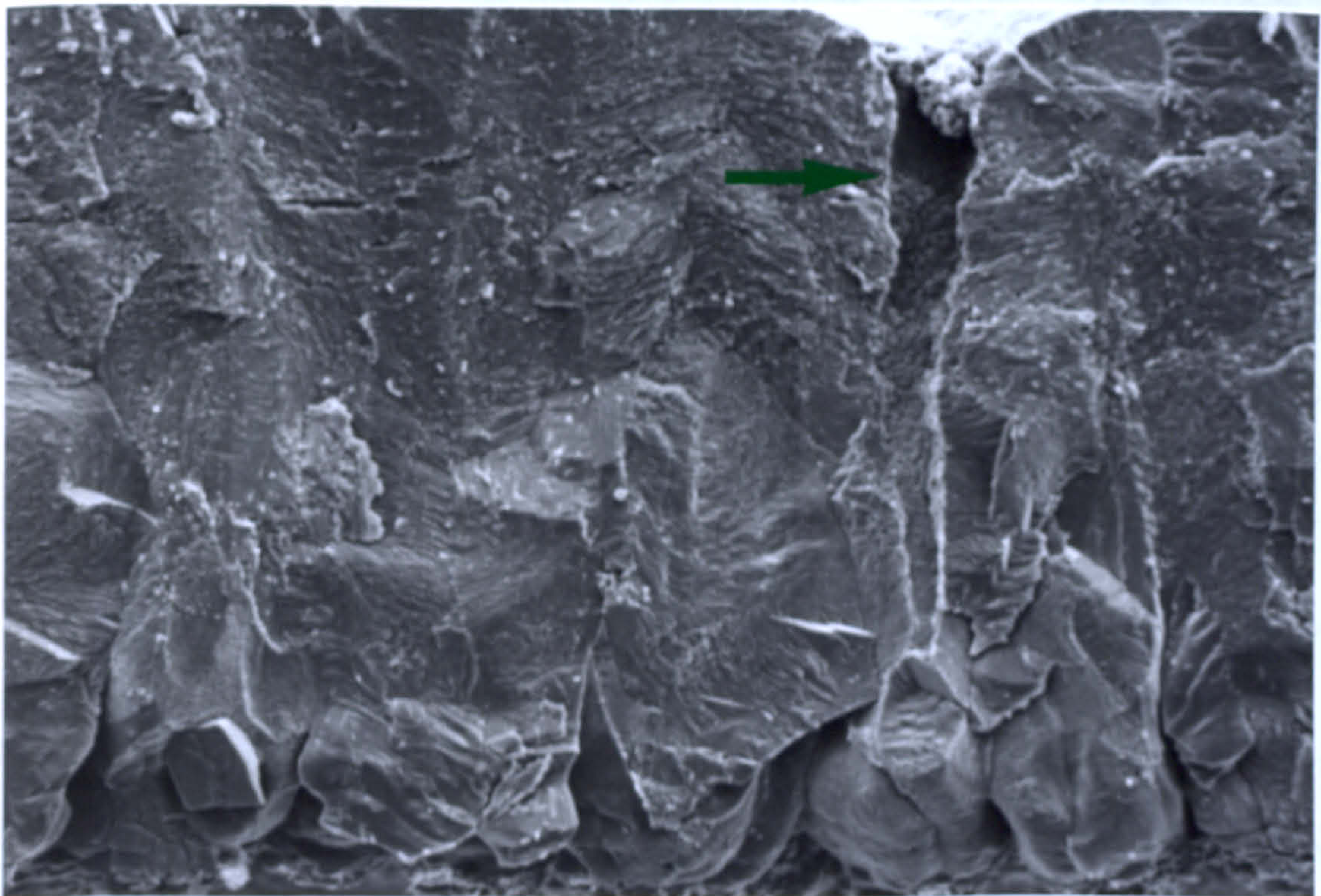


Figure 9: Transverse section of a fully formed shell showing a pore canal (arrow) (360 X).

A significant ($P < 0.001$) correlation (overall coefficient of 0.42) exists between penetration and pore numbers (table 15) in eggs with no membranes attached, in an overall groups evaluation, but no individual age group has shown a significant ($P < 0.05$) correlation coefficient.

2.3.4. THE MEMBRANES

The O.S.M. showed interstices larger than bacterial dimensions, according to Garibaldi and Stokes (1958) and Lifshitz *et al.* (1964), but these spaces might not be directly connected to the shell pore canals (Haines and Moran, 1940).

Ultrastructural assessment of the paired membranes (O.S.M. and I.S.M.) (table 6) (figure 10) showed that most (> 90 %) of them were abnormal or pitted, irrespective of the sampling date. They did however impede bacterial transfer even in this state (table 7), nevertheless bacteria can and do attach to the membranes and having reached this point (figure 11), can easily be dragged into the egg contents during handling. According to Stokes and Osborne (1956), the membranes protect bacteria against the toxic action of a suspending fluid, eventually even stimulating extensive growth of bacteria by supplying them with nutrients (proteins and related compounds), thus providing a nidus from which infection can spread to the yolk and albumen (Board, 1966). It was shown by Doyle (1984) that some *C. jejuni* could penetrate the shell and membranes of sound uncracked refrigerated eggshells, but not the egg contents, reinforcing the protective role of the shell against spoilage.

Board and Fuller (1974) stated that the membranes are capable of imposing only a temporary barrier to the inward movement of bacteria, and once they have passed through the shell membranes, the viscosity of the albumen ensures that they remain in a "clump".

Earlier, Board (1964) and Board and Ayres (1965) suggested that the antimicrobial properties of the albumen were primarily responsible for confining bacterial multiplication to the shell membranes, but that it could be easily overcome when the yolk makes contact with the I.S.M.. It is more prone to happen in eggs held at room temperature (Board, 1964), in aged

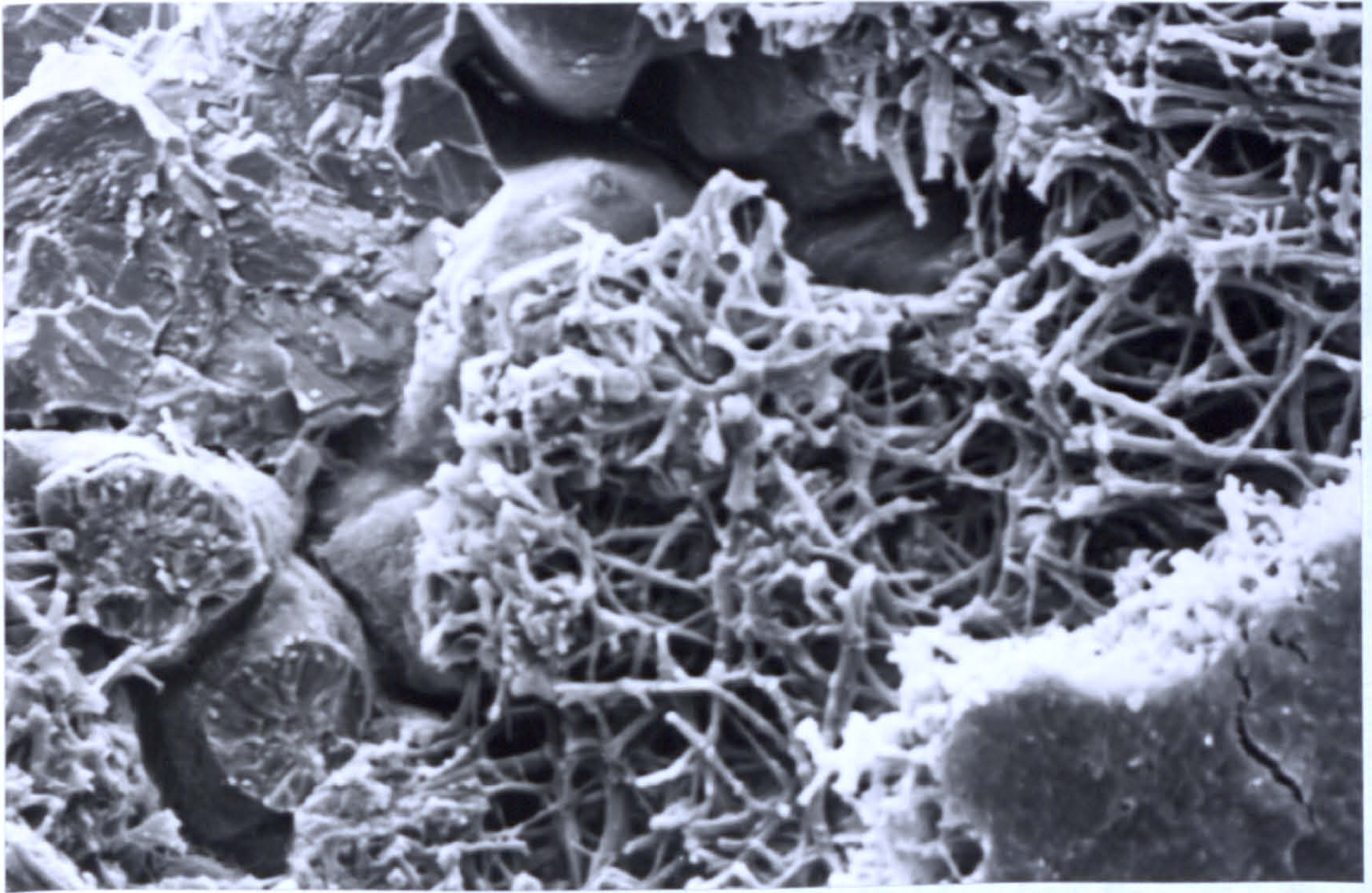


Figure 10: Scanning electron micrograph showing the relationship between the organic and the inorganic fractions of the shell (720 X).

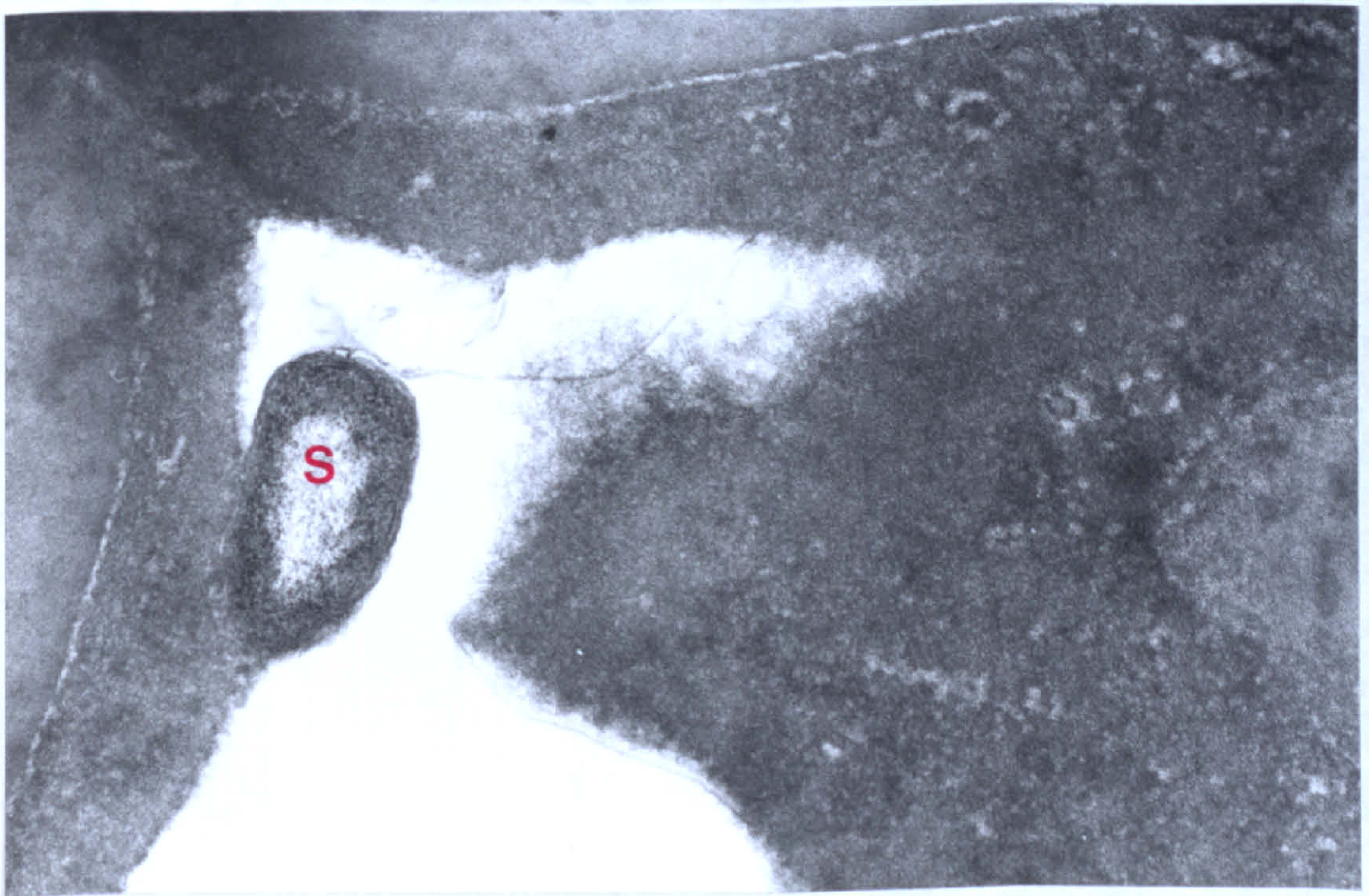


Figure 11: *S. enteritidis* (S) on the shell membranes (45,000 X).

eggs (Hartung and Stadelman, 1963) or even when eggs are turned or positioned improperly.

Membrane resistance is quickly breached when large inocula are used (Brooks, 1960; Hartung and Stadelman, 1962; Board, 1964 and Board, Henden and Board, 1968), especially when eggs are held at 37 ^{o}C (Board and Ayres, 1965), with the microorganisms having been recovered from the inner surface of the I.S.M. within minutes of the challenge (Williams *et al.*, 1968).

Hartung and Stadelman (1963), Brown *et al.* (1965) and Candlish (1972) suggested that bacteria could find their way through the membranes by proteolysis, a possibility that Wedral (1971) refutes.

In the present experiment, there was no overall significant ($P > 0.05$) correlation between the occurrence of abnormal membrane and penetration (table 15), except at the beginning of lay in eggs with only the O.S.M. left (coefficient of -0.68 and $P < 0.01$). Surprisingly, this correlation was negative, suggesting that a pitted membrane can cope with the bacterial challenge, if the pitting is isolated.

In summary, membranes appear to limit the movement of bacteria, which is in line with findings of Haines and Moran (1940), but in the case of no membranes left, there was still variability in the rate of transfer, indicating a variation in shell quality, which will be analysed subsequently.

	BEGINNING OF LAY	MIDDLE OF LAY	END OF LAY	"BAD" GROUP
NORMAL	2 (6.25 %)	1 (3.12 %)	3 (9.38 %)	0 (0 %)
ABNORMAL	30 (93.75 %)	31 (96.88 %)	29 (90.62 %)	5 (100 %)
TOTAL	32 (100 %)	32 (100 %)	32 (100 %)	5 (100 %)

Table 6: S.E.M. evaluation of integrity of shell membranes (O.S.M. and I.S.M.). Membranes classified as abnormal if they were pitted, and as normal if they were intact.

	Overall	Beginning	Middle	End	"Bad"	[mid. x end]	(mid. x bad)	{end x bad}
% Penetration (with both membranes)	NS	0.0 ± 0.0	0.0 ± 0.0 NS	0.0 ± 0.0 NS	0.0 ± 0.0 NS	[NS]	(NS)	{NS}
% Penetration (with O.S.M. only)	NS	0.16 ± 0.62	0.21 ± 0.83 NS	0.0 ± 0.0 NS	0.0 ± 0.0 NS	[NS]	(NS)	{NS}

Table 7: Mean and S.D. values for % of penetration for the beginning, middle and end of lay and poor quality ("bad") group, with both I.S.M. and O.S.M. attached and with only O.S.M. left.

NS = Not significant ($P > 0.05$).

[] = middle x end comparisons.
() = middle x bad comparisons.
{} = end x bad comparisons.

2.3.5. THE TIER EFFECT

A significant difference ($P < 0.05$) was observed (tables 8, 9 and 10) between the bottom position in the battery and the top and middle, in terms of penetration at beginning and middle of lay. Eggs from the bottom were less easily invaded than the other two. This agrees with the results from Sparks (1991), who found that birds in the top tier produced the majority of cuticle-less eggs. Solomon (1990a) also reported the occurrence of tier effects, but then the top tier eggs were structurally superior.

This pattern was repeated in terms of total structural score, when the bottom position showed a significantly ($P < 0.01$) lower score than the other two at middle of lay, (according to this system, the lower the score the better is shell quality). At middle of lay, the bottom position showed significantly ($P < 0.01$) fewer pores.

The reasons for this effect are, at this point, obscure, but may reflect in-house variations in light and/or temperature (Solomon, personal communication).

BEGINNING OF LAY

	Overall	Top	Middle	Bottom	[mid.x bot.]
% Penetration	NS	15.66 ± 5.48	13.67 ± 6.57 NS	8.67 ± 4.94 *	[NS]
Mamm. Density	NS	88.21 ± 12.96	81.48 ± 13.21 NS	80.46 ± 15.92 NS	[NS]
Total Score	NS	28.88 ± 4.12	27.19 ± 4.85 NS	26.50 ± 4.21 NS	[NS]
Pores (number)	NS	28.12 ± 18.53	12.00 ± 9.44 NS	21.71 ± 20.83 NS	[NS]

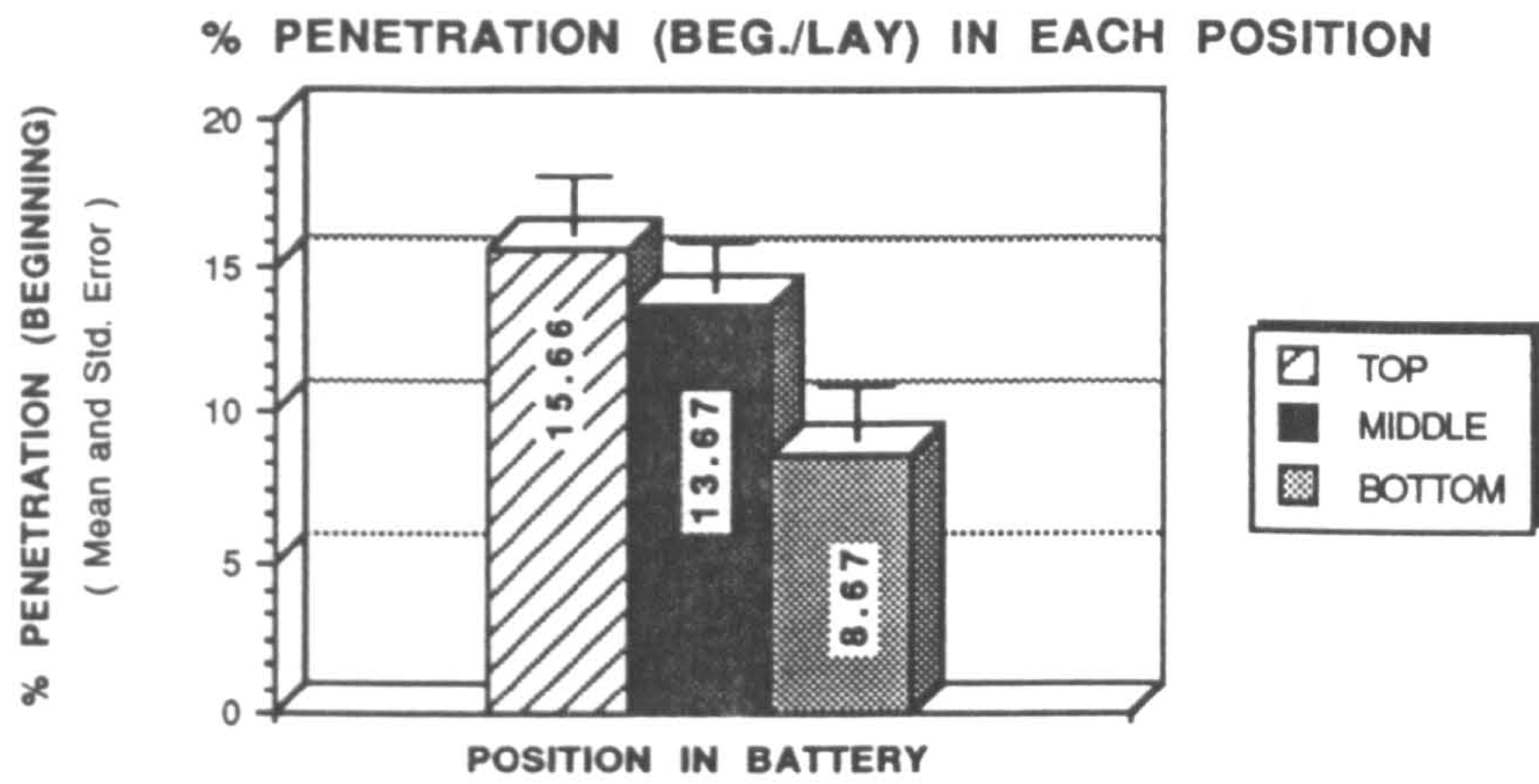
MIDDLE OF LAY

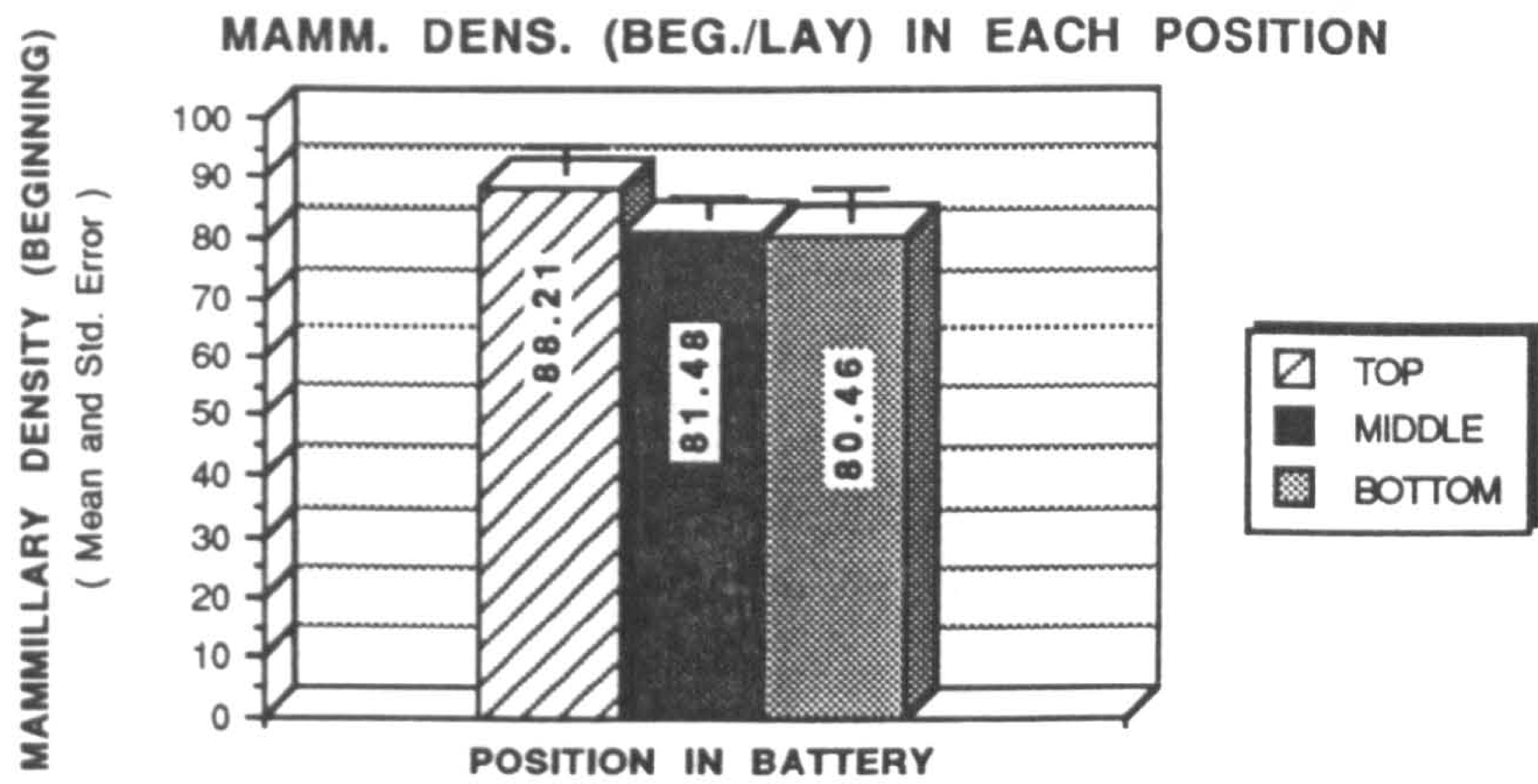
	Overall	Top	Middle	Bottom	[mid.x bot.]
% Penetration	*	26.09 ± 14.88	20.59 ± 14.48 NS	8.36 ± 4.73 **	[*]
Mamm. Density	NS	78.25 ± 11.64	79.58 ± 17.95 NS	76.92 ± 5.93 NS	[NS]
Total Score	*	36.00 ± 6.76	32.62 ± 5.70 NS	28.50 ± 1.77 **	[NS]
Pores (number)	**	167.25 ± 79.24	117.94±59.74 NS	64.12 ± 45.97 **	[*]

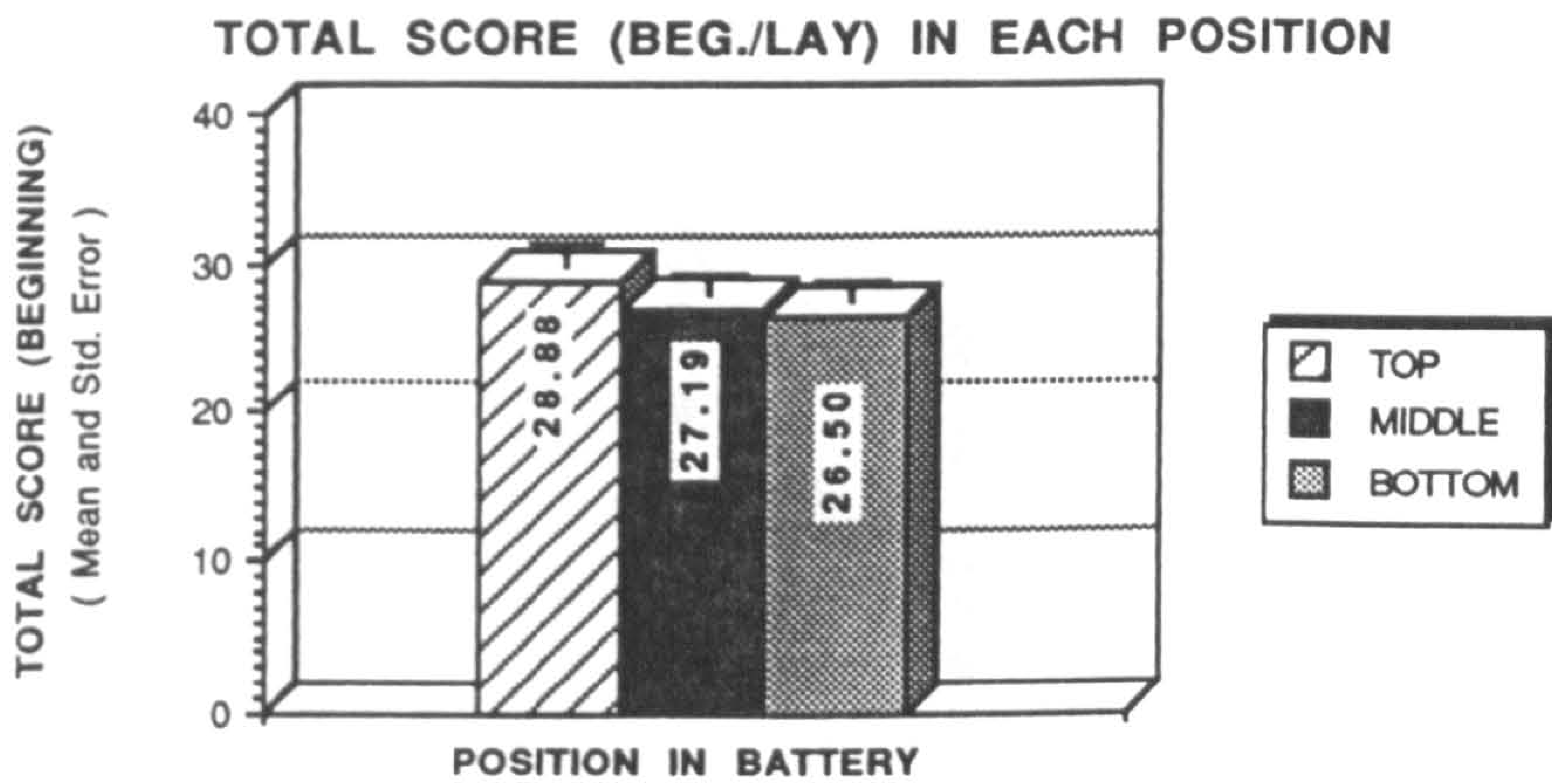
Table 8: Mean and S.D. values for % penetration,mamm.dens.,total structural score and pore number for each position in the battery system, at beginning and middle of lay. Significance is given by ANOVA.

** = Highly significant at a 1 % level ($P < 0.01$)
* = Significant at a 5 % level ($P < 0.05$)
NS = Not significant ($P > 0.05$)

[] = middle x bottom comparisons.







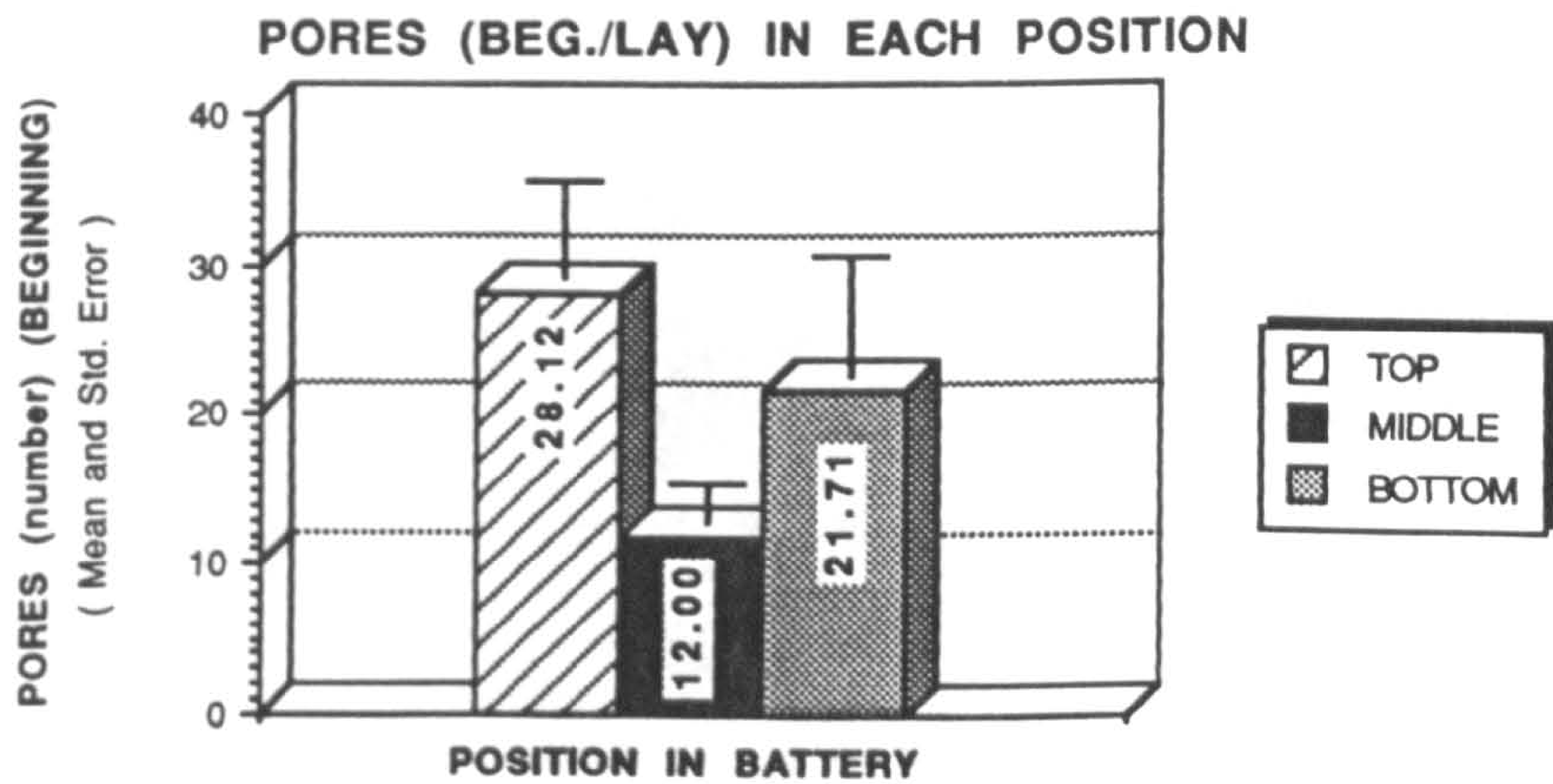
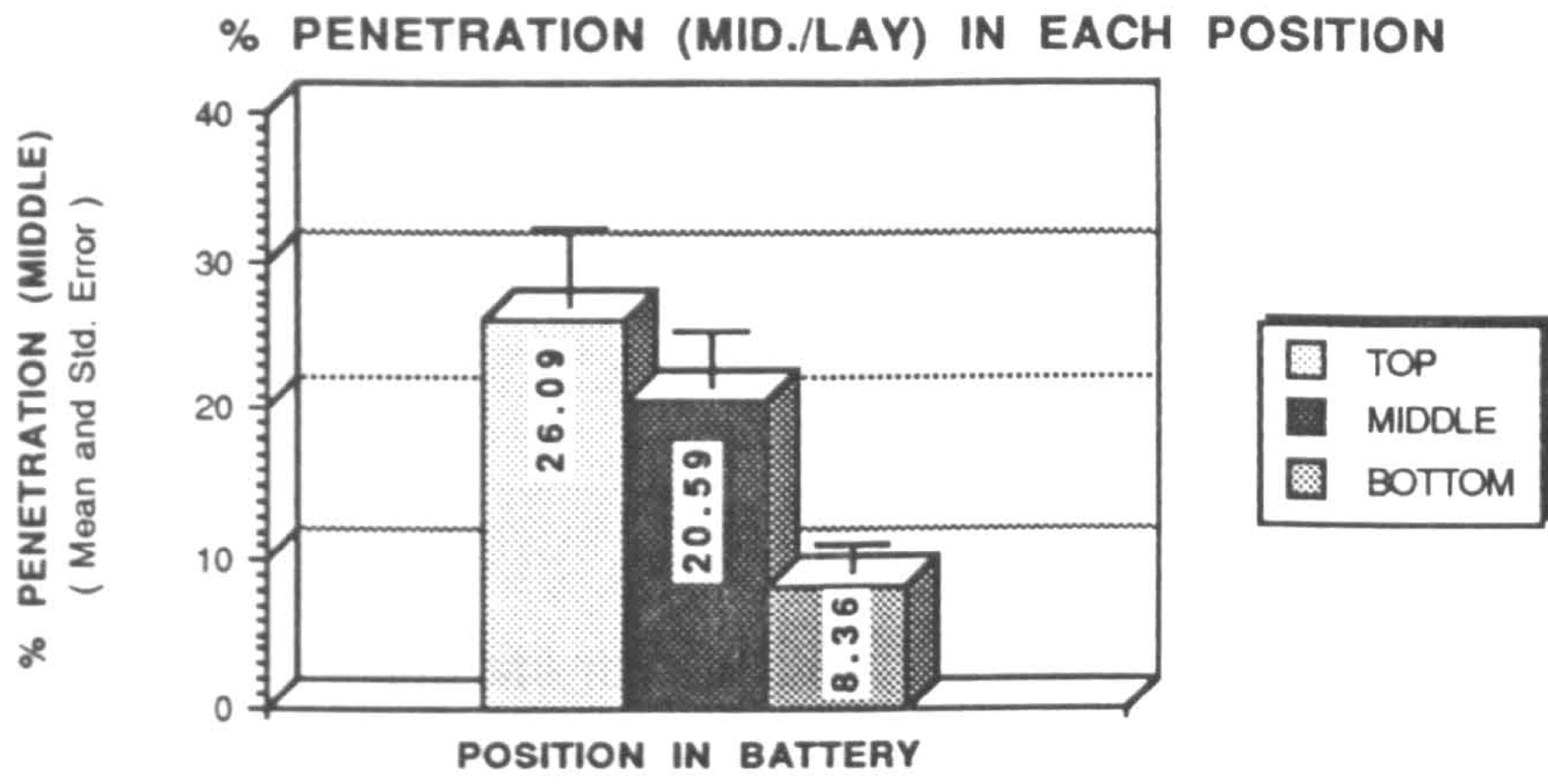
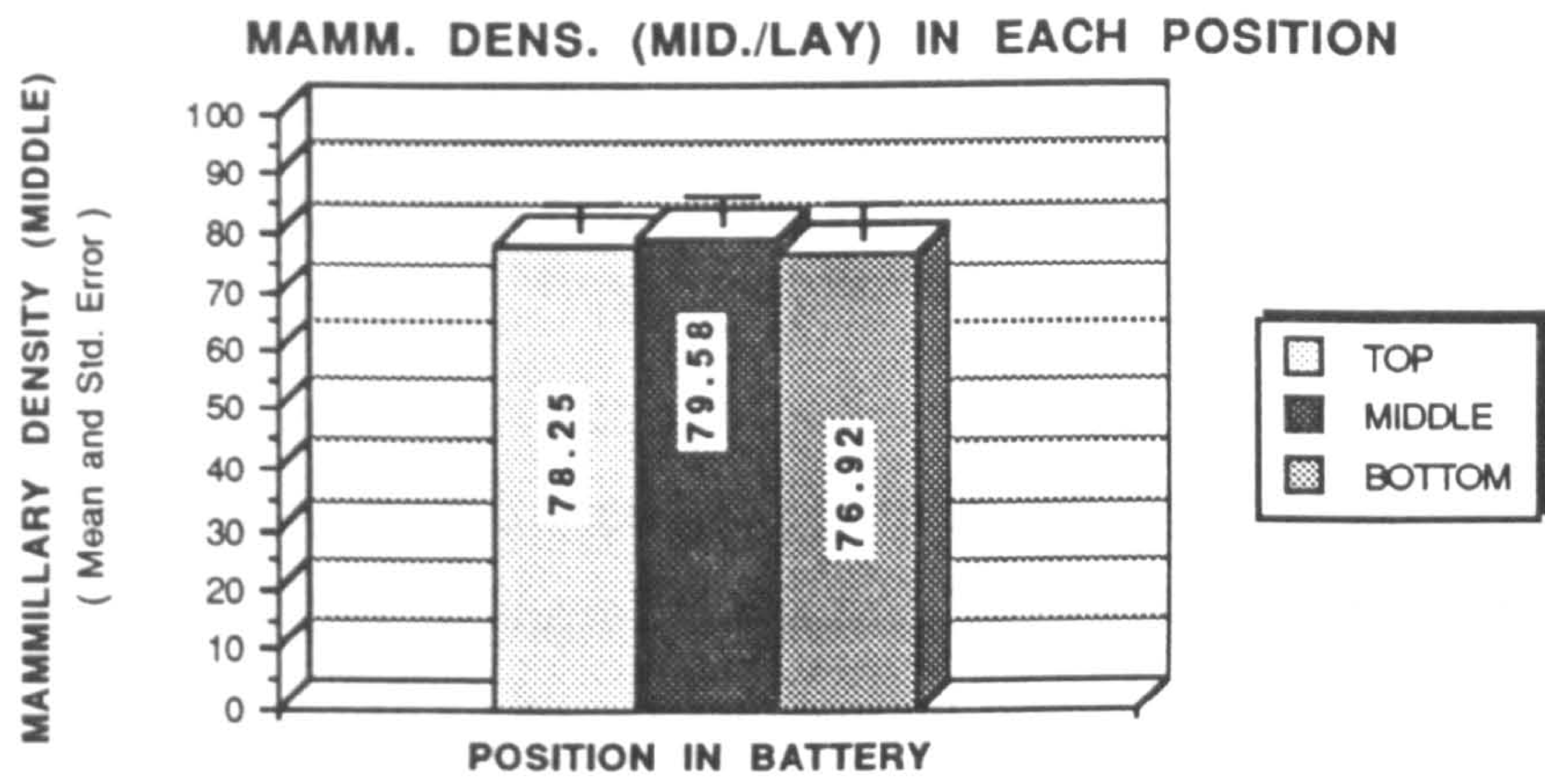
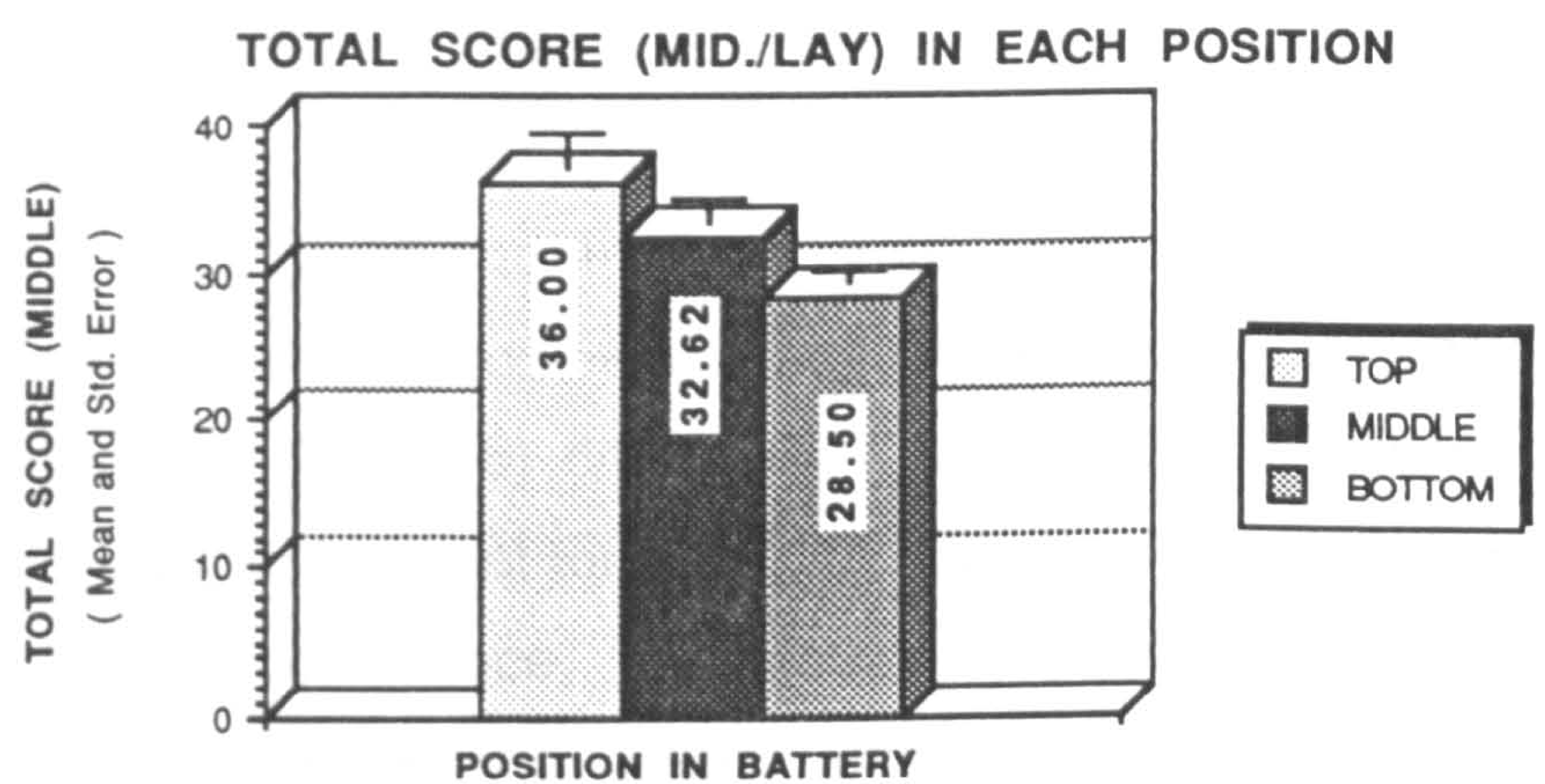


Table 9: Bar histograms showing the mean and s.e. for some features in relation to the position in the battery at the beginning of lay.







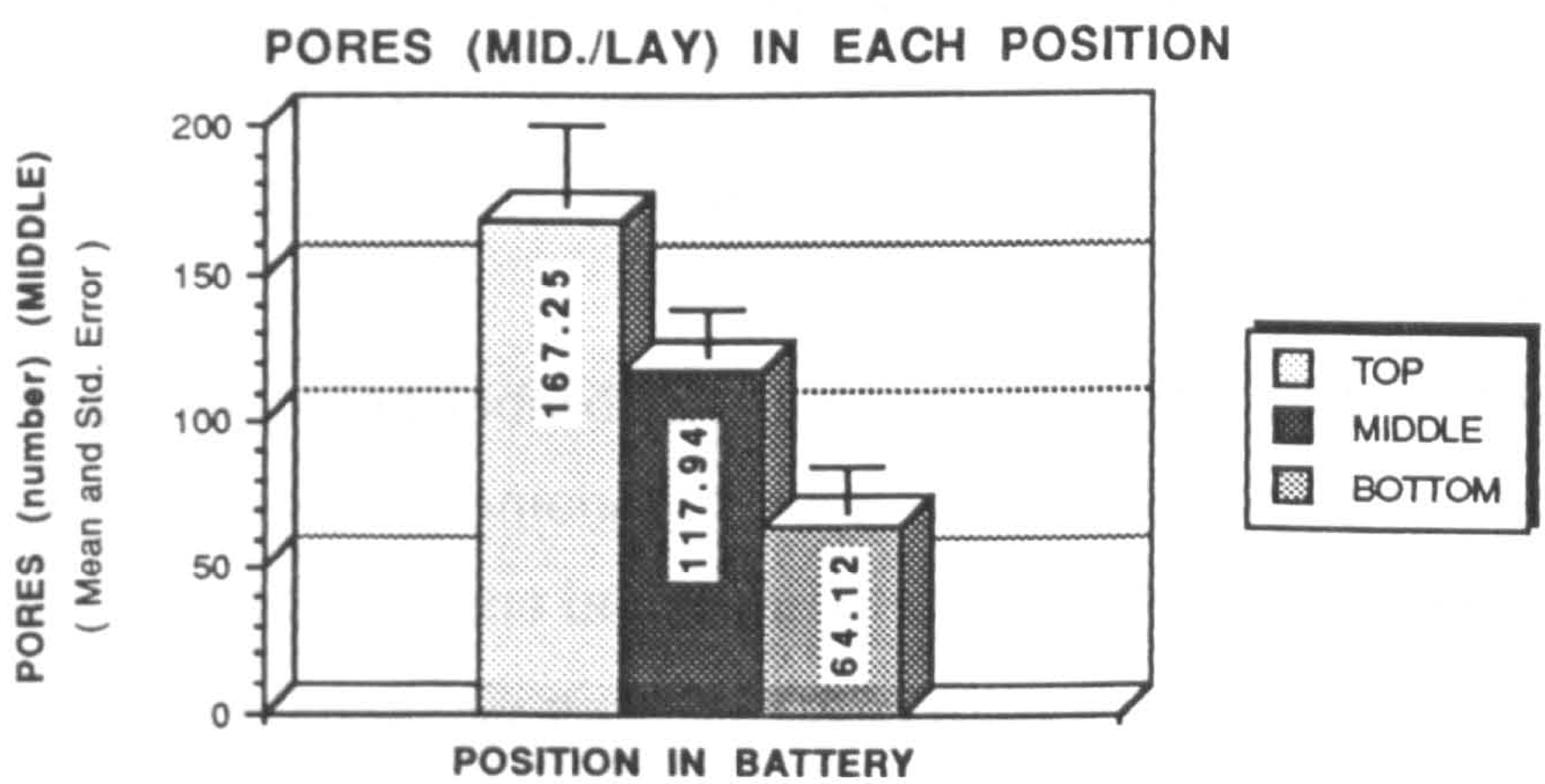


Table 10: Bar histograms showing the mean and s.e. for some features in relation to the position in the battery at the middle of lay.

2.3.6. EGGSHELL STRUCTURAL QUALITY AND BACTERIAL PENETRATION

The results herein presented indicate that shell quality decreases with age, thus substantiating the works of Tullett (1987) and Solomon (1987), and also that bacterial penetration is correlated positively with age, which is in accordance with Lifshitz (1963), Bruce and Johnson (1978) and Bruce and Drysdale (1991). The results are presented in a number of different forms to take account of a number of features which it is considered worthwhile debating. Thus, table 11 shows the distribution of faults within the score system used (see score sheet at figure 4). The pie charts are used to illustrate the increasing incidence of specific structural variance with age. The bar histograms illustrate score means presented in table 13 for each characteristic with respect to bird age.

Bacterial penetration, analysed over all age groups, increased significantly ($P< 0.05$) as the birds aged (tables 13 and 14), although between the end of lay group and the "bad" group (a random sample of poor quality shells from end of lay) and between middle and end of lay, the results were not significantly ($P> 0.05$) different. Since the latter did not differ significantly ($P> 0.05$) in terms of total structural score, however, penetration results are perfectly in accordance. The penetration results were very similar to those reported by Berrang *et al.* (1991), who found a rate of recovery of less than 50 % of the bacteria (*Salmonella*) initially inoculated in the shell, when suspended in water saline (rates of recovery of up to 100 % were achieved when bacteria were in a chicken faecal paste).

In terms of comparing individual structural characteristics (tables 11 and 12), the confluence phenomenon (figure 12) showed a significant ($P< 0.01$) decrease from 90.6 % at the beginning of lay to 52.4 % at the end of lay and in the "bad" group (appendix 2, x^2 1). With regard to comparisons of the structural scores (figure 4) for each characteristic (tables 13 and 14), which

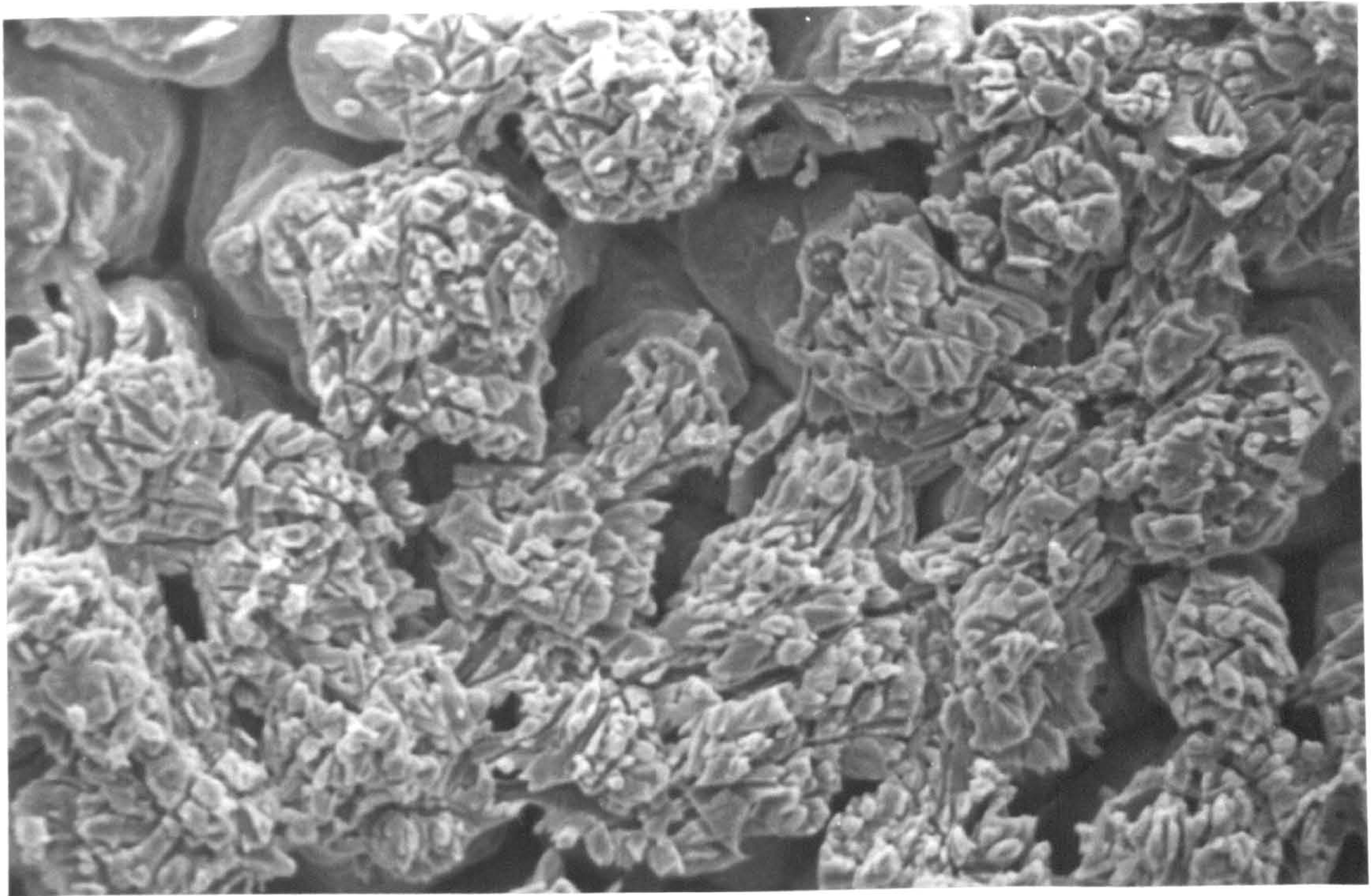


Figure 12: Individual mammillary bodies are difficult to define, because of extensive confluence (720 X).

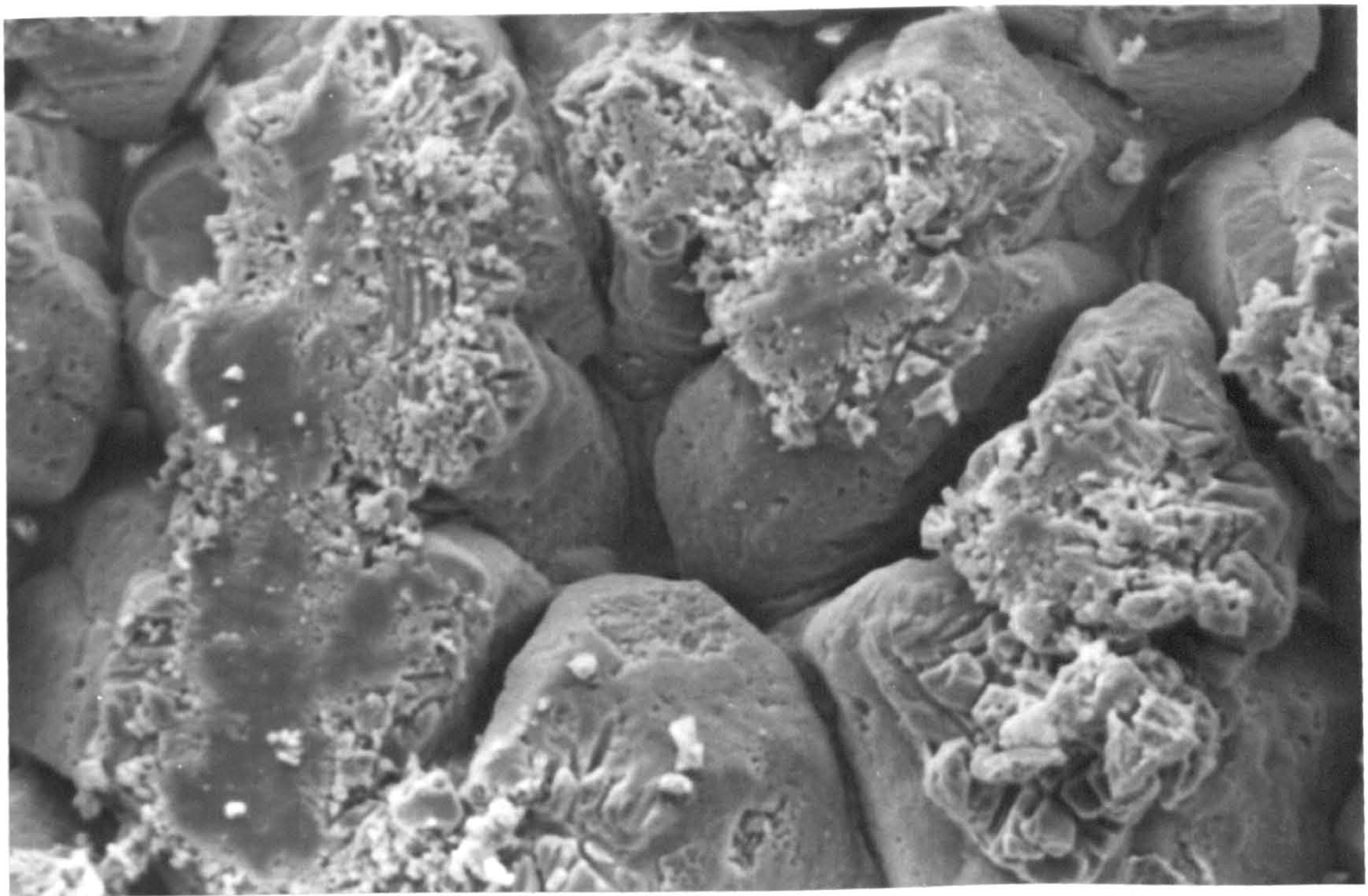


Figure 13: Poor quality caps, which allow no firm attachment with the membranes (720 X).

weight the importance of the level of occurrence of each in terms of influence over the soundness of the eggshell architecture, the results were very similar, differing significantly from those expressed in table 11 only in the confluence characteristic, in which the x^2 test revealed a highly significant difference ($P < 0.01$) in occurrence between the four groups. The ANOVA test, however, showed no significance ($P > 0.05$) for this particular feature. This may be explained by considering inherent difficulties in the scoring system. With regard to confluence characterisation, no confluence is awarded three points; isolated four; moderate six and extensive only one. When this system was developed, the intention was to penalise the shell with extensive structural variation, but with respect to confluence, extensive occurrence was considered to be beneficial, because of its homogeneous distribution, and its strong contact with the membrane fibres. In any case, confluence was shown by Solomon (1991) to often occur in eggs laid by stressed birds, and is believed to alter the pore distribution and the formation of the palisade layer.

In terms of bacterial resistance, confluence is to be welcomed, serving as one more barrier to penetration by closing the gaps between columns. The results (tables 13 and 14) have given a misleading impression that, at the end of lay, eggs had less (mean score of 3.66) confluence than at the beginning (mean score of 3.94) or middle (mean score of 4.16). In fact, at the end of lay many eggs displayed no confluence, while at the beginning and middle only a few scored three points, with more coming into the isolated/moderate category.

In terms of correlation between penetration and shell structure characteristics (table 15), the analysis of results for confluence indicates that, since the same scores were also used to analyse the correlation coefficient, this characteristic suffered the same problem already described for the ANOVA, and only eggs from beginning of lay had a significant ($P < 0.01$) correlation coefficient (0.48) when crossed with penetration (graph 1).

In terms of cap appearance, the experiment suggests a very highly

significant ($P< 0.001$) increment in abnormality from 0 % at the beginning to 43.75 % at the middle and 57.14 % at the end of lay and "bad" group (appendix 2, x^2 2) (tables 11, 12, 13 and 14). There is also a very highly significant ($P< 0.001$) and high correlation (overall coefficient of 0.73) with penetration (table 15) (graph 2), emphasising the fact (graph 3) that poor quality caps (figure 13) do not interact with the shell membranes in terms of creating a strong bond, thus spaces are created through which microorganisms can make their way into the egg contents. The cause of poor basal caps has been related to oviducal dysfunction (Bain, 1990).

Type B abnormalities (figures 14 and 15) increased significantly ($P< 0.01$) from 21.9 % with moderate or extensive occurrence at the beginning and middle of lay to 57.1 % at the end of lay and in the "bad" group (appendix 2, x^2 3) (tables 11, 12, 13 and 14). This structural modification shows a very highly significant ($P< 0.001$) correlation (overall coefficient of 0.52) with penetration (table 15) (graphs 4 and 5). These aberrant crystal forms provide no meaningful contribution to the palisade layer and in terms of poor membrane attachment, create open spaces (figures 16 and 17). Such open spaces, with no membrane attachment areas are obviously contributing towards the ease of bacterial penetration. These changes in crystal morphology have been attributed to environmental stress (Watt, 1989) and are also believed to decrease the shell's fracture toughness, as demonstrated by Bain (1990). In stressed birds "normal" core sites are absent, and random mineralisation, with an increased deposition of type B's is the norm (Watt, 1989). These bodies are characteristic of thin shelled eggs and also more common in eggs from older birds, where they account for a large proportion of the mammillary layer.

The occurrence of pitted (depression (fig. 18), erosion (fig. 19) and through hole (fig. 20)) areas increased significantly ($P< 0.01$) from 0 % at the beginning to 21.43 % at the end of lay and in the "bad" group (appendix 2, x^2 4) (tables 11 and 12). There was also a very significant ($P< 0.01$) although low (overall coefficient of 0.27) correlation with penetration (table 15) (graph

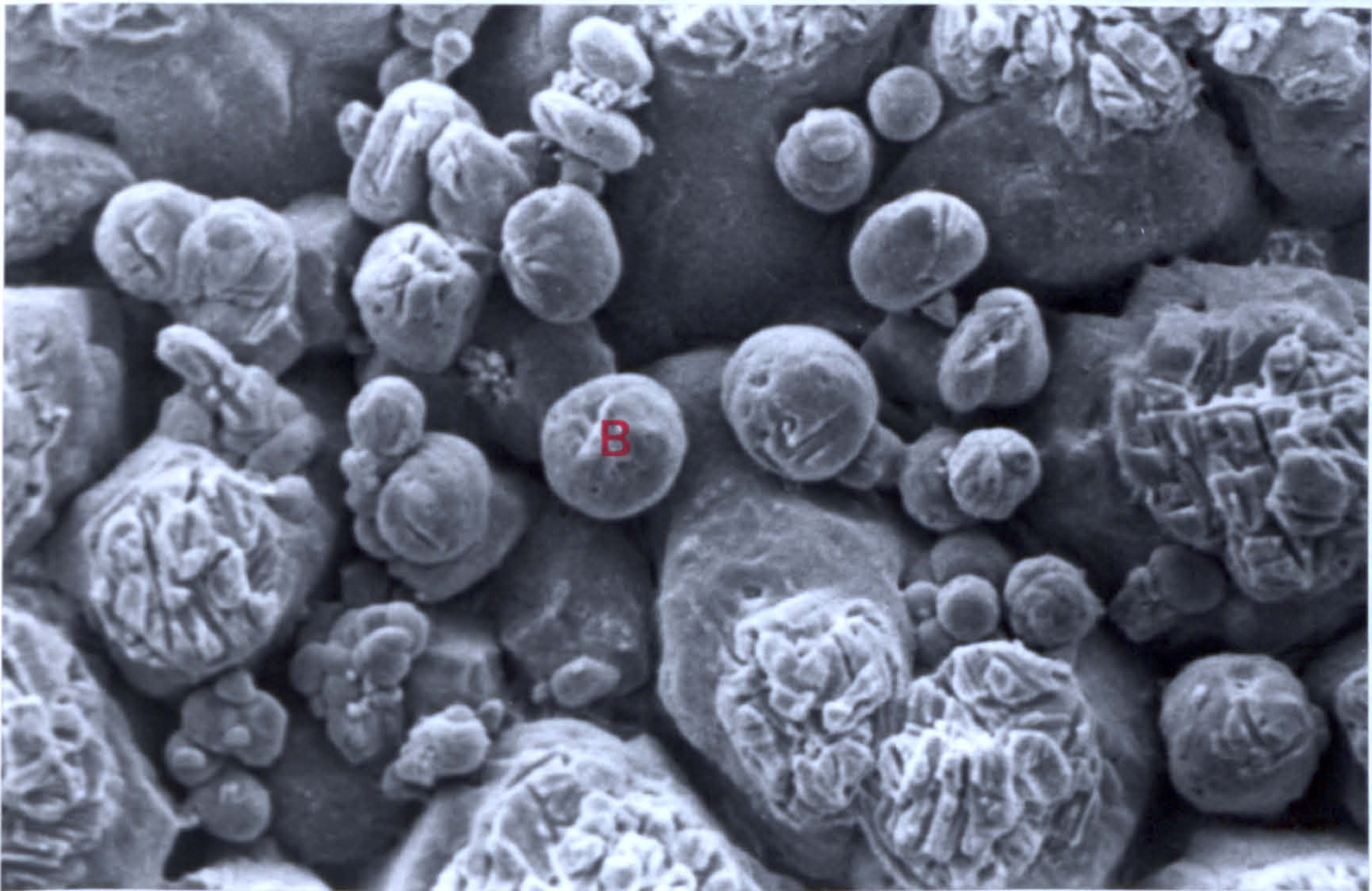


Figure 14: Rounded type "B" bodies make no contribution to the palisade layer (720 X).

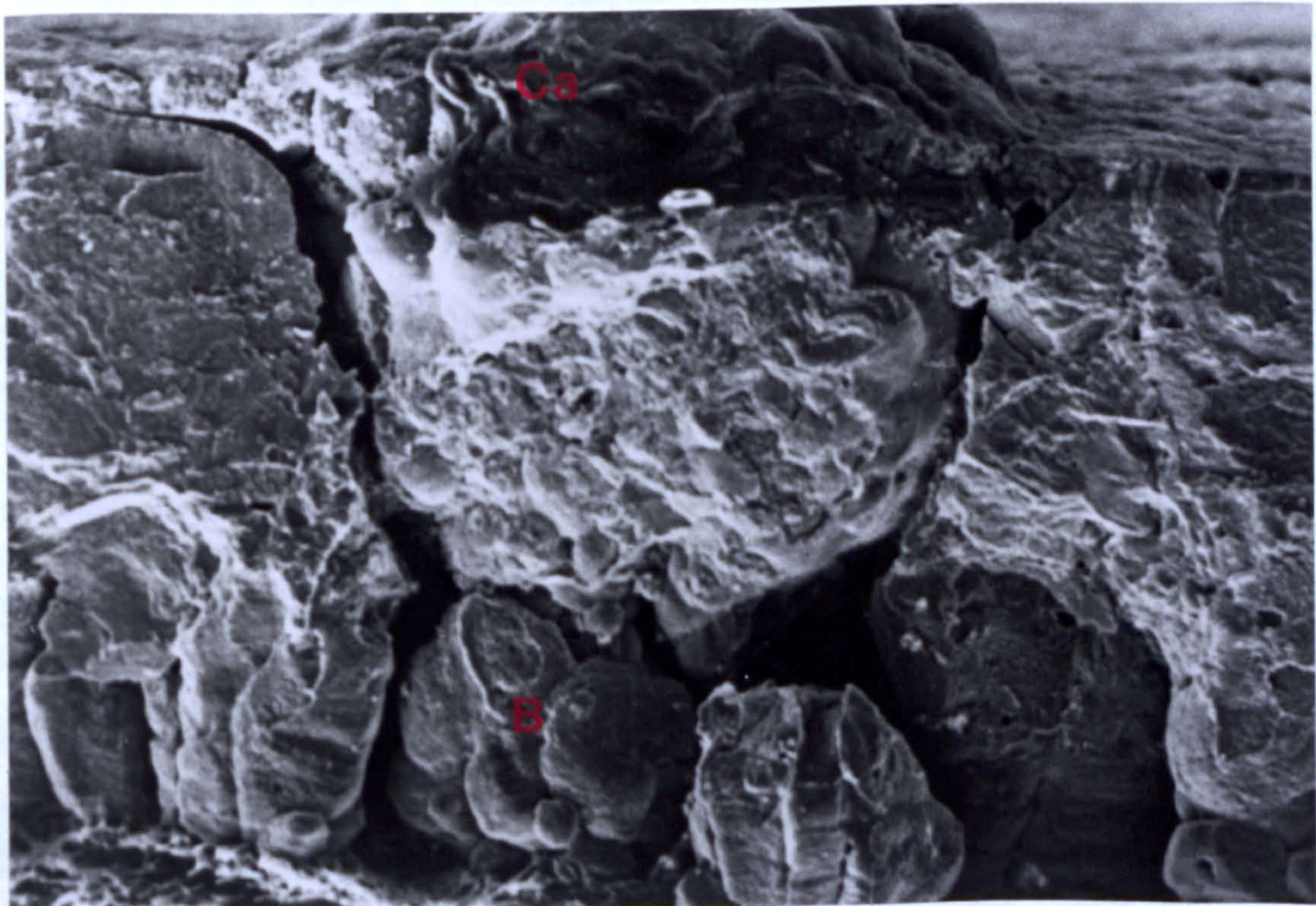


Figure 15: Type "B" bodies and calcium accretion (an excessive Ca deposit) in a transverse section of an eggshell. Note the open framework at the level of the mammillary layer (720 X).

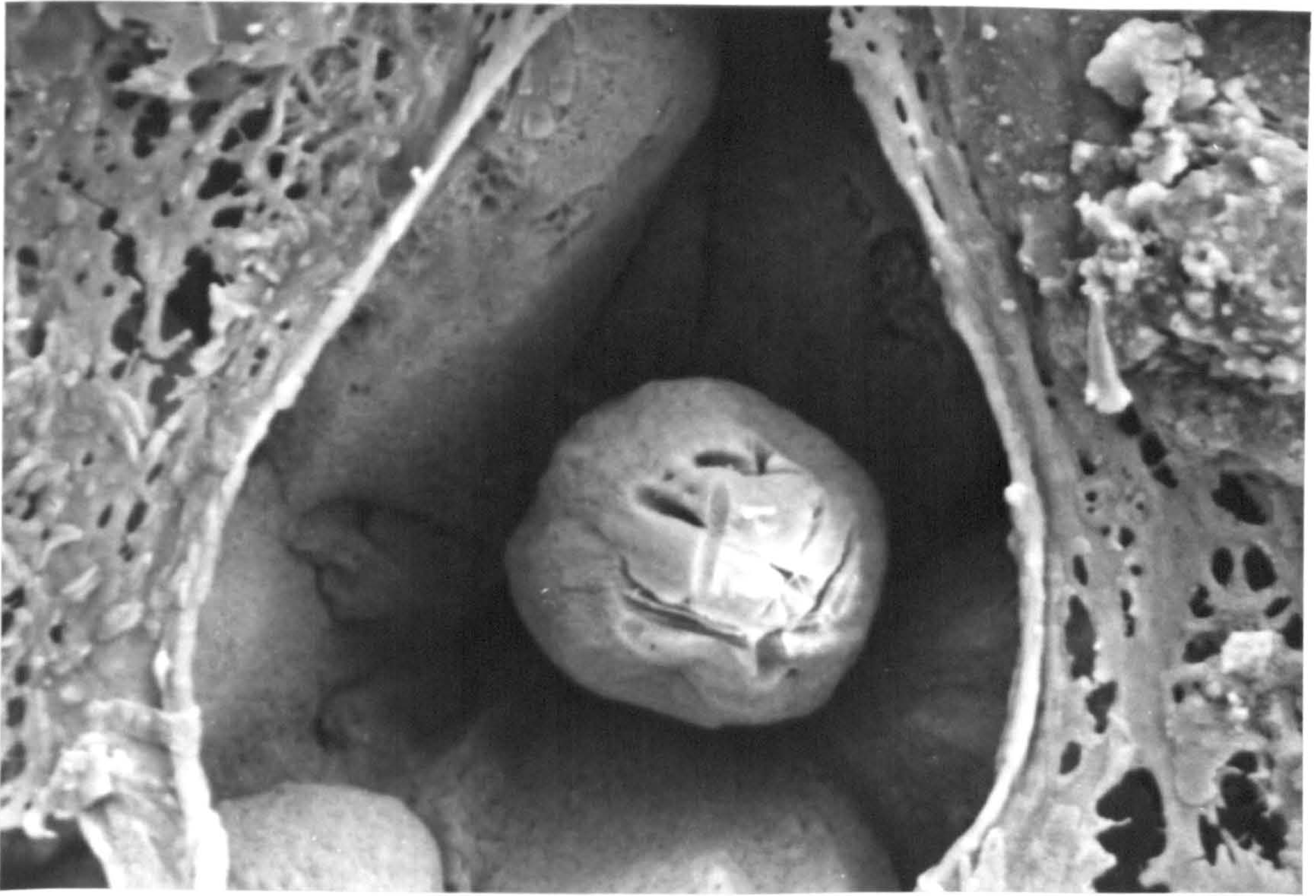


Figure 16: Membrane attachment failure occurring just over a type "B" mammillary body (1,440 X).

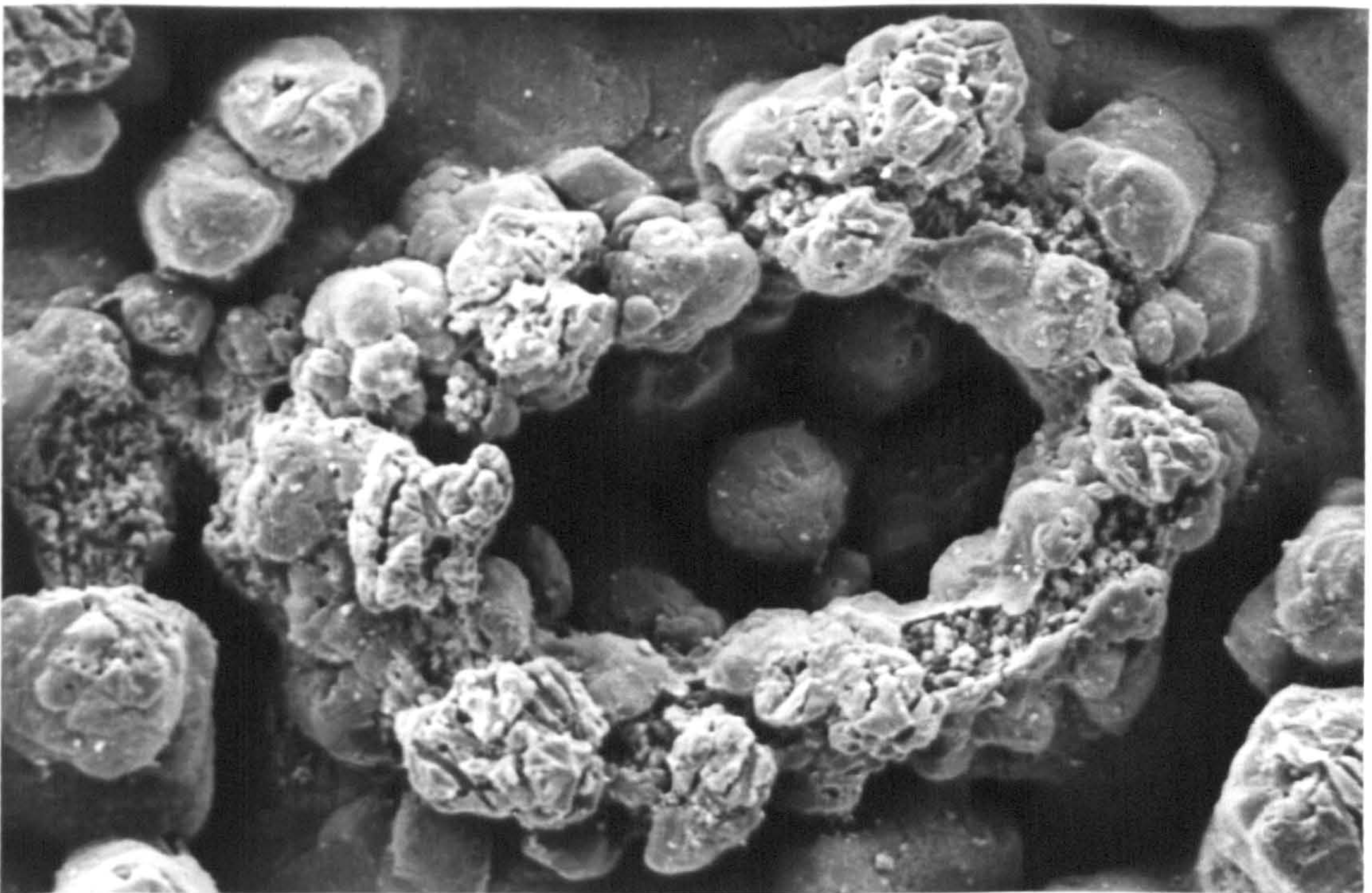


Figure 17: Type "B" mammillary bodies. Their configuration and disposition suggests a fault initiated at the level of the isthmus (720 X).

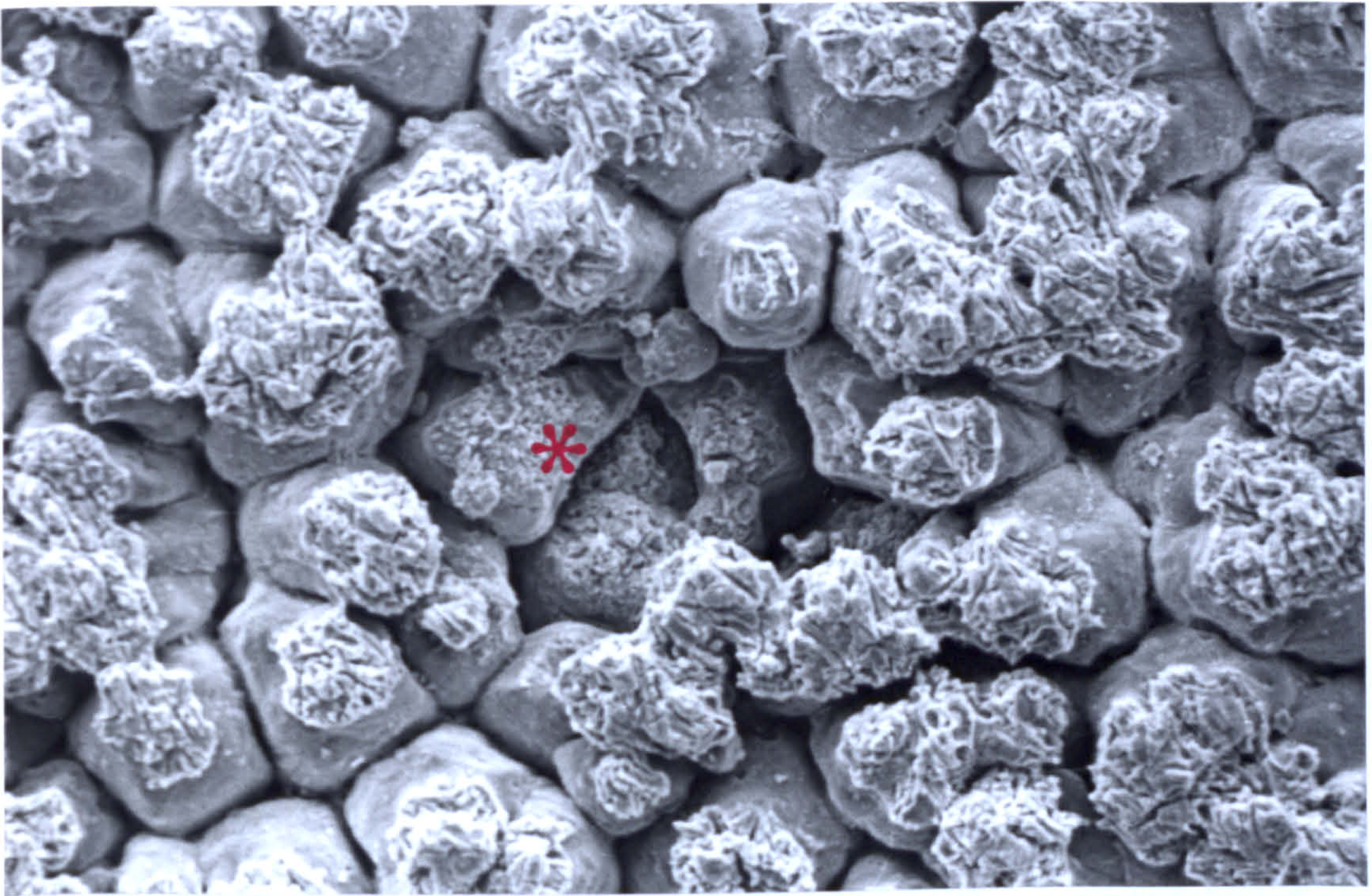


Figure 18: Depression (360 X).*

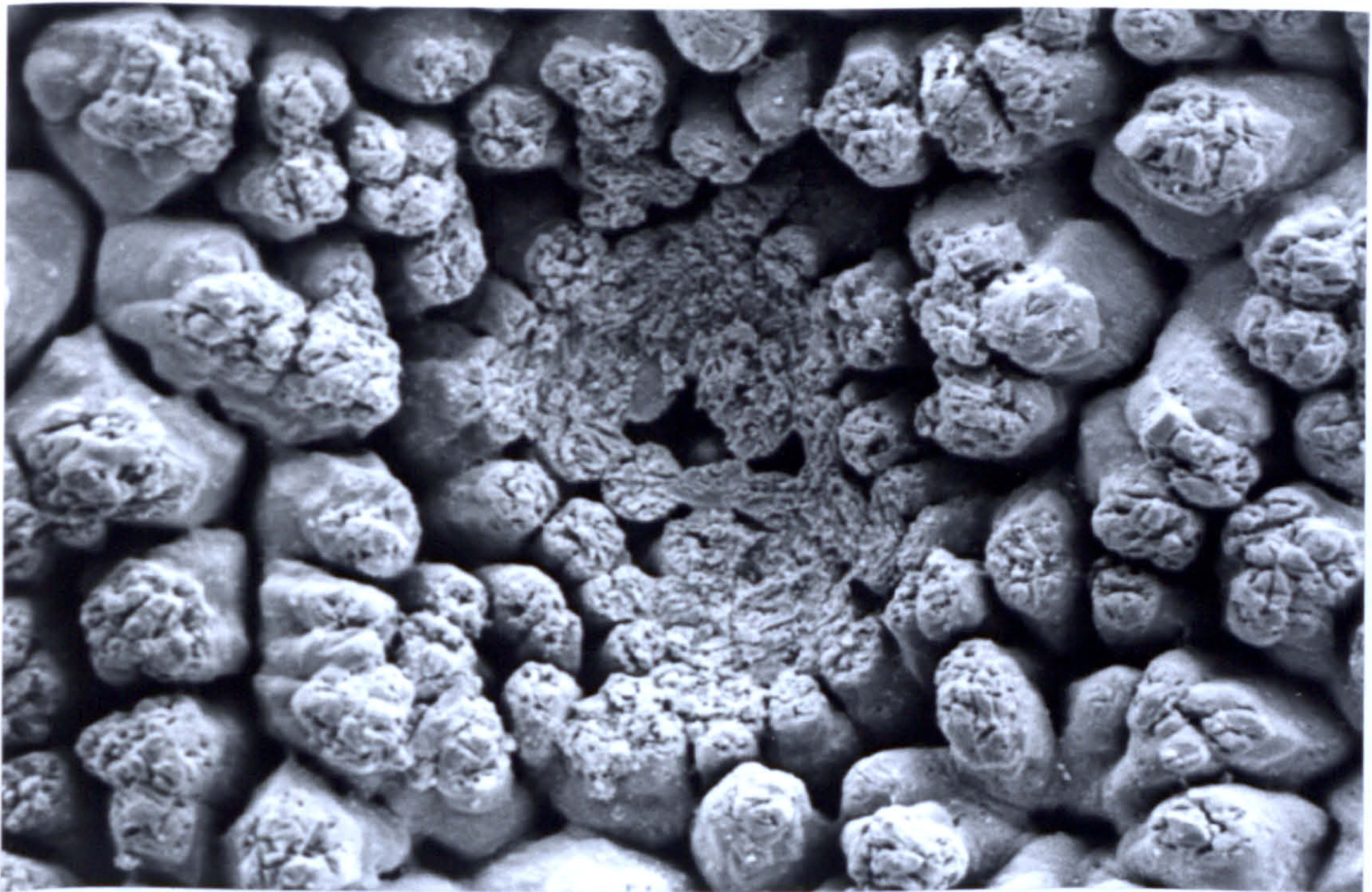


Figure 19: Erosion (360 X).

Figures 18 and 19: Different levels of disruption of the mammillary layer.

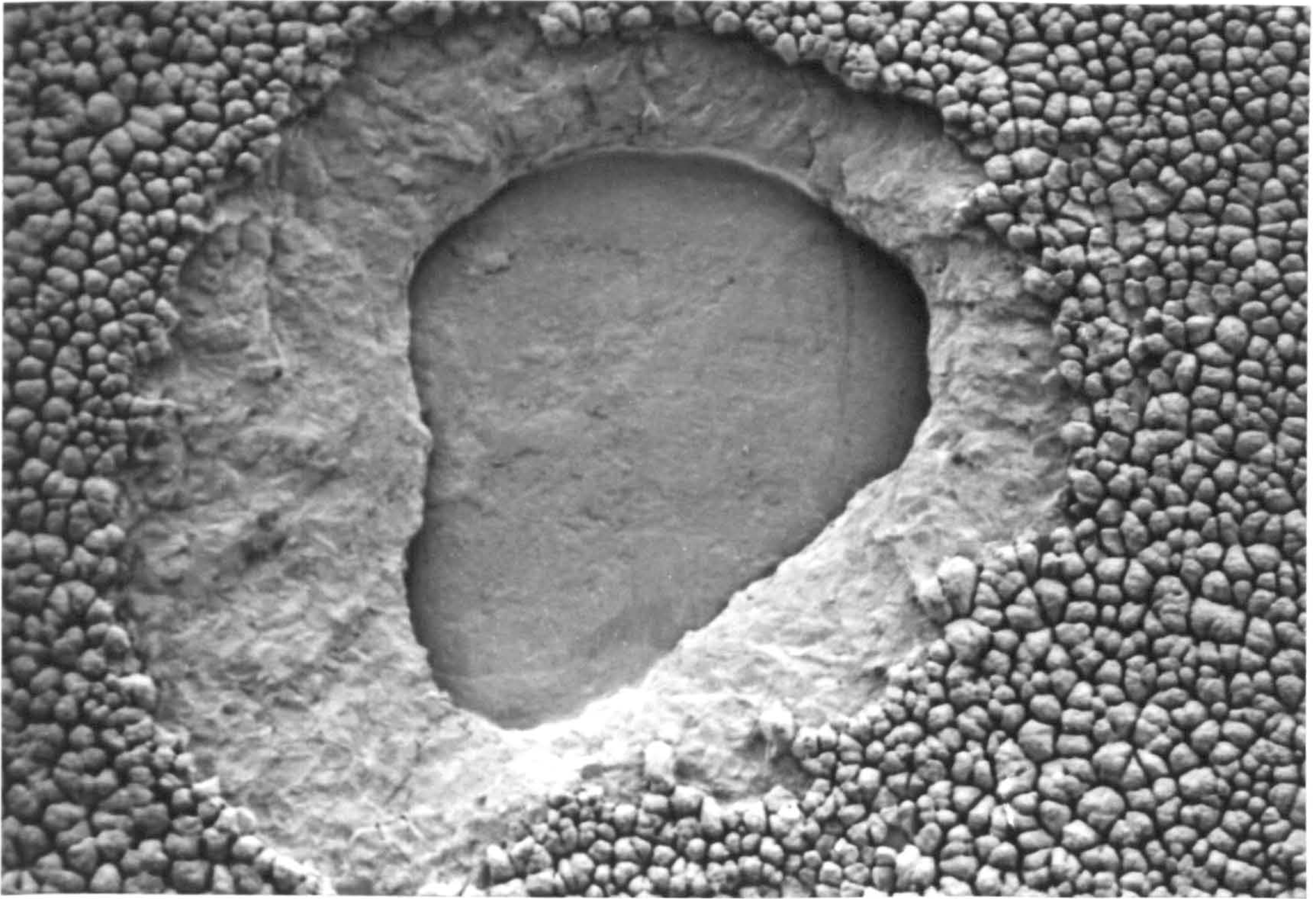


Figure 20: Pin holes are characterised by the presence of a well defined area of discontinuity within the shell (45 X).

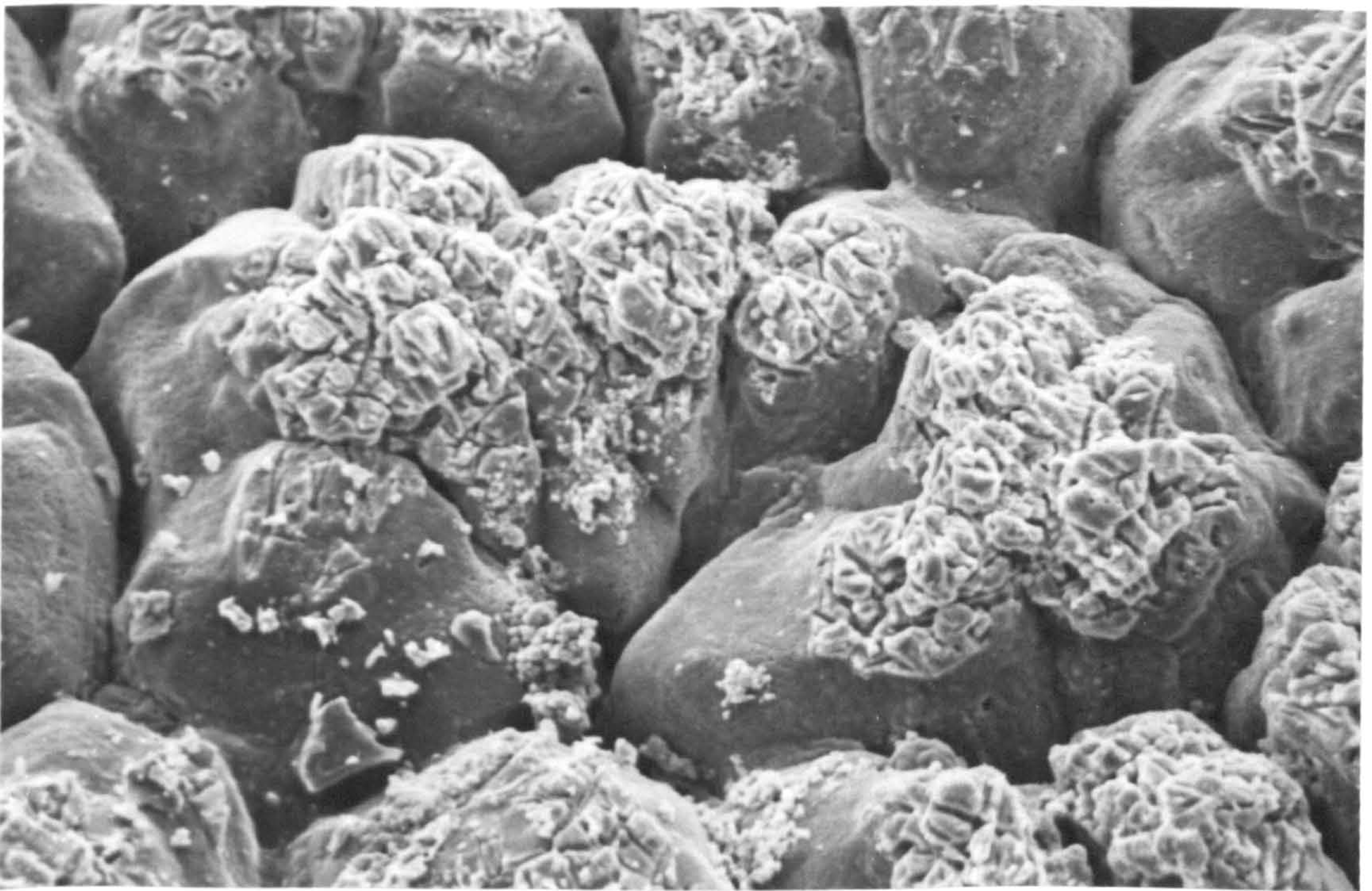


Figure 21: Early fusion of the crystal columns (720 X).

6). It is more than obvious that this kind of abnormality has a strong effect in damaging the eggshell's capacity to restrain microbial entry (graph 7), and the reason for this relatively low correlation may be explained by the fact that a comparatively general lower incidence of pitting was found in the shells examined (in comparison with earlier works by Watt (1989) and Bain (1990)). According to Solomon (1991), pin holes can originate in two different ways. One is of oviducal origin, which the author hypothesises as being caused by an air bubble from the oviducal fluid that might have become trapped on the surface of the membrane fibres, and as it is seeded around by calcium salts, adjacent nucleation sites are structurally modified. Consequently, when the egg cools at oviposition, a clear passageway is left between the mammillary layer and the cuticular surface. The other is caused by cage insult, and differs from the oviducal type by disturbing the cuticular layer without necessarily affecting the mammillary layer.

In terms of the level of occurrence of early fusion (figure 21) and late fusion (figure 22) of the mammillary columns, despite the fact that they were not significantly ($P > 0.05$) different from group to group (tables 11, 12, 13 and 14), they were, for different reasons, very highly significantly ($P < 0.001$) correlated (overall coefficients of 0.37 and 0.50, respectively) with penetration (table 15) (graphs 8, 9 and 10). Because extensive early fusion is desirable in a good shell, leading to a closer framework with fewer free spaces and increased fracture toughness (Bain, 1990), shells with isolated early fusion are penalised with a higher score, which explains the correlation between low early fusion and high penetration. The late fusion mechanism works in the opposite way, *i.e.* extensive late fusion is considered as undesirable and therefore it received a high score, which was found to correlate with higher penetration. Late fusion of the adjacent crystal columns permits easy passage of water into the shell (Watt, 1989) and decreases the shell's fracture toughness (Bain, 1990).

Alignment of the mammillae (figures 23 and 24), although not significantly ($P > 0.05$) different between age groups (tables 11, 12, 13 and

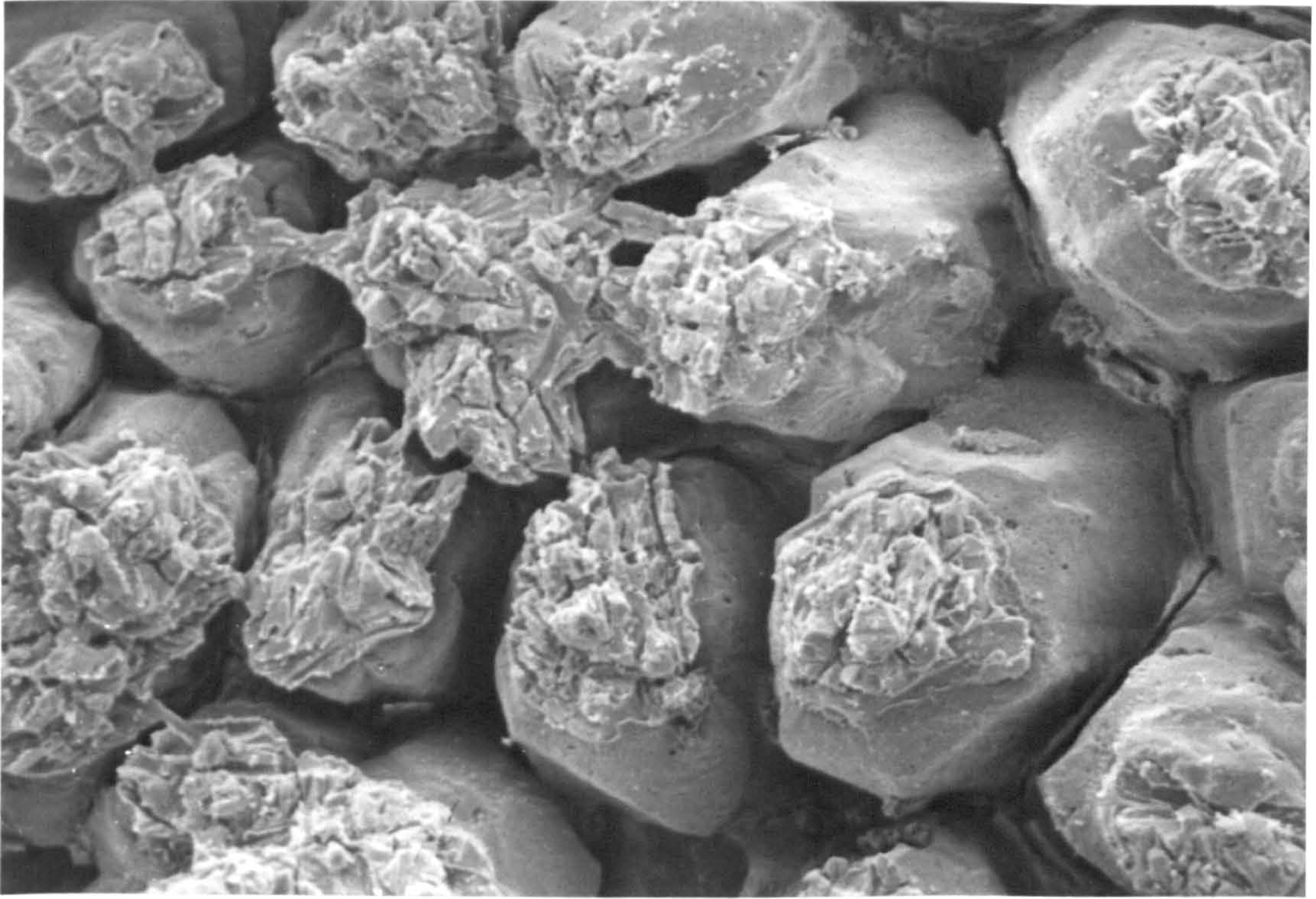


Figure 22: Late fusion of the crystal columns permits easy passage of water into the shell (720 X).

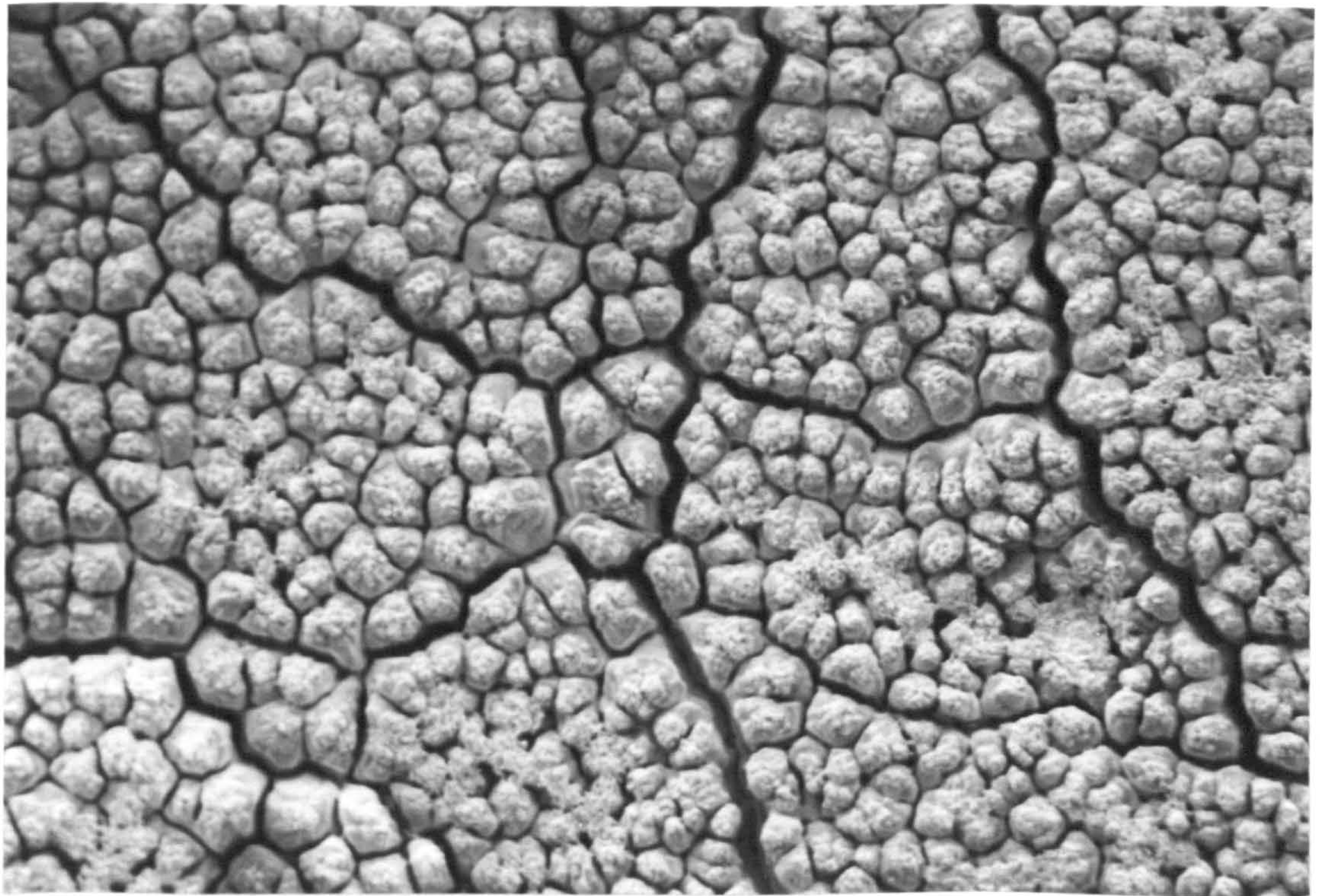


Figure 23: Alignment of the mammillae (90 X).

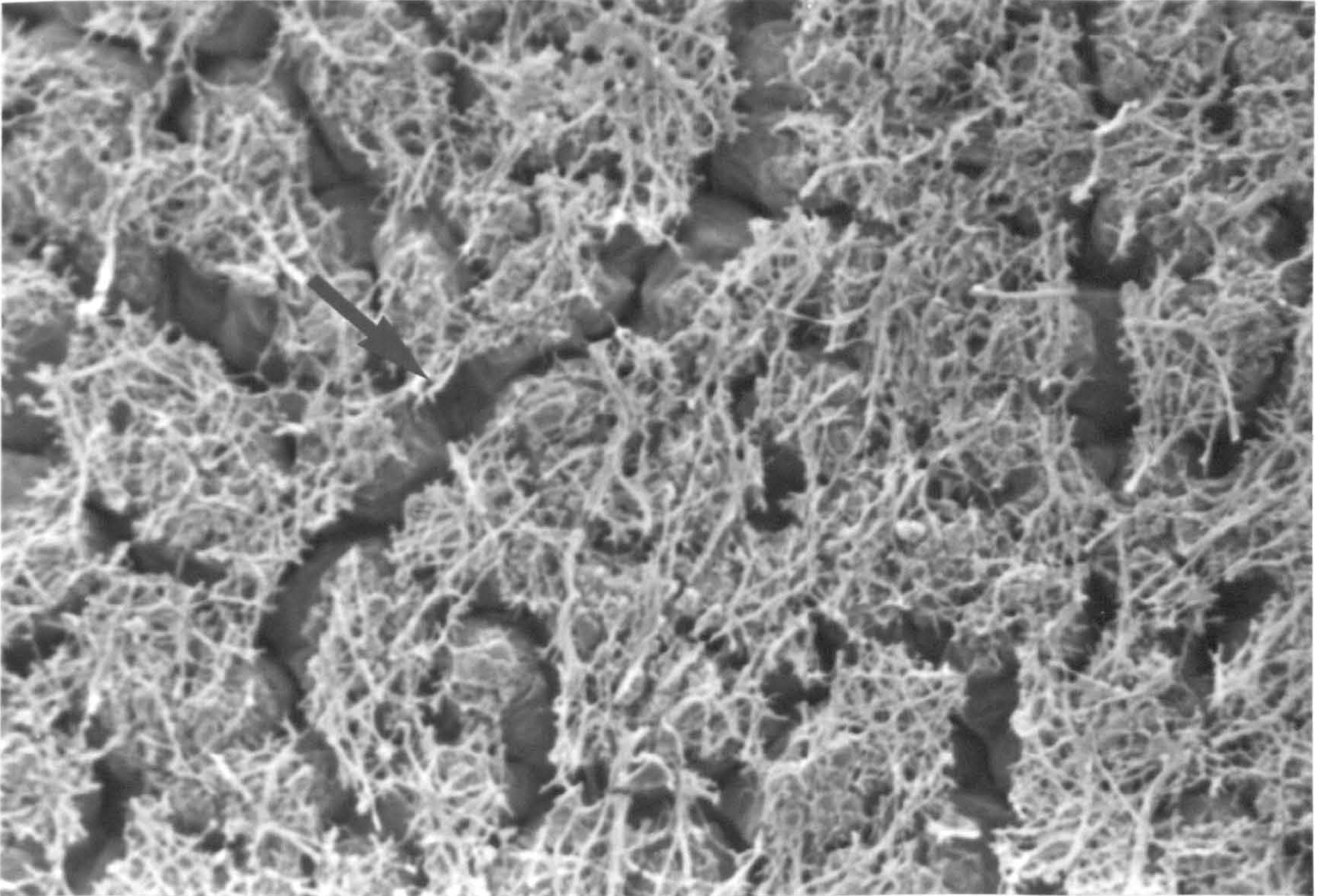


Figure 24: The mammillary layer is covered by a mat of changed membrane fibres. Nevertheless, the underlining alignment of the mammillary bodies is obvious from the crack lines (arrow) (360 X).

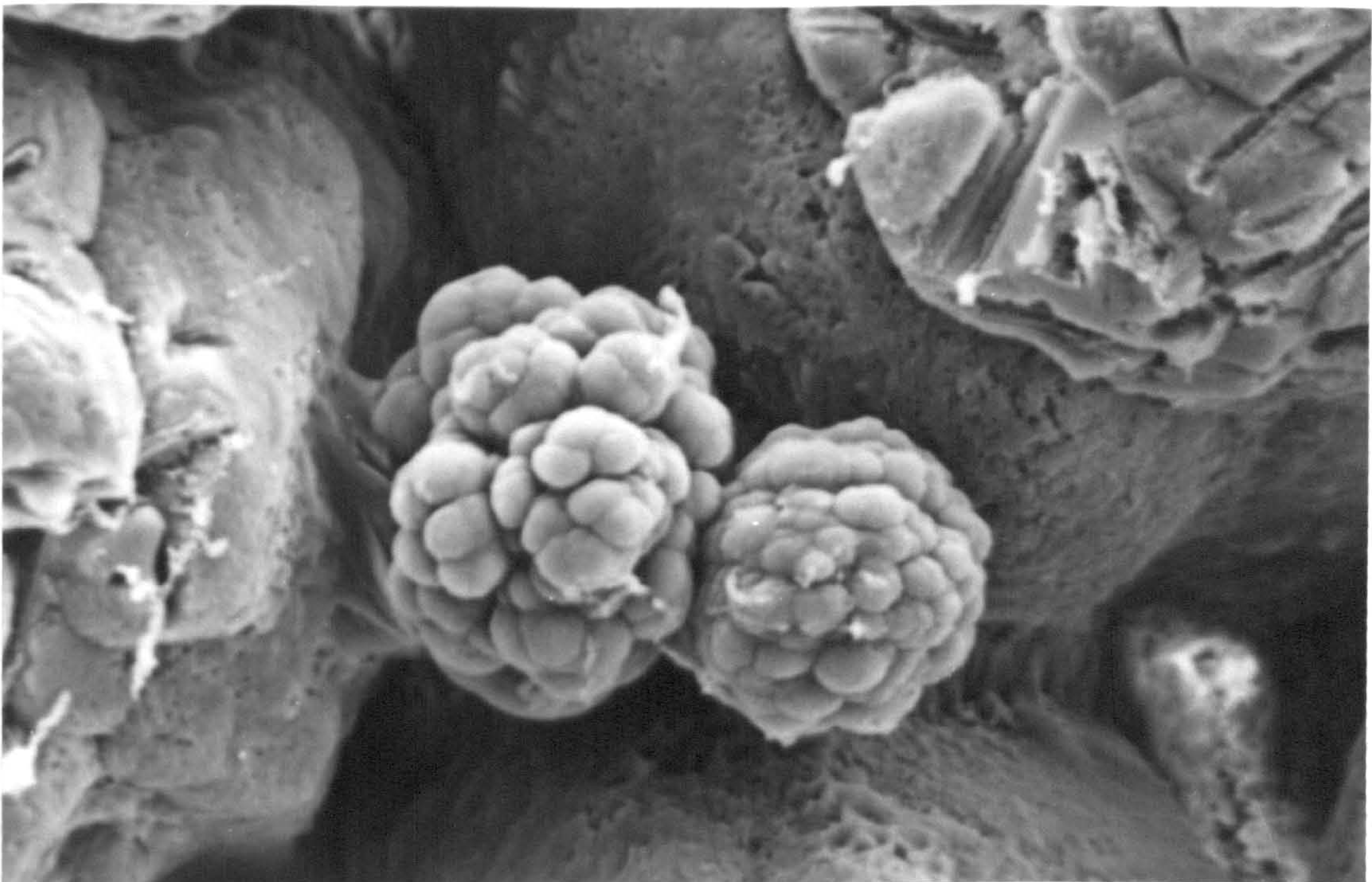


Figure 25: Aragonite crystals (2,813 X).

14), was found to be very highly significantly ($P < 0.001$) correlated (overall coefficient of 0.44) with bacterial penetration (table 15) (graph 11). Ordered mammillae provide the ideal site for crack propagation (Bain, 1990). In a survey of cracked eggs, Solomon (1985a) found that there was a much higher proportion of structural abnormalities in the cone layer associated with the crack line than with other parts of the intact shell. Alignment also causes more open spaces, with the lines having almost the same effect as late fusion, except that it is a localised effect.

The presence of the aragonite aberrant crystal form (figure 25) increased very significantly ($P < 0.001$) from 3.1 % at the beginning to 69 % at the end of lay and in the "bad" group (appendix 2, x^2 5) (tables 11, 12, 13 and 14). It was also found to bear a very highly significant ($P < 0.001$) correlation (overall coefficient of 0.55) with penetration (table 15) (graphs 12 and 13). As with many of the other defects, it causes a more open framework, with poor membrane attachment and consequently increased permeability to bacteria and decreased fracture toughness (Bain, 1990). Aragonite structural changes have also been correlated with environmental stress (Watt, 1989). There are many factors influencing the rate of Ca deposition, *viz.* pH, temperature and impurity ions. Calcite deposition is indicative of a slow, steady rate of mineralisation, while the elongated crystal forms of aragonite (normally present in reptilian shells) are the result of a fast phase of growth, and when present, render shells loosely organised and functionally weak.

Type A bodies, which have no true cap (figure 26), increased very highly significantly ($P < 0.001$) from 31.2 % at the beginning to 87.5 % at middle of lay, with a slight decrease to 83.3 % at the end of lay and in the "bad" group (appendix 2, x^2 6) (tables 11, 12, 13 and 14). This structural variation was also very highly significantly ($P < 0.001$) correlated (overall coefficient of 0.42) with penetration (table 15) (graphs 14 and 15). The lack of establishment between shell and membrane fibre at this point obviously creates the ideal portal for bacterial penetration. Occurrence of stress is believed to increase type A occurrence (Watt, 1989).

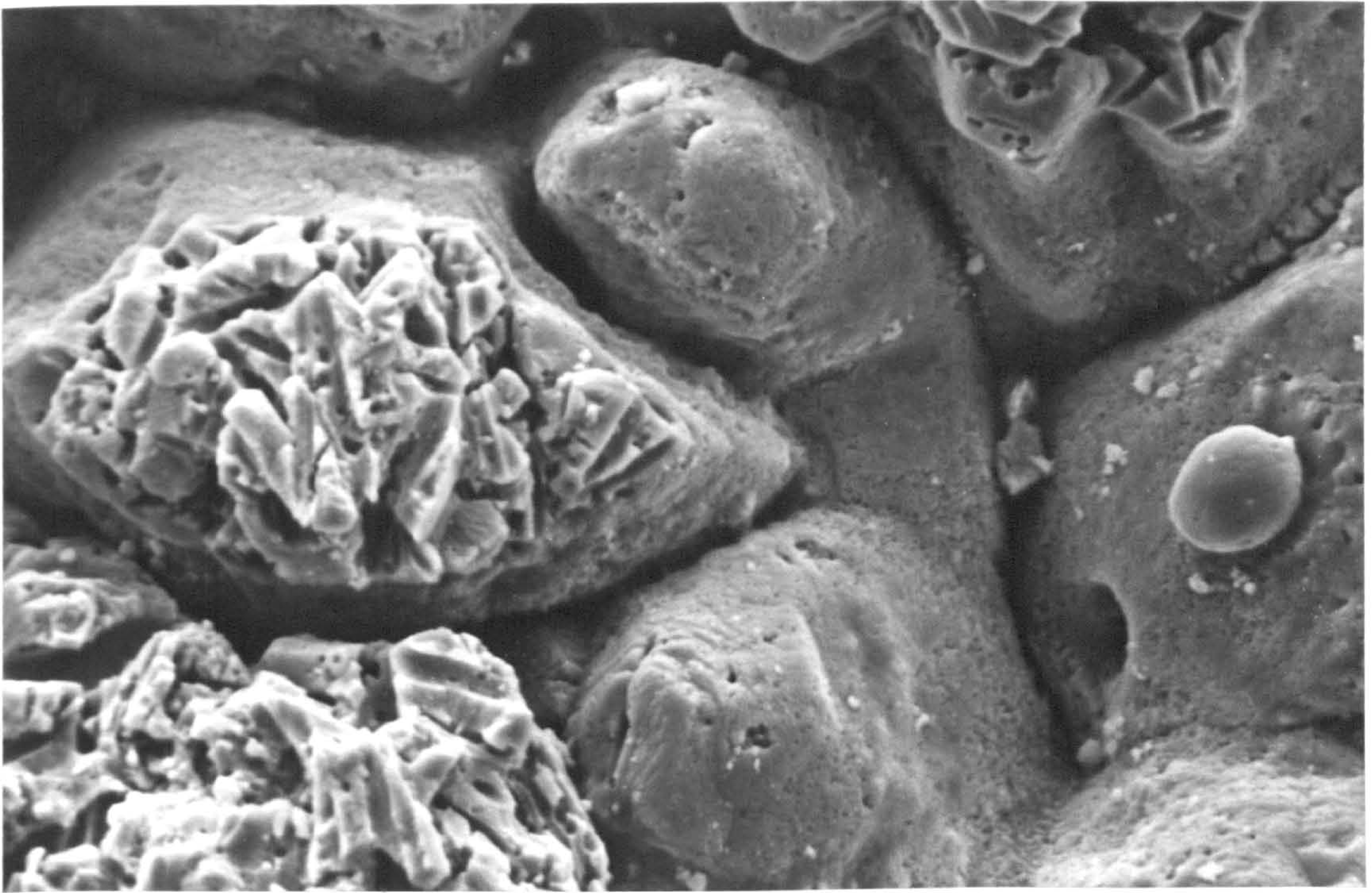


Figure 26: Type "A" mammillary body, which has no cap (1,440 X).

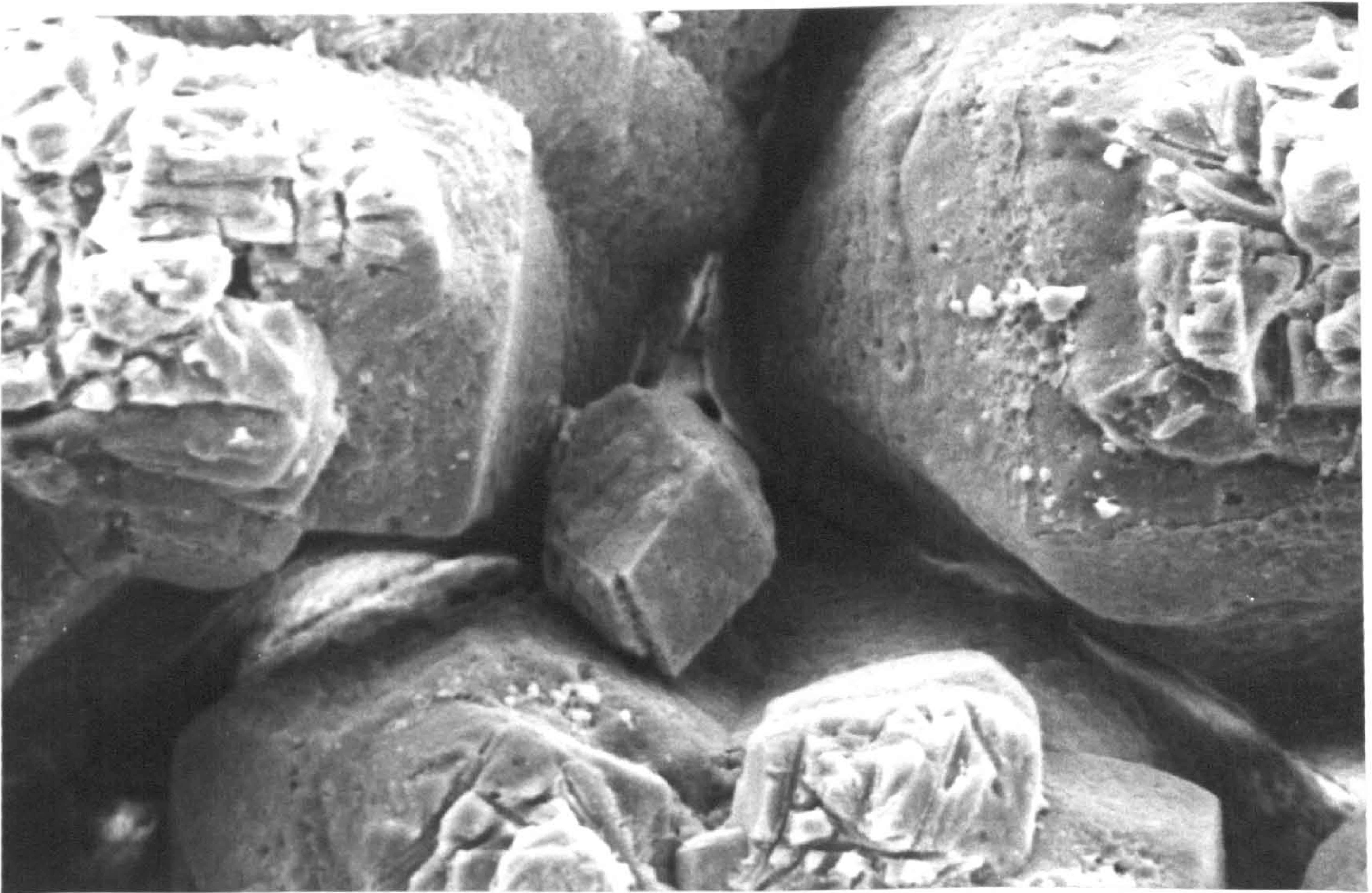


Figure 27: Cubic crystal (free growing calcite) within the mammillary layer (1,440 X).

Cubic crystal forms (figure 27), a typical form of free growing calcite (caused by a change in the rate of mineralisation), did not show any significant difference (P> 0.05) between the groups tested (appendix 2, x^2 7) (tables 11, 12, 13 and 14), nor was the low correlation of occurrence (overall coefficient of 0.17) significant with respect to penetration (table 15).

Cuffing (figure 28), a secondary nucleation of calcium carbonate occurring around and between adjacent mammillary knobs (which also reflects a change in the rate of crystal growth), was found to have significantly (P< 0.01) decreased from 46.9 % at the beginning of lay to 19 % at the end of lay and in the "bad" group (appendix 2, x^2 8) (tables 11, 12, 13 and 14). However, it had no significant (P> 0.05) correlation (overall coefficient of 0.09) with penetration (table 15). Its occurrence is believed to increase shell quality, and the mean score found for this characteristic was in accordance to those reported by Bain (1990) for the same commercial strain.

In terms of occurrence of changed membrane (figure 29), defined as a shell membrane which has suffered both morphological and chemical changes, *i.e.* a high sulphur content (Watt, 1985), thus contrasting with the normal membrane composition, which is mainly potassium, magnesium, sodium and phosphorus (Wedral *et al.*, 1974), it was not found to differ significantly (P> 0.05) from group to group (appendix 2, x^2 9) (tables 11, 12, 13 and 14). Changed membrane was also not considered to be significantly (P> 0.05) correlated (overall coefficient of 0.13) with penetration (table 15), although a significant (P< 0.05) correlation (coefficient of 0.44) was observed in the eggs from beginning of lay (graph 16). In severe cases, the membranes lose contact with the basal caps, following the production of a molten, pitted mass of sulphur and potassium rich material on the surface of individual mammillary caps (Watt, 1985). Changed membrane is believed to be related to oviducal dysfunction, according to Bain (1990) and can also be induced by stress, according to Solomon (1991). Under conditions of stress, the isthmus secretes primarily acidic substances.

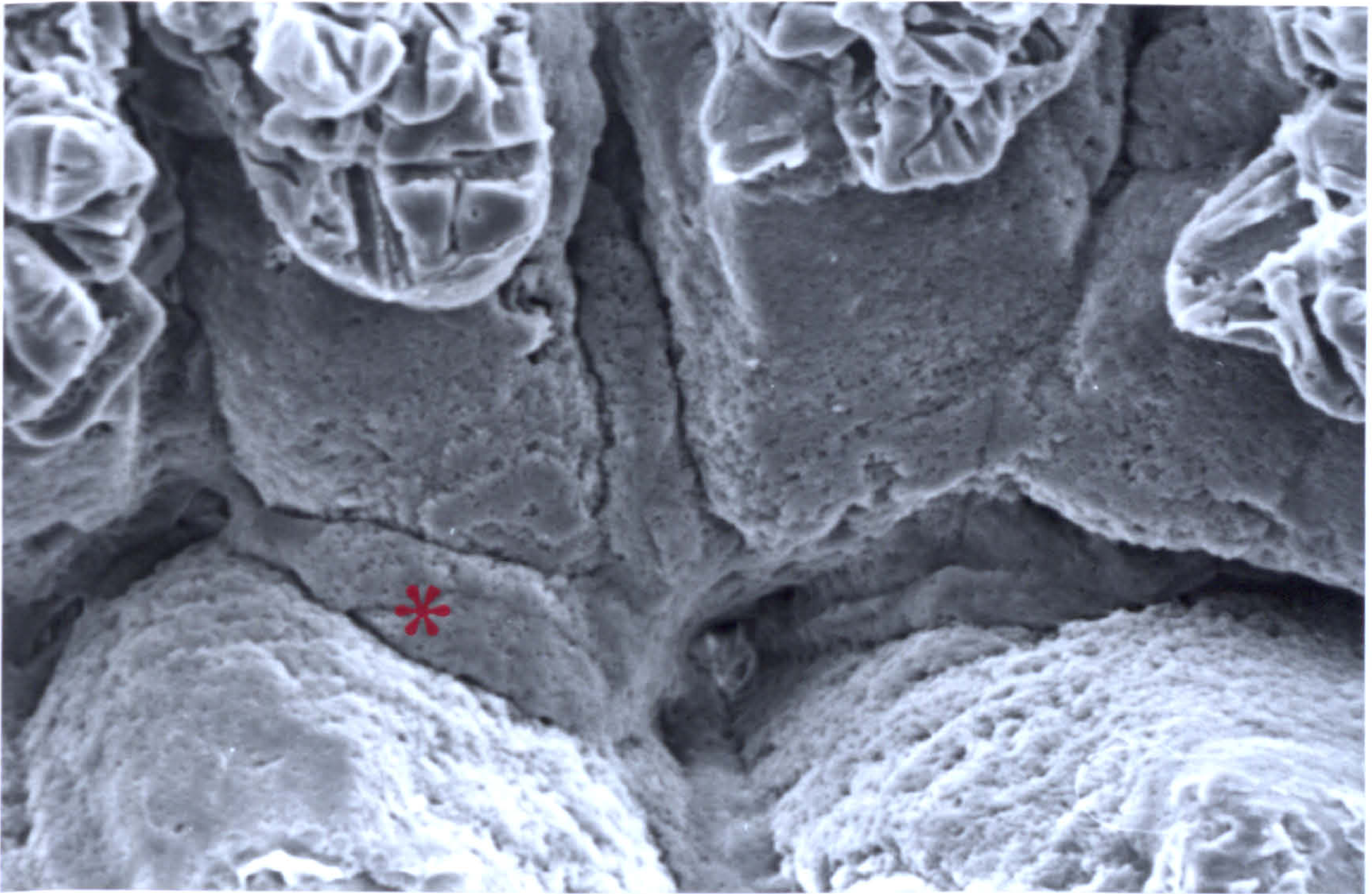


Figure 28: A secondary nucleation of calcium carbonate (cuffing) occurs around and between adjacent mammillary knobs (1,440 X).*

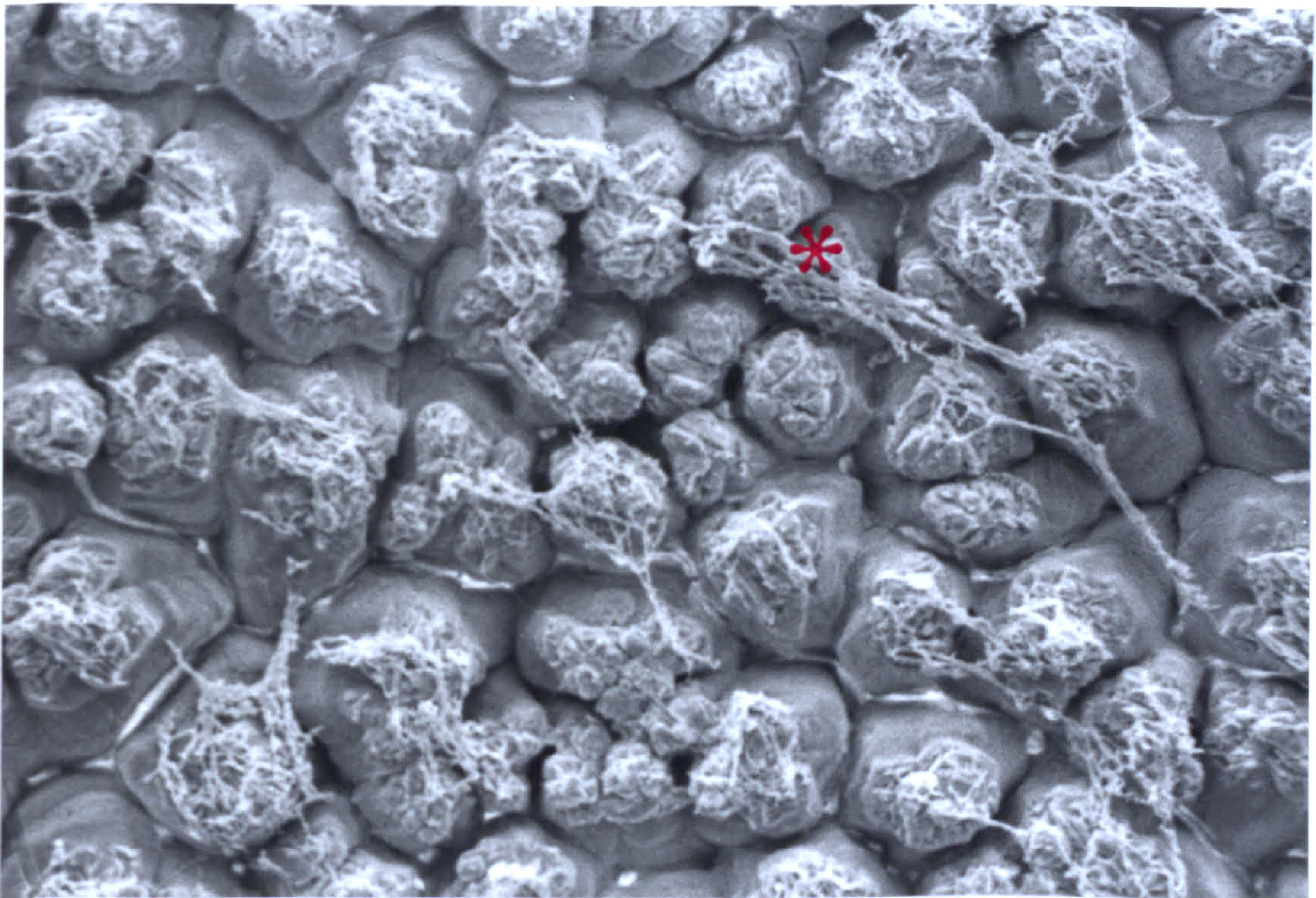


Figure 29: Changed membrane, a shell membrane which has suffered both morphological and chemical changes (360 X).*

As regards mammillary density, there was a decrease from the beginning to the end of lay and in the poor quality ("bad") group, with differences between the latter and the others being very obvious and significant (P< 0.01) (tables 13 and 14). A high mammillary density seems to be a feature of a good quality shell, which is in accordance with observations of Robinson and King (1970); Simons (1971); King and Robinson (1972) and Bunk and Balloun (1978), but contradicts the work of van Toledo *et al.* (1982). In terms of correlation between mammillary density and penetration, it was not found to have any significance (P> 0.05) (table 15). Bain (1990) suggested that mammillary density alone does not influence the fracture toughness of shells, and the same could be suggested in terms of bacterial penetration.

Eggs become progressively and highly significantly (P< 0.001) lower in ultrastructural quality at the level of mammillary layer as the bird ages, with 65.6 % considered as being excellent or good quality at beginning of lay, compared with 34.4 % in the same situation at middle of lay (appendix 2, x^2 10) (tables 11, 12, 13 and 14). These results confirm those found by Watt (1989).

The most remarkable and significant (P< 0.001) correlation (overall coefficient of 0.90) was the relationship between the total structural score and bacterial penetration (table 15) (graphs 17 and 18). Understood as a general parameter of the structural condition of the shell's architecture, it proved to be a reliable means of evaluating the tendency of a poor quality shell to fail in its responsibility of restraining microbial penetration. As no one feature can be singled out as the causative factor of lower resistance, and as the perfect shell as such does not exist, only the overall magnitude of the individual characteristics, represented by the total structural score, could ultimately determine the probable performance of the shell as a barrier to bacterial penetration. This is corroborated by the work of Solomon (1985b and 1991), in which she claims that the mammillary layer is often altered in inherently

weak and low quality shells. Eggs of excellent shell quality were also found by Sauter and Petersen (1974) to be considerably more resistant to penetration by various *Salmonellae* than were eggs of lower shell quality. A calcium accretion on the cuticular layer, may be indicative of a direct path from the outside of the shell to the shell membranes, providing an easy way for microorganisms to invade the egg (figure 15).

Solomon (1988b) and Watt (1989) indicated the importance of the effects of disturbance or stress situations in causing eggshell abnormalities, which may consequently decrease its capacity to resist bacterial invasion. In an earlier work, Solomon *et al.* (1987) showed that a single subcutaneous injection of adrenaline administered to the laying hen will lead to the second and third eggs after treatment being structurally abnormal, showing severe structural disorganisation at all levels, from the mammillary caps (causing poor contact with membrane fibres) up to the cuticular layer (generating a rough, patchy cuticle), only returning to "normality" 20 days after treatment.

CONFLUENCE [**]	BEG	MID	END	BAD
NONE	3	7	18	2
ISOLATED	16	19	8	4
MODERATE	8	6	5	4
EXTENSIVE	5	-	1	-

CAPS [***]	BEG	MID	END	BAD
GOOD	17	6	-	-
GOOD-	15	12	15	3
POOR+	-	12	15	6
POOR	-	2	2	1
POOR-	-	-	-	-

EARLY FUSION [NS]	BEG	MID	END	BAD
ISOLATED	4	6	3	-
MODERATE	19	23	22	7
EXTENSIVE	9	3	7	3

LATE FUSION [NS]	BEG	MID	END	BAD
ISOLATED	1	1	-	1
MODERATE	16	12	15	1
EXTENSIVE	15	19	17	8

ALIGNMENT [NS]	BEG	MID	END	BAD
NONE	2	1	2	-
ISOLATED	19	16	24	5
MODERATE	11	11	3	4
EXTENSIVE	-	4	3	1

TYPE B's [**]	BEG	MID	END	BAD
NONE	5	4	2	1
ISOLATED	20	21	11	4
MODERATE	7	4	15	5
EXTENSIVE	-	3	4	-

PITTING [**]	BEG	MID	END	BAD
NONE	32	30	26	7
DEPRESSION	-	2	5	3
EROSION	-	-	1	-
HOLE	-	-	-	-

ARAGONITE [***]	BEG	MID	END	BAD
NONE	31	28	11	2
ISOLATED	1	3	12	2
MODERATE	-	1	9	6

Table 11: Frequencies of structural scores within each group of eggs assessed by S.E.M., with [*] or [NS] indicating whether there is a significant difference between the four groups.

[***] = Very highly significant at a 0.1 % level ($P < 0.001$)
[**] = Highly significant at a 1 % level ($P < 0.01$)
[*] = Significant at a 5 % level ($P < 0.05$)
[NS] = Not Significant ($P > 0.05$)

TYPE A's [***]	BEG	MID	END	BAD
NONE	22	4	6	1
ISOLATED	10	28	26	9

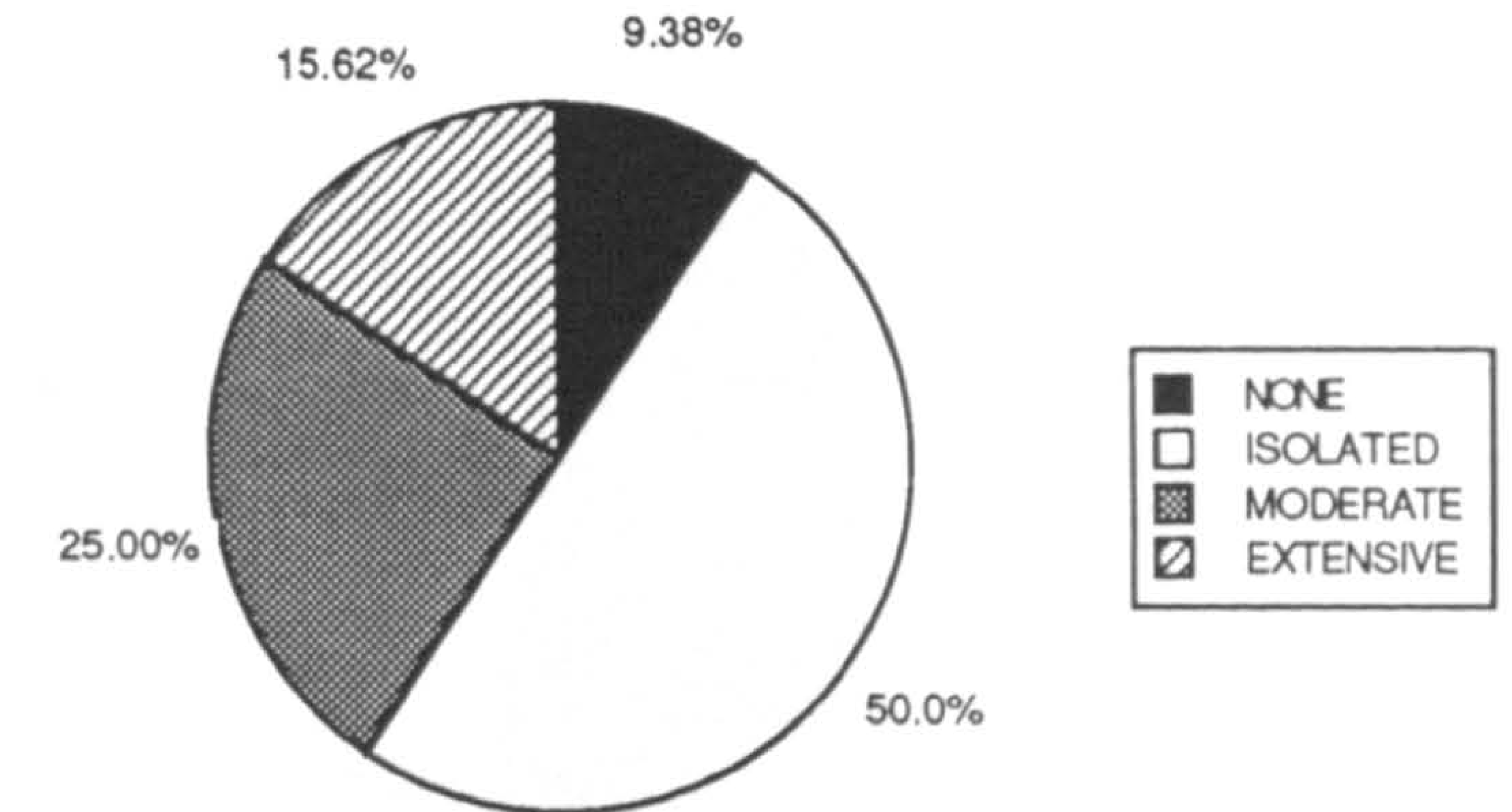
CUBICS [NS]	BEG	MID	END	BAD
NONE	25	22	22	5
ISOLATED	7	7	10	5
MODERATE	-	3	-	-

CUFFING [**]	BEG	MID	END	BAD
NONE	17	14	28	6
ISOLATED	14	14	4	4
MODERATE	1	4	-	-

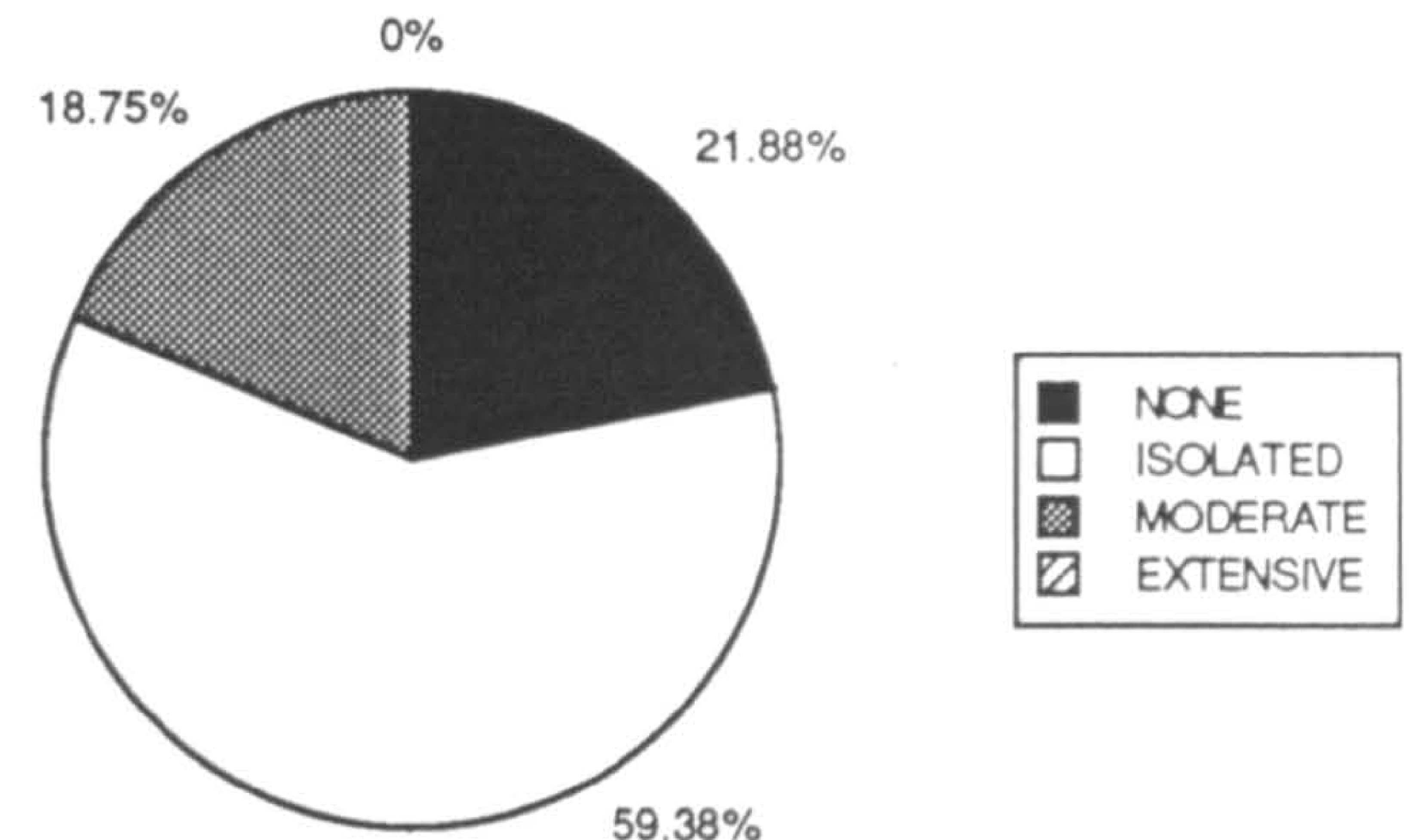
CHANGED MEMBRANE [NS]	BEG	MID	END	BAD
NONE	31	30	32	10
ISOLATED	1	2	-	-
MODERATE	-	-	-	-
EXTENSIVE	-	-	-	-

TOTAL SCORE [***]		BEG	MID	END	BAD
E	≤ 19	2	-	-	-
G	20-29	19	11	6	-
G-	30-34	9	11	10	2
P+	35-39	2	6	9	4
P	40-49	-	4	7	4
P-	≥ 50	-	-	-	-
TOTAL No. EGGS		32	32	32	10

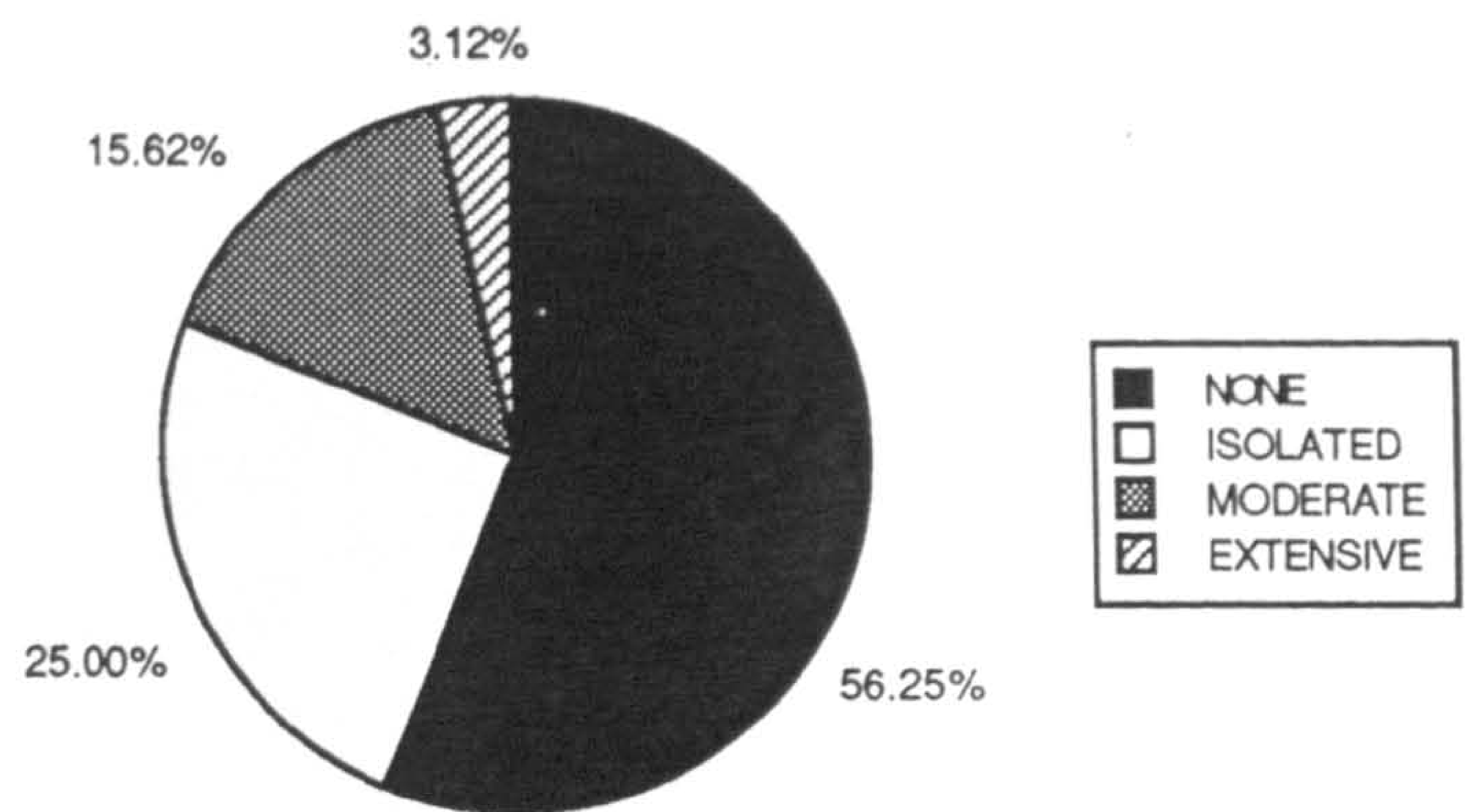
Table 11: (continued)



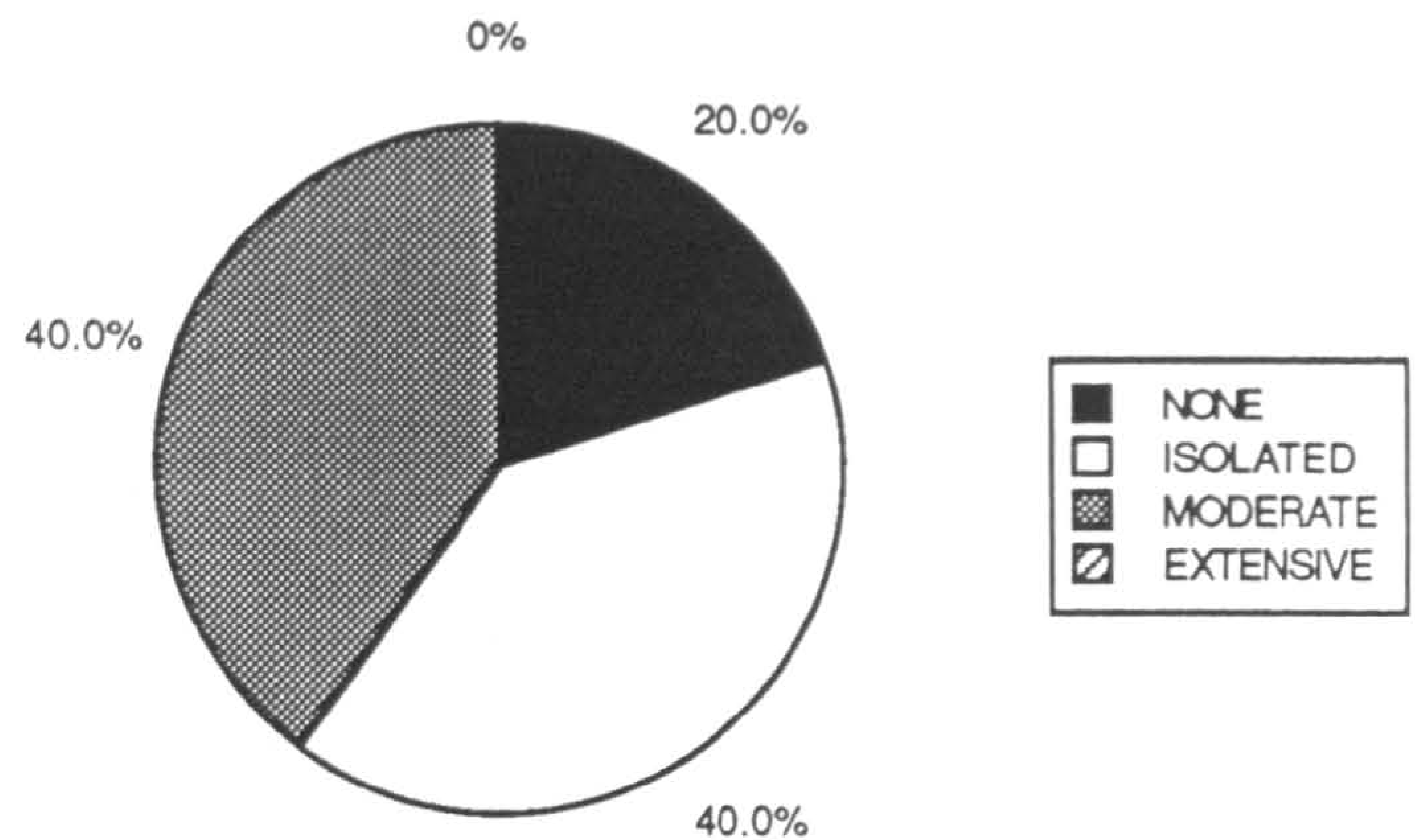
OCCURR. OF CONFLUENCE AT BEGINNING OF LAY



OCCURR. OF CONFLUENCE AT MIDDLE OF LAY

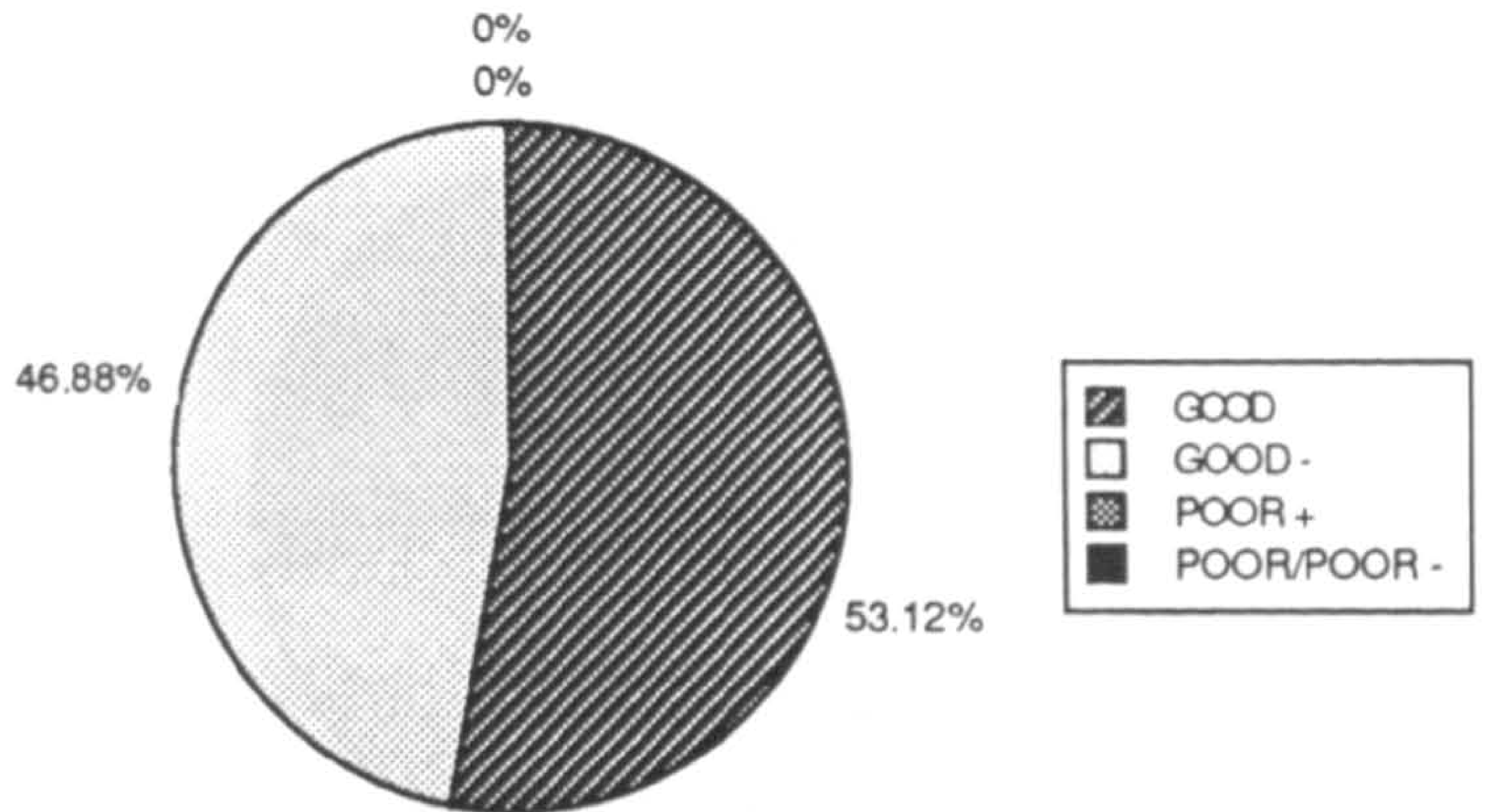


OCCURR. OF CONFLUENCE AT END OF LAY

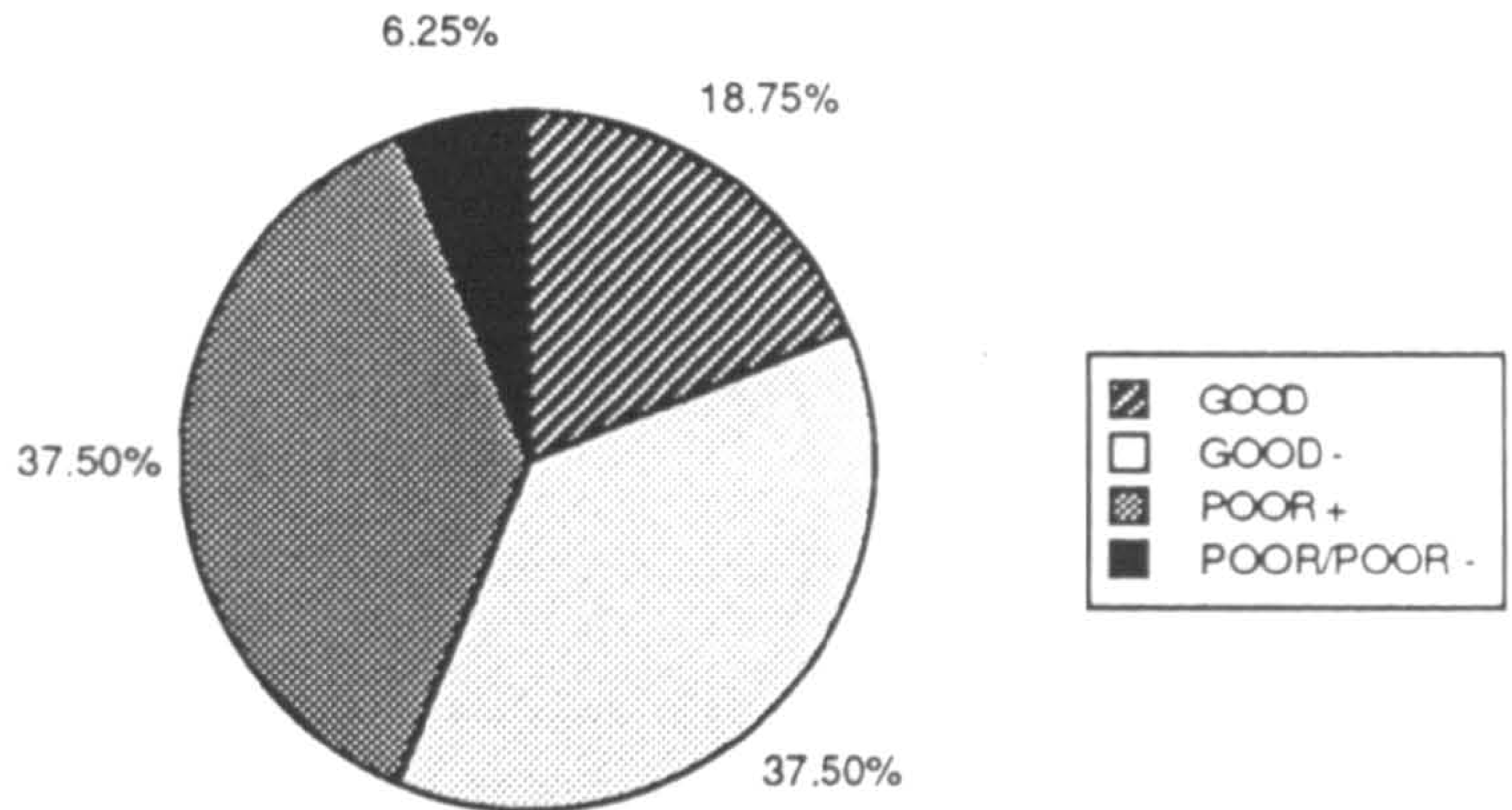


OCCURR. OF CONFLUENCE IN THE "BAD" GROUP

Table 12: Illustrates the results of table 11. Each page represents one structural characteristic, in terms of occurrence of each category over the four groups examined.



CONDITION OF CAPS AT BEGINNING OF LAY



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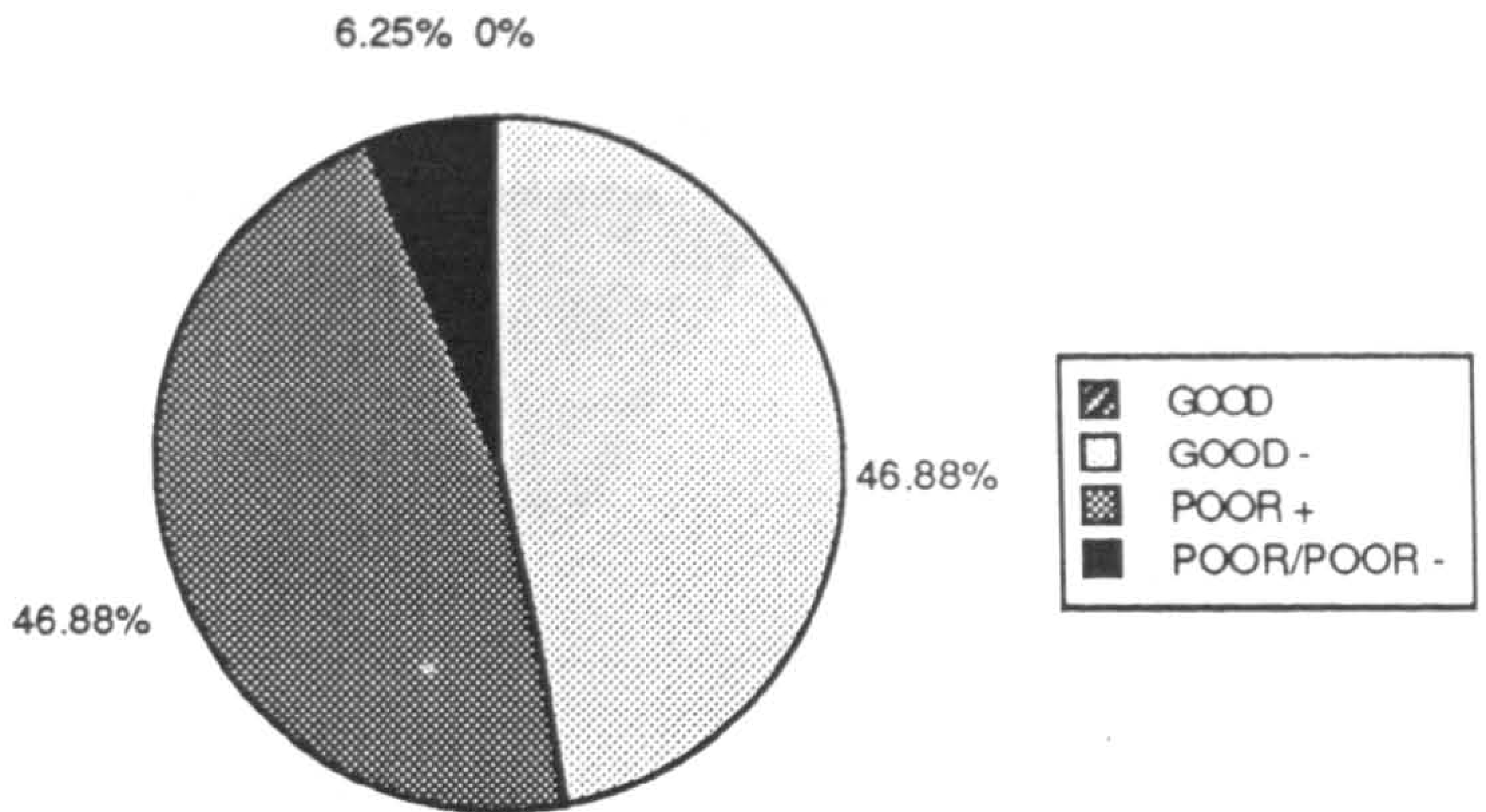
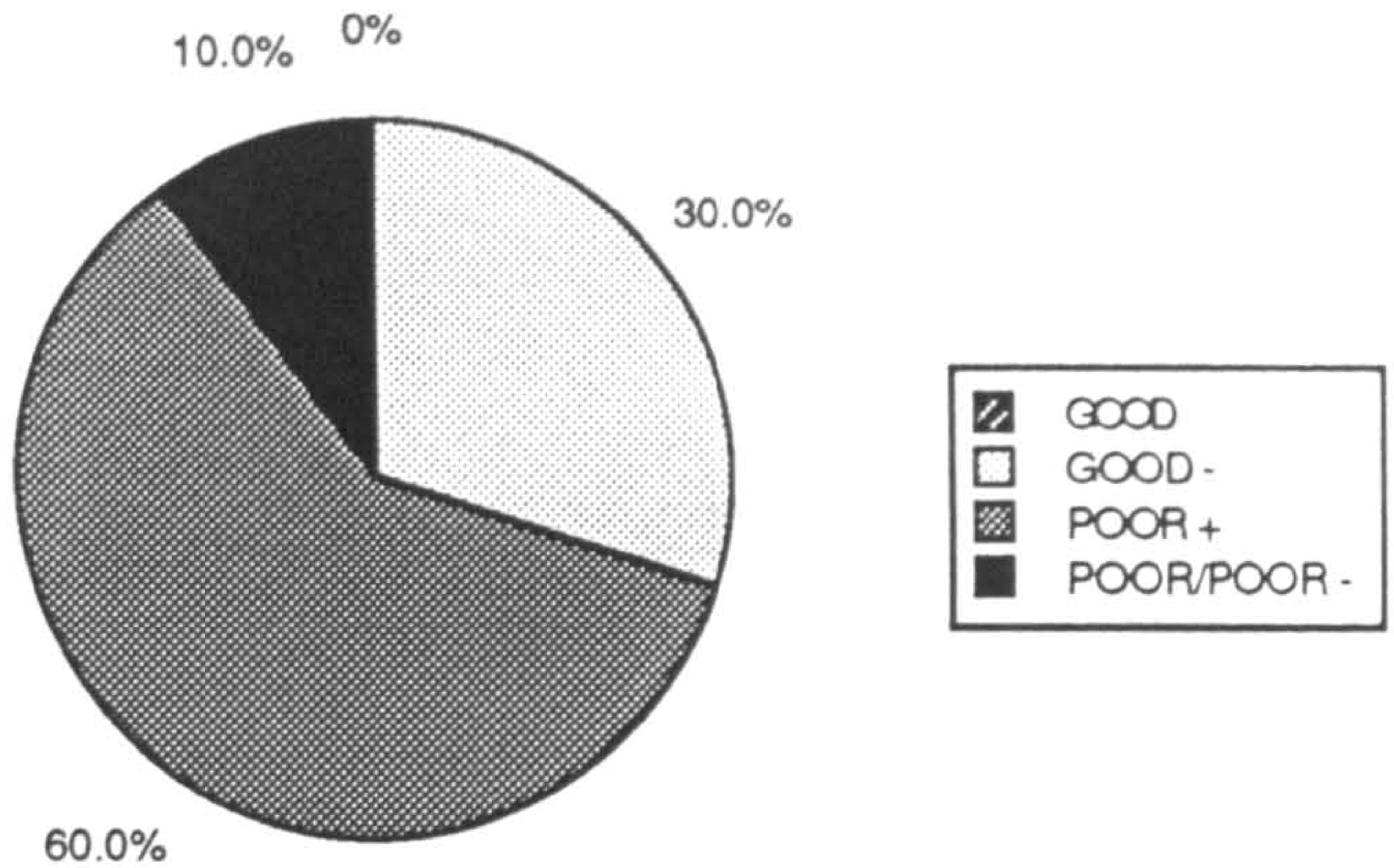
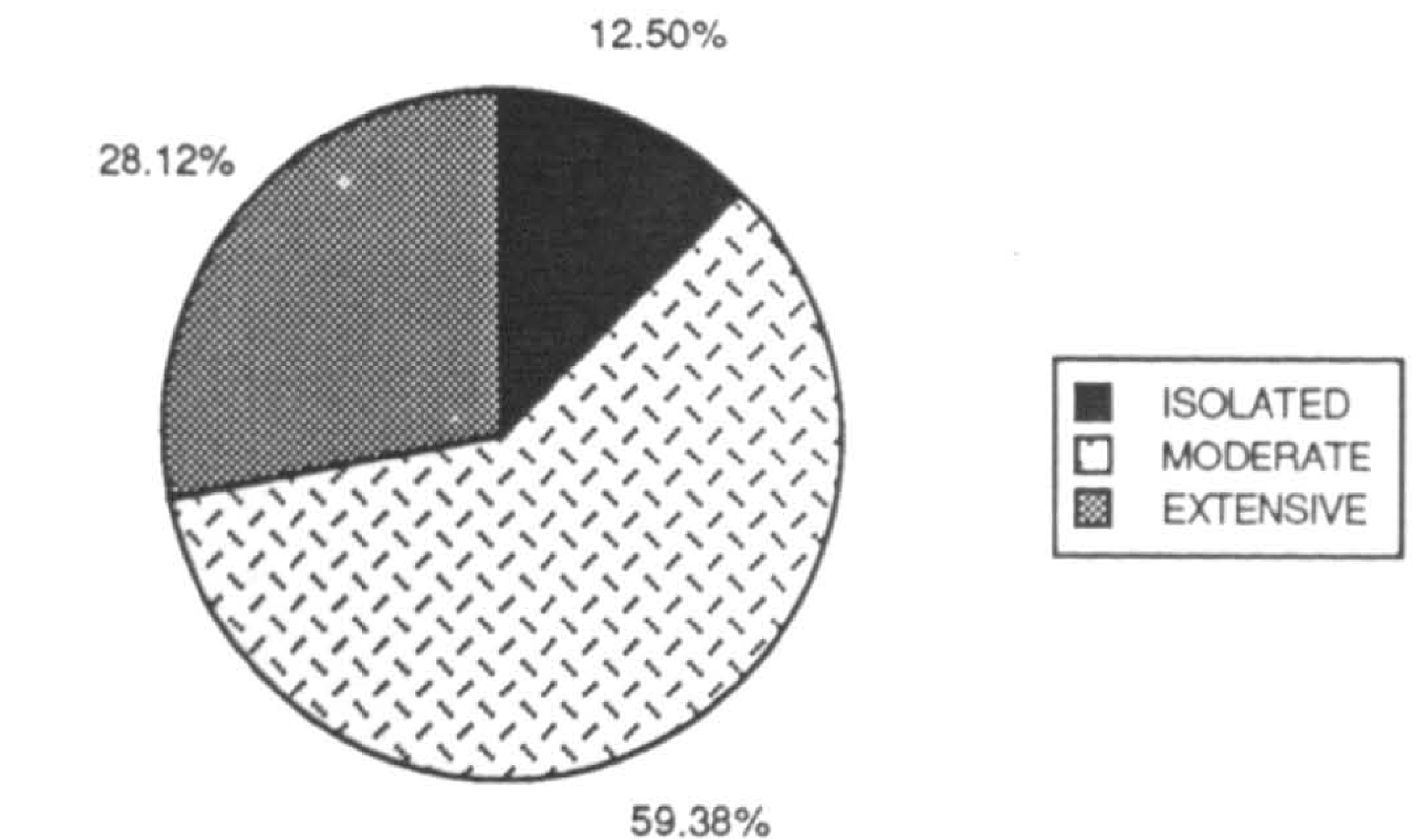


CONDITION OF CAPS AT END OF LAY

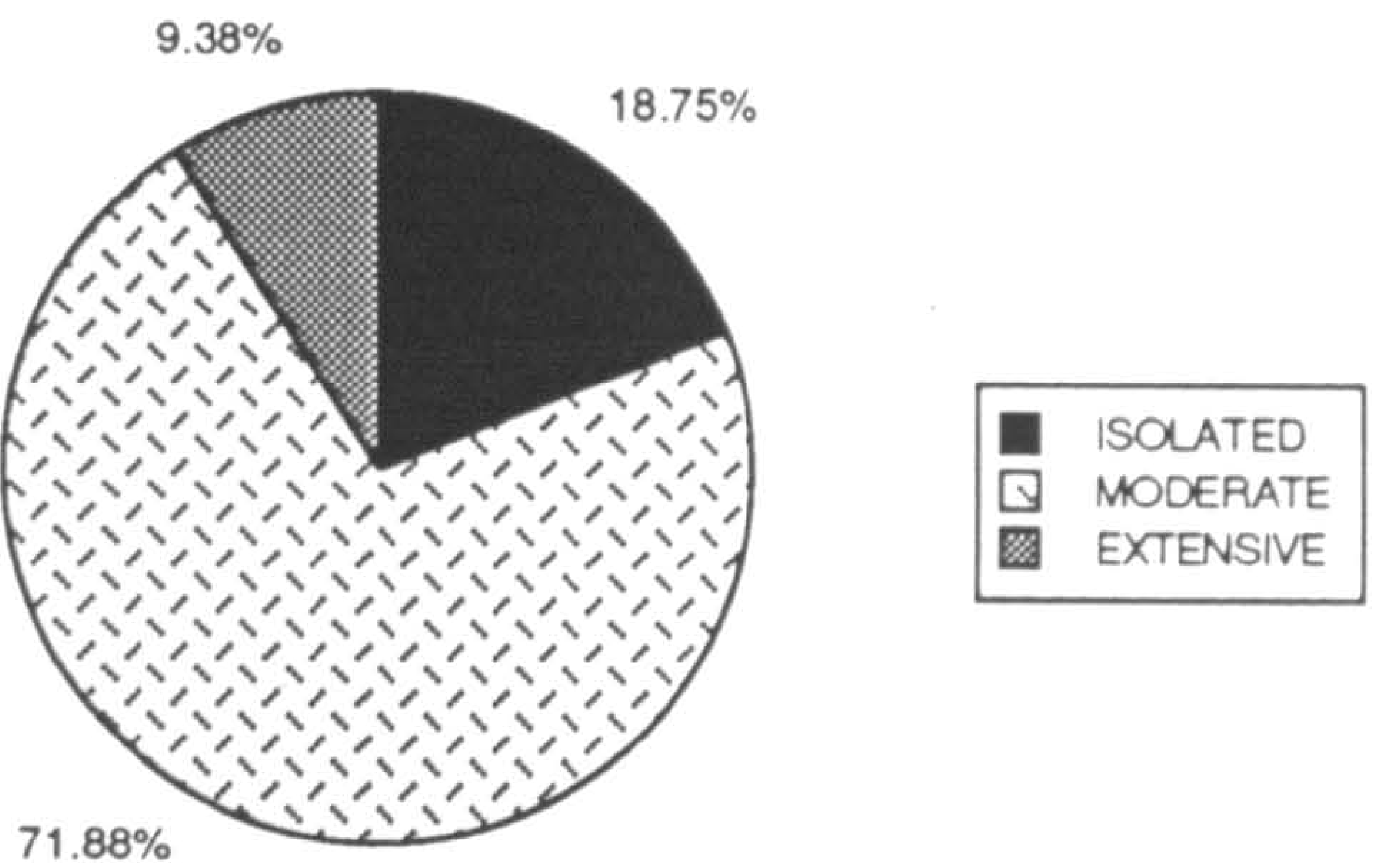


CONDITION OF CAPS IN THE "BAD" GROUP

Table 12: Cont.



OCCURR. OF EARLY FUS. AT BEGINNING OF LAY



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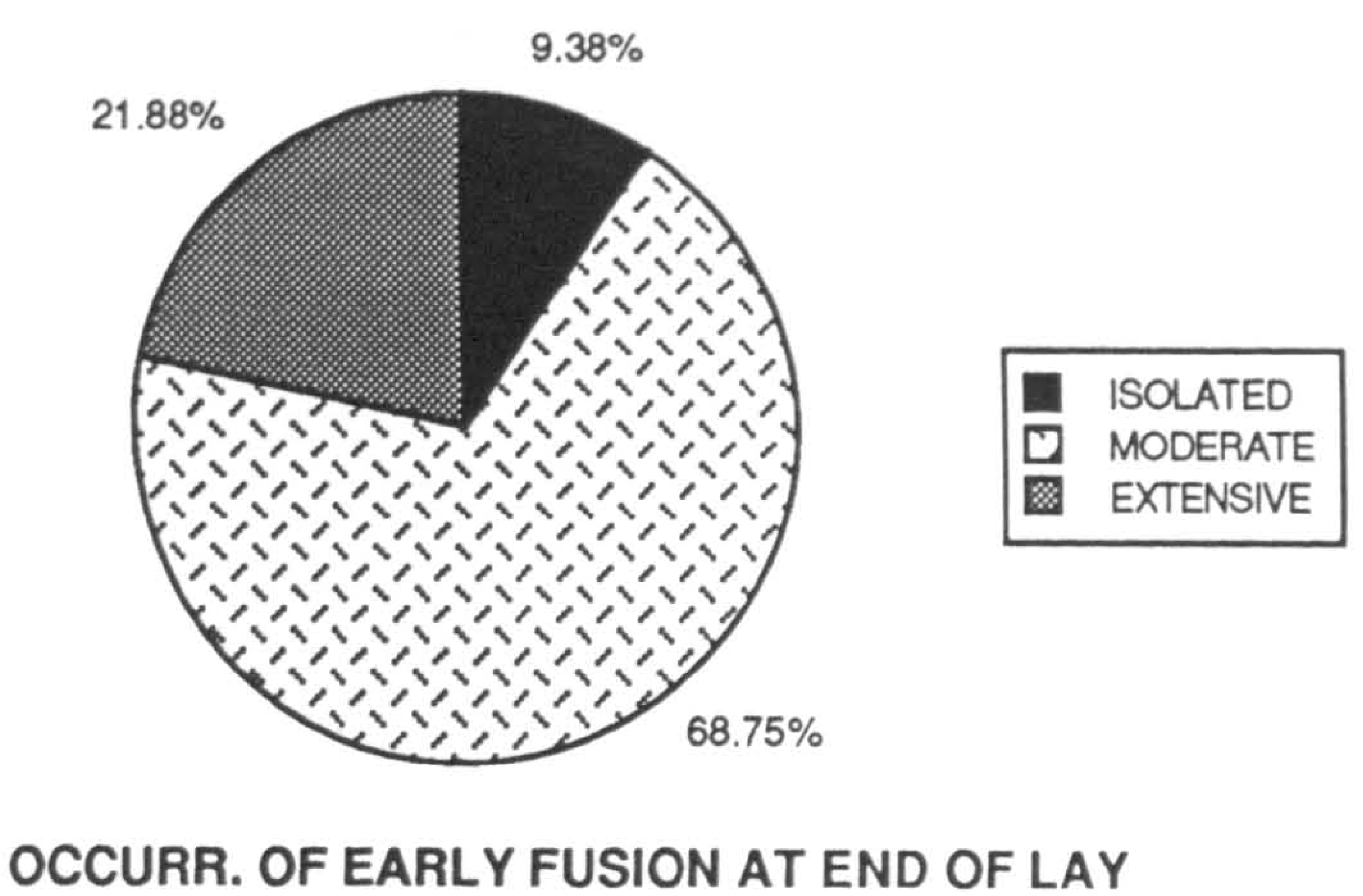

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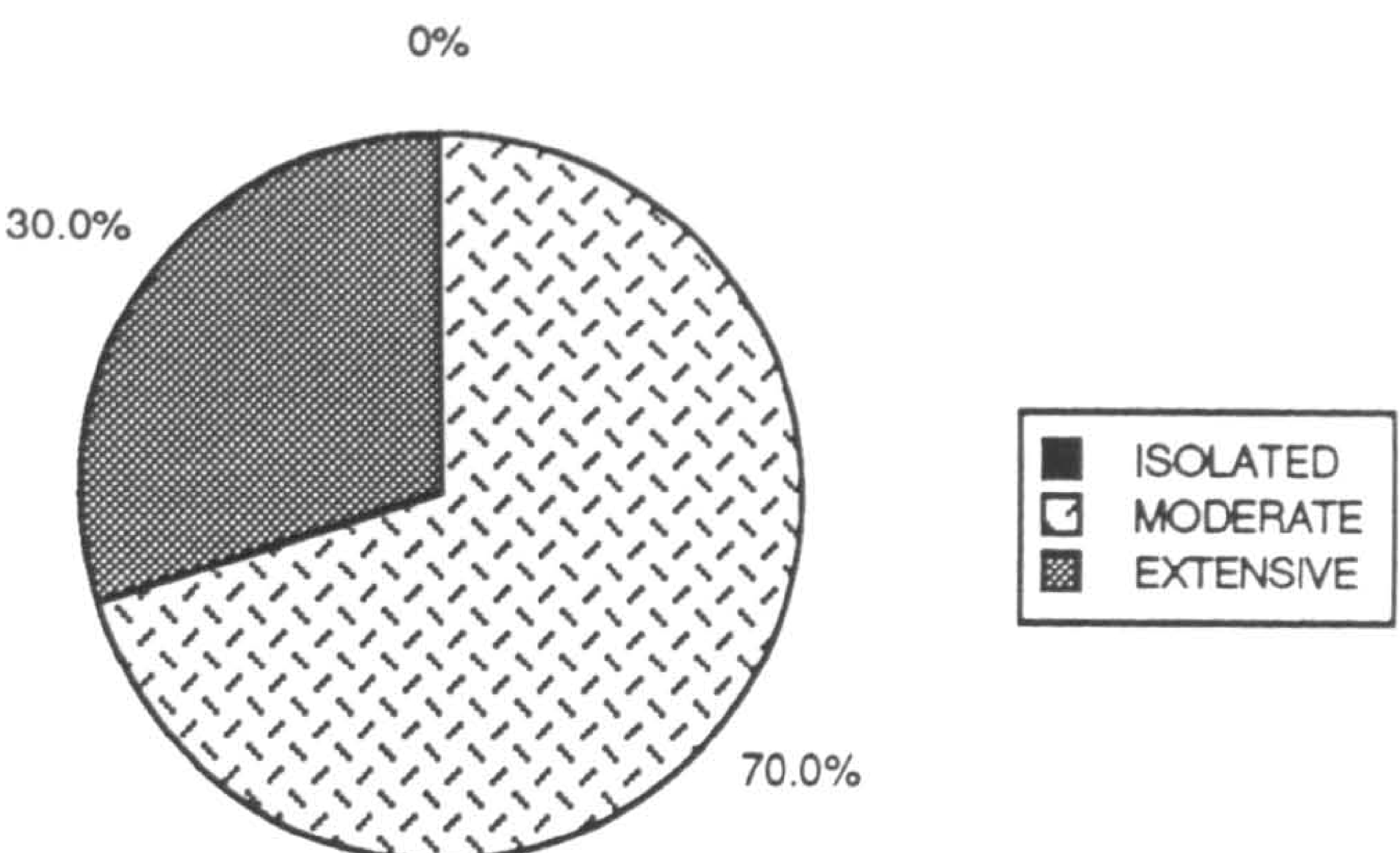
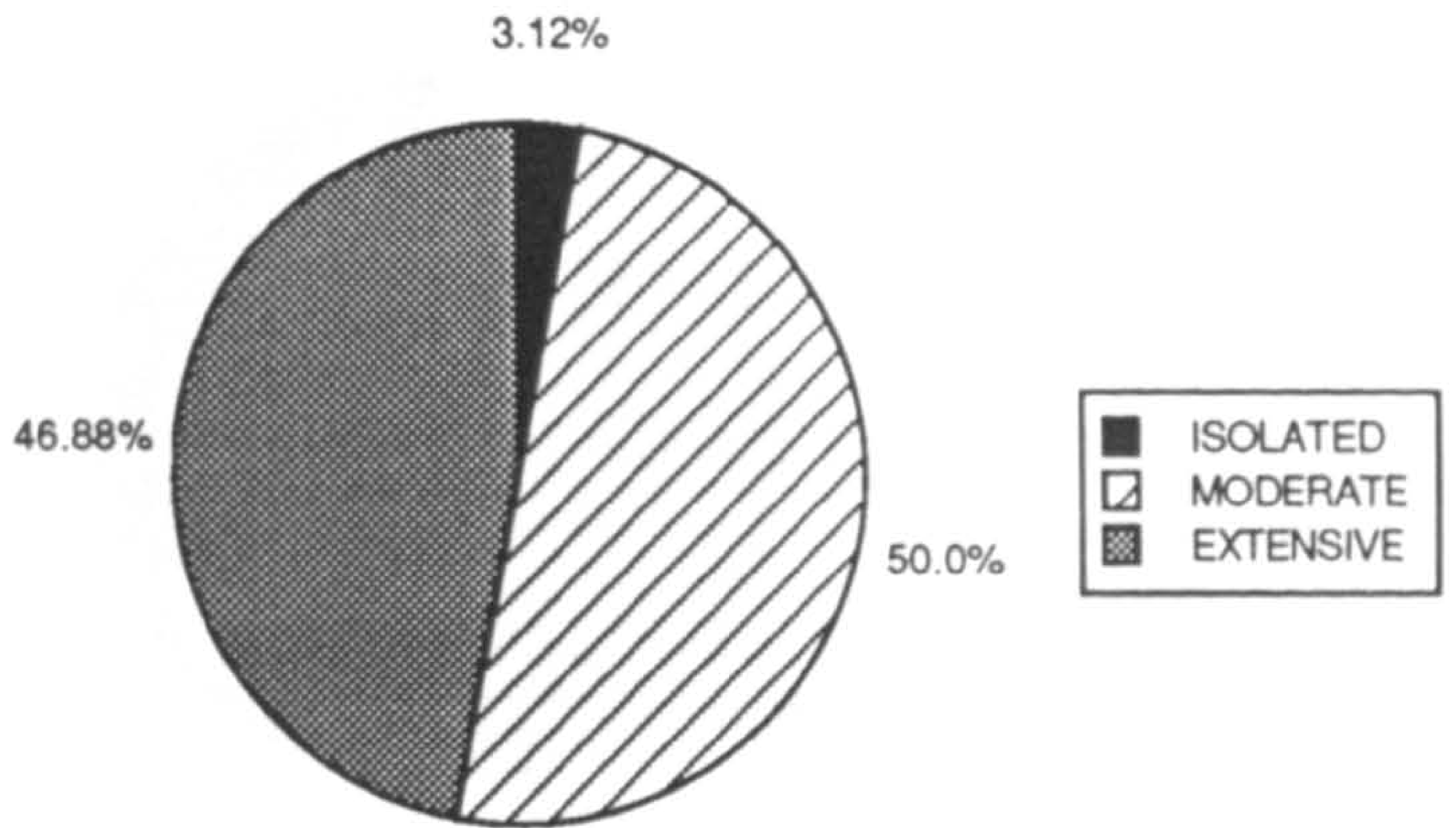
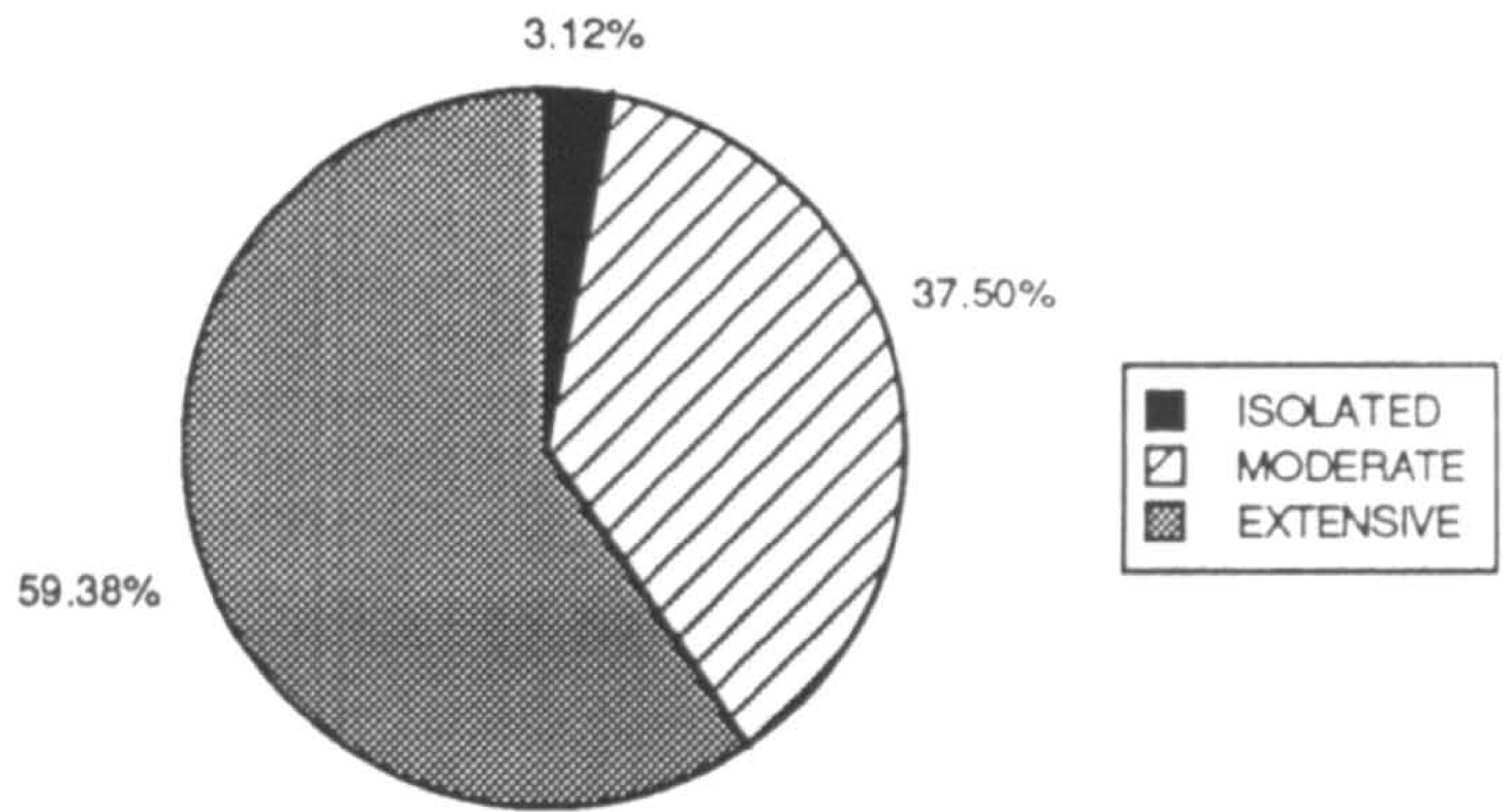


OCCURR. OF EARLY FUS. IN THE"BAD" GROUP

Table 12: Cont.



OCCURR. OF LATE FUSION AT BEGINNING OF LAY



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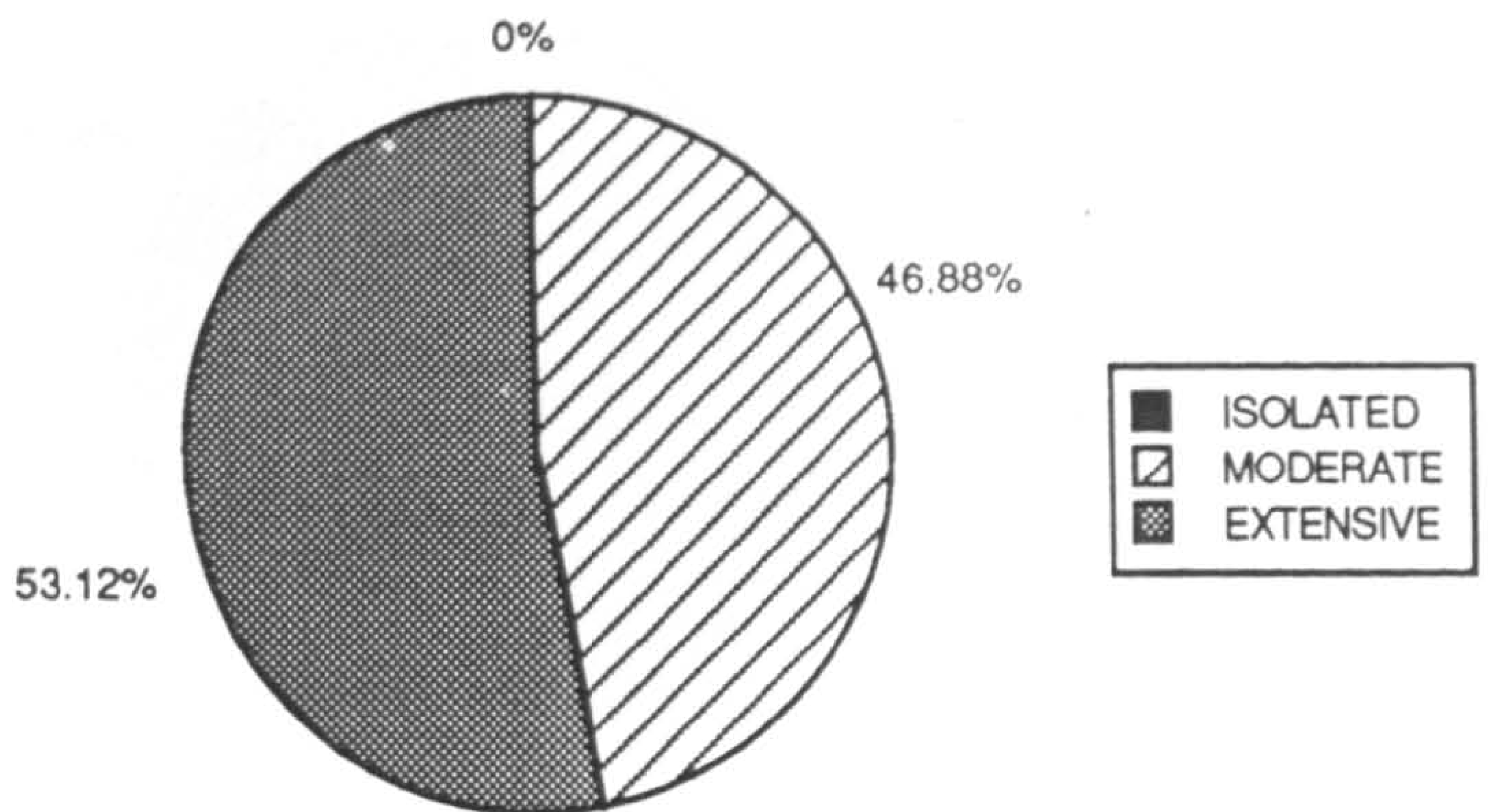

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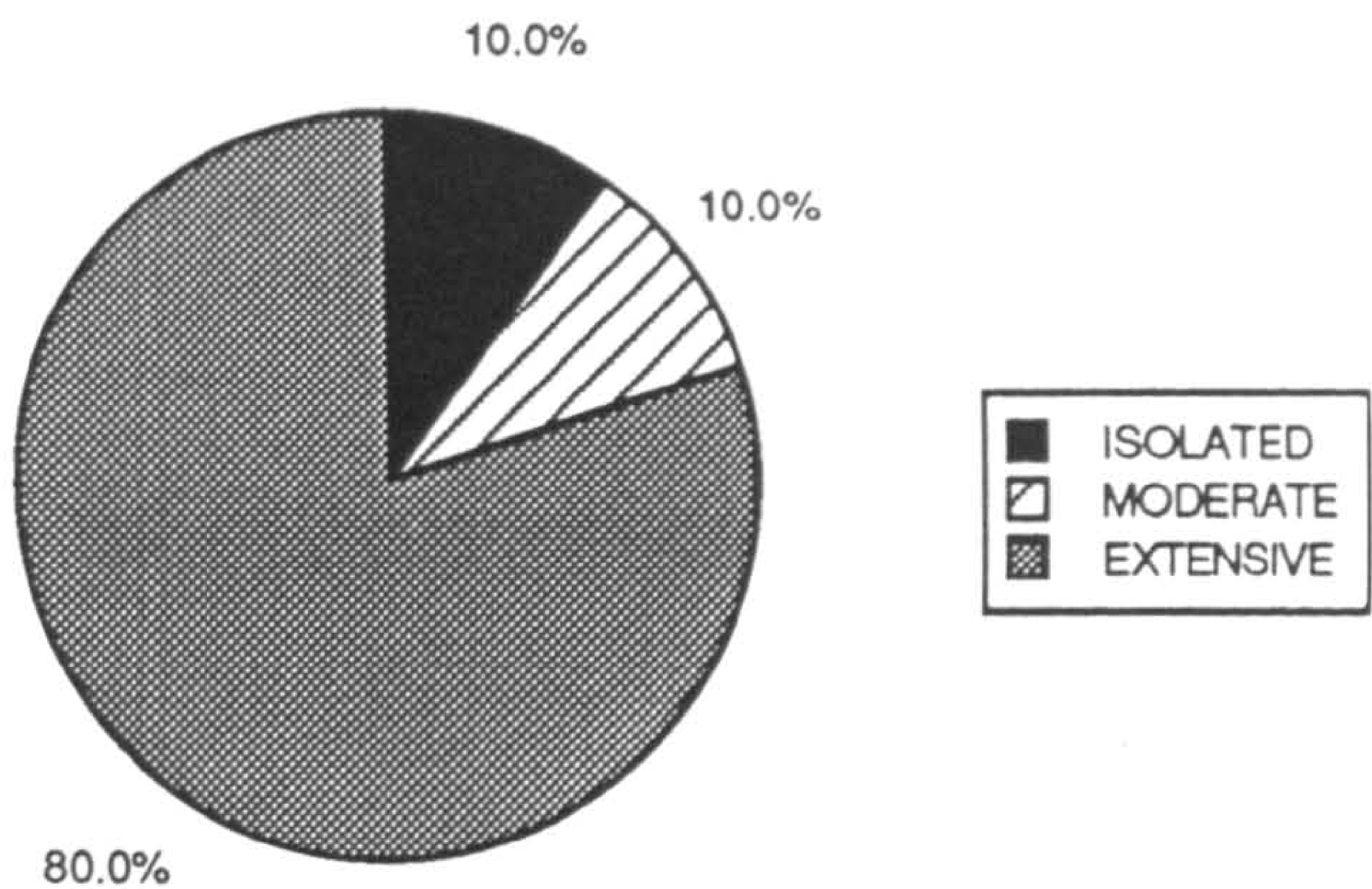
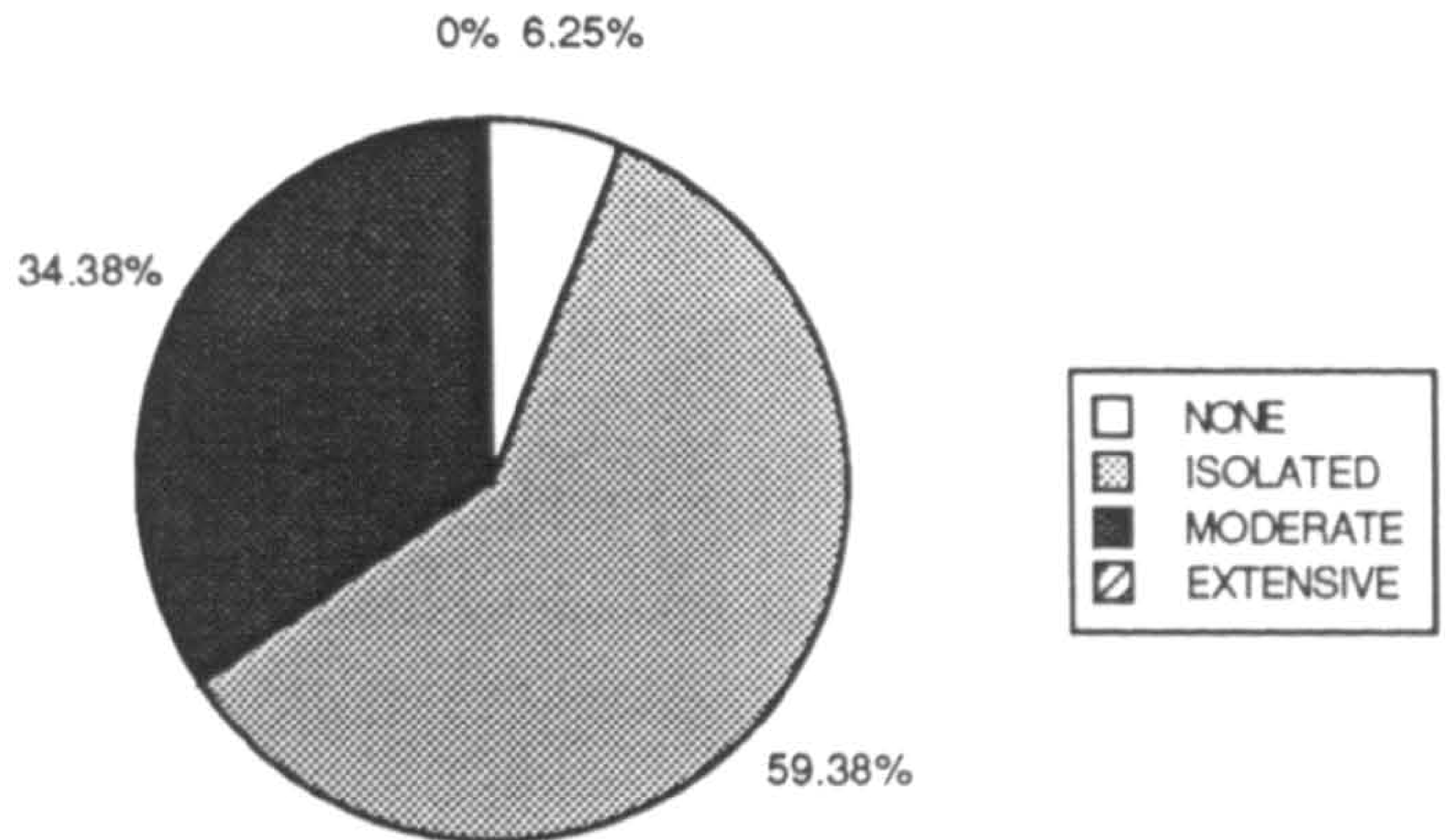
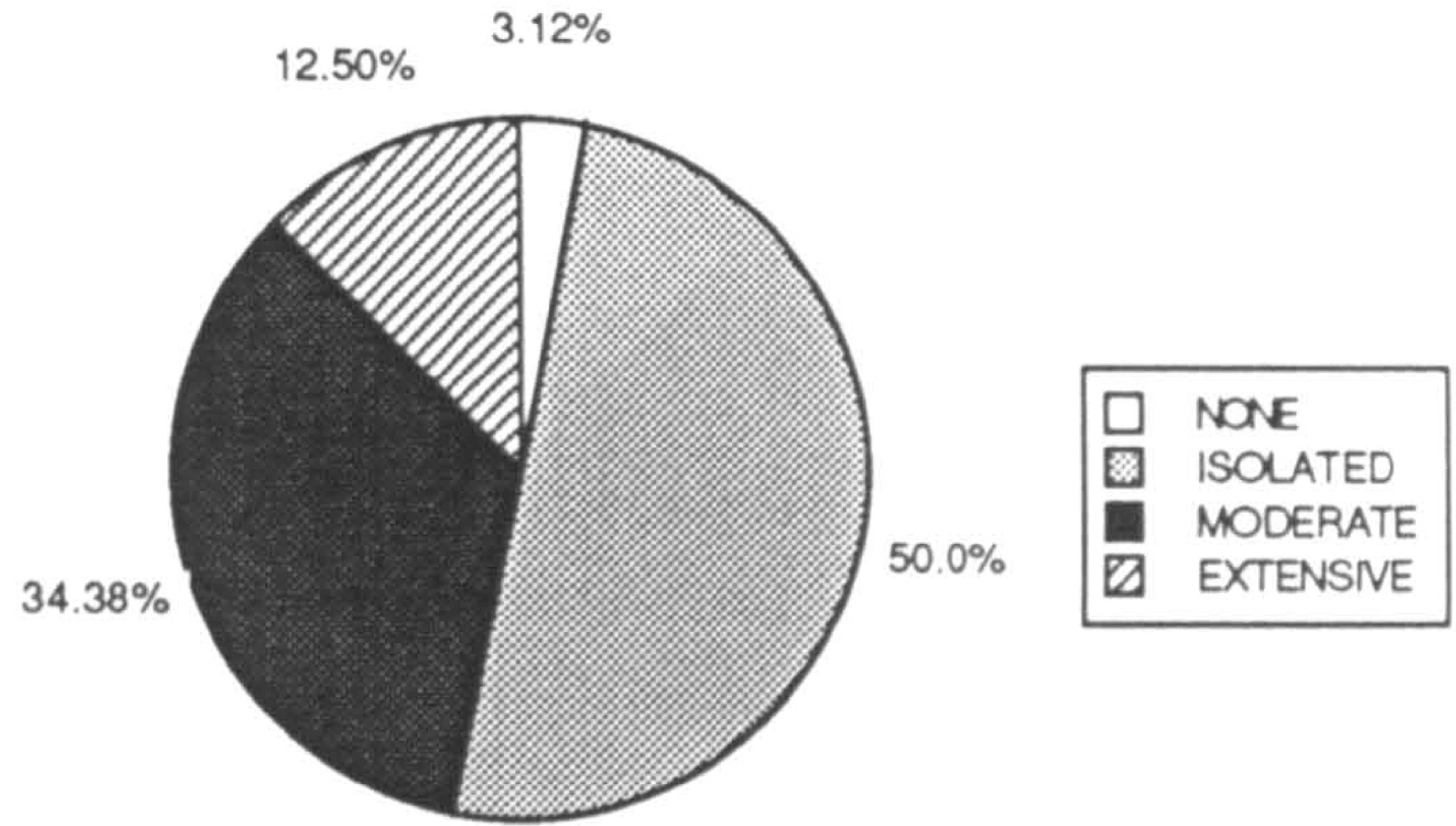


OCCURR. OF LATE FUSION IN THE "BAD" GROUP

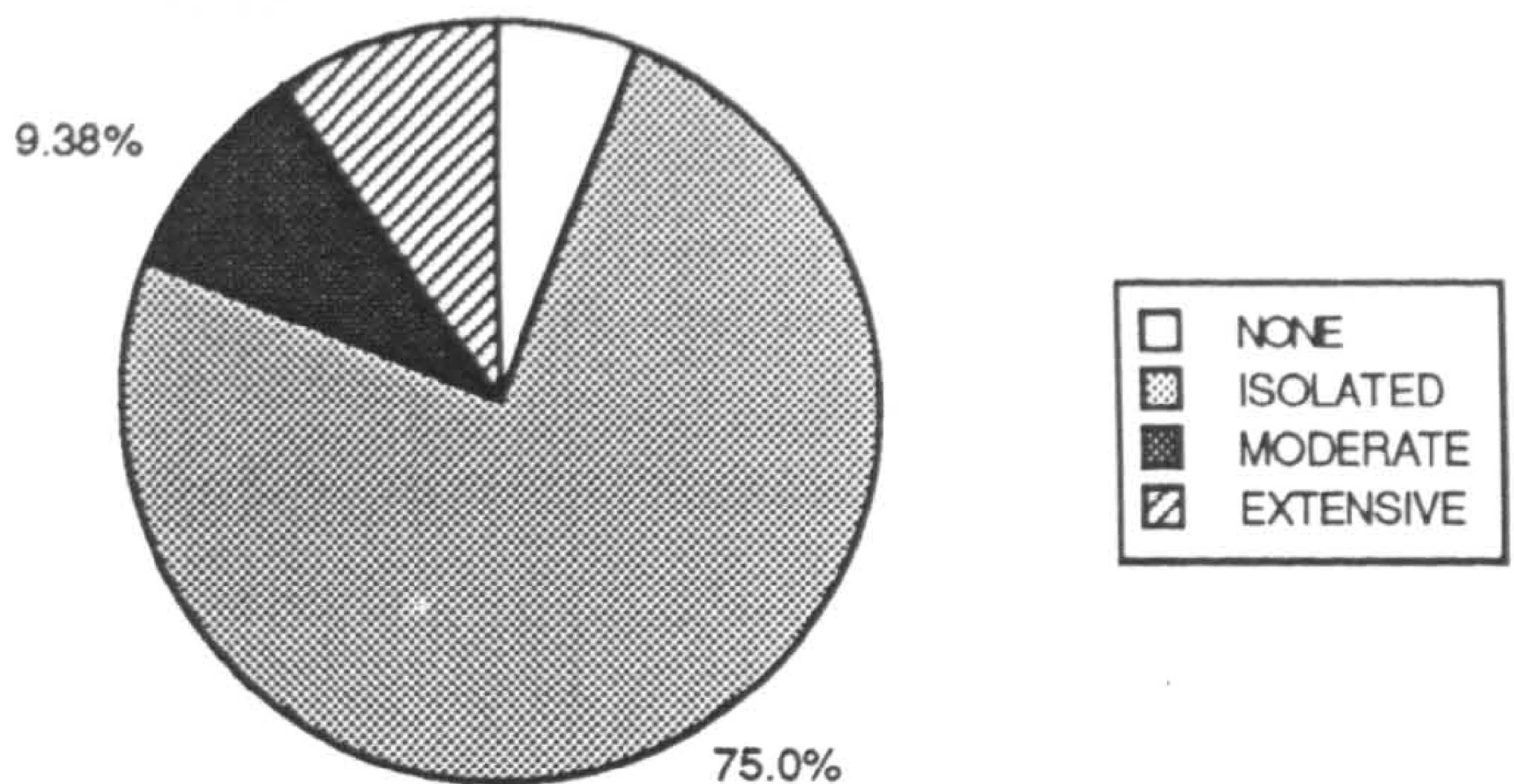
Table 12: Cont.



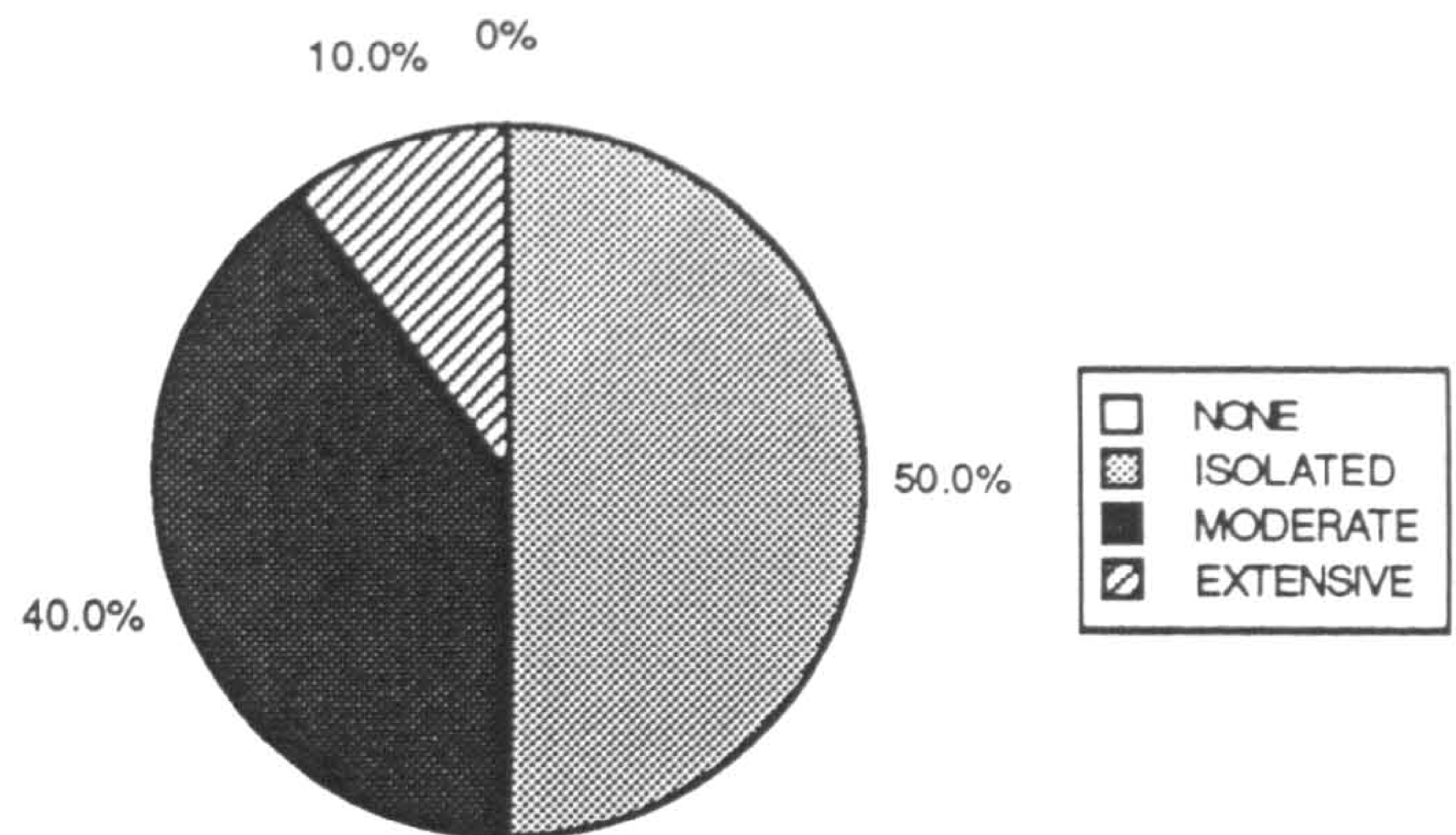
OCCURR. OF ALIGNMENT AT BEGINNING OF LAY



OCCURR. OF ALIGNMENT AT MIDDLE OF LAY

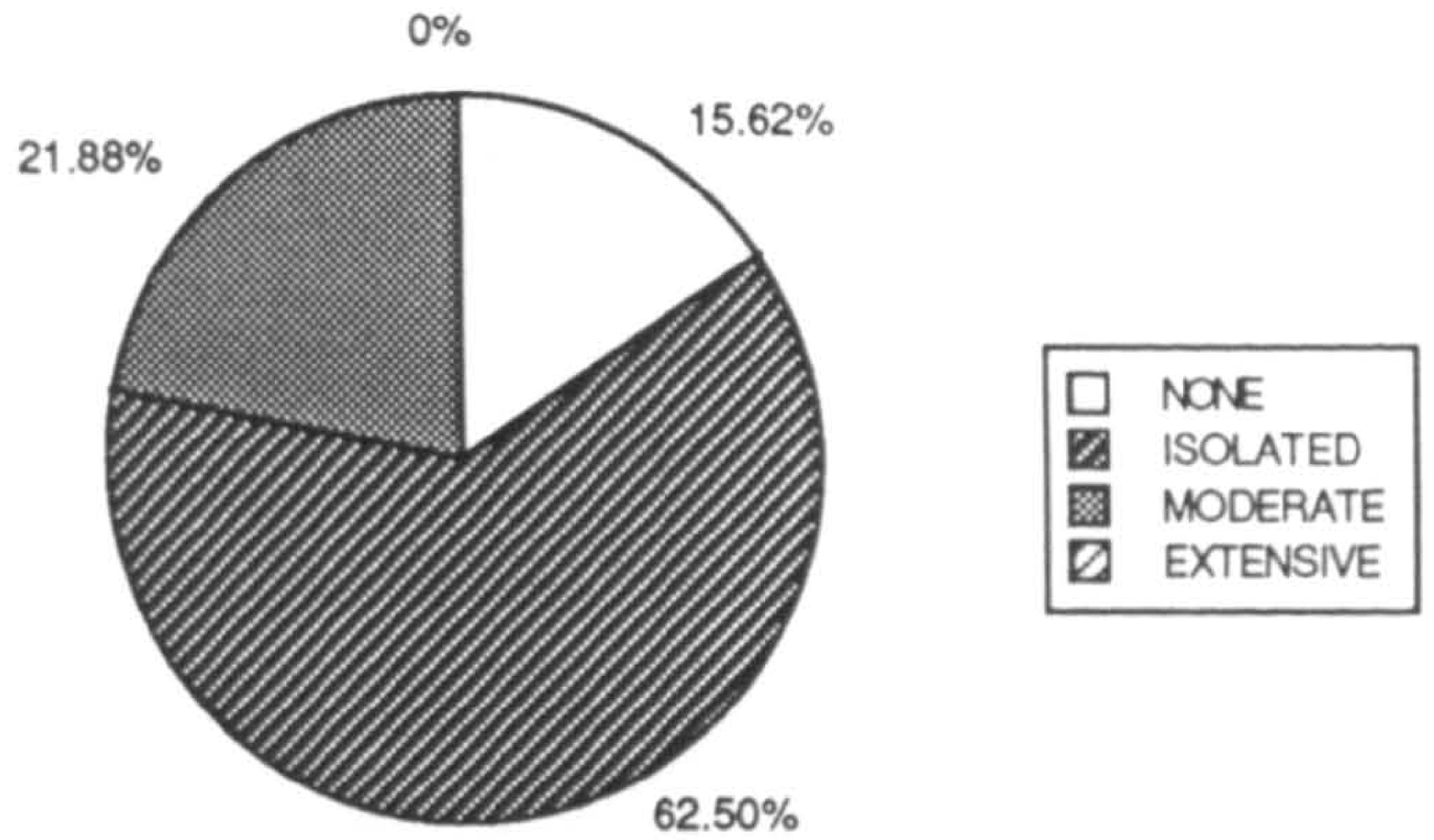


OCCURRENCE OF ALIGNMENT AT END OF LAY

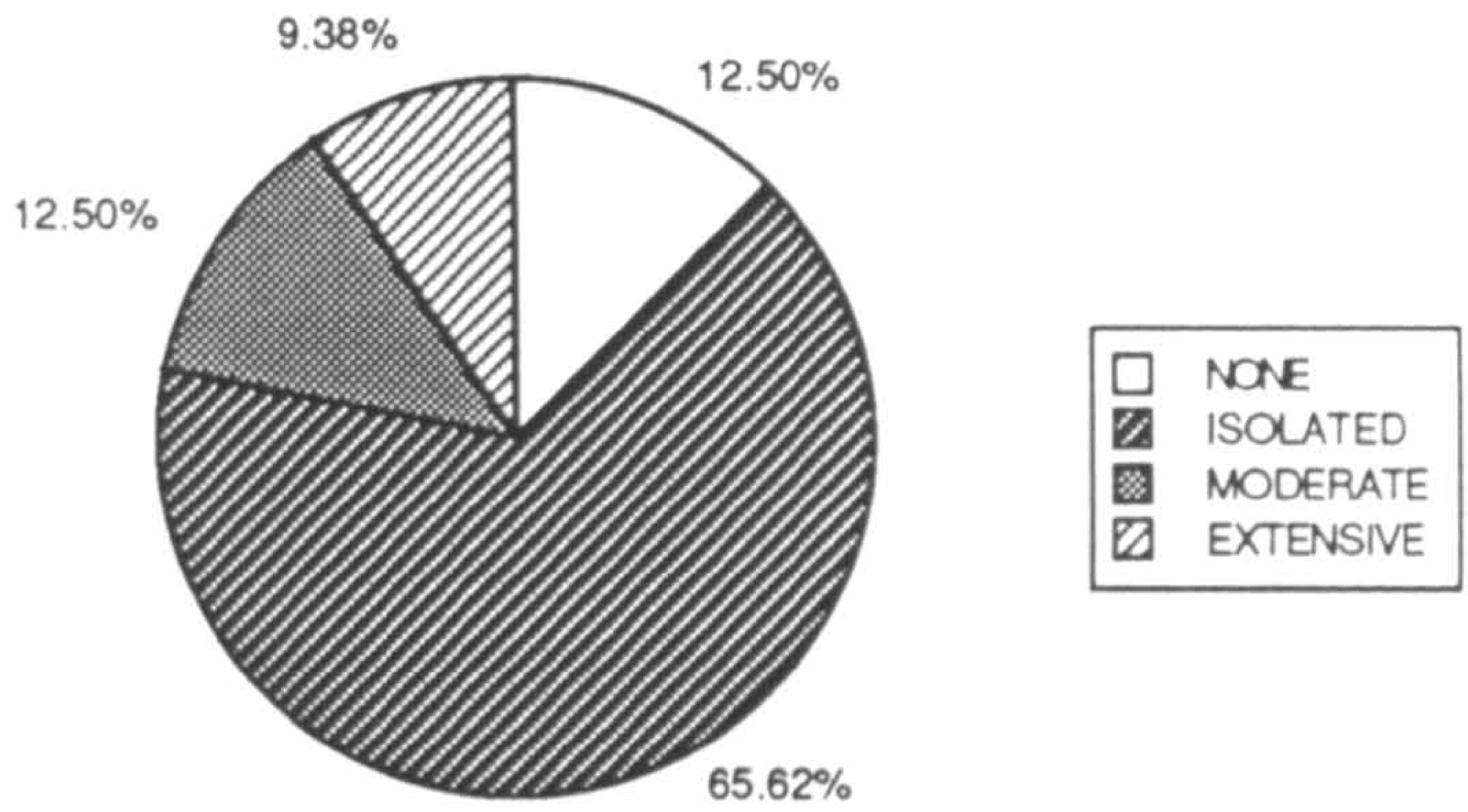


OCCURR. OF ALIGNMENT IN THE "BAD" GROUP

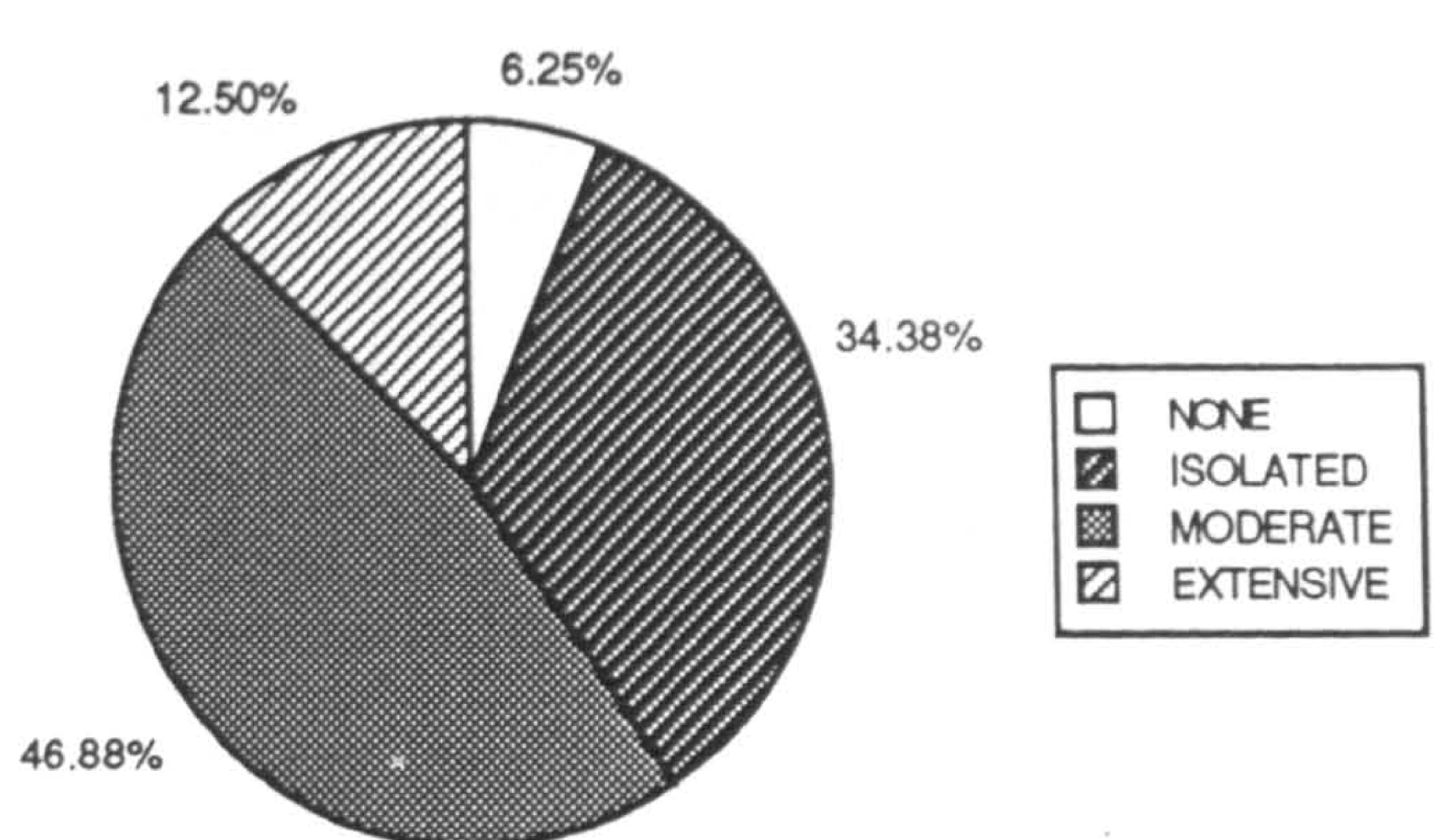
Table 12: Cont.



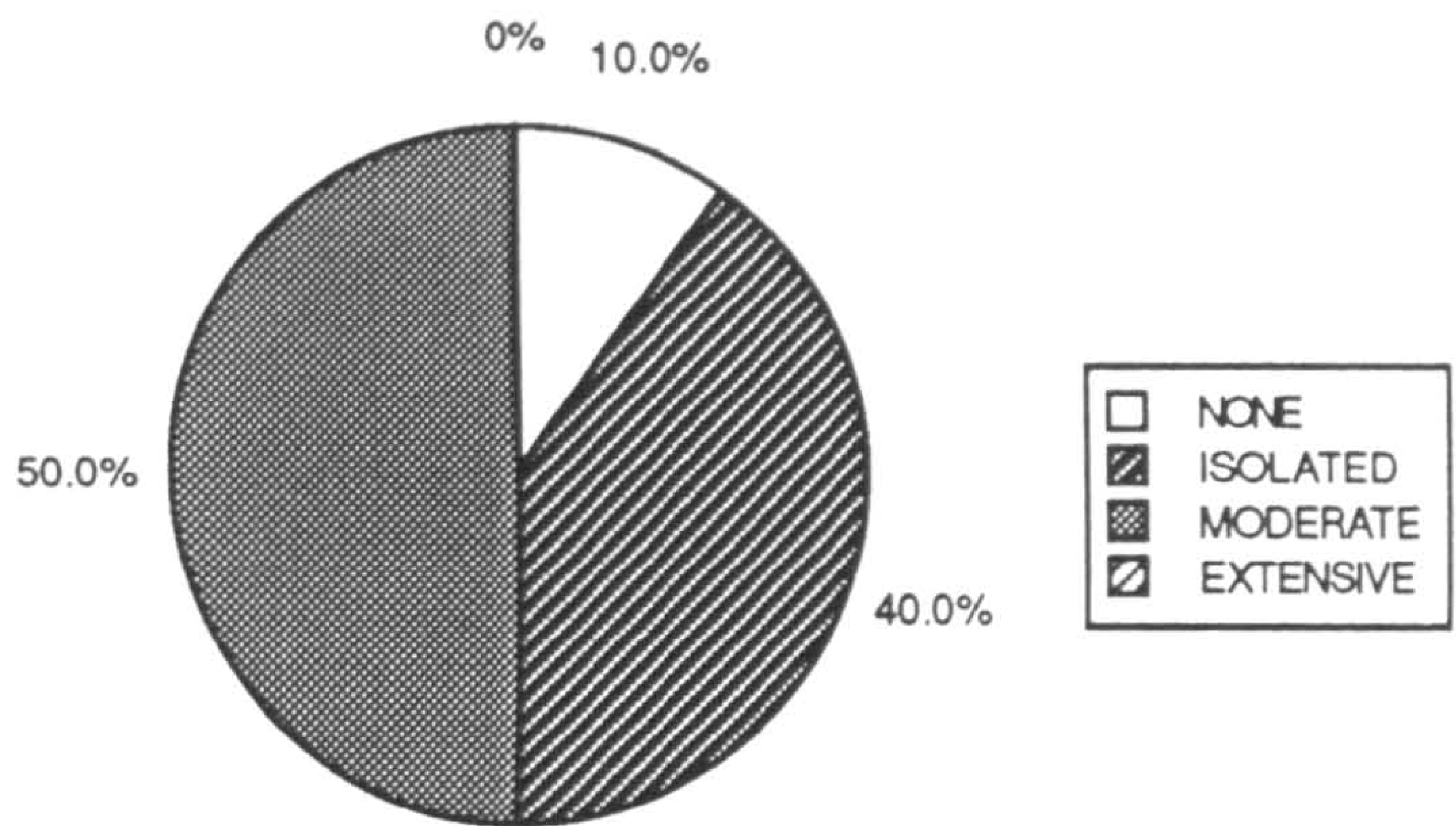
OCCURR. OF TYPE B's AT BEGINNING OF LAY



OCCURR. OF TYPE B's AT MIDDLE OF LAY

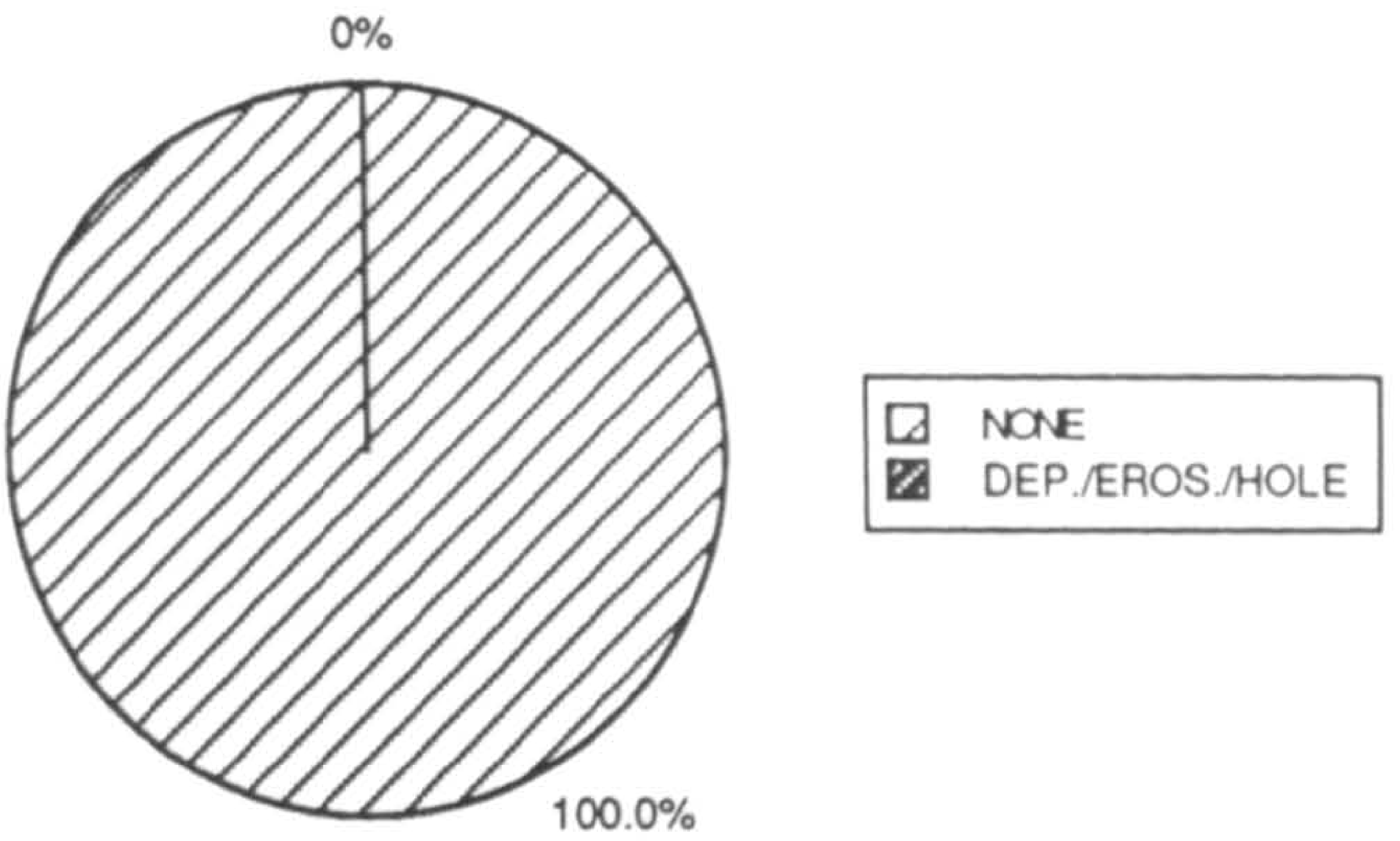


OCCURRENCE OF TYPE B's AT END OF LAY

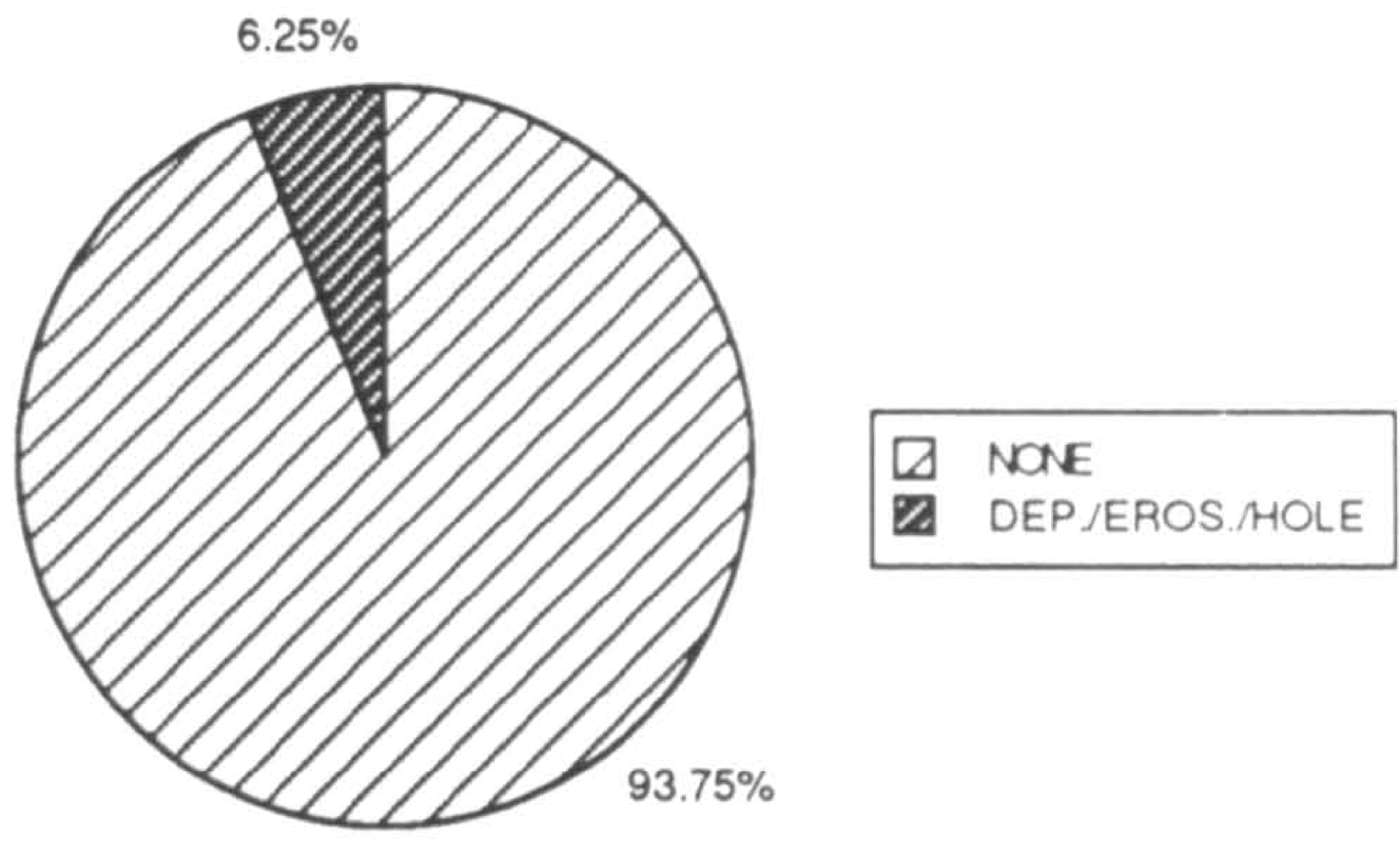


OCCURR. OF TYPE B's IN THE "BAD" GROUP

Table 12: Cont.



OCCURR. OF PITTING AT BEGINNING OF LAY



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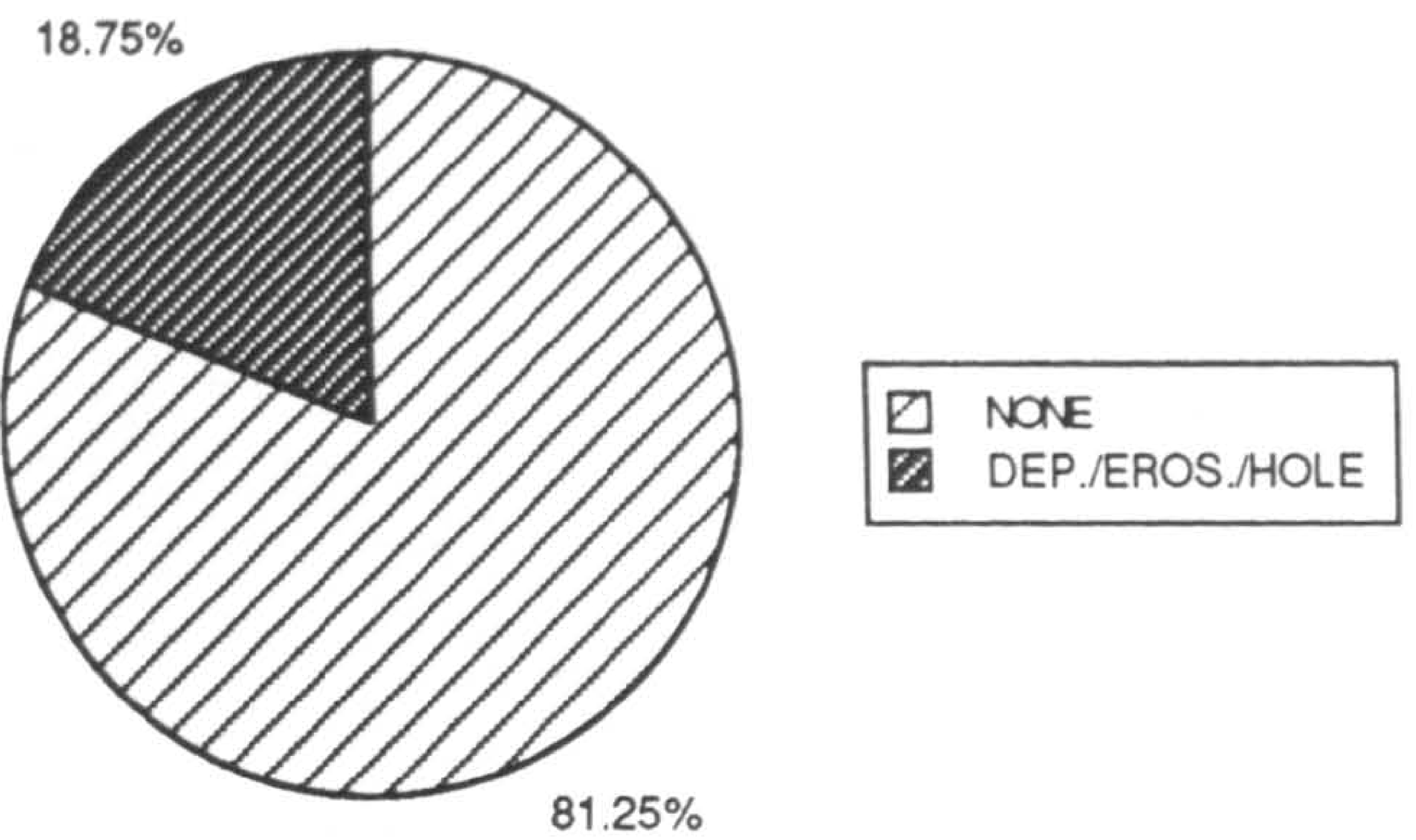

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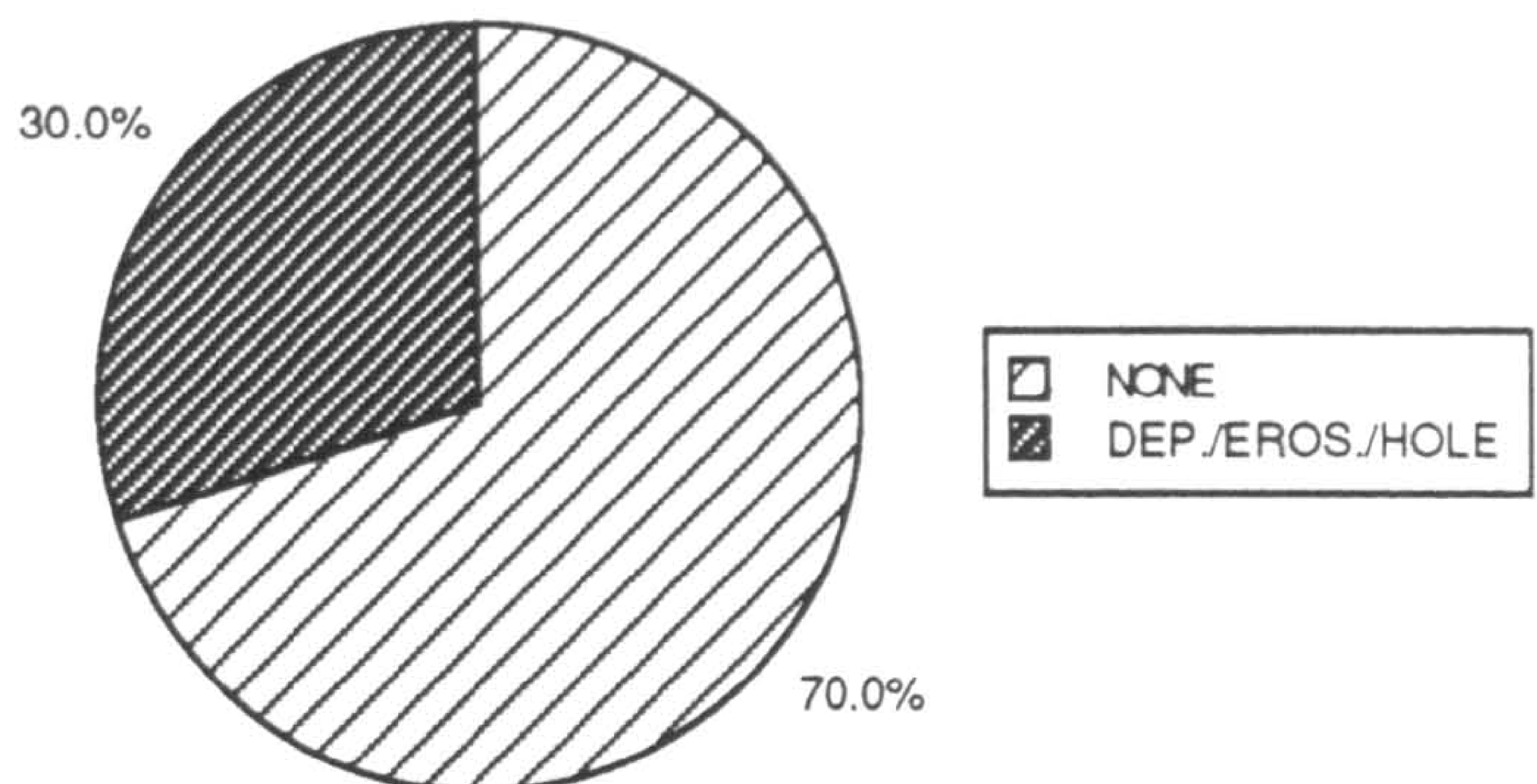
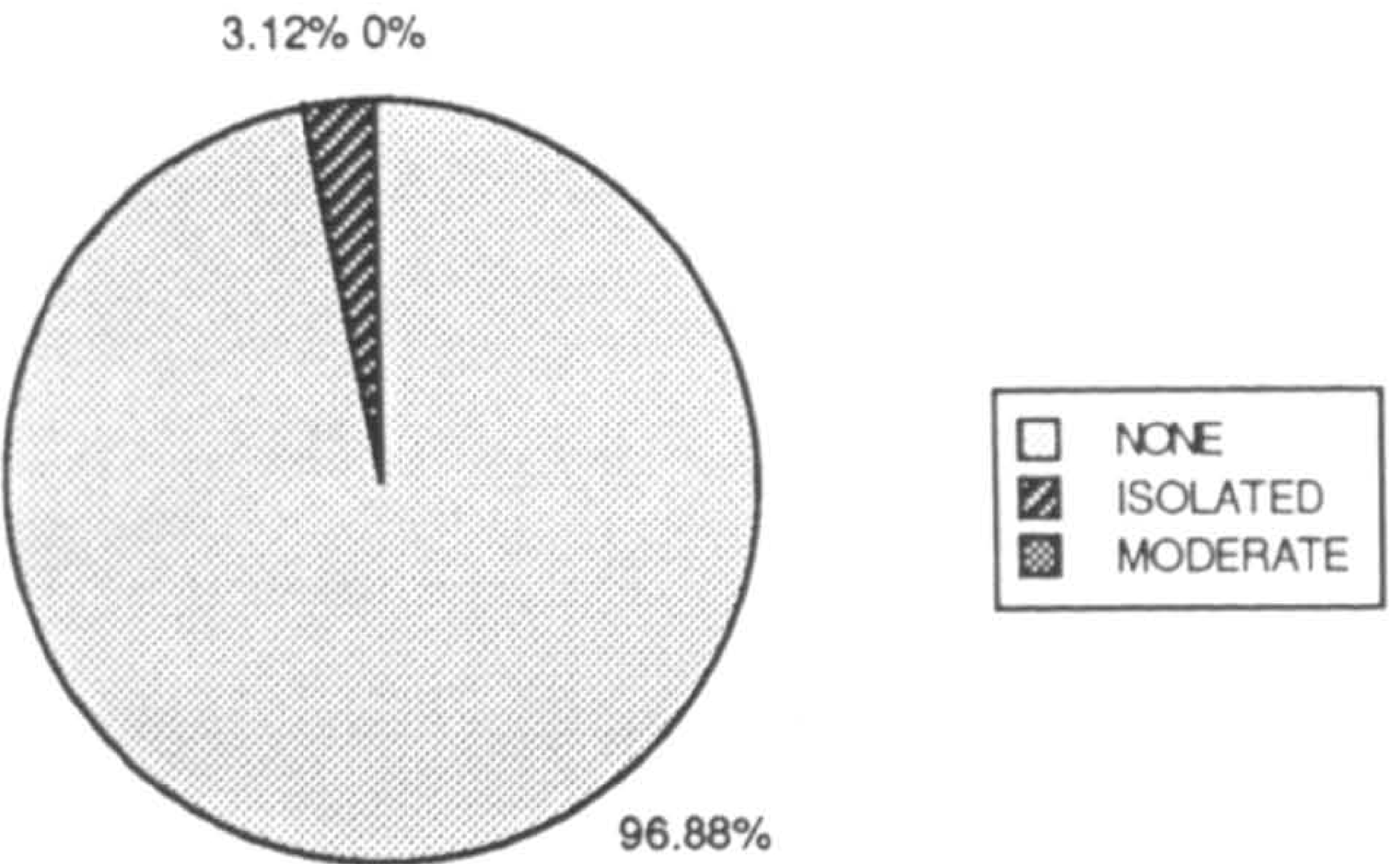
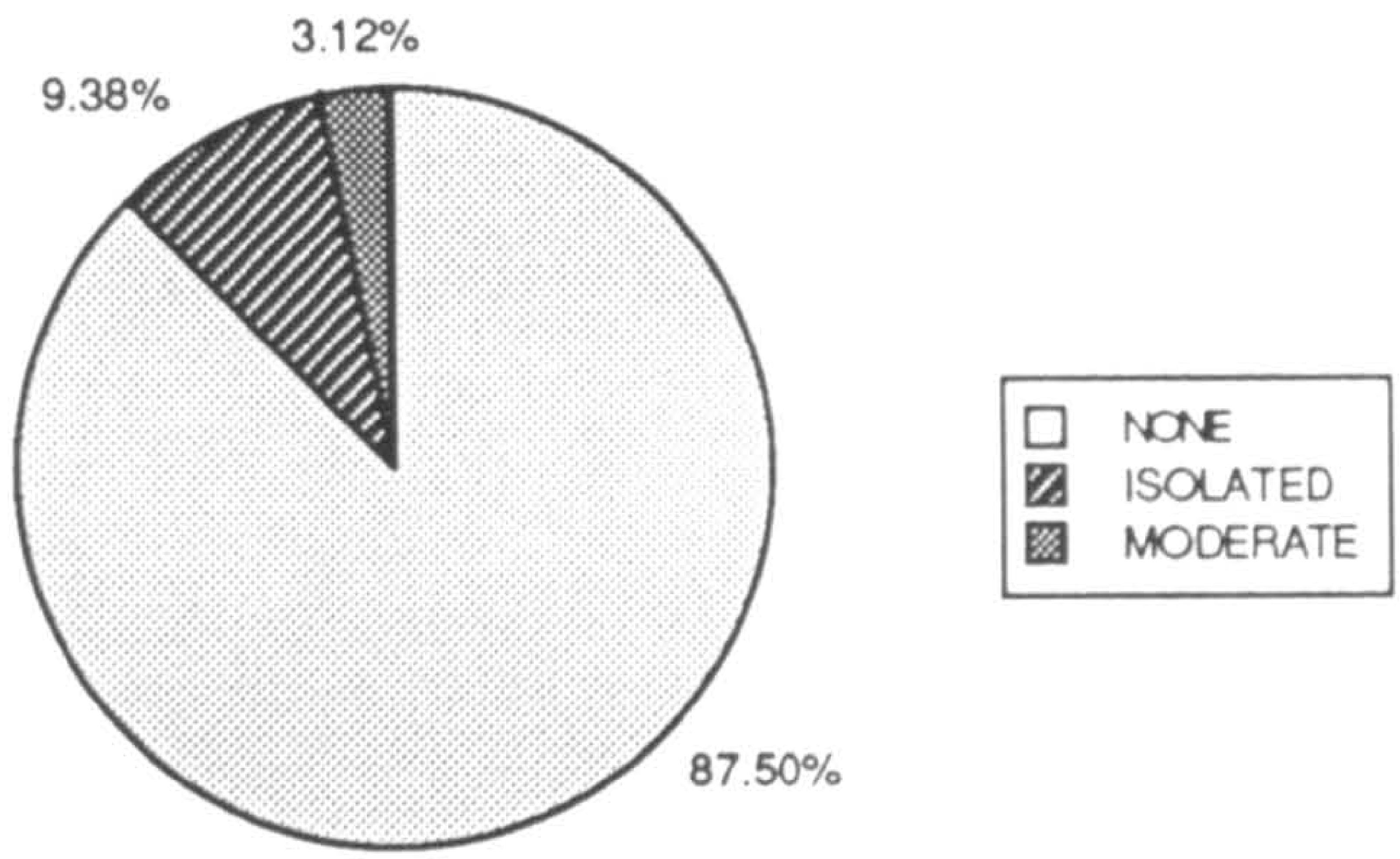


OCCURR. OF PITTING IN THE "BAD" GROUP

Table 12: Cont.



OCCURR. OF ARAGONITE AT BEGINNING OF LAY



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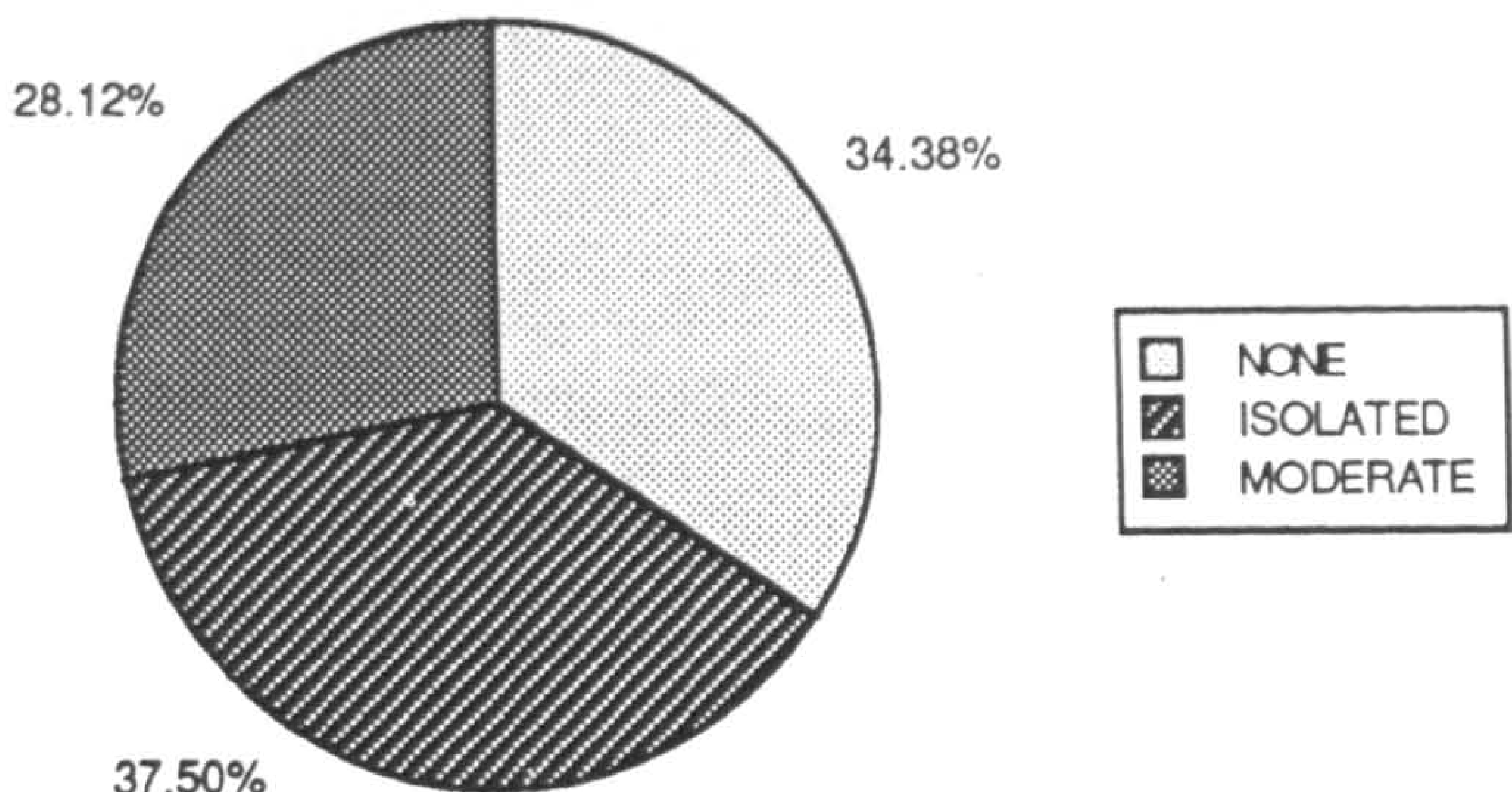
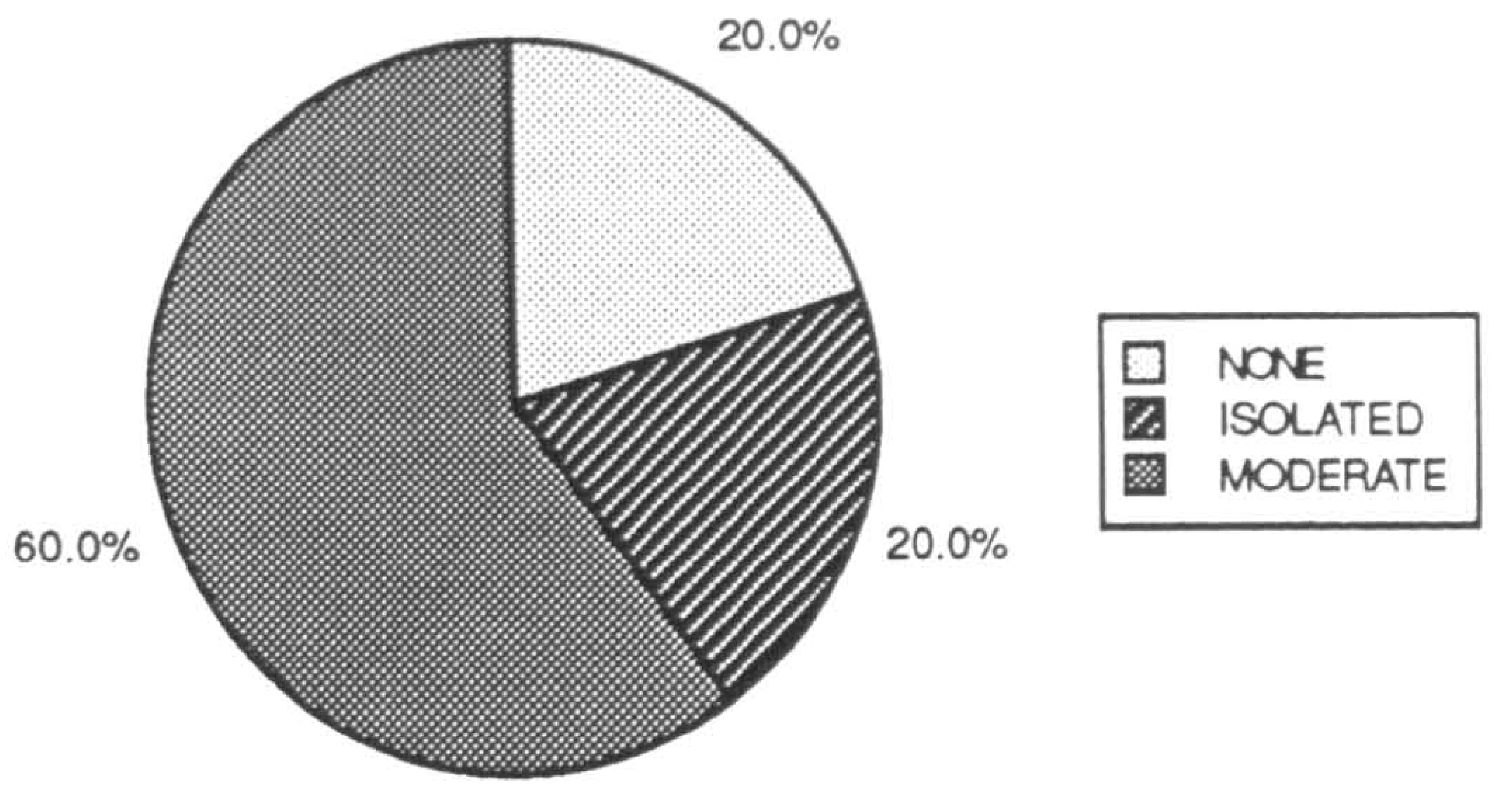
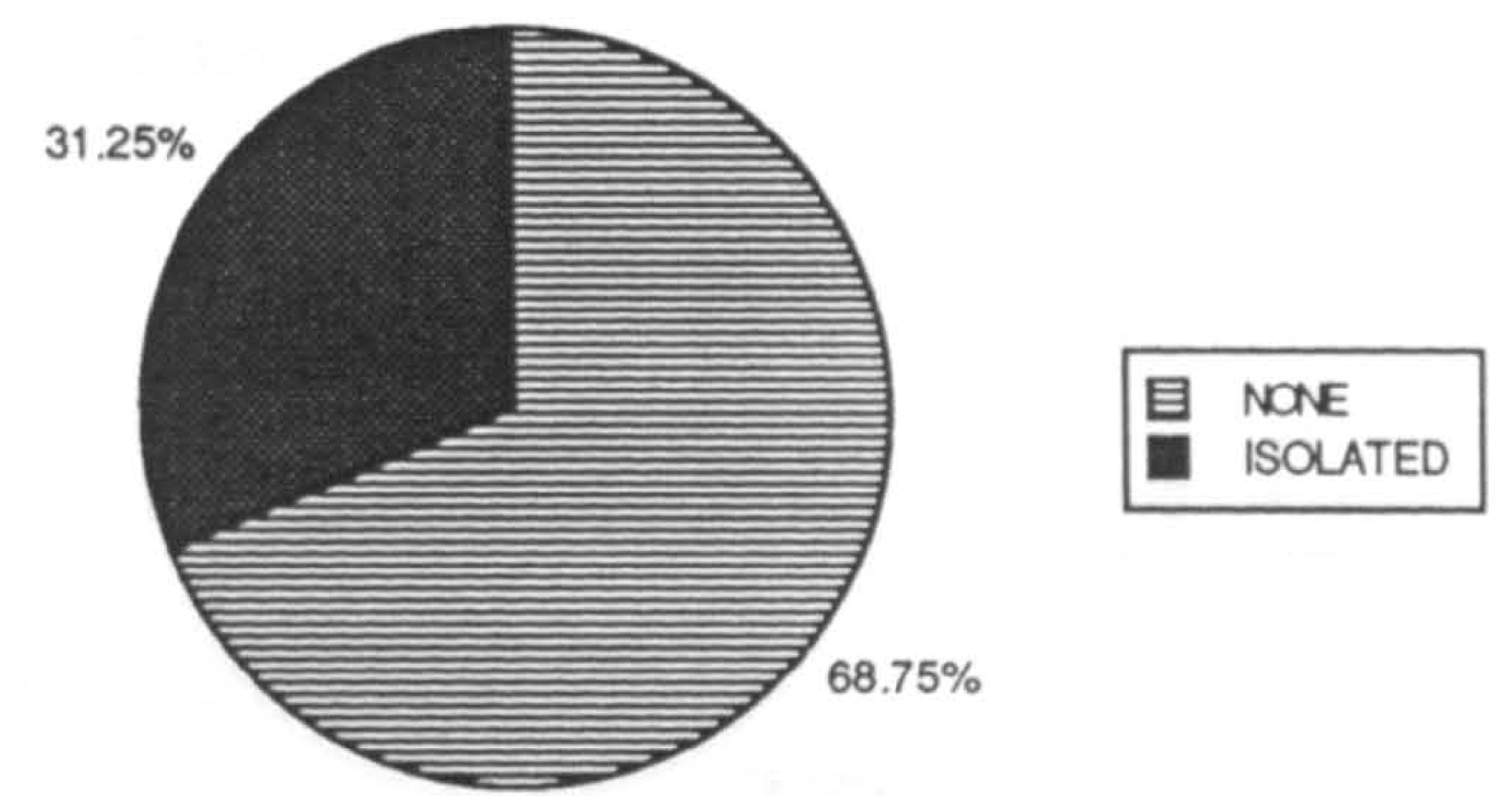


OCCURR. OF ARAGONITE AT END OF LAY

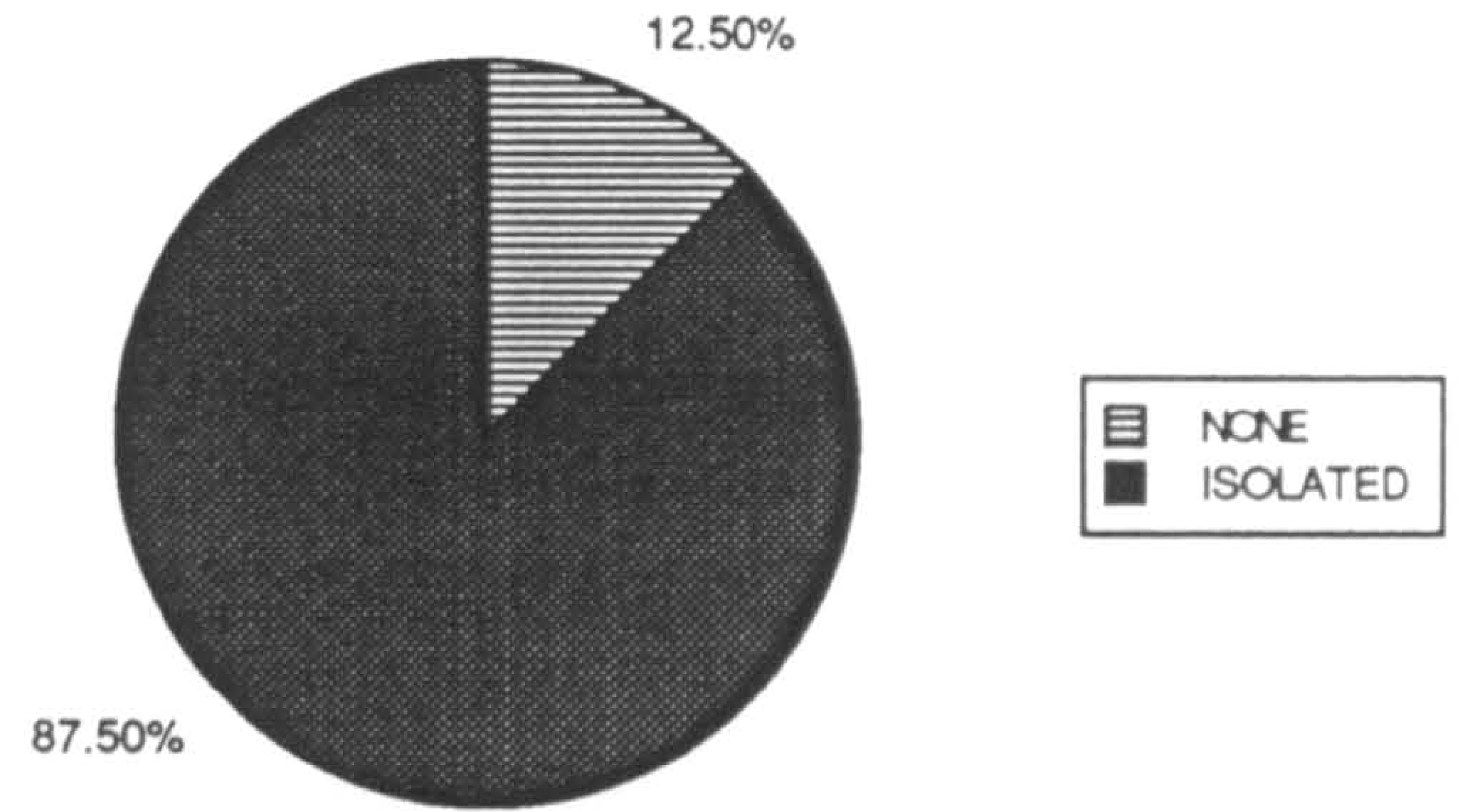


OCCURR. OF ARAGONITE IN THE "BAD" GROUP

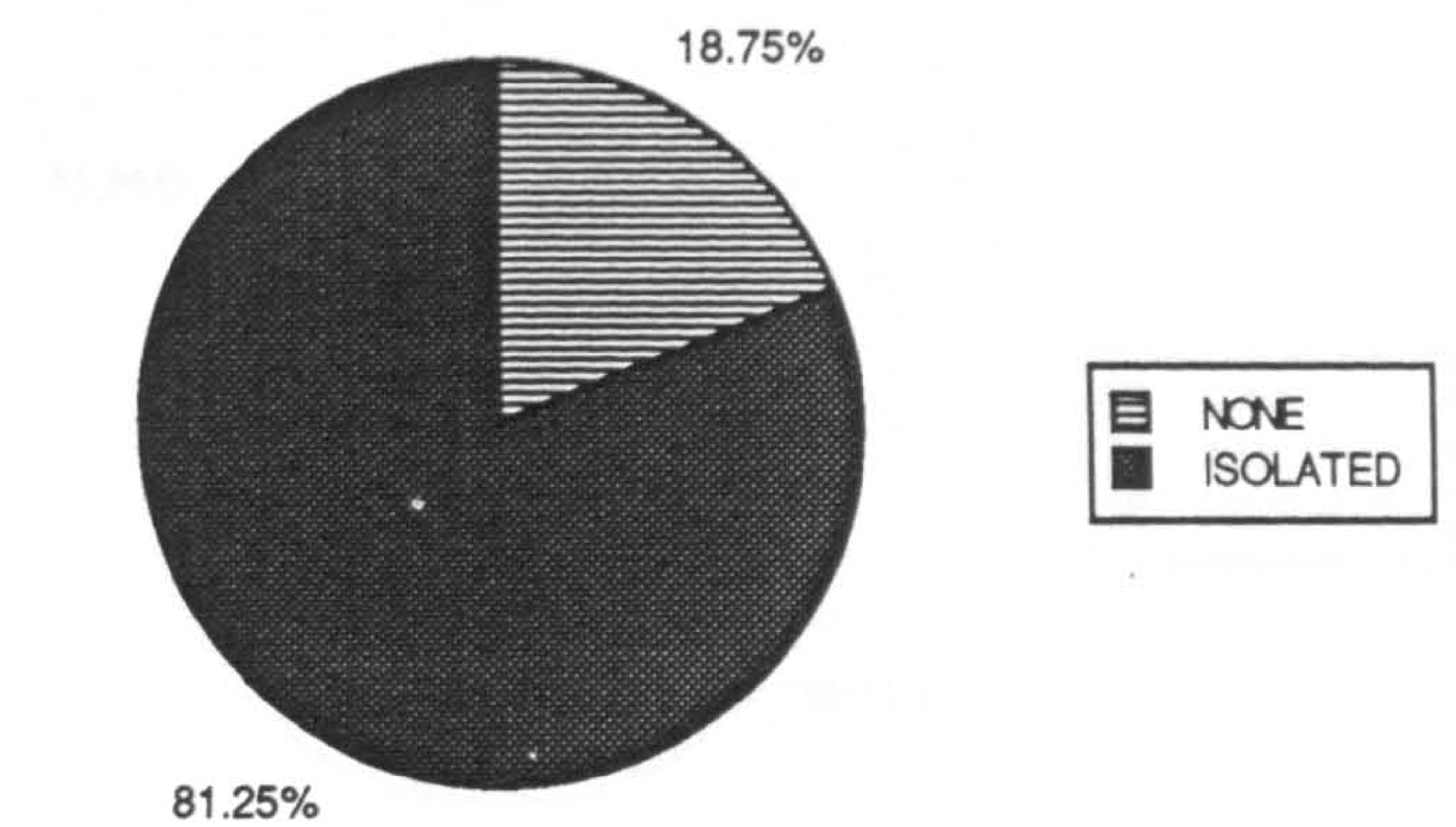
Table 12: Cont.



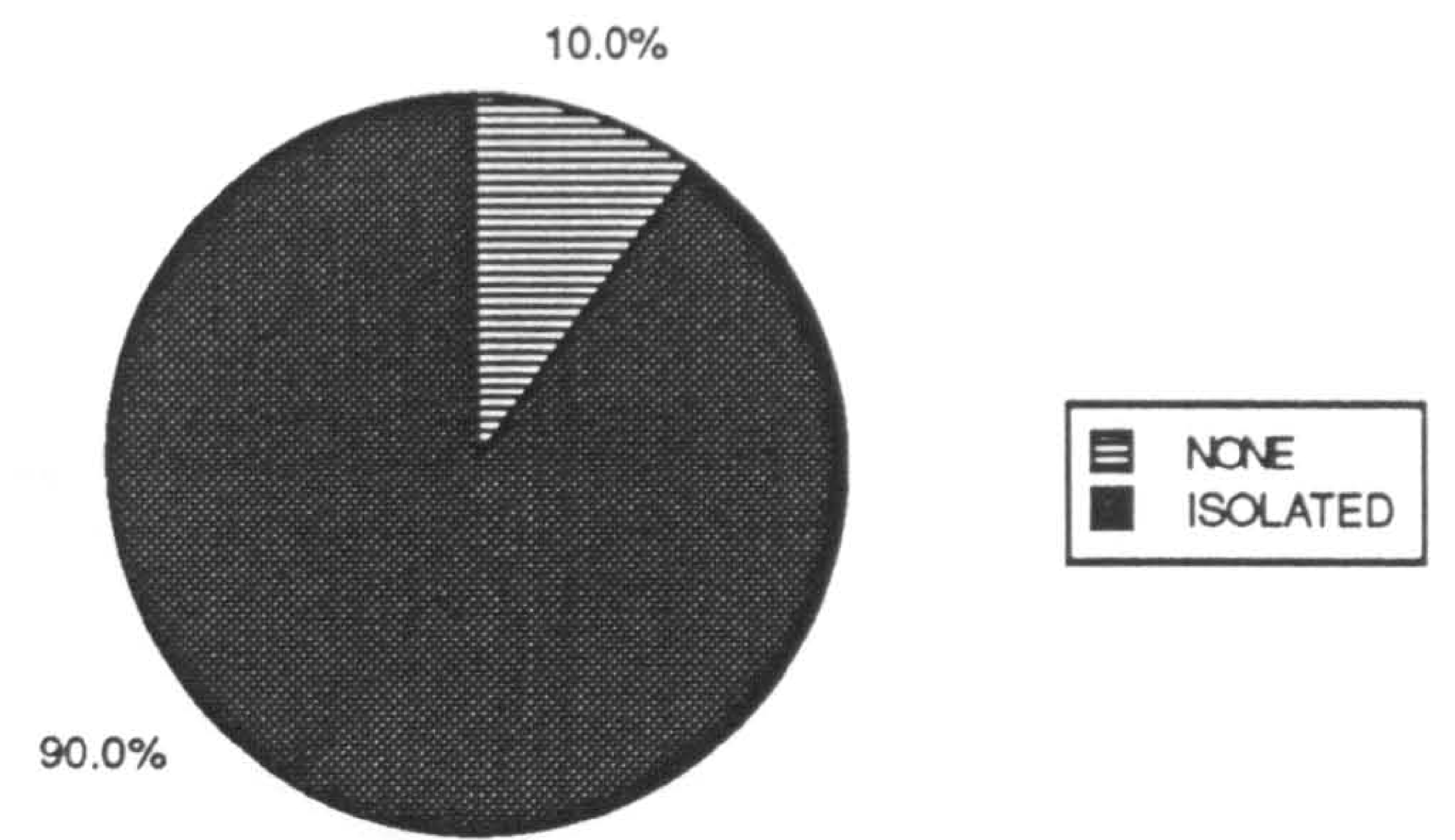
OCCURR. OF TYPE A's AT BEGINNING OF LAY



OCCURR. OF TYPE A's AT MIDDLE OF LAY

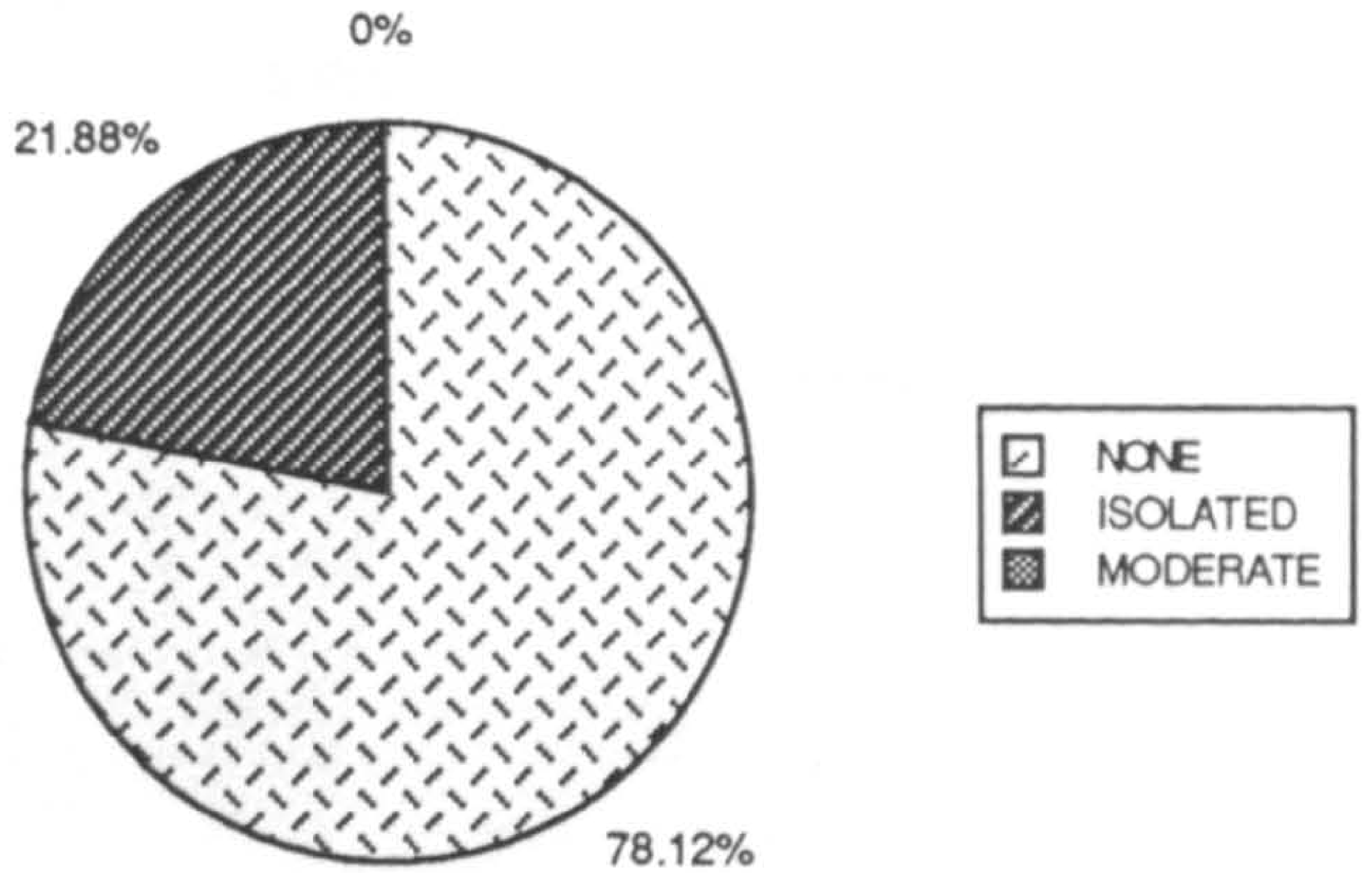


OCCURR. OF TYPE A's AT END OF LAY

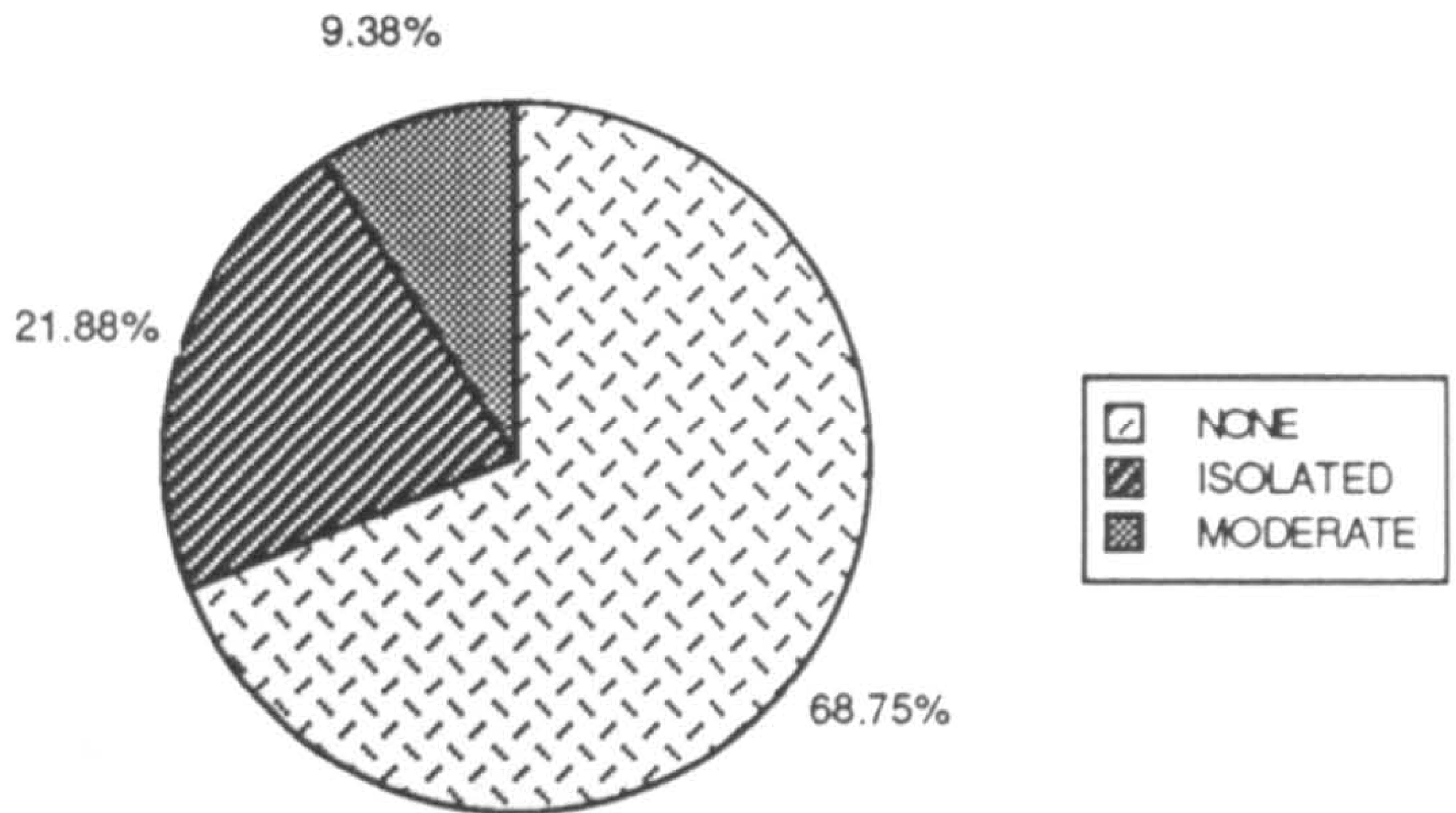


OCCURR. OF TYPE A's IN THE "BAD" GROUP

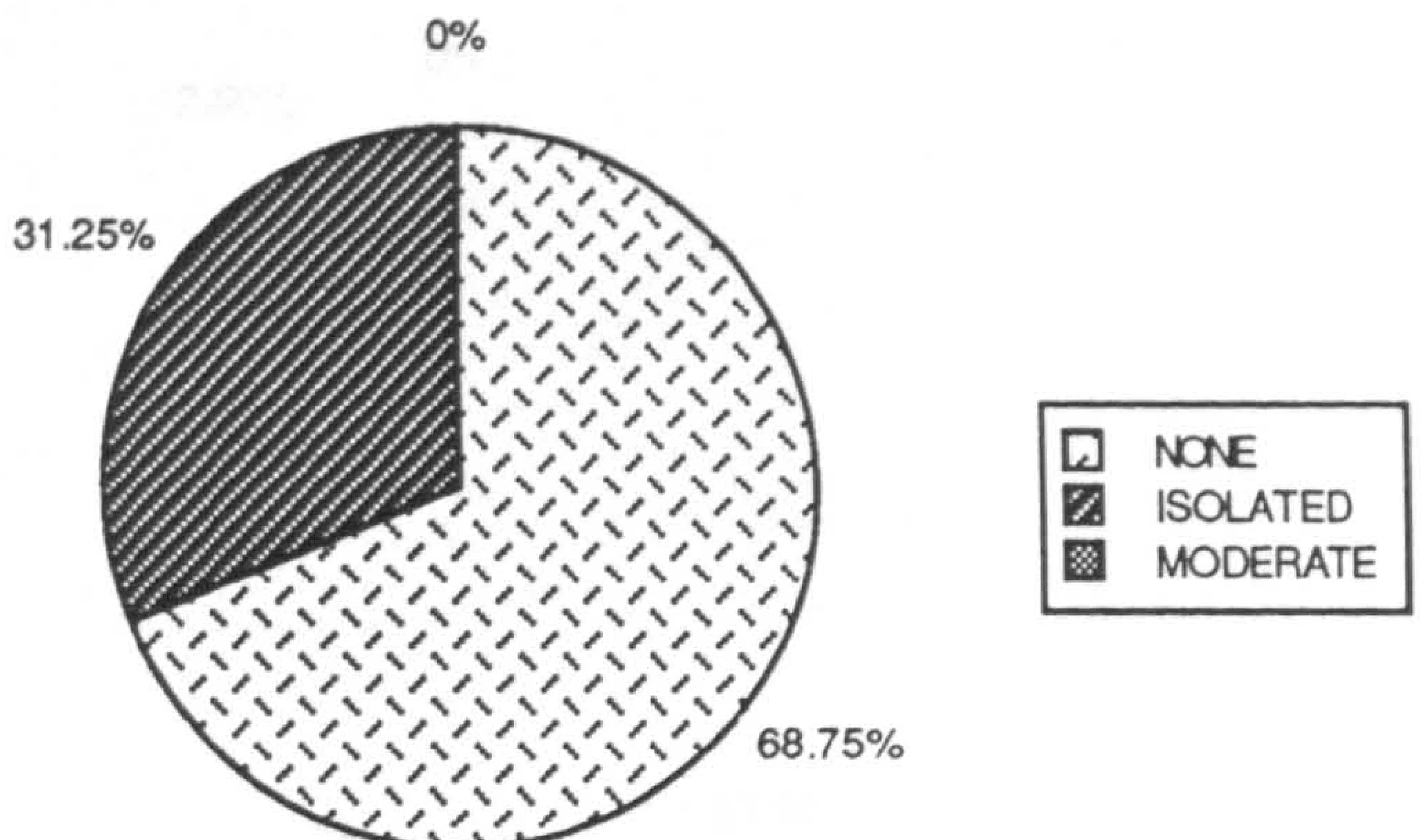
Table 12: Cont.



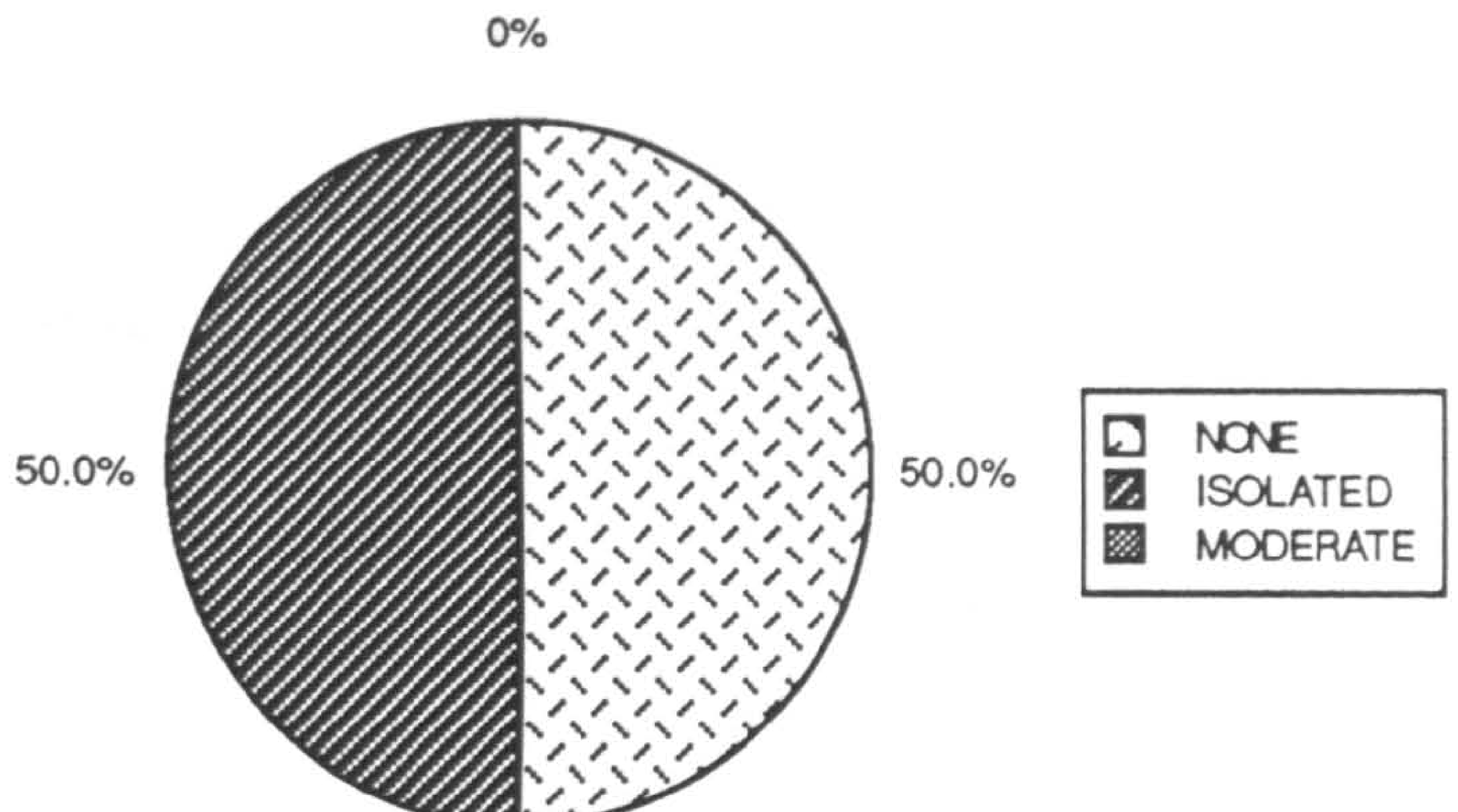
OCCURR. OF CUBICS AT BEGINNING OF LAY



OCCURR. OF CUBICS AT MIDDLE OF LAY

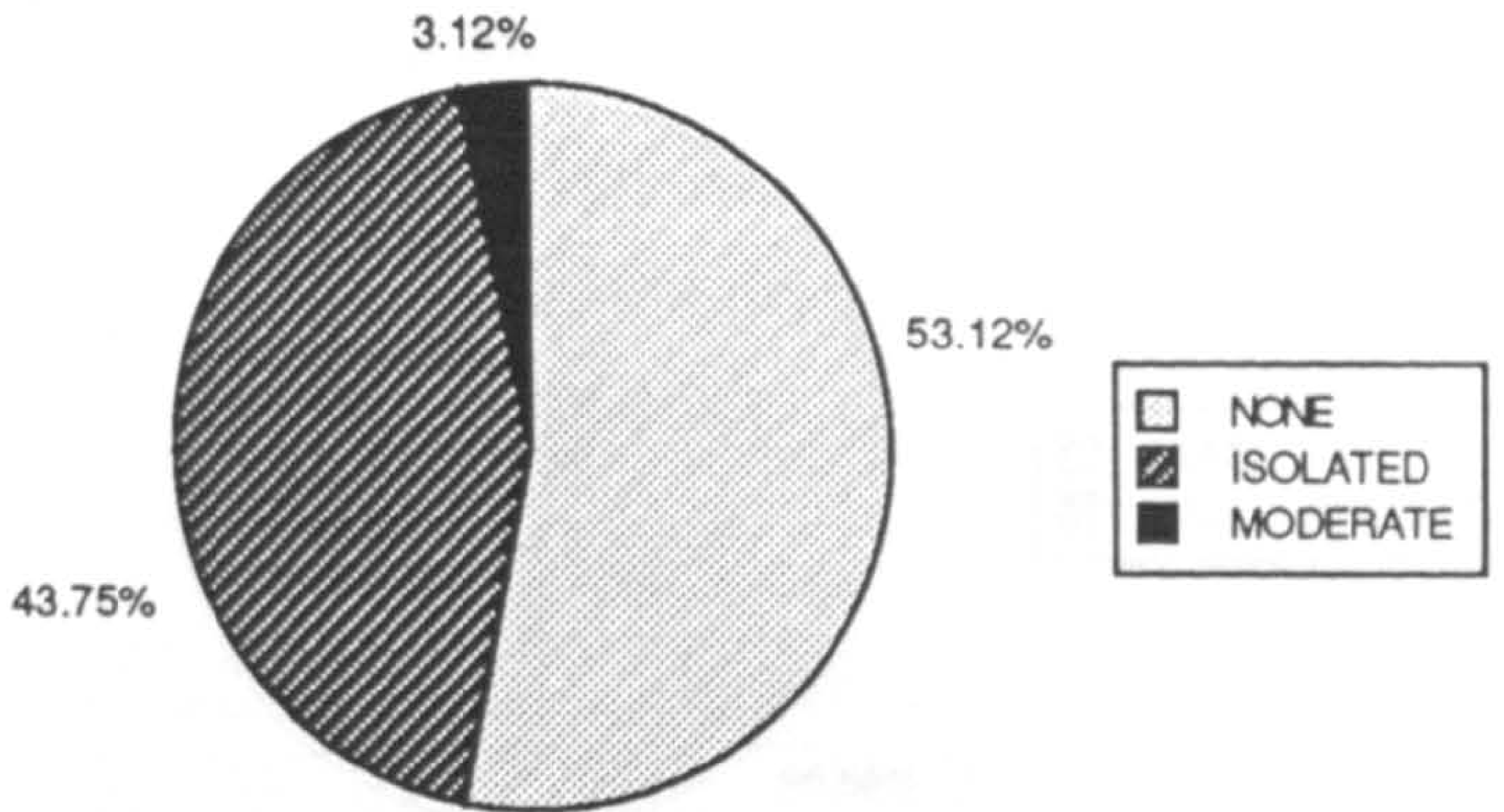


OCCURRENCE OF CUBICS AT END OF LAY

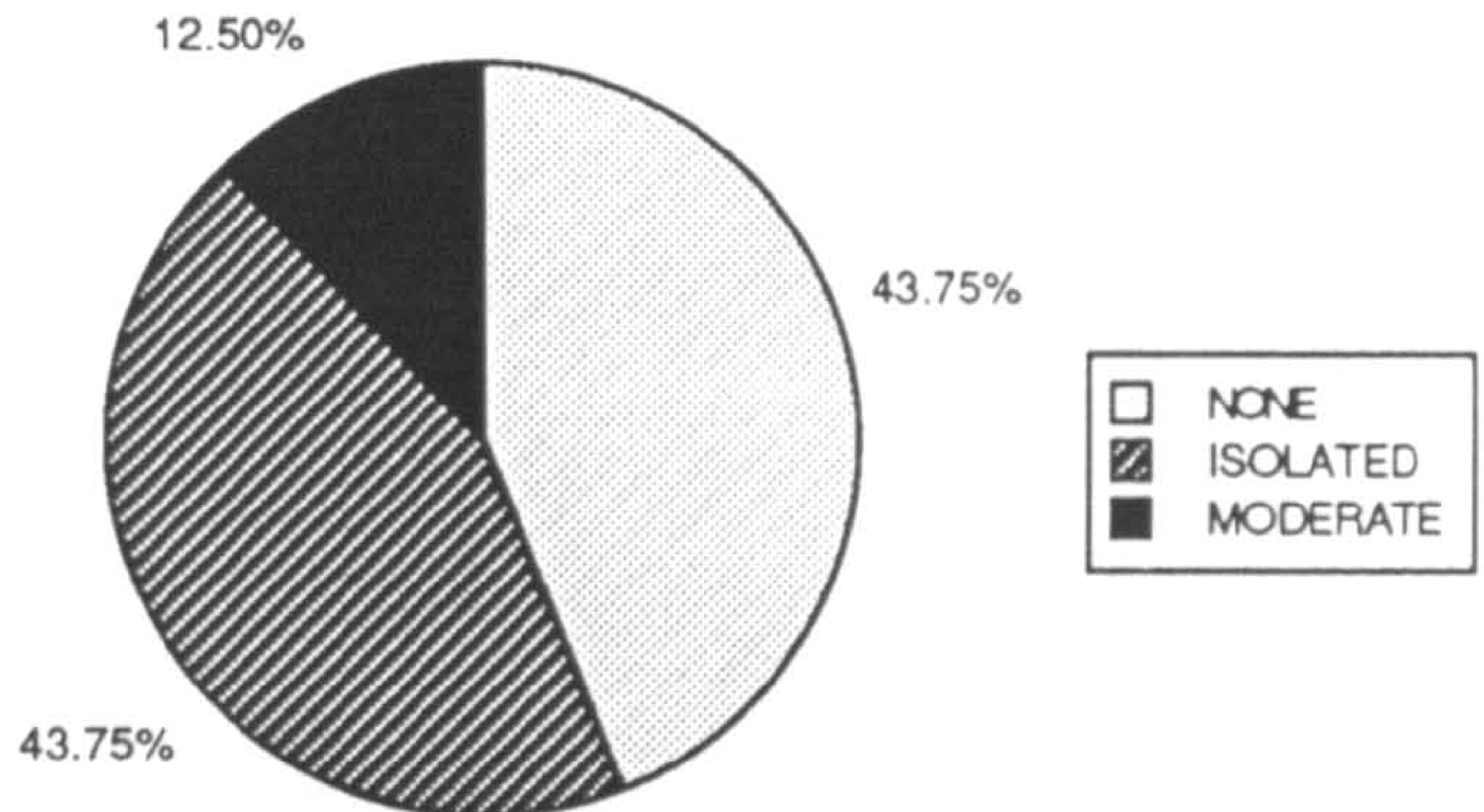


OCCURR. OF CUBICS IN THE "BAD" GROUP

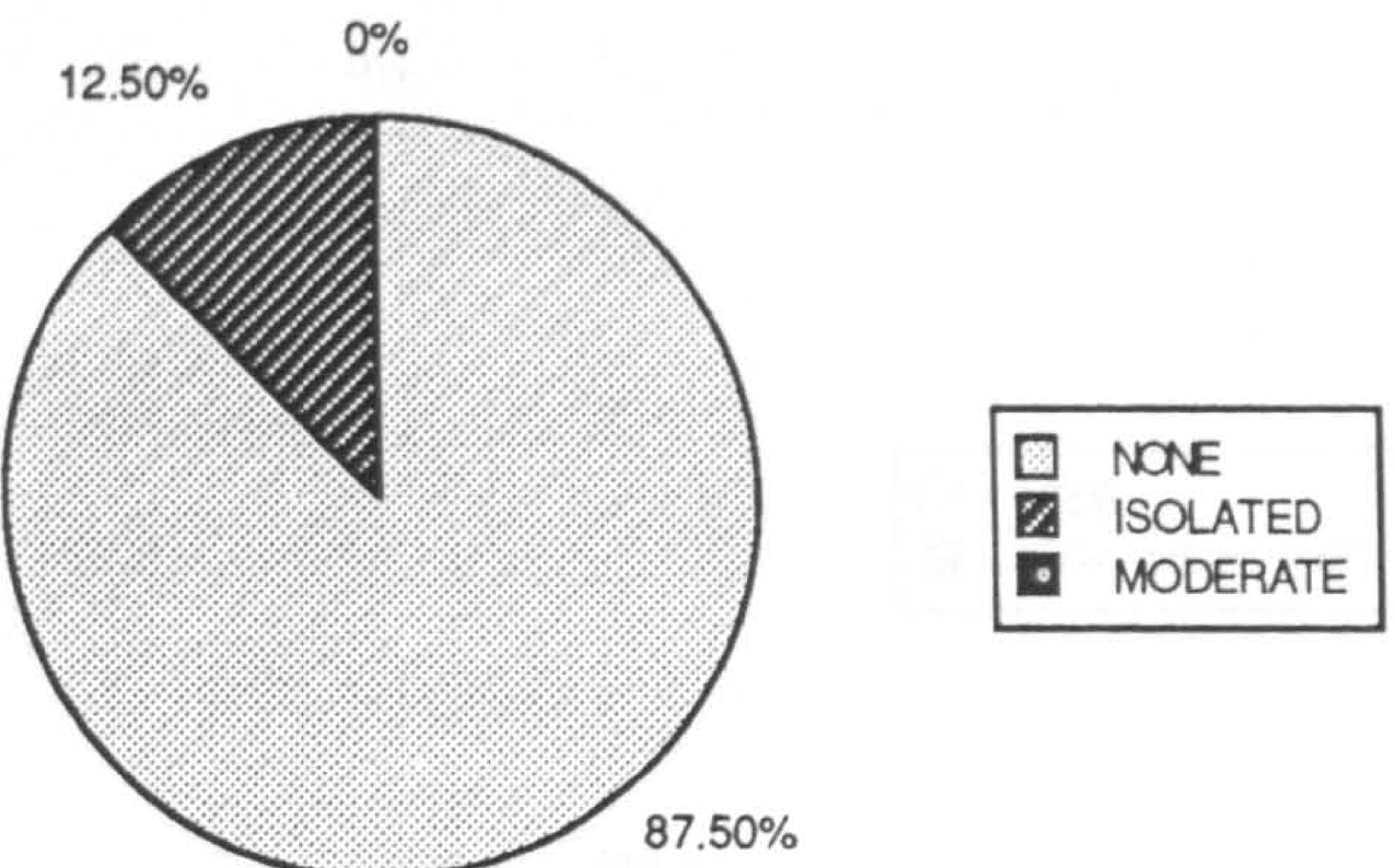
Table 12: Cont.



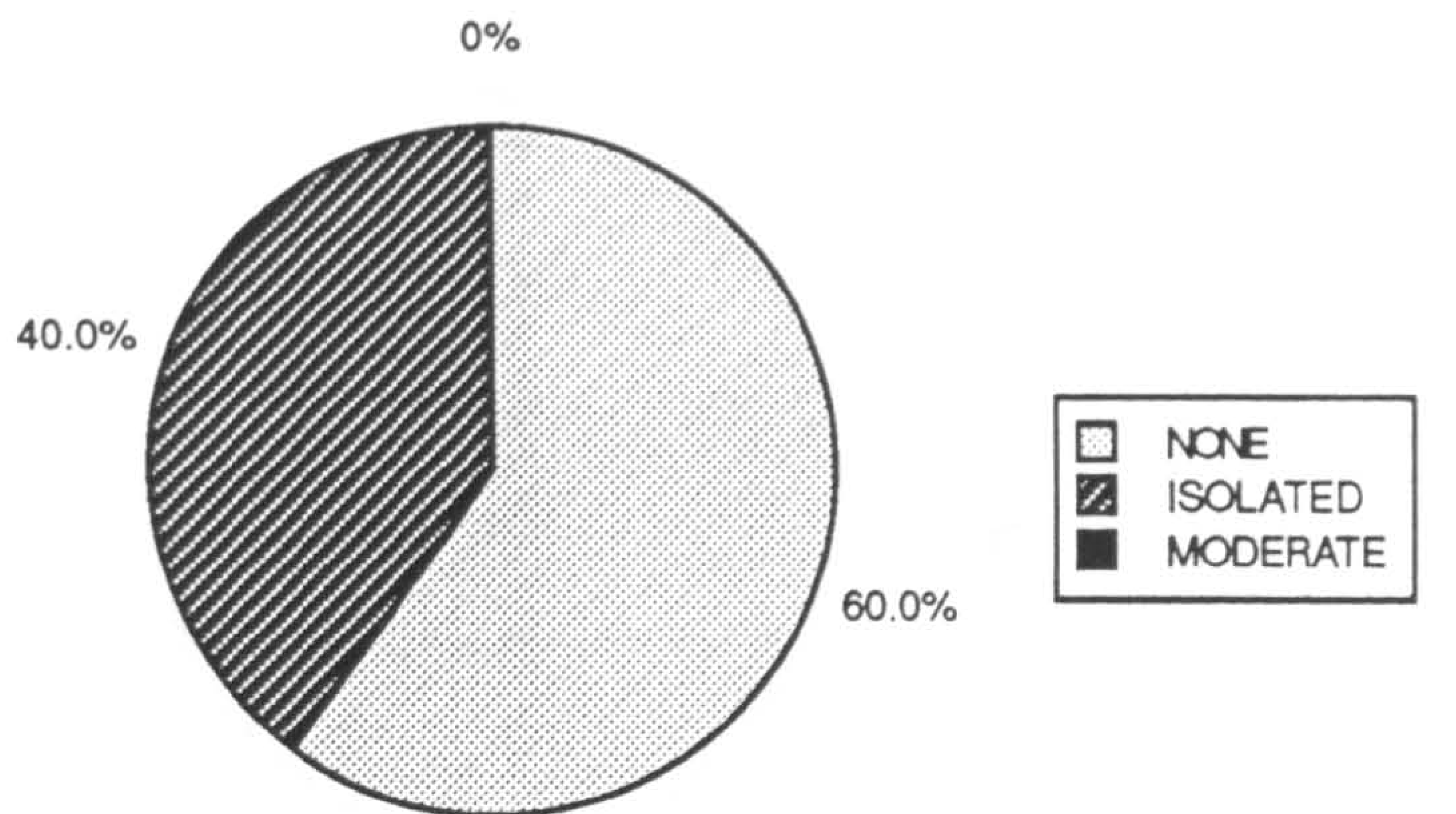
OCCURR. OF CUFFING AT BEGINNING OF LAY



OCCURR. OF CUFFING AT MIDDLE OF LAY

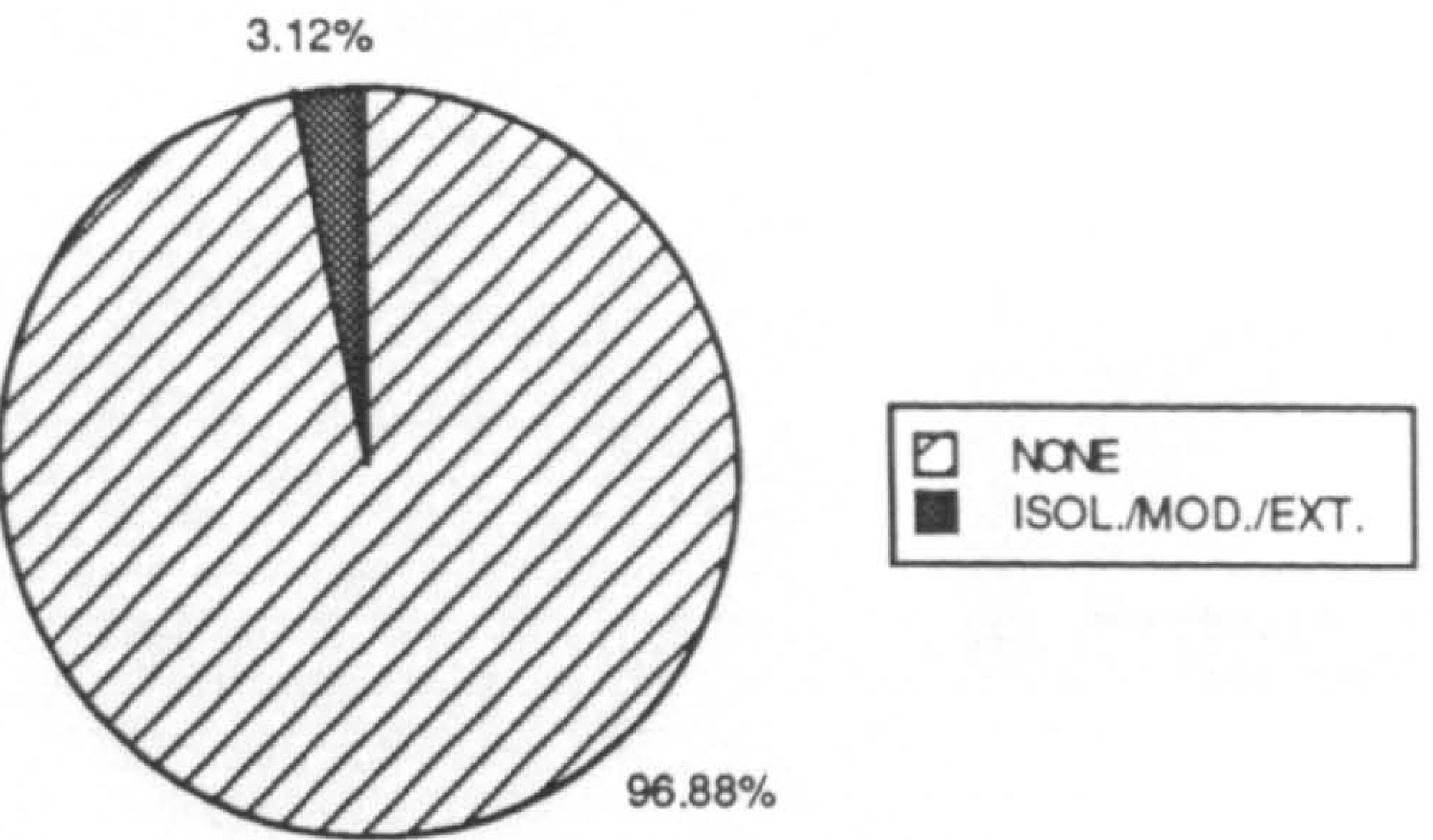


OCCURR. OF CUFFING AT END OF LAY

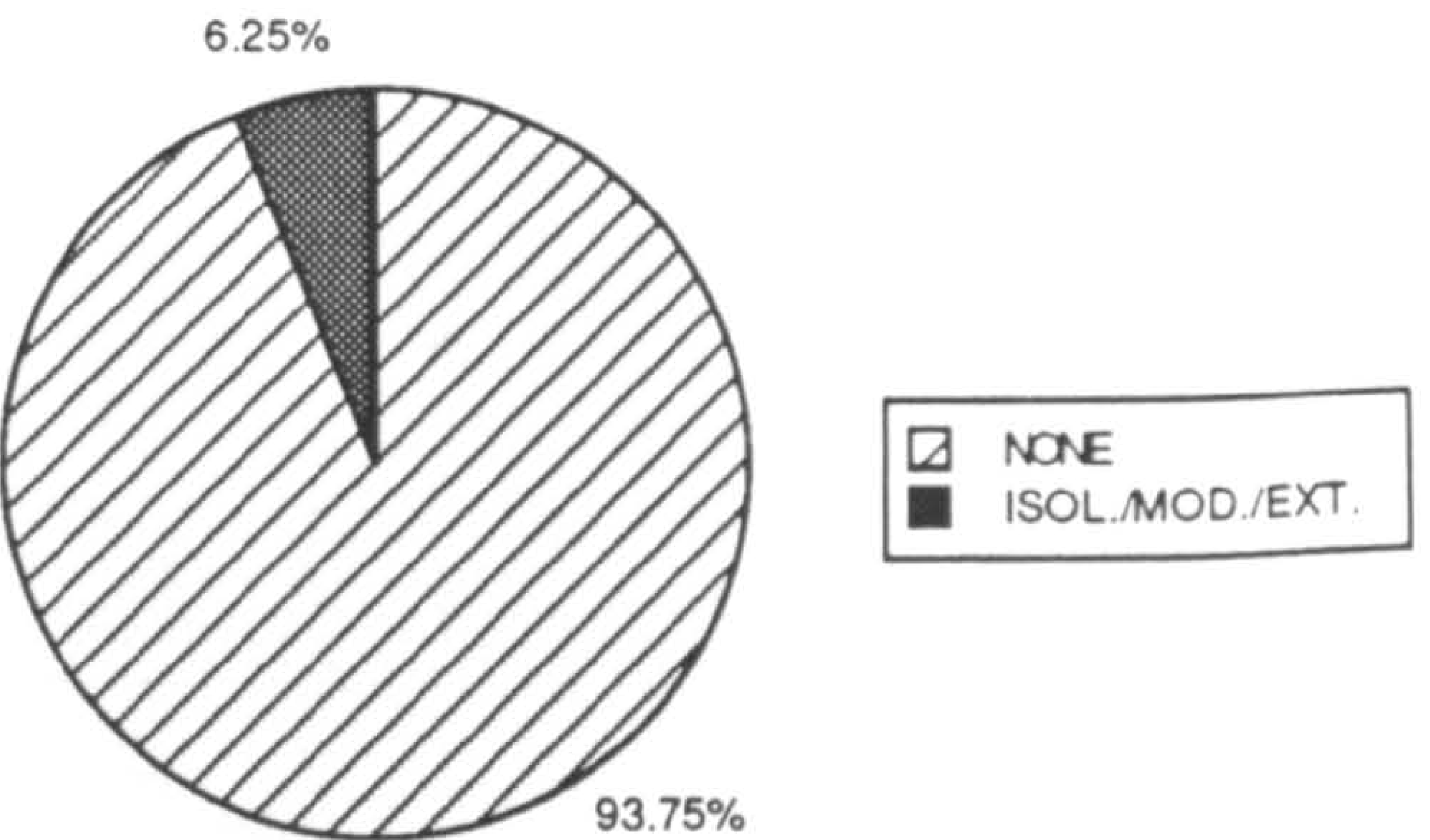


OCCURR. OF CUFFING IN THE "BAD" GROUP

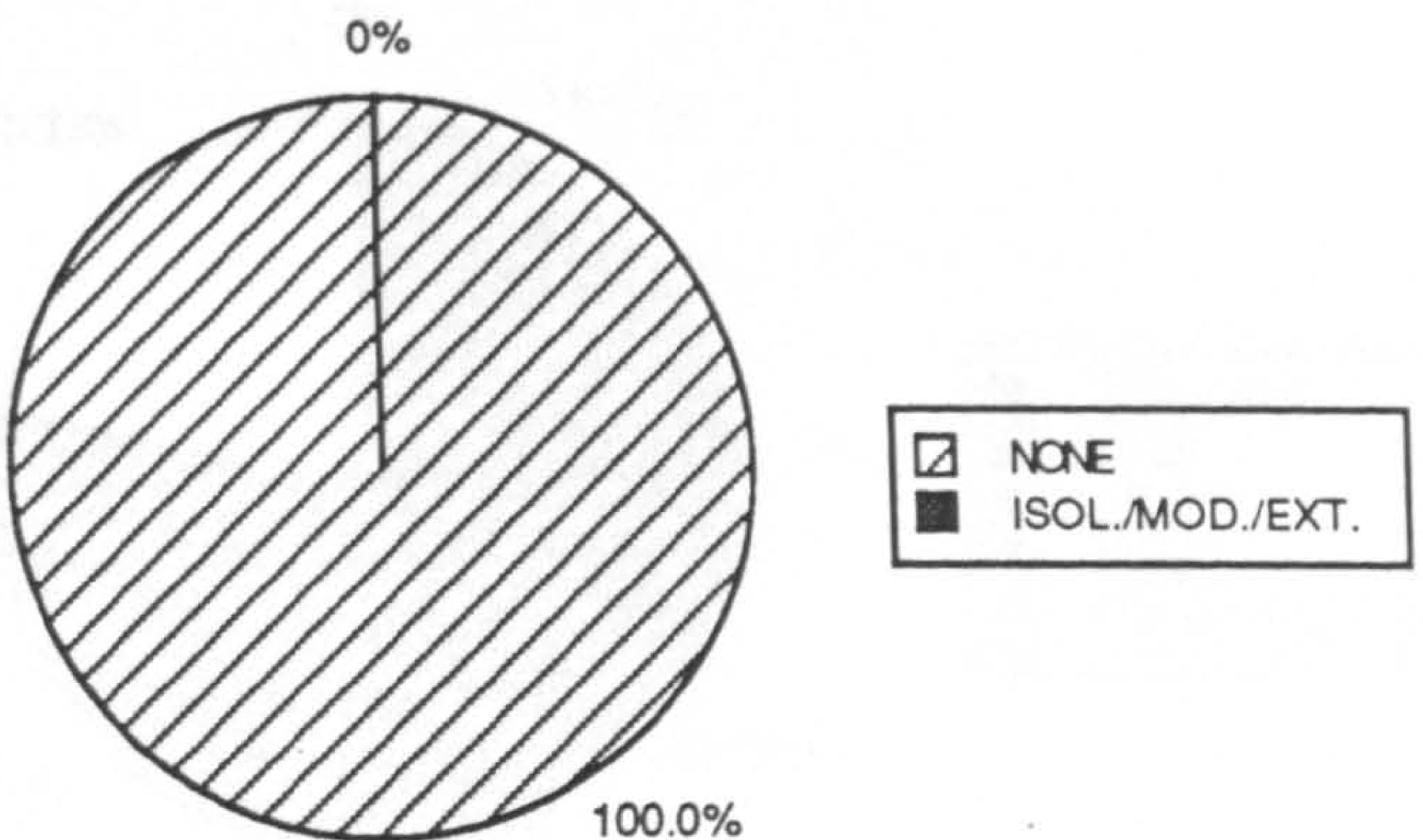
Table 12: Cont.



OCCURR. OF CHANGED MEMB. AT BEGIN. OF LAY



OCCURR. OF CHANGED MEMB. AT MIDDLE OF LAY



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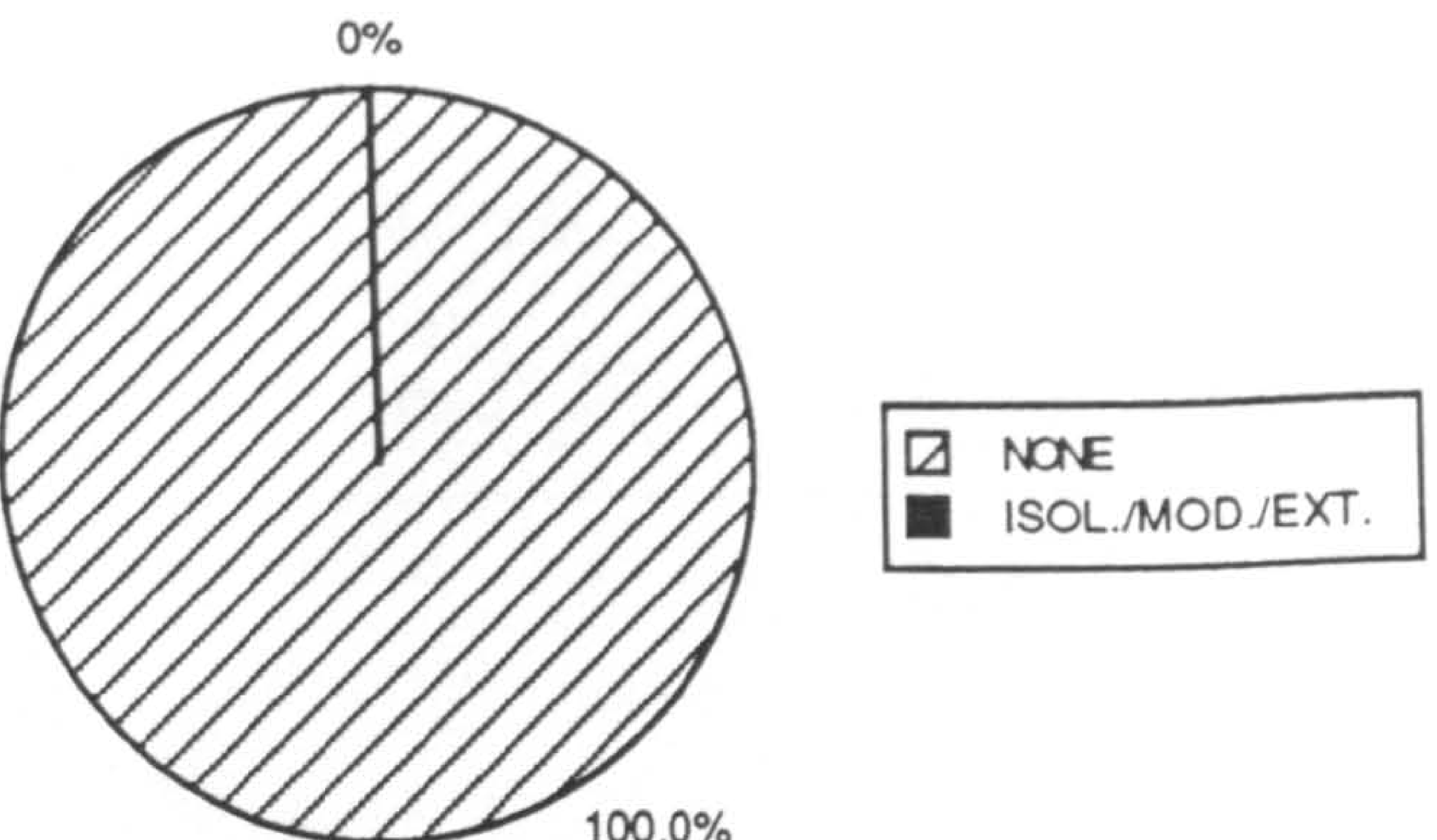
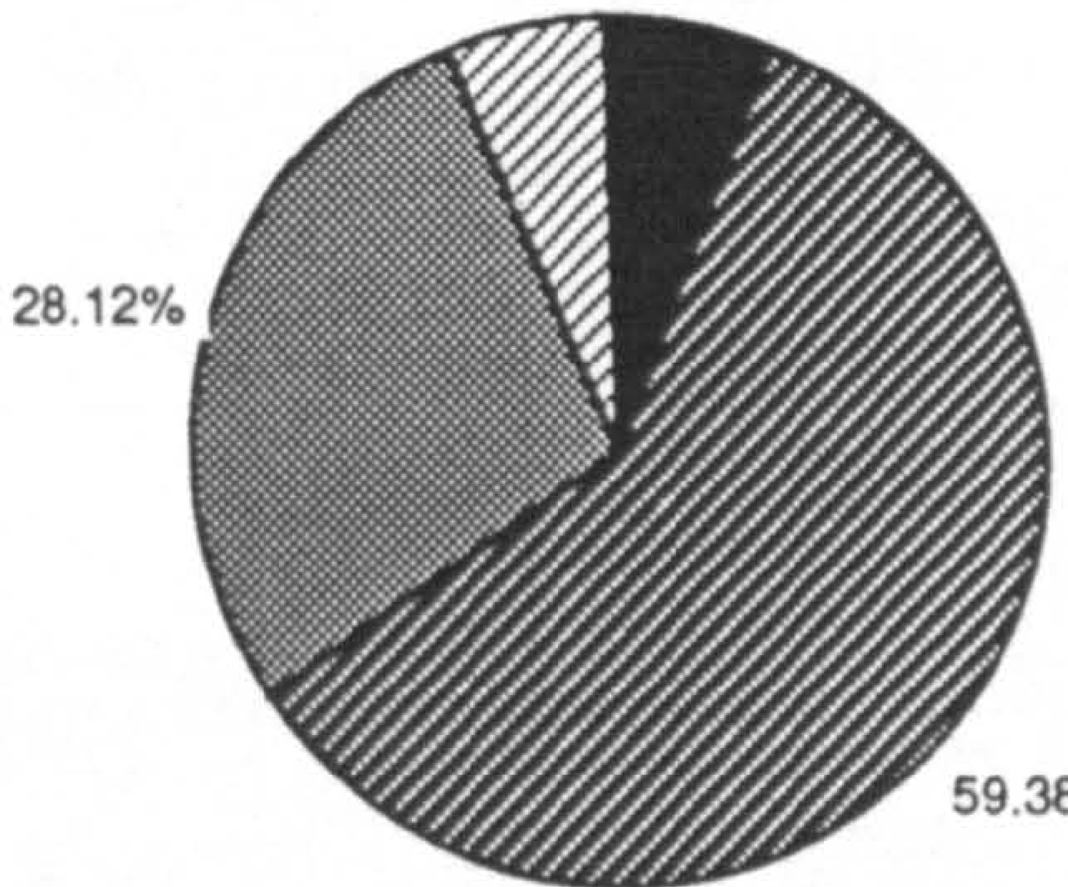
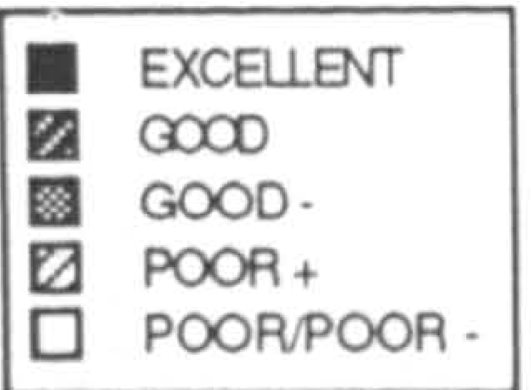


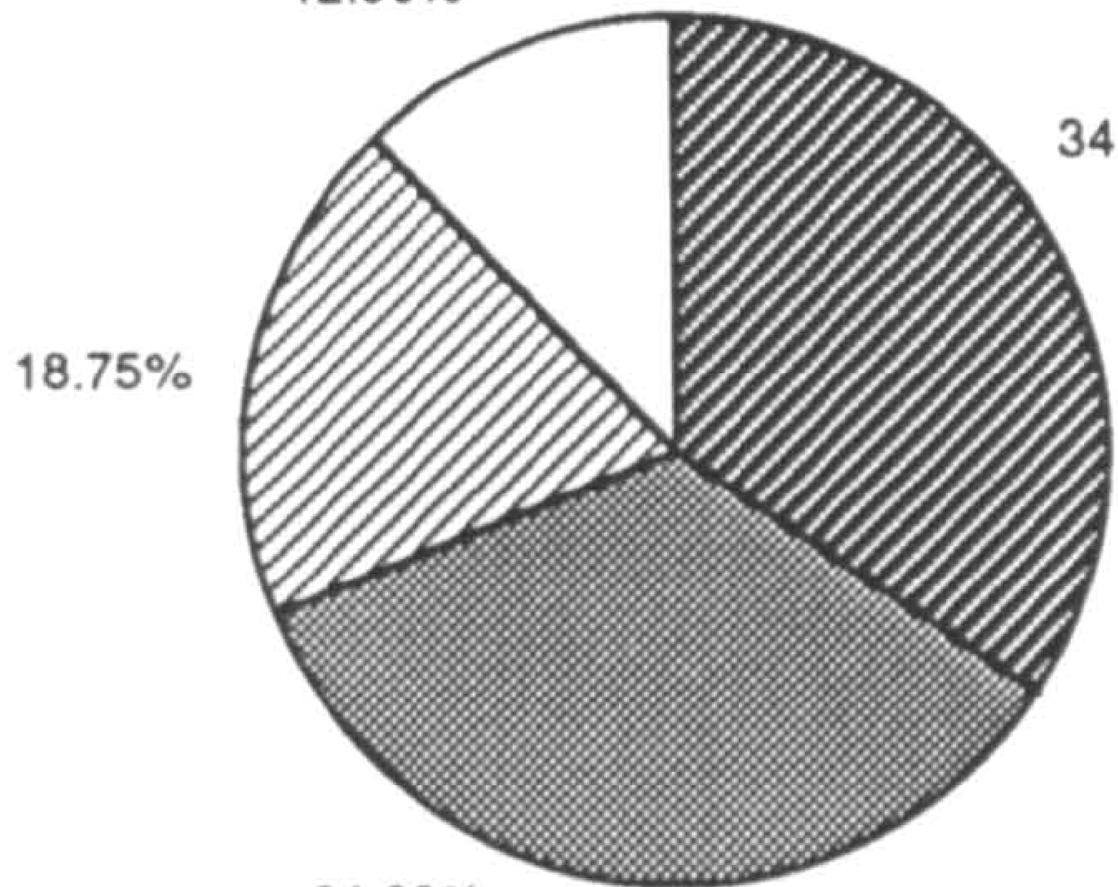
OCCURR. OF CHANGED MEMB. IN THE "BAD" GROUP

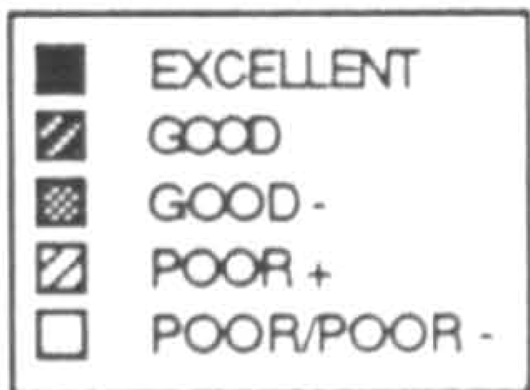
Table 12: Cont.



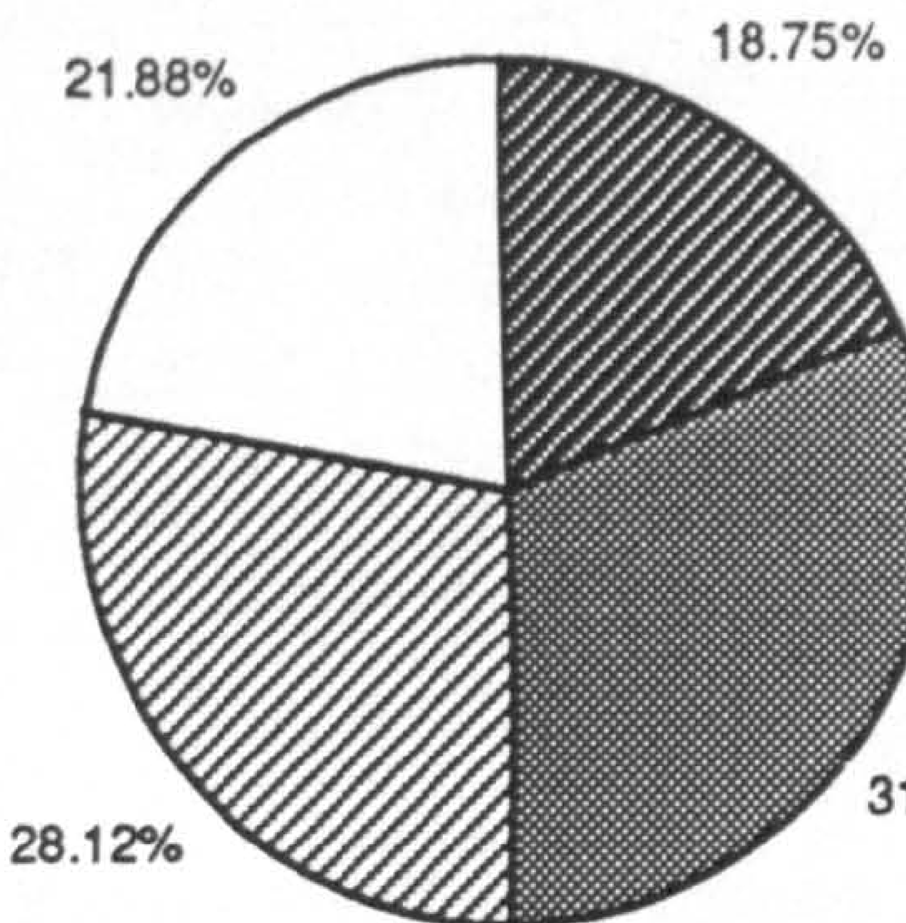


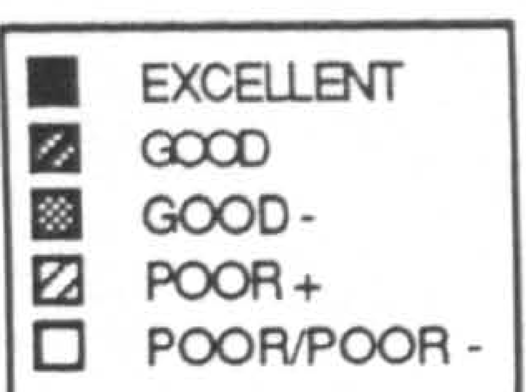
TOTAL SCORE (CATEGORIES) AT BEGIN. OF LAY



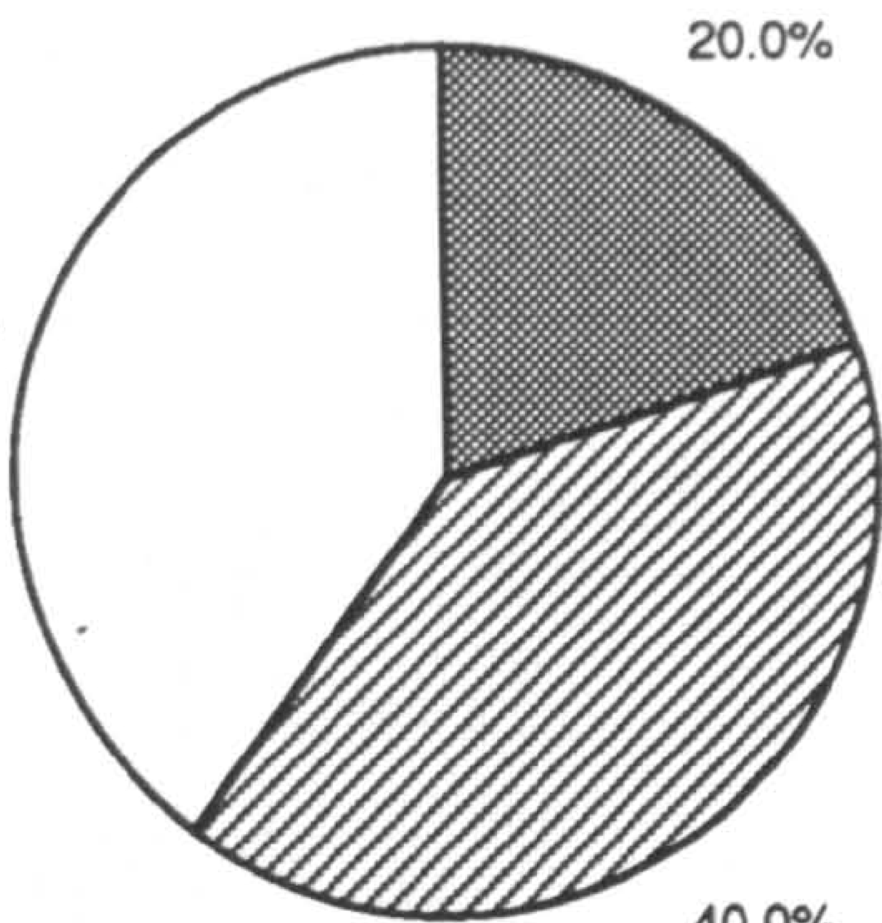


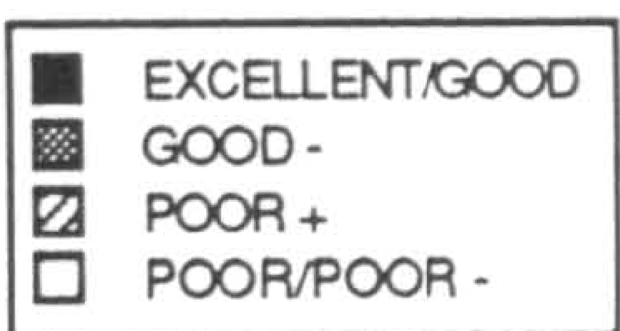
TOTAL SCORE (CATEGORIES) AT MIDDLE OF LAY





TOTAL SCORE (CATEGORIES) AT END OF LAY





TOTAL SCORE (CATEGORIES) IN THE "BAD" GROUP

Table 12: Cont.

	Overall	Beginning	Middle	(Be x M)	End	(Be x E)	Bad	(Be x Ba)	(M x E)	(M x Ba)	(E x Ba)
CONFLUENCE	NS	3.94 ± 1.60	4.16 ± 0.99	NS	3.66 ± 1.18	NS	4.60 ± 1.26	NS	NS	NS	*
CAPS	***	1.94 ± 1.01	4.06 ± 2.17	***	4.72 ± 1.71	***	5.30 ± 1.70	***	NS	NS	NS
EARLY FUSION	NS	1.97 ± 0.90	2.28 ± 0.89	NS	1.97 ± 0.78	NS	1.70 ± 0.48	NS	NS	NS	NS
LATE FUSION	NS	4.34 ± 1.62	4.72 ± 1.61	NS	4.59 ± 1.52	NS	5.20 ± 1.75	NS	NS	NS	NS
MAMM. ALIGNMENT	NS	2.62 ± 1.04	3.28 ± 1.73	NS	2.59 ± 1.58	NS	3.30 ± 1.64	NS	NS	NS	NS
TYPE B's	**	2.50 ± 1.39	2.81 ± 2.02	NS	4.09 ± 2.13	***	3.40 ± 1.71	NS	*	NS	NS
PITTING	*	1.00 ± 0.00	1.25 ± 0.98	NS	1.81 ± 1.75	*	2.20 ± 1.93	***	NS	*	NS
ARAGONITE	***	1.03 ± 0.18	1.22 ± 0.75	NS	2.50 ± 1.65	***	3.60 ± 1.84	***	***	***	NS
TYPE A's	***	1.31 ± 0.47	1.88 ± 0.34	***	1.81 ± 0.40	***	1.90 ± 0.32	***	NS	NS	NS
CUBICS	NS	1.22 ± 0.42	1.59 ± 1.19	NS	1.31 ± 0.47	NS	1.50 ± 0.53	NS	NS	NS	NS
CUFFING	**	4.44 ± 0.80	4.06 ± 1.27	NS	4.88 ± 0.34	**	4.60 ± 0.52	NS	***	NS	NS
CHANGED MEMBR.	NS	1.09 ± 0.53	1.19 ± 0.74	NS	1.00 ± 0.00	NS	1.00 ± 0.00	NS	NS	NS	NS
TOTAL SCORE	***	27.44 ± 4.47	32.44 ± 5.84	***	34.78 ± 6.66	***	38.50 ± 4.72	***	NS	**	NS
MAMM. DENSITY	***	82.91 ± 13.70	78.58 ± 15.80	NS	75.93 ± 17.20	NS	59.60 ± 11.70	***	NS	**	**
PORES (number)	***	18.57 ± 16.30	116.80 ± 70.70	***	174.97 ± 119.90	***	278.70 ± 95.90	***	*	***	**
% PENETRATION	***	12.92 ± 6.33	18.91 ± 14.14	*	24.97 ± 14.00	***	34.78 ± 18.40	***	NS	**	NS

Table 13: Structural scores (mean ± s.d.) for each individual characteristic of the cone layer in eggs, number of pores and percentage of penetration. Values correspond to beginning, middle, end of lay and the poor quality ("bad") group.

*** = Very highly significant at a 0.1 % level (P< 0.001)
** = Highly significant at a 1 % level (P< 0.01)
* = Significant at a 5 % level (P< 0.05)
NS = Not Significant (P> 0.05)

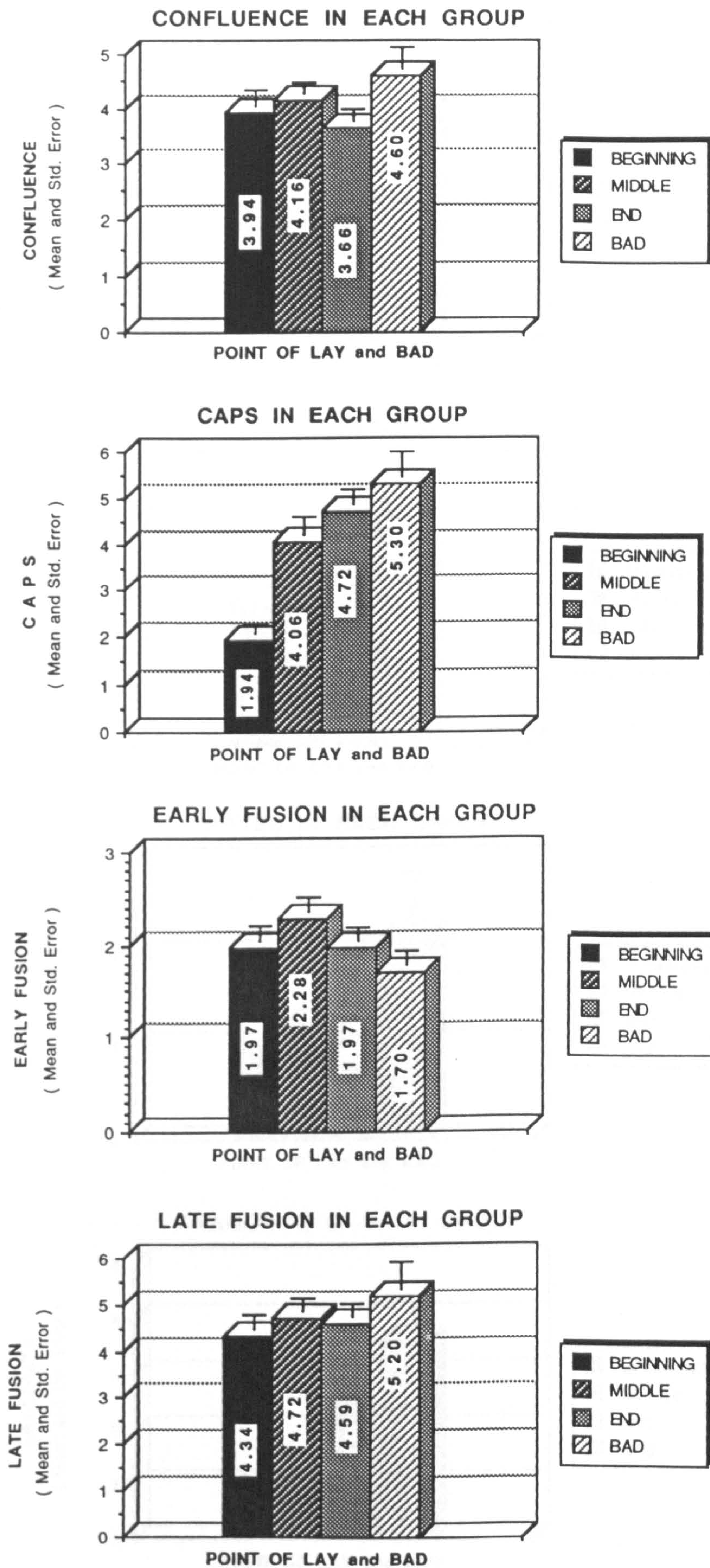
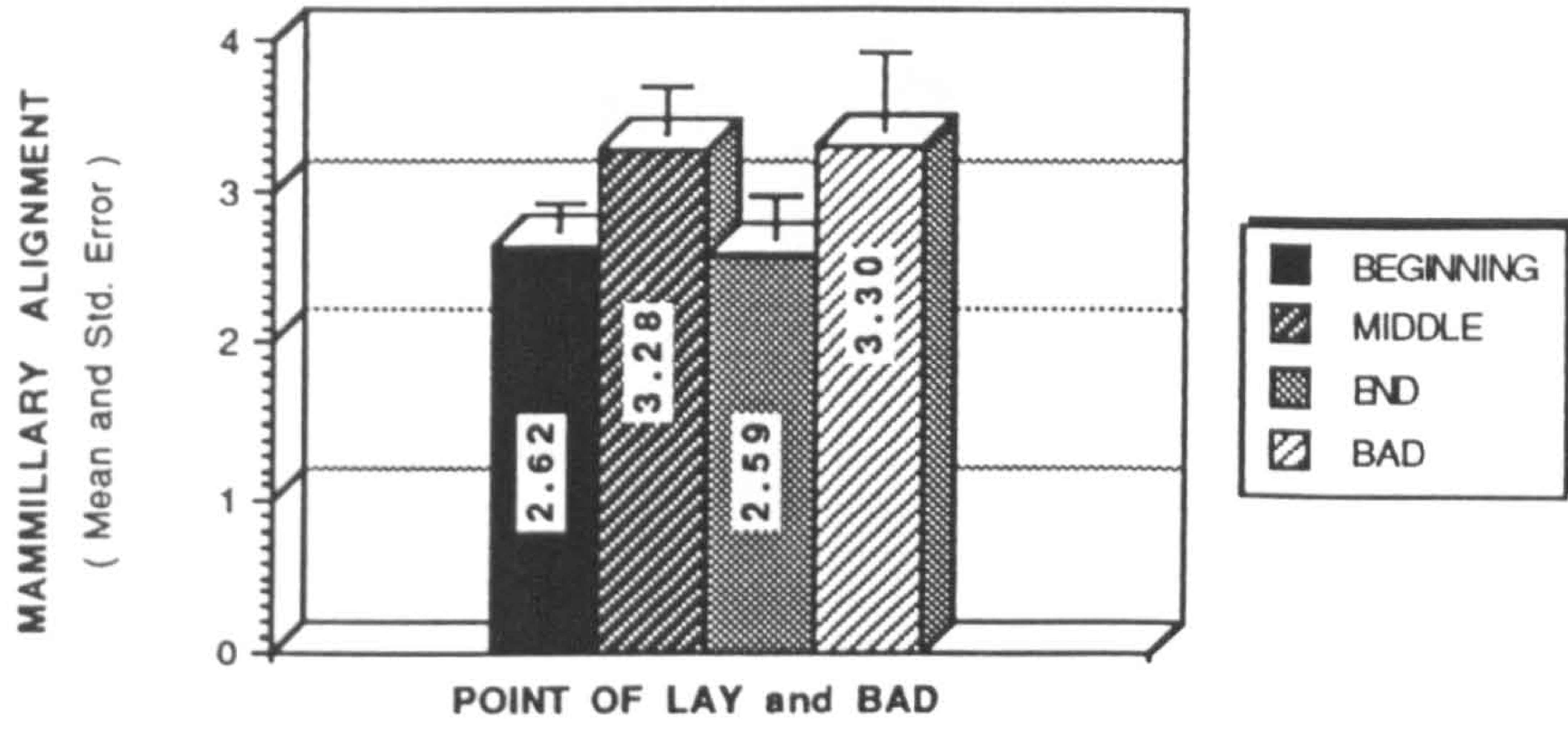
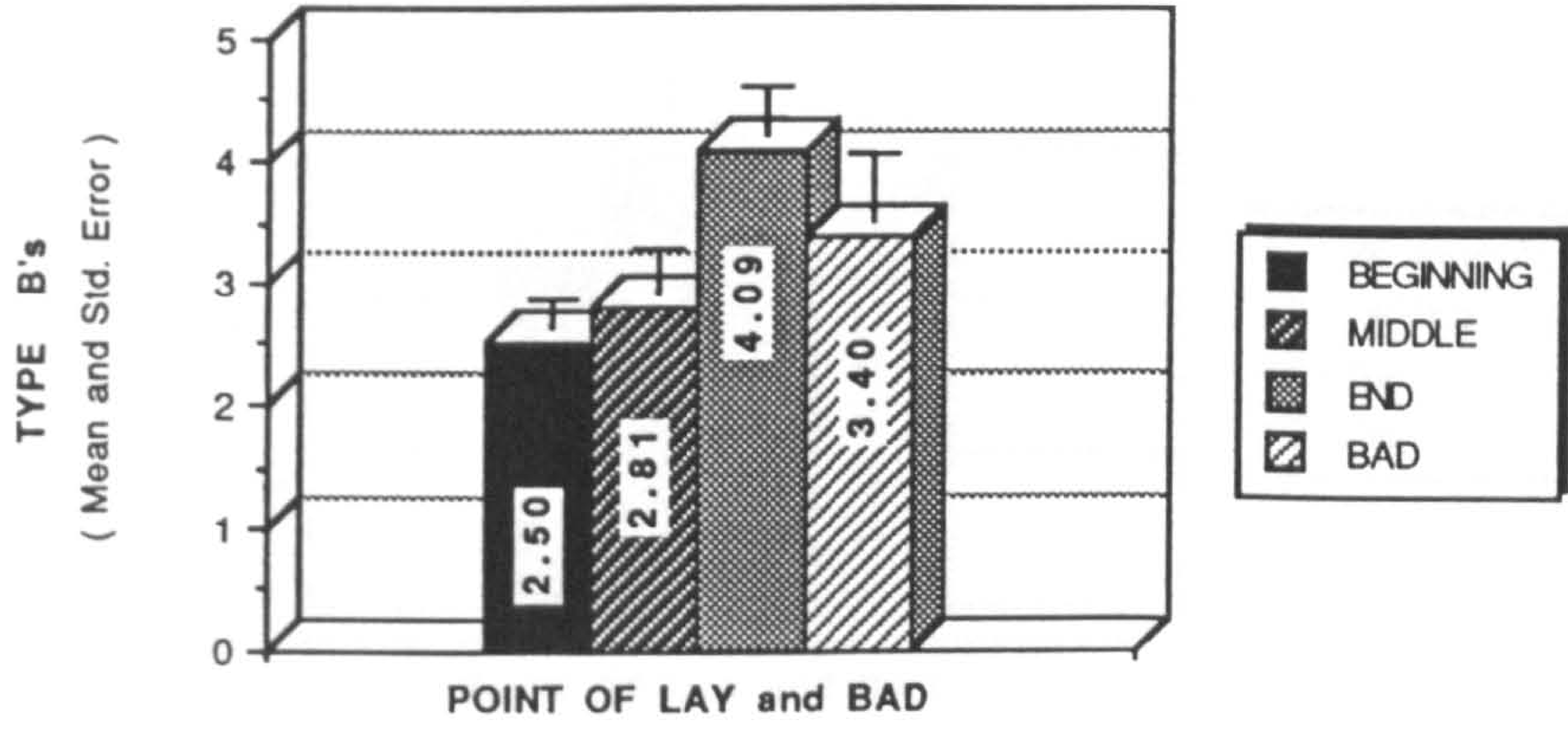
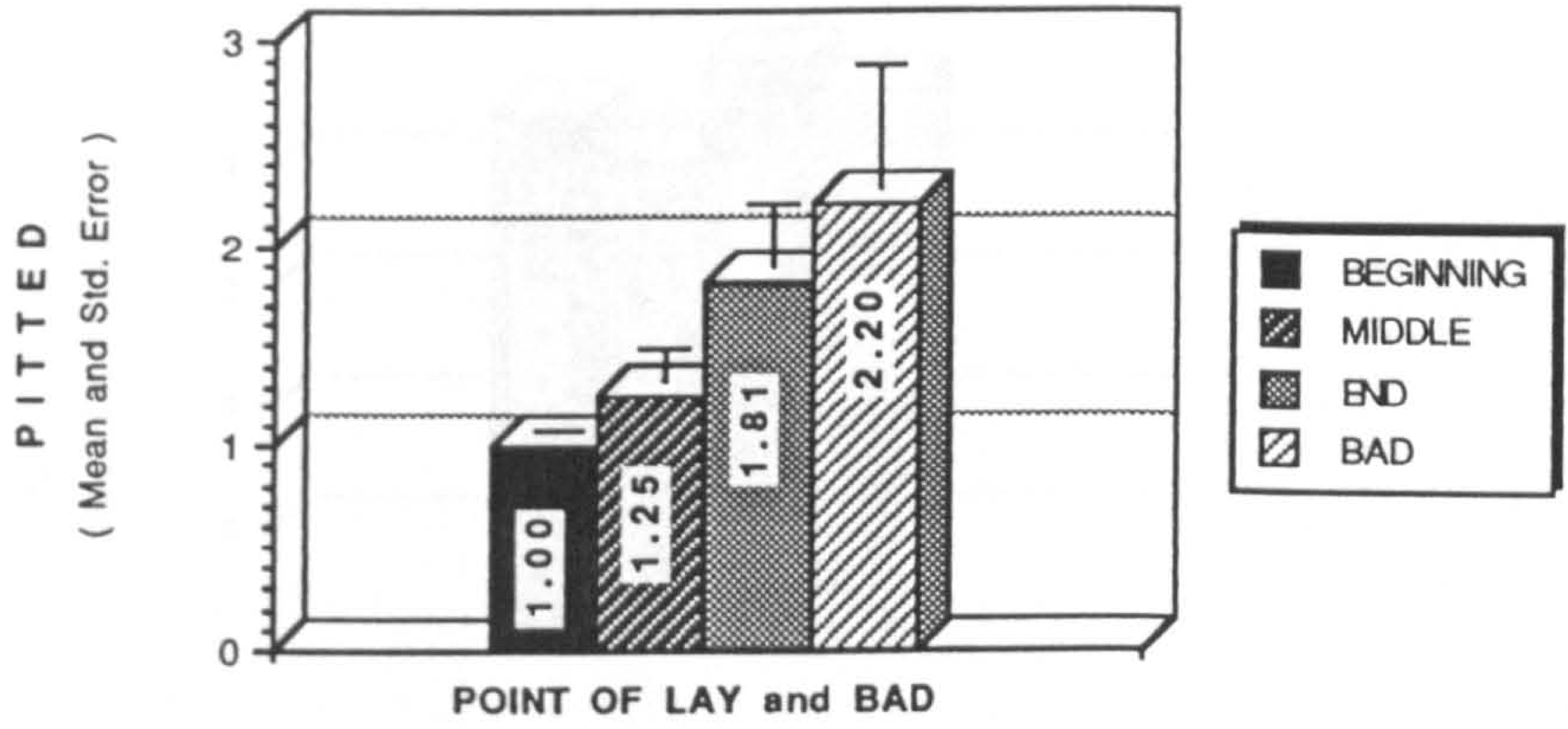


Table 14: Bar histograms illustrate table 13 results and show the mean score and standard error (S.E.) for each characteristic in each group.







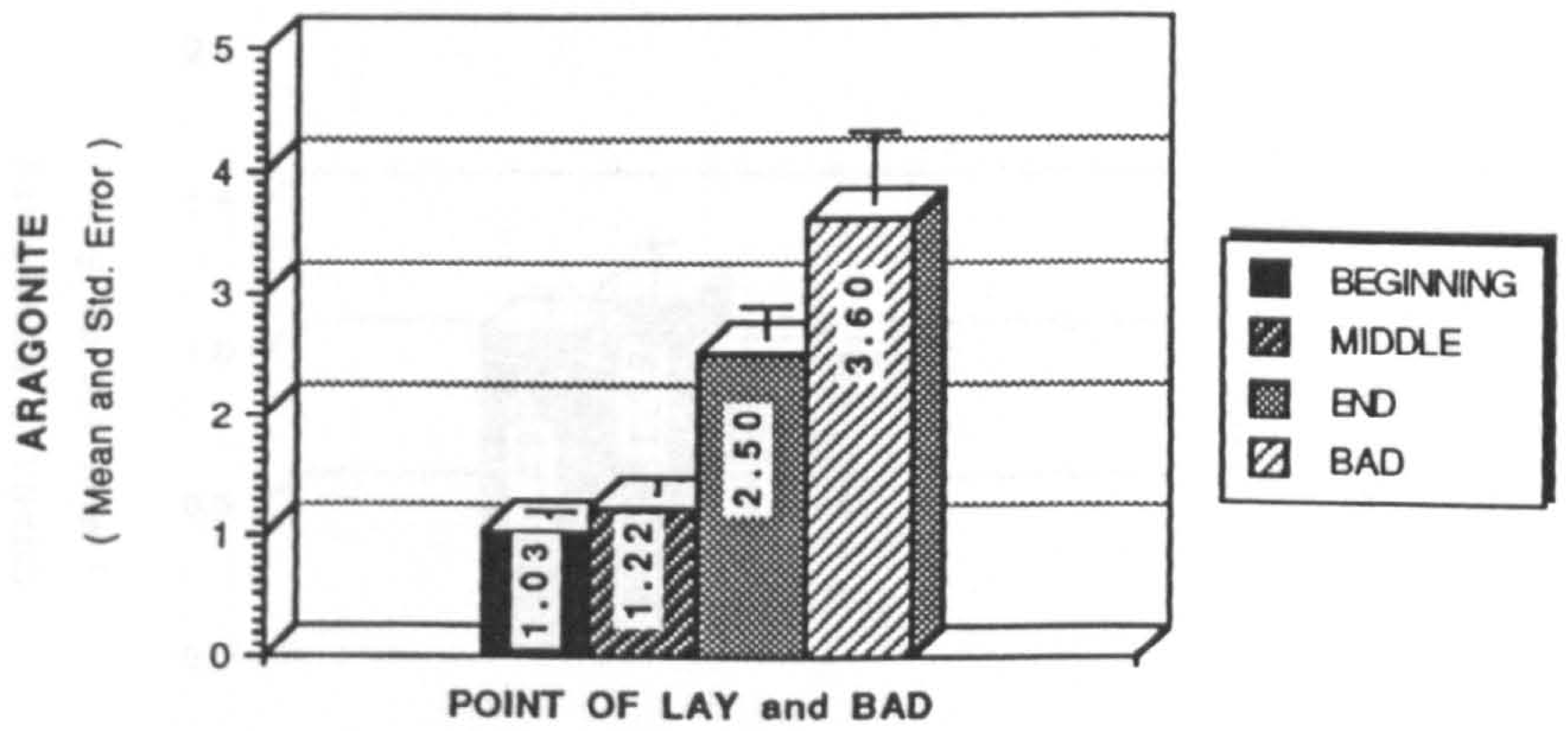
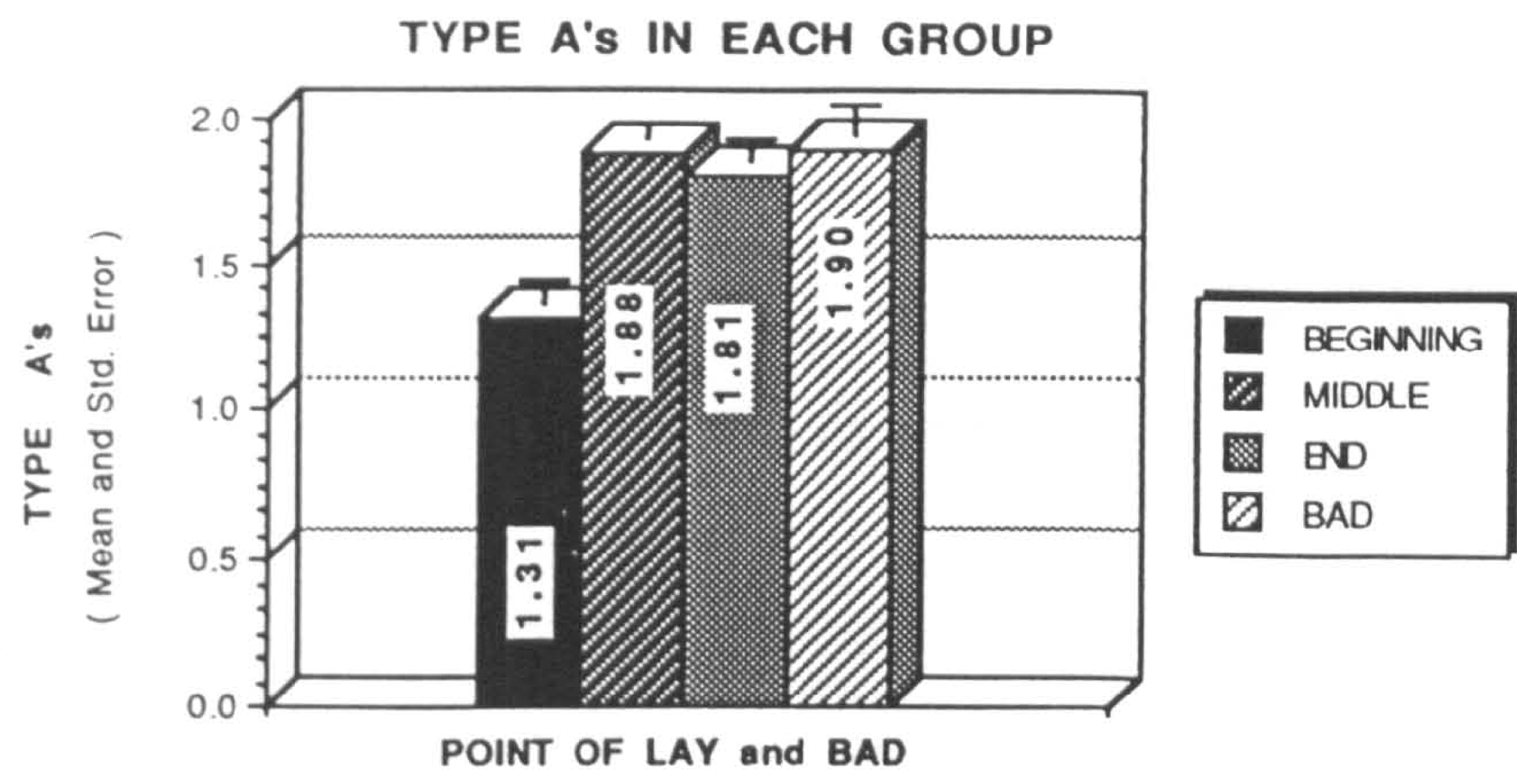
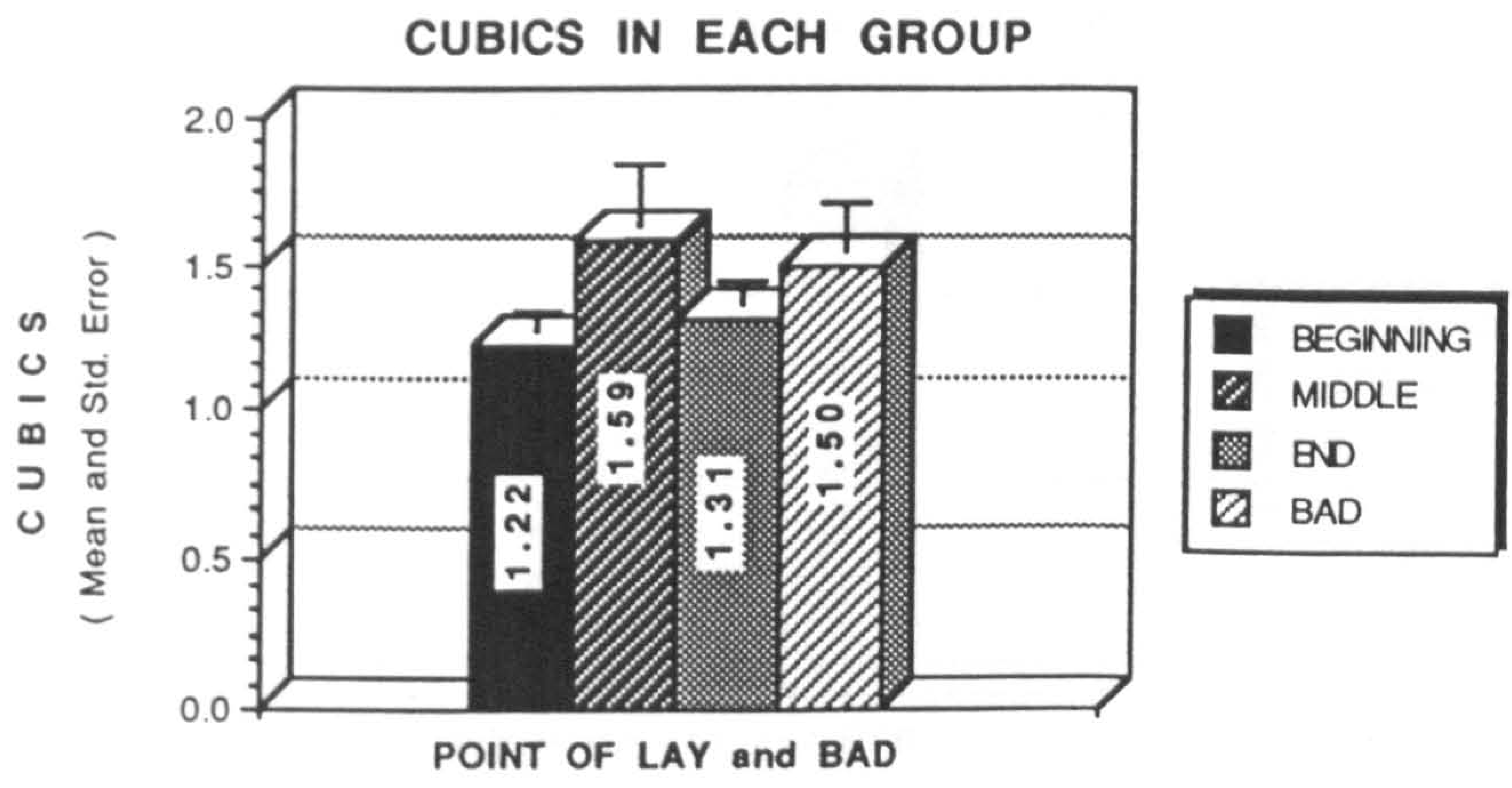
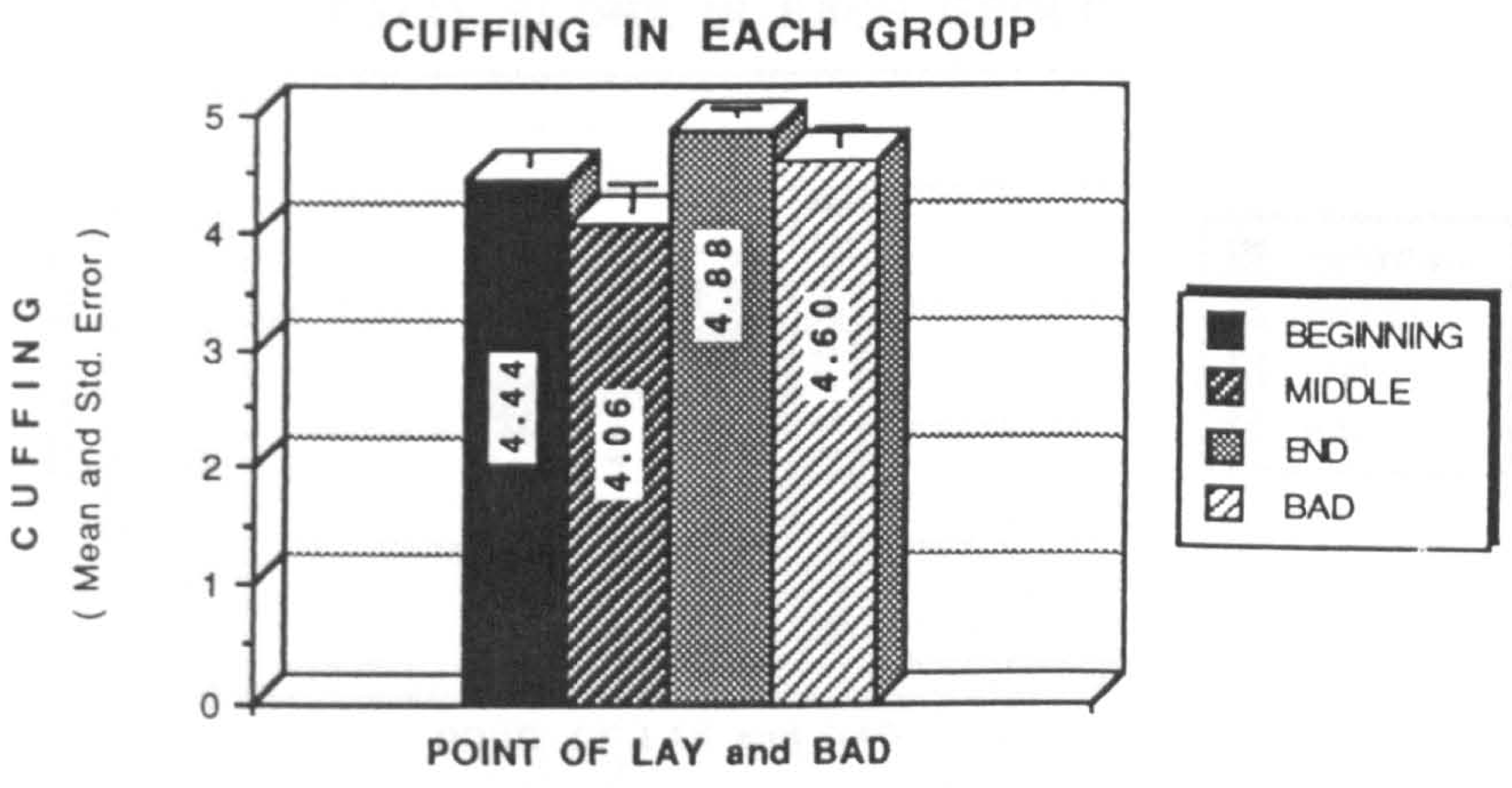


Table 14: Cont.







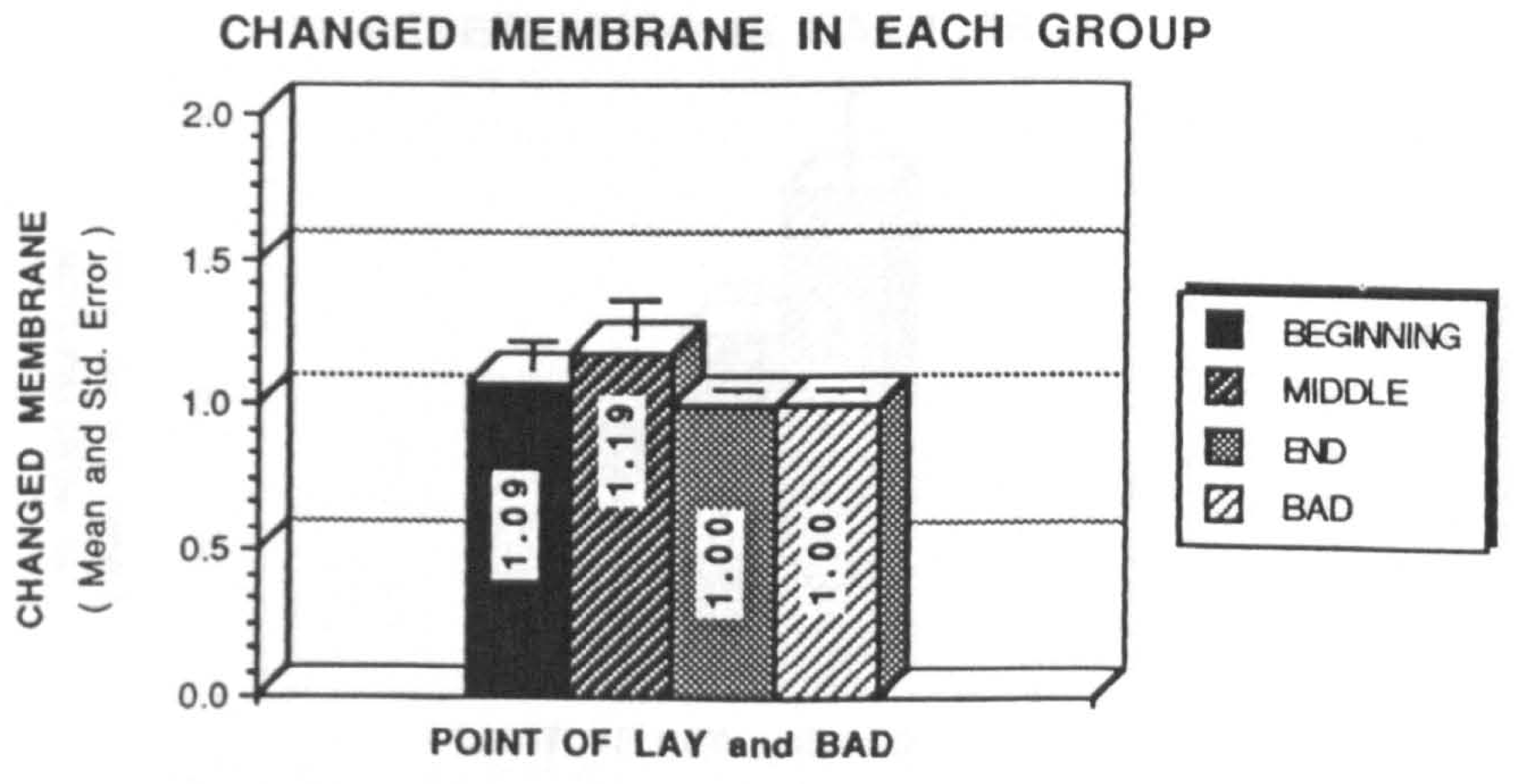
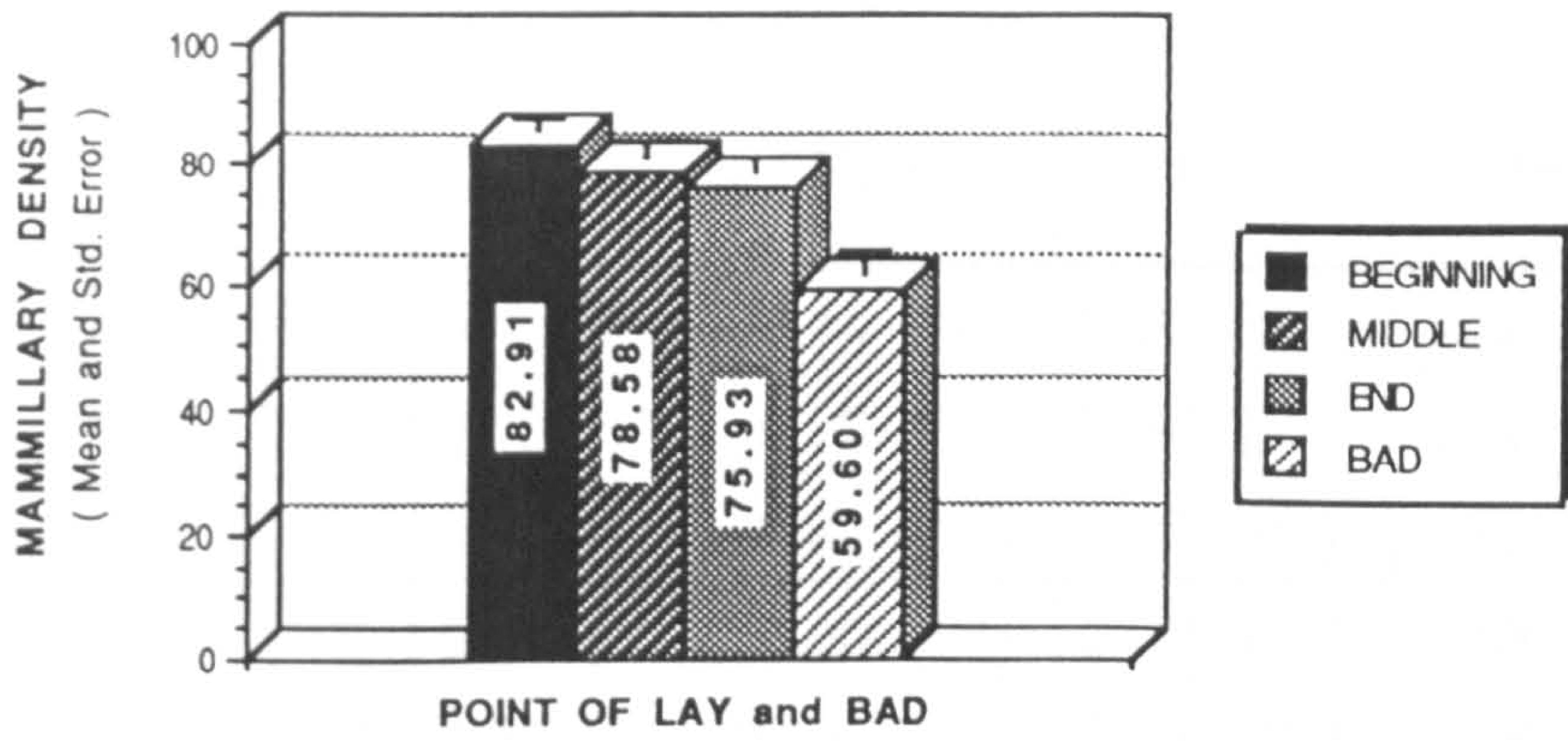
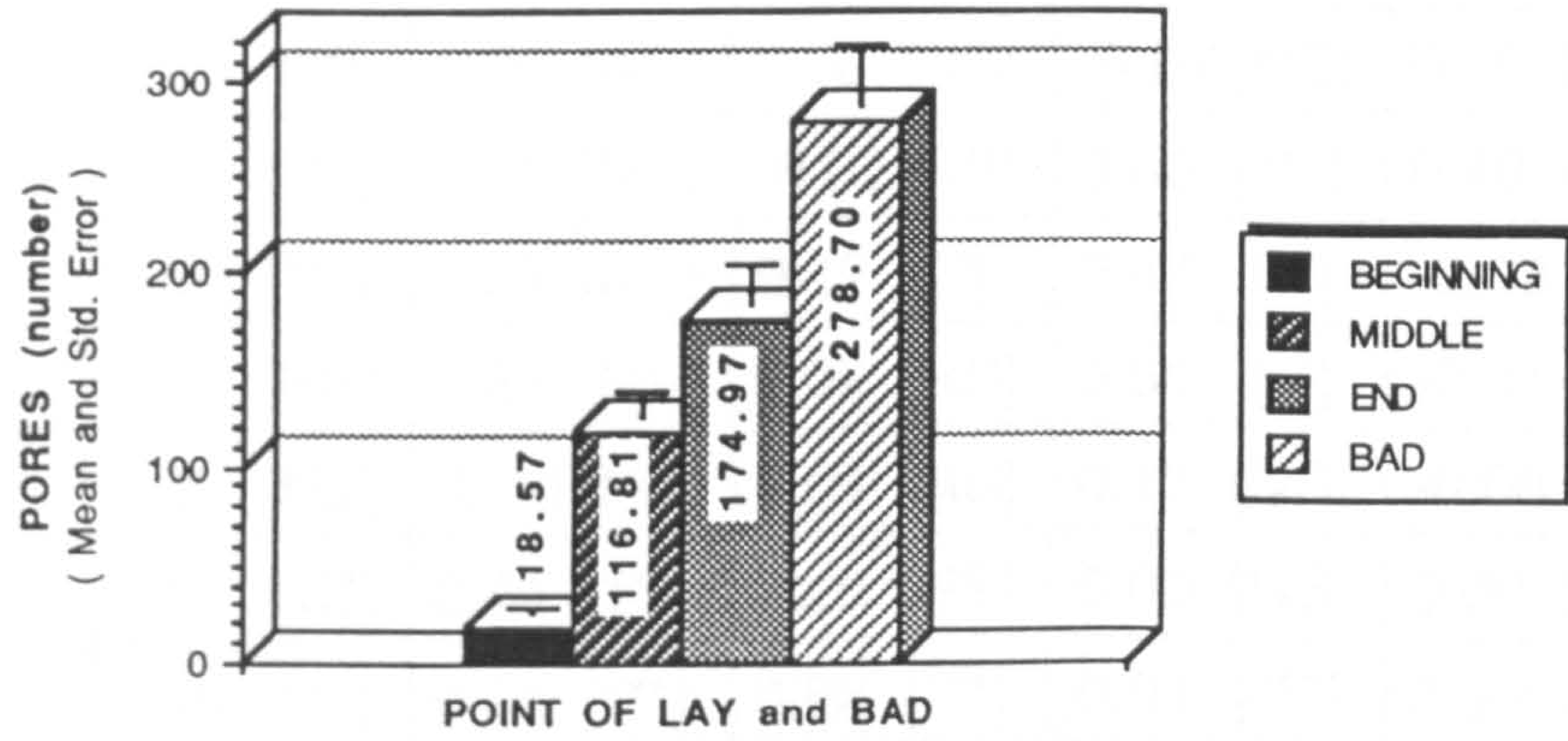
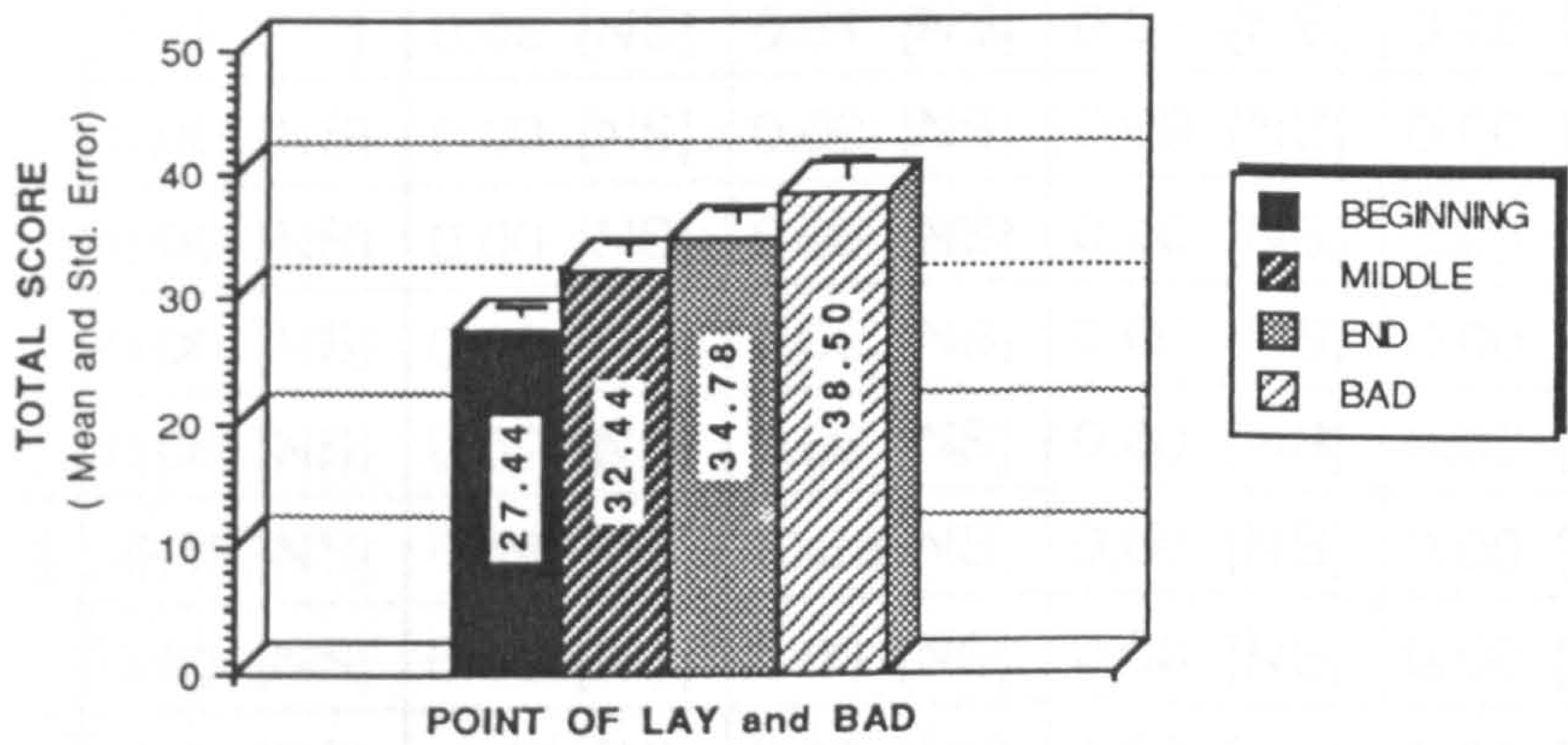


Table 14: Cont.







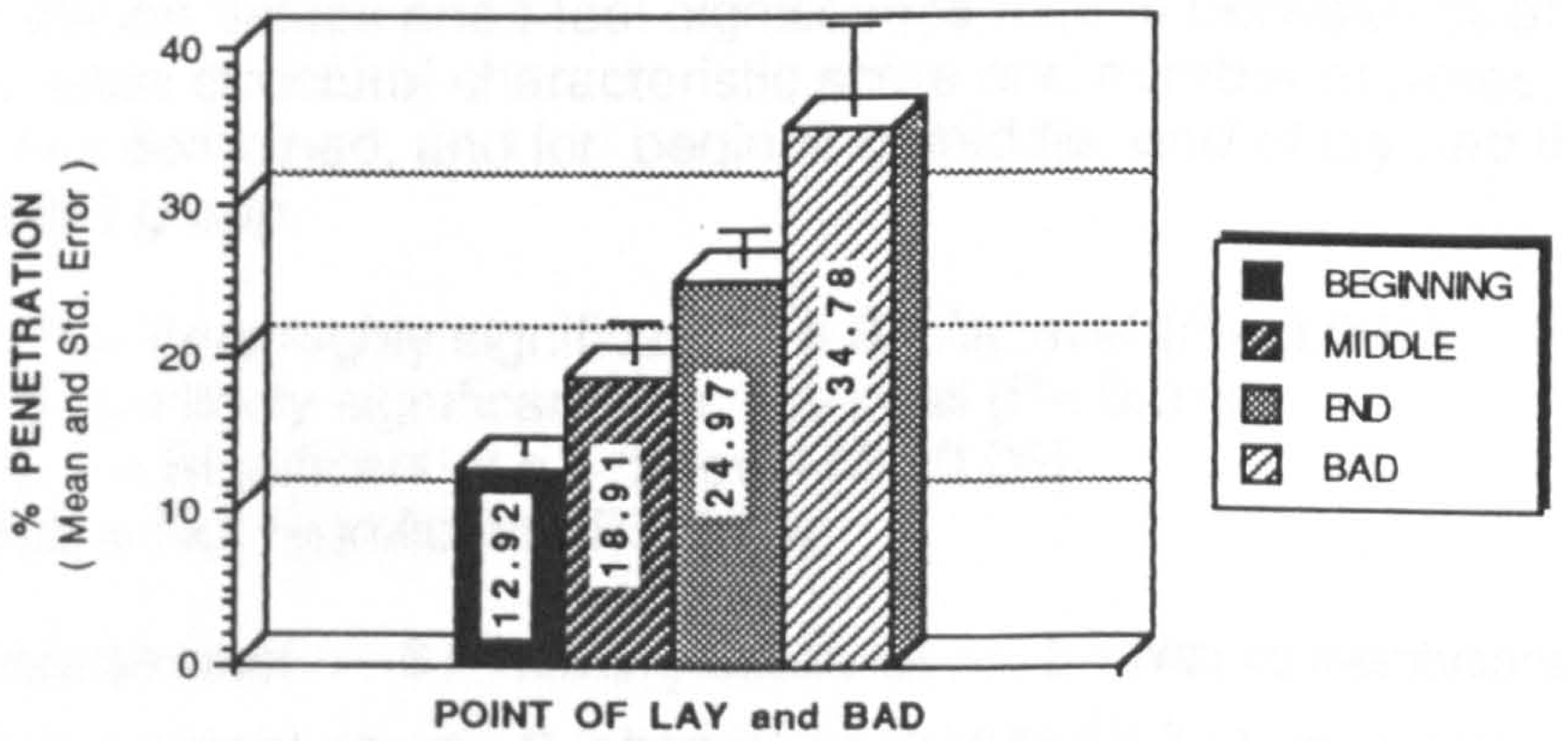


Table 14: Cont.

% Penetration against ...	Overall	Beginning	Middle	End	"Bad"
Confluence	0.07 [NS]	0.48 [**]	0.14 [NS]	0.01 [NS]	0.02 [NS]
Caps	0.73 [***]	0.45 [*]	0.80 [***]	0.56 [***]	0.72 [*]
Early Fusion	0.37 [***]	0.44 [*]	0.62 [***]	0.46 [**]	0.55 [NS]
Late Fusion	0.50 [***]	0.64 [***]	0.60 [***]	0.60 [***]	0.13 [NS]
Mamm. Align.	0.44 [***]	0.63 [***]	0.63 [***]	0.57 [***]	-0.20 [NS]
Type B's	0.52 [***]	0.04 [NS]	0.25 [NS]	0.78 [***]	0.77 [**]
Pitting	0.27 [**]	0.00 [NS]	0.30 [NS]	-0.07 [NS]	0.50 [NS]
Aragonite	0.55 [***]	0.16 [NS]	-0.02 [NS]	0.60 [***]	0.49 [NS]
Type A's	0.42 [***]	0.25 [NS]	0.42 [*]	0.47 [**]	-0.03 [NS]
Cubics	0.17 [NS]	0.25 [NS]	0.07 [NS]	0.51 [**]	-0.01 [NS]
Cuffing	0.09 [NS]	-0.18 [NS]	0.04 [NS]	0.13 [NS]	-0.08 [NS]
Chang. Membr.	0.13 [NS]	0.44 [*]	0.25 [NS]	0.00 [NS]	0.00 [NS]
Total Score	0.90 [***]	0.84 [***]	0.92 [***]	0.91 [***]	0.93 [***]
Mamm. Density	-0.08 [NS]	-0.02 [NS]	0.07 [NS]	0.10 [NS]	0.54 [NS]
Pores (number) †	0.42 [***]	0.02 [NS]	0.31 [NS]	0.07 [NS]	0.29 [NS]
Cuticle †	0.00 [NS]	0.00 [NS]	0.00 [NS]	0.06 [NS]	0.00 [NS]
Cuticle - I	0.00 [NS]	0.00 [NS]	0.00 [NS]	0.00 [NS]	0.00 [NS]
Cuticle - S	0.00 [NS]	0.00 [NS]	0.00 [NS]	0.00 [NS]	0.00 [NS]
Pores (number) - I	0.00 [NS]	0.00 [NS]	0.00 [NS]	0.00 [NS]	0.00 [NS]
Pores (number) - S	-0.13 [NS]	0.59 [*]	-0.28 [NS]	0.00 [NS]	0.00 [NS]
I. S. M. - I	0.00 [NS]	0.00 [NS]	0.00 [NS]	0.00 [NS]	0.00 [NS]
O. S. M. - S	-0.19 [NS]	-0.68 [**]	0.07 [NS]	0.00 [NS]	0.00 [NS]

Table 15: Correlation values and t-test significance results between % of penetration and each structural characteristic score and number of pores, for the four categories combined, and for beginning, middle, end of lay and the poor quality ("bad") group.

[***] = Very highly significant at a 0.1 % level (P< 0.001)
[**] = Highly significant at a 1 % level (P< 0.01)
[*] = Significant at a 5 % level (P< 0.05)
[NS] = Not Significant (P> 0.05)

I = With both membranes intact. S = With only O.S.M. left. † = With no membranes left

Cuticle assessment score= 2 (absent) / 1 (patchy) / 0 (complete)
I.S.M. assessment score= 1 (pitted) / 0 (intact)
O.S.M. assessment score= 1 (pitted) / 0 (intact)

Structural variations in the eggshell which increase resistance to bacterial penetration:

Early fusion
Good cap formation (*i.e.*, close bonding between organic and inorganic fractions of shell)
Cuffing
Confluent mammillae
A high mammillary density

Structural variations in the eggshell which decrease resistance to bacterial penetration:

Late fusion
Type B's
Type A's
Aragonite
Pitting: depressions, erosions, pin holes.
Alignment of mammillae
Cubics
Changed membrane
A low mammillary density

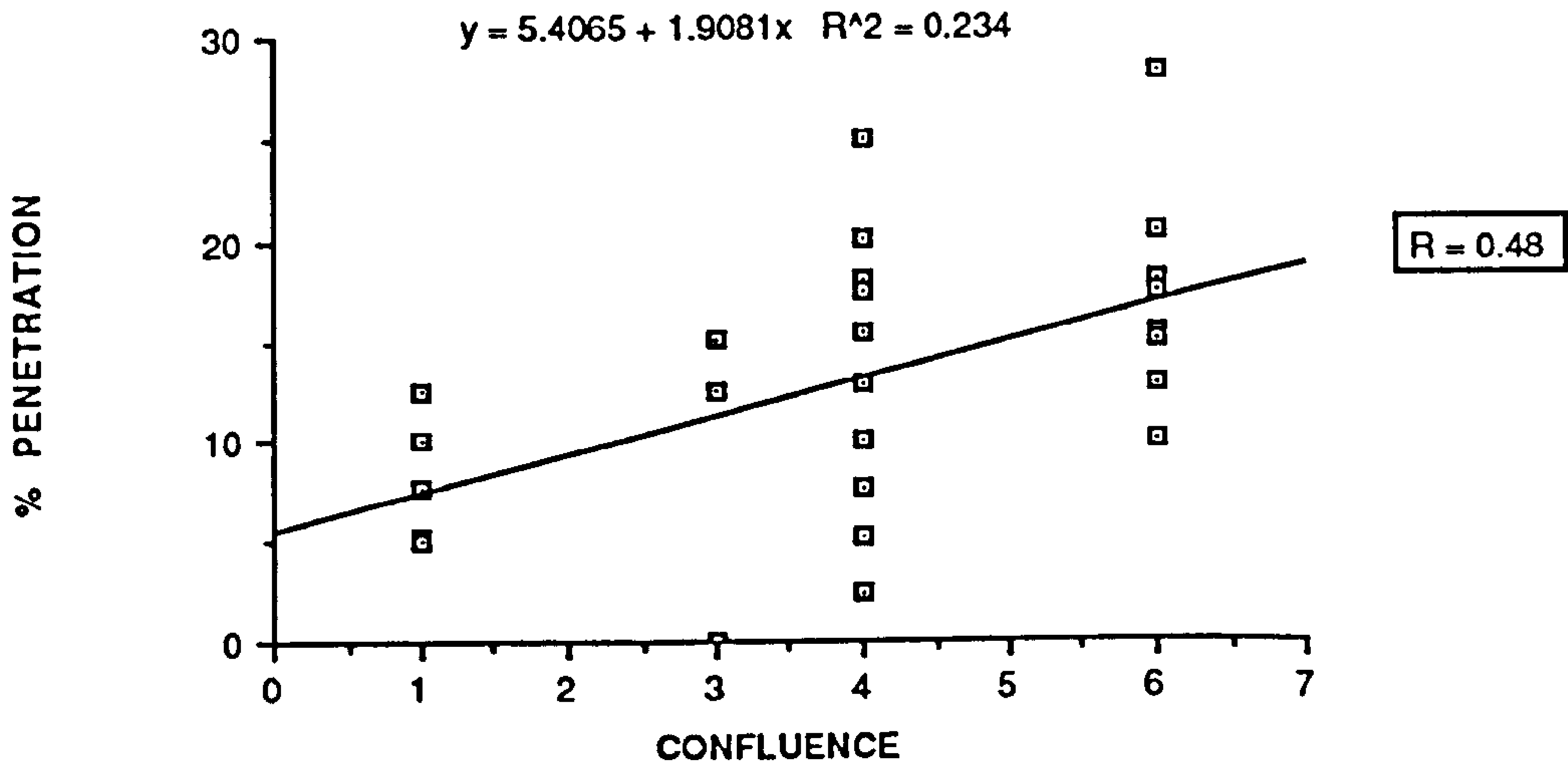
Table 16: Categorisation of mammillary layer characteristics into those which increase, and those which decrease the resistance of the eggshell to bacterial penetration.

CHARACTERISTIC	BEG.	MID.	END	BAD	PROBABILITY
BETTER CAP APPEARANCE	X				$P<0.001$
MORE ARAGONITE			X	X	$P<0.001$
LESS TYPE A's	X				$P<0.001$
BETTER TOTAL SCORE	X				$P<0.001$
LOWER MAMMILLARY DENSITY				X	$P<0.01$
LOWER NUMBER OF PORES	X				$P<0.01$
HIGHER NUMBER OF PORES				X	$P<0.05$
LOWER BACT. PENETRATION	X				$P<0.05$

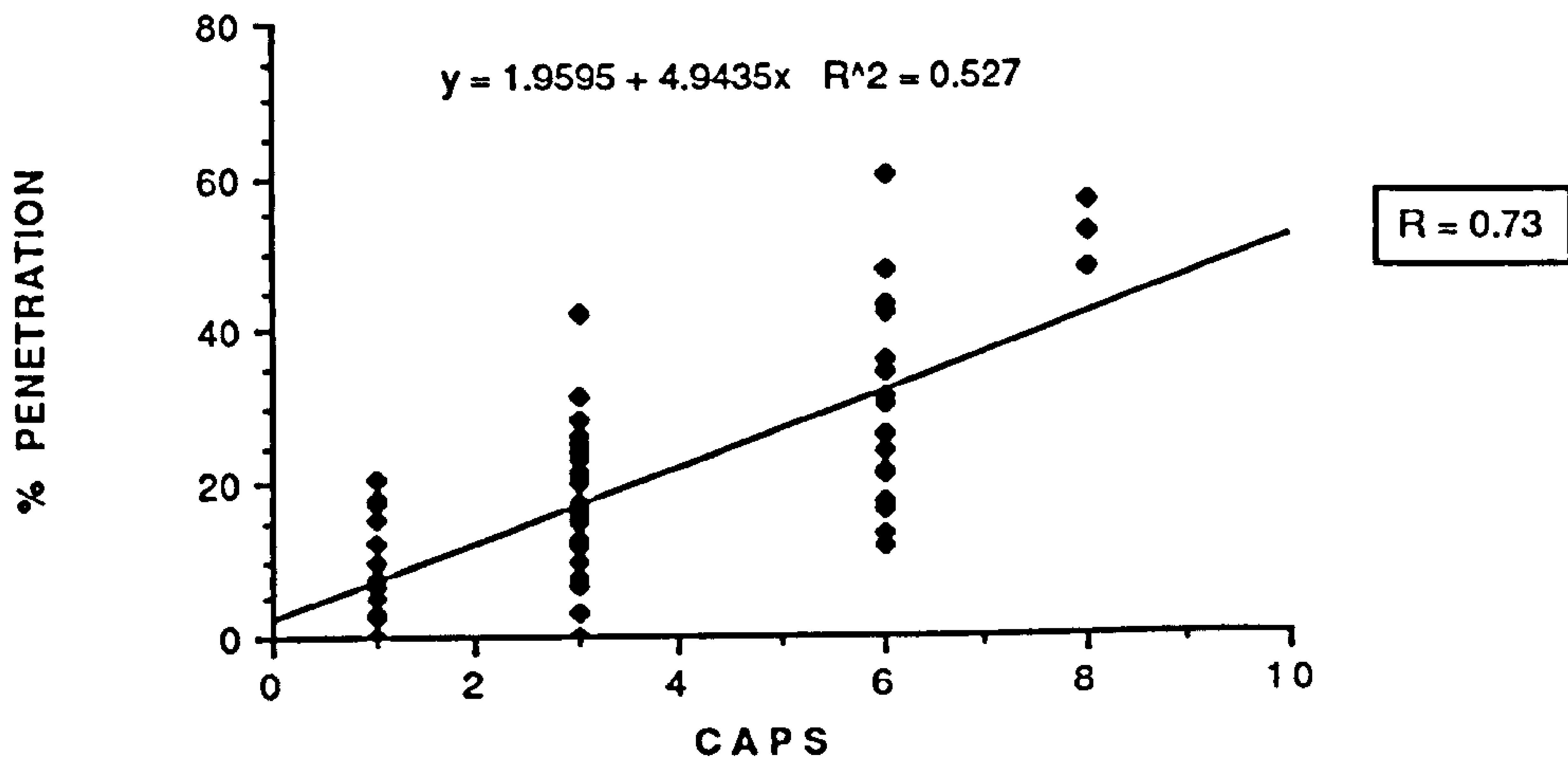
Table 17: Comparison of ultra-structural scores (specific characteristics only), pore number and bacterial penetration between each sampling period and the poor quality ("bad") group.

- X denotes the group displaying the significant characterisitic.

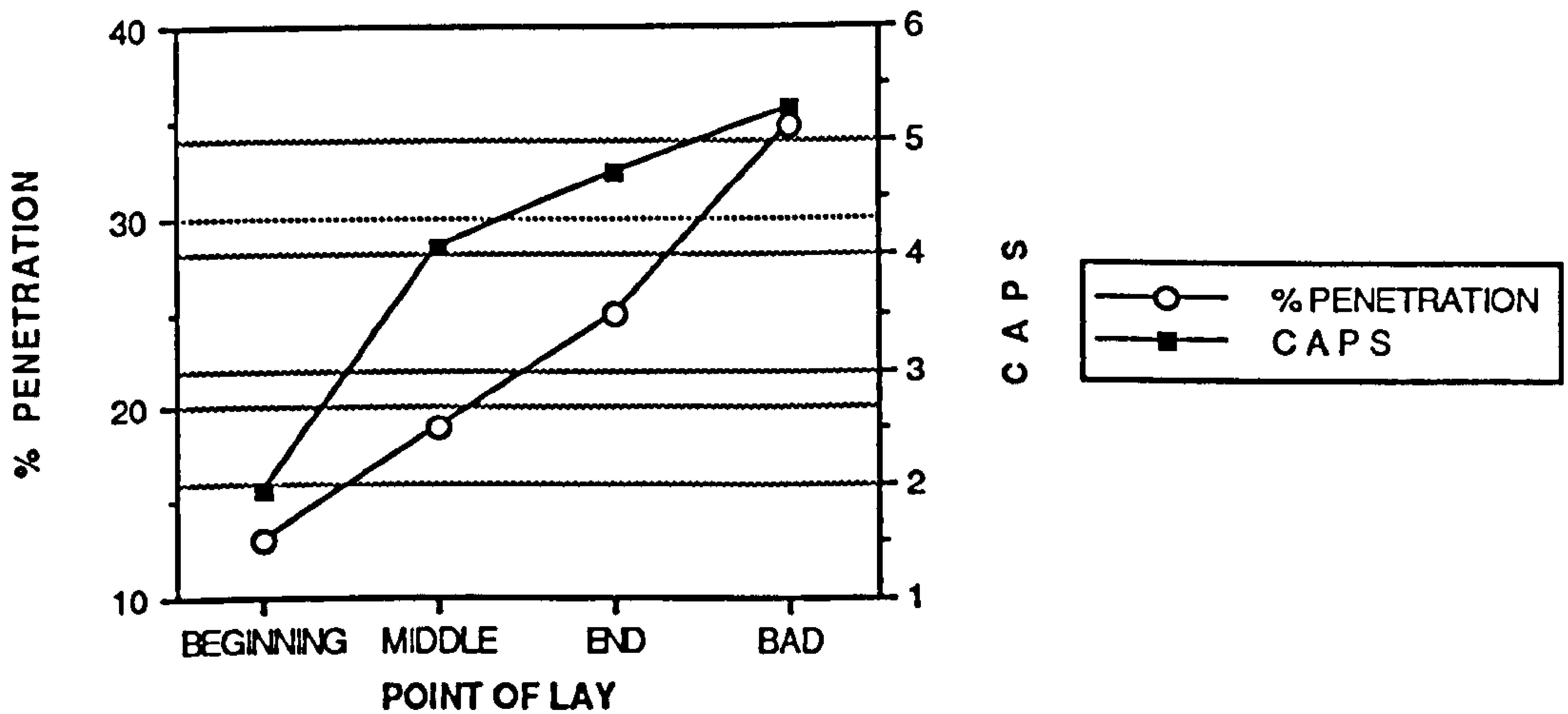
GRAPH 1: LINE OF BEST FIT (CORREL. % PENETR. X CONFLUENCE (BEG))



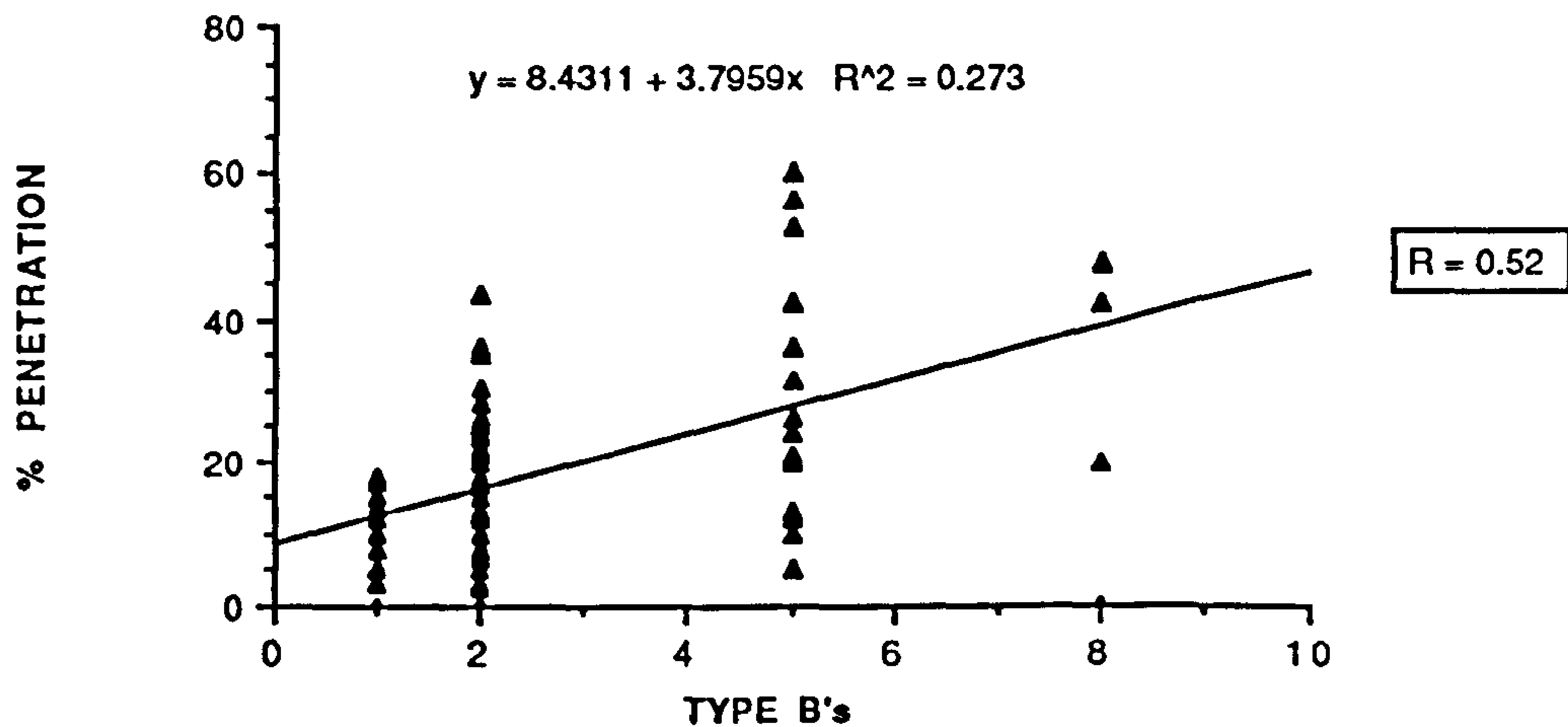
GRAPH 2: LINE OF BEST FIT (CORREL. % PENETR. X CAPS (TOTAL))



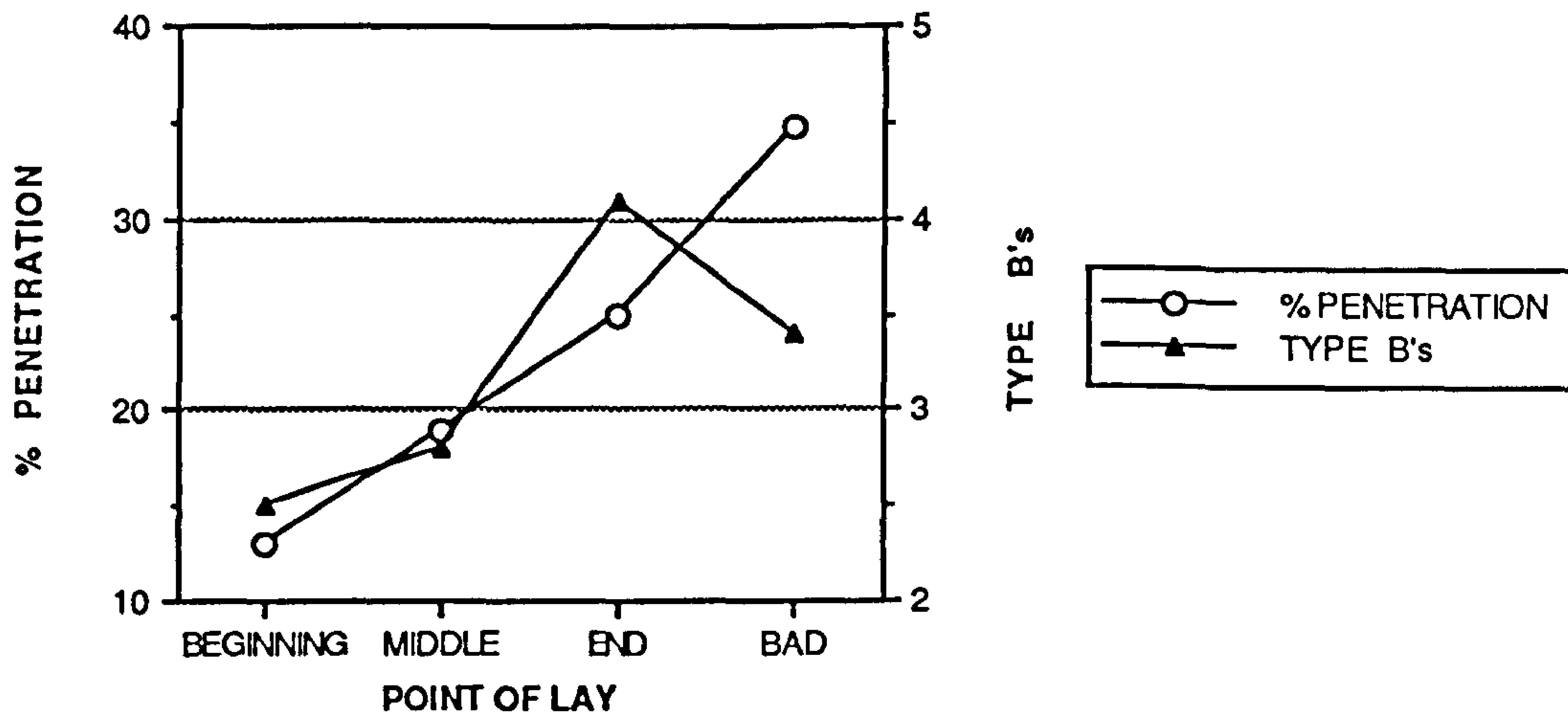
GRAPH 3: C A P S X % PENETRATION IN EACH GROUP



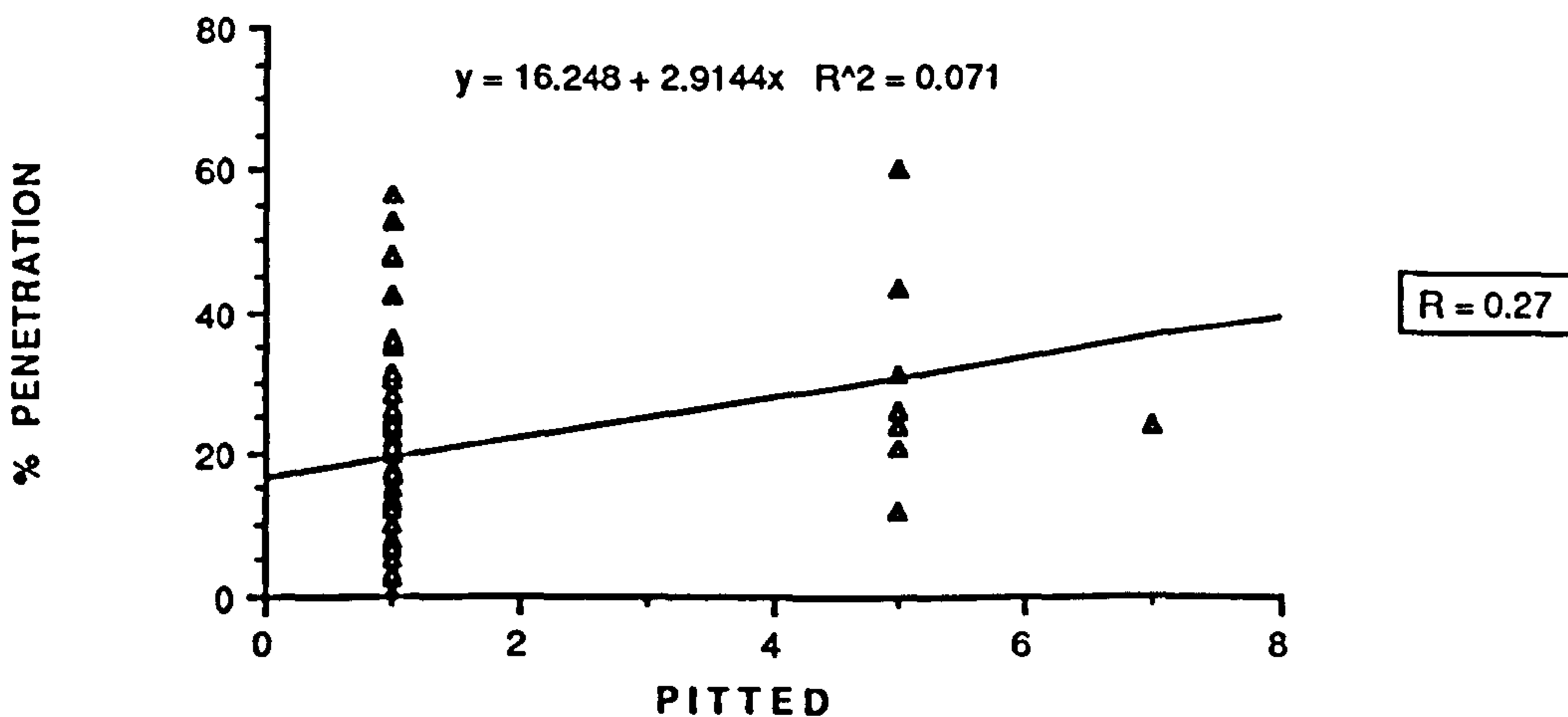
GRAPH 4: LINE OF BEST FIT (CORREL. % PENETR. X TYPE B's (TOTAL))



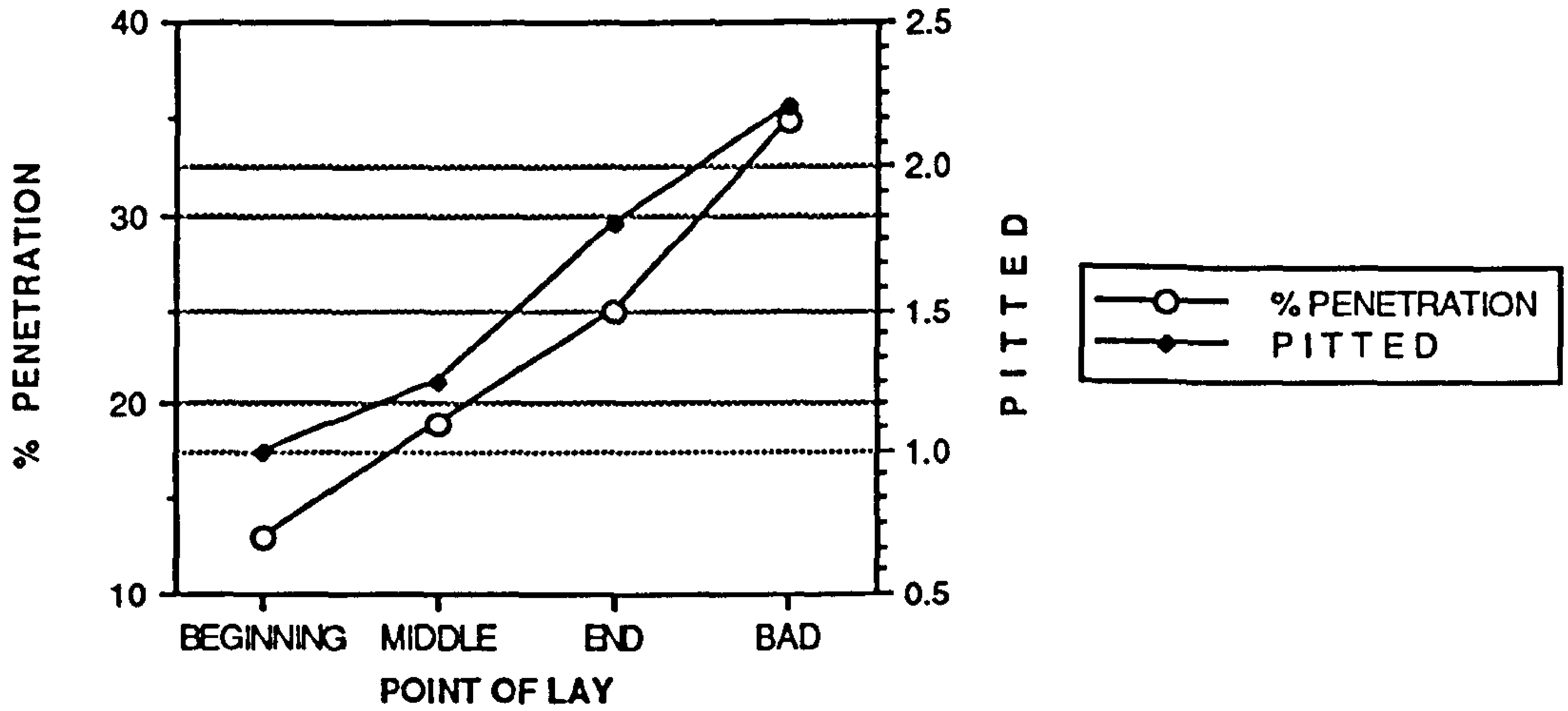
GRAPH 5: TYPE B's X % PENETRATION IN EACH GROUP



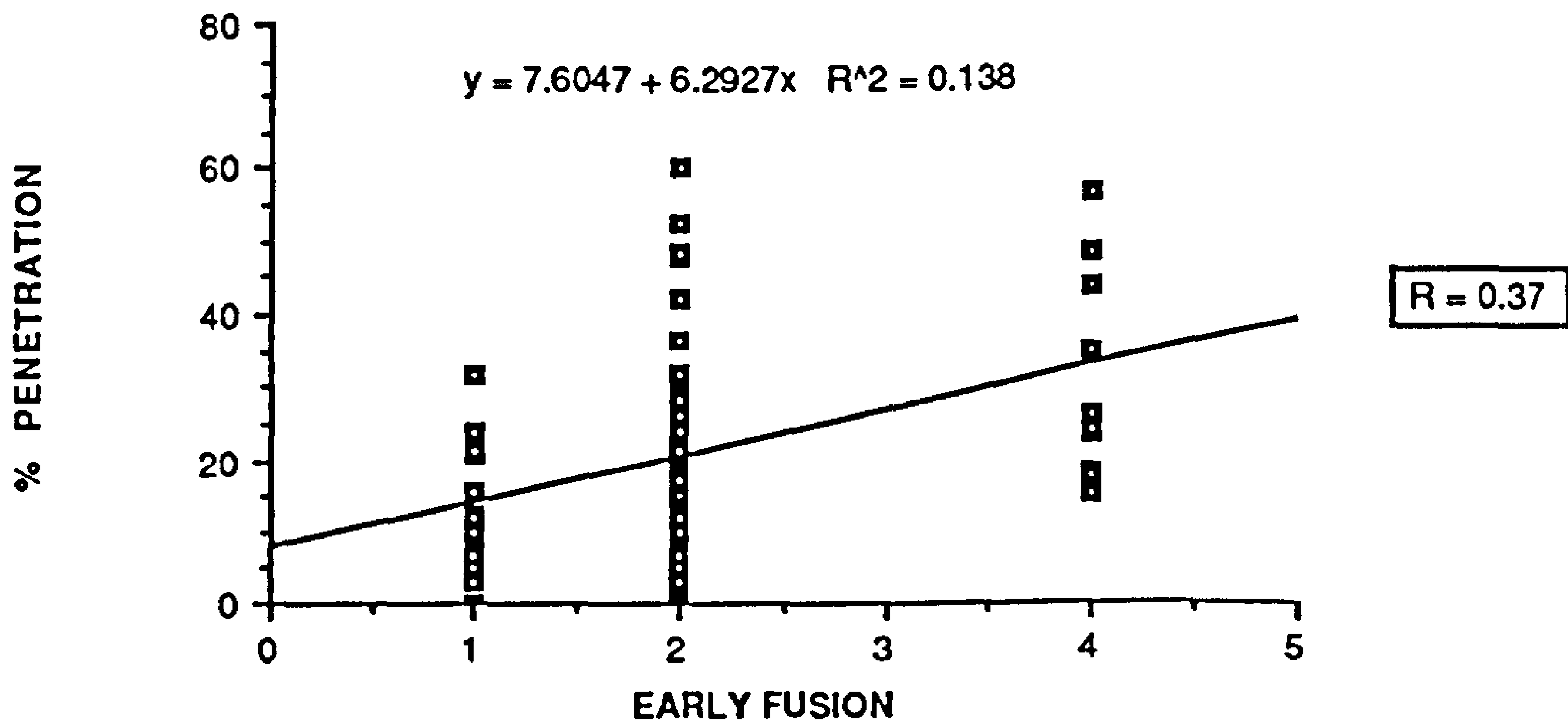
GRAPH 6: LINE OF BEST FIT (CORREL. % PENETR. X PITTED (TOTAL))



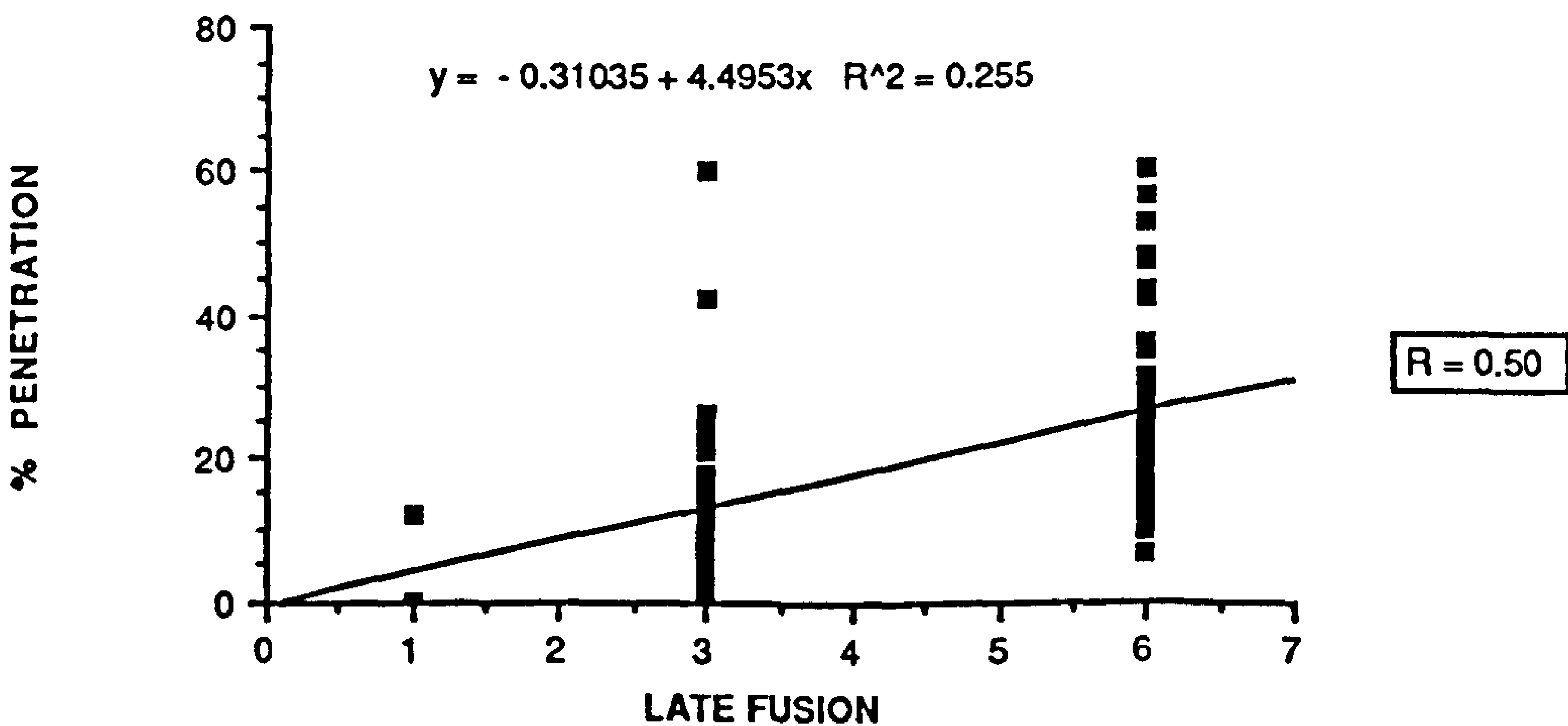
GRAPH 7: PITTED X % PENETRATION IN EACH GROUP



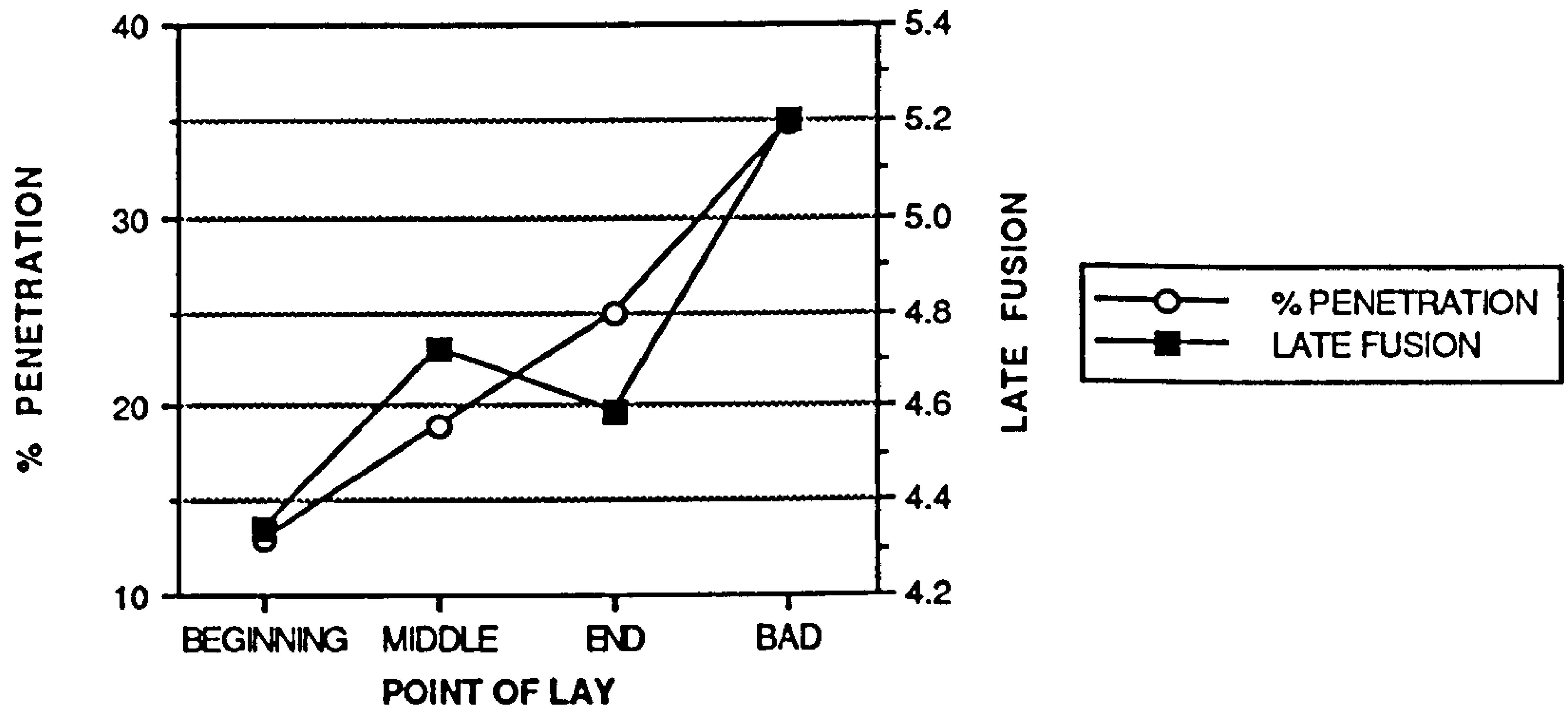
GRAPH 8: LINE OF BEST FIT (CORR. % PENET. X EARLY FUS. (TOTAL))



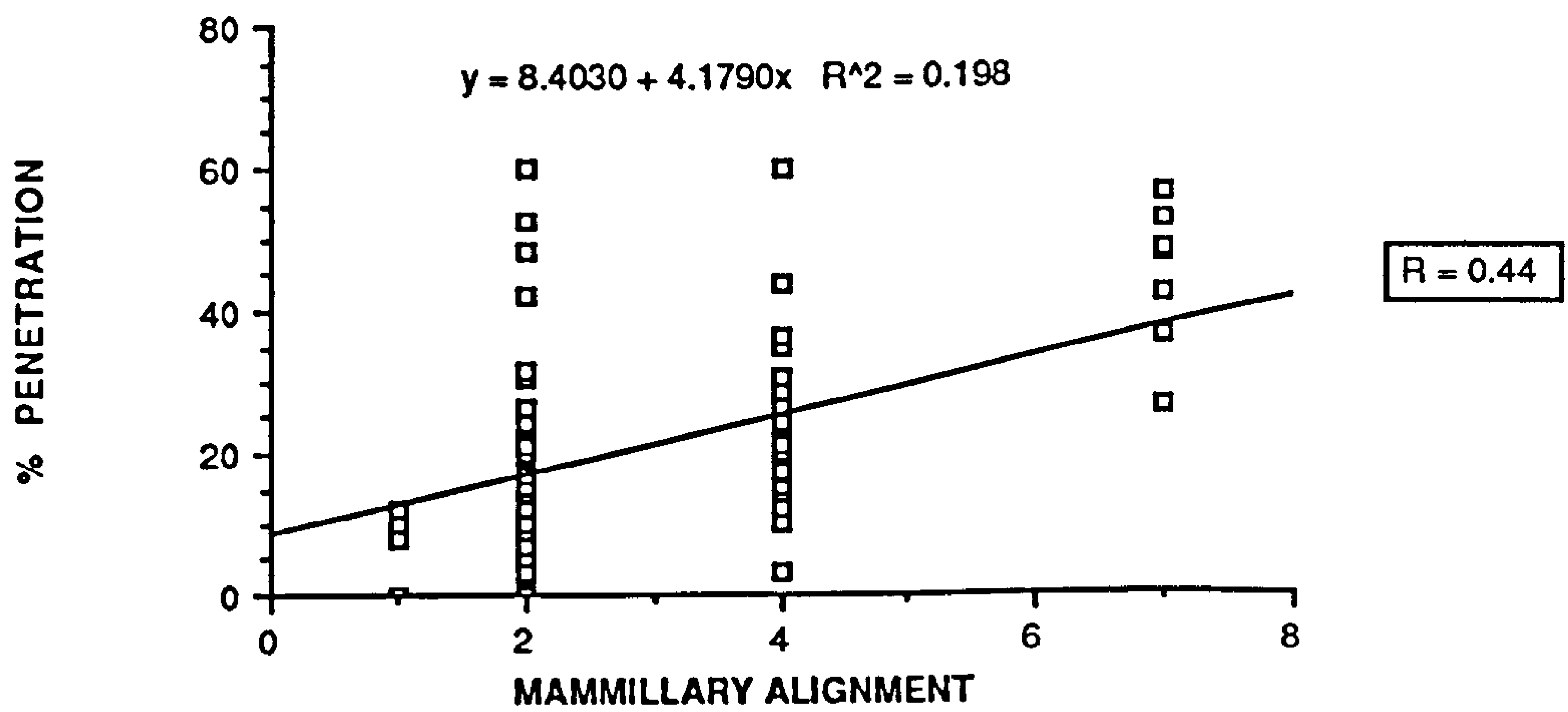
GRAPH 9: LINE OF BEST FIT (CORREL. % PENETR. X LATE FUS. (TOTAL))



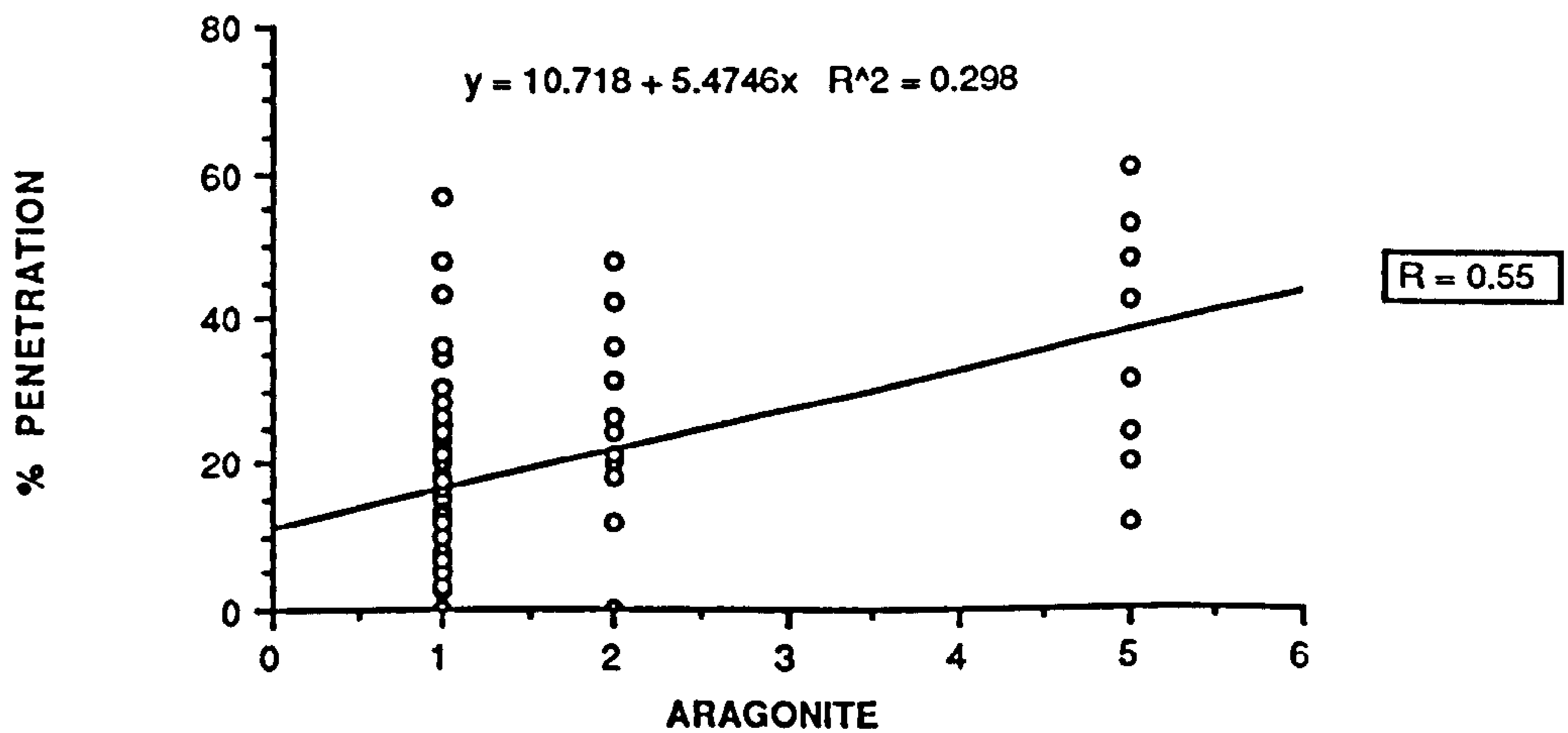
GRAPH 10: LATE FUSION X % PENETRATION IN EACH GROUP



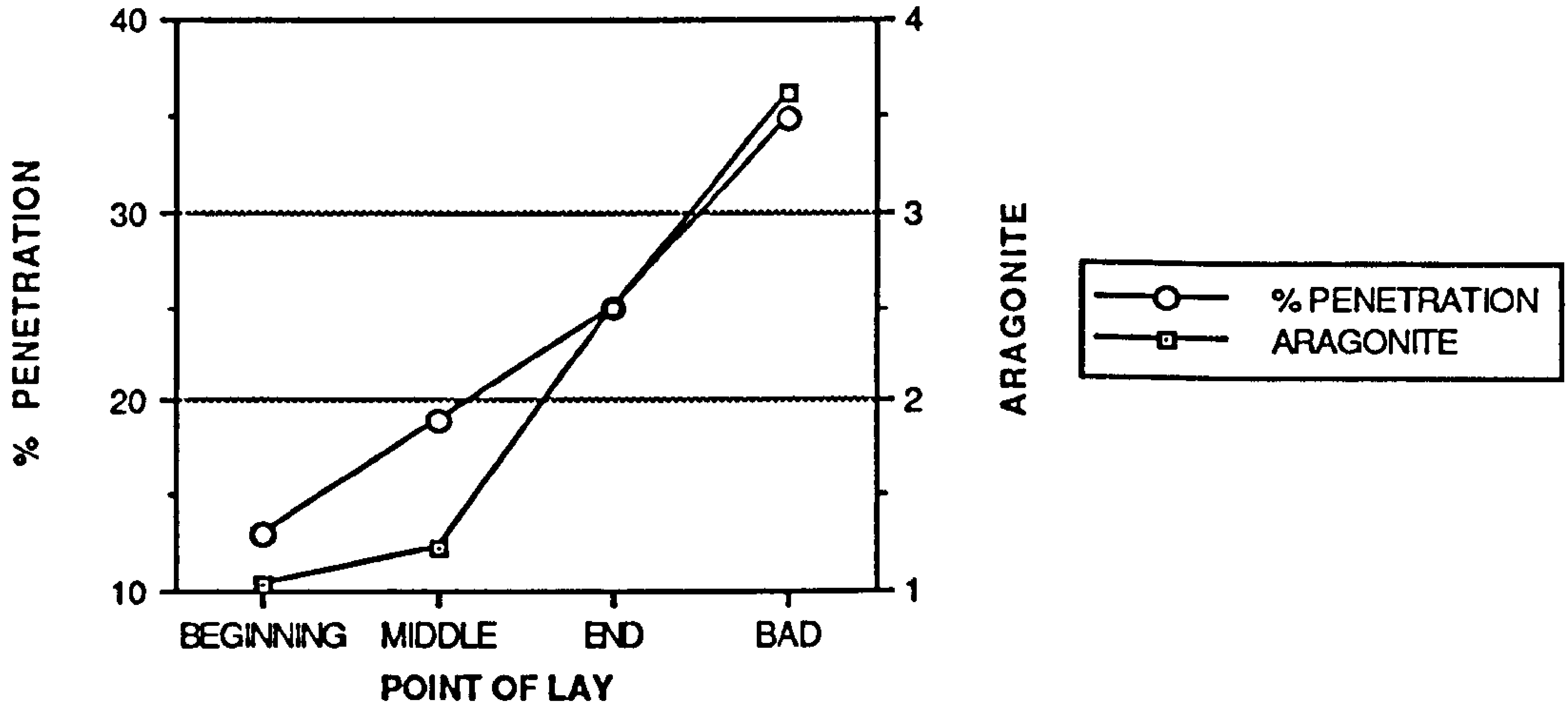
GRAPH 11: LINE OF BEST FIT (CORR. % PENET. X MAMM. ALIGN. (TOTAL))



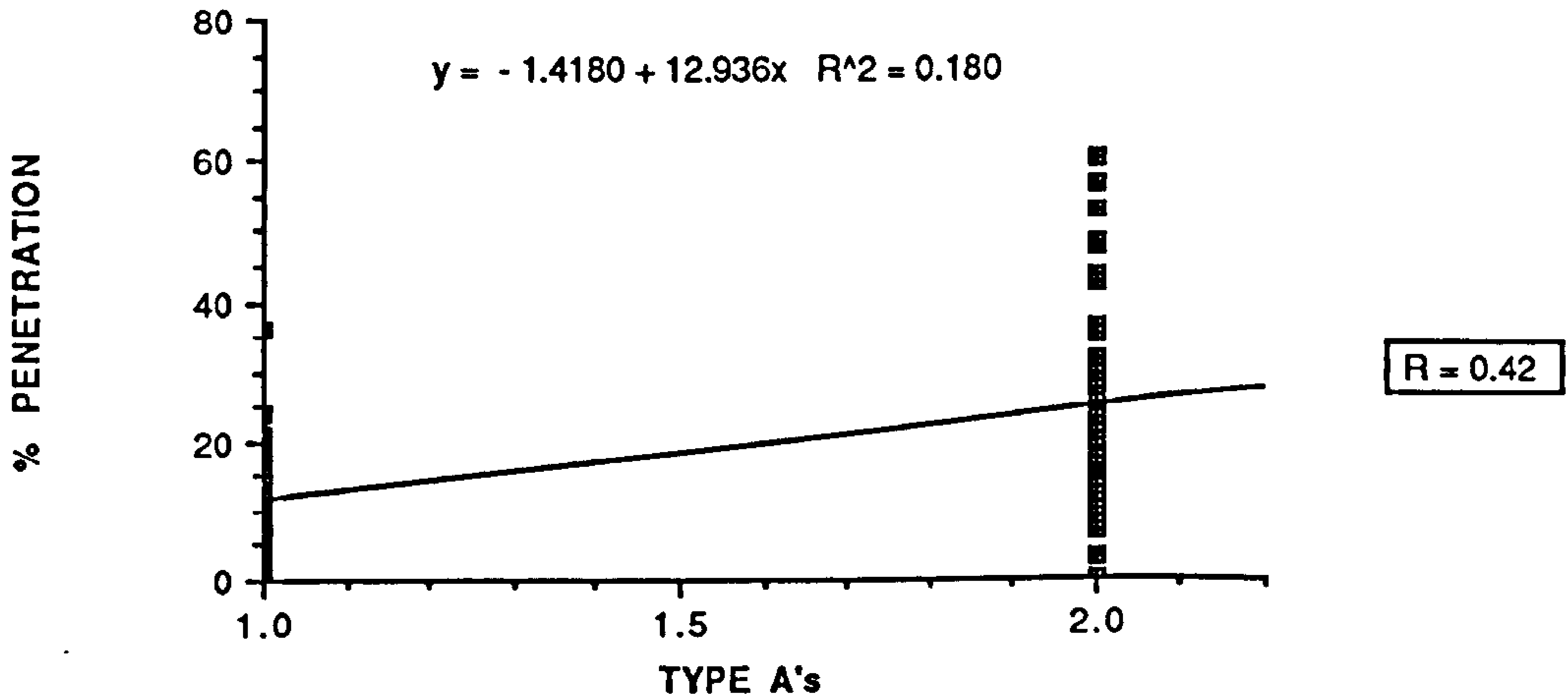
GRAPH 12: LINE OF BEST FIT (CORREL. % PENET. X ARAGONITE (TOTAL))



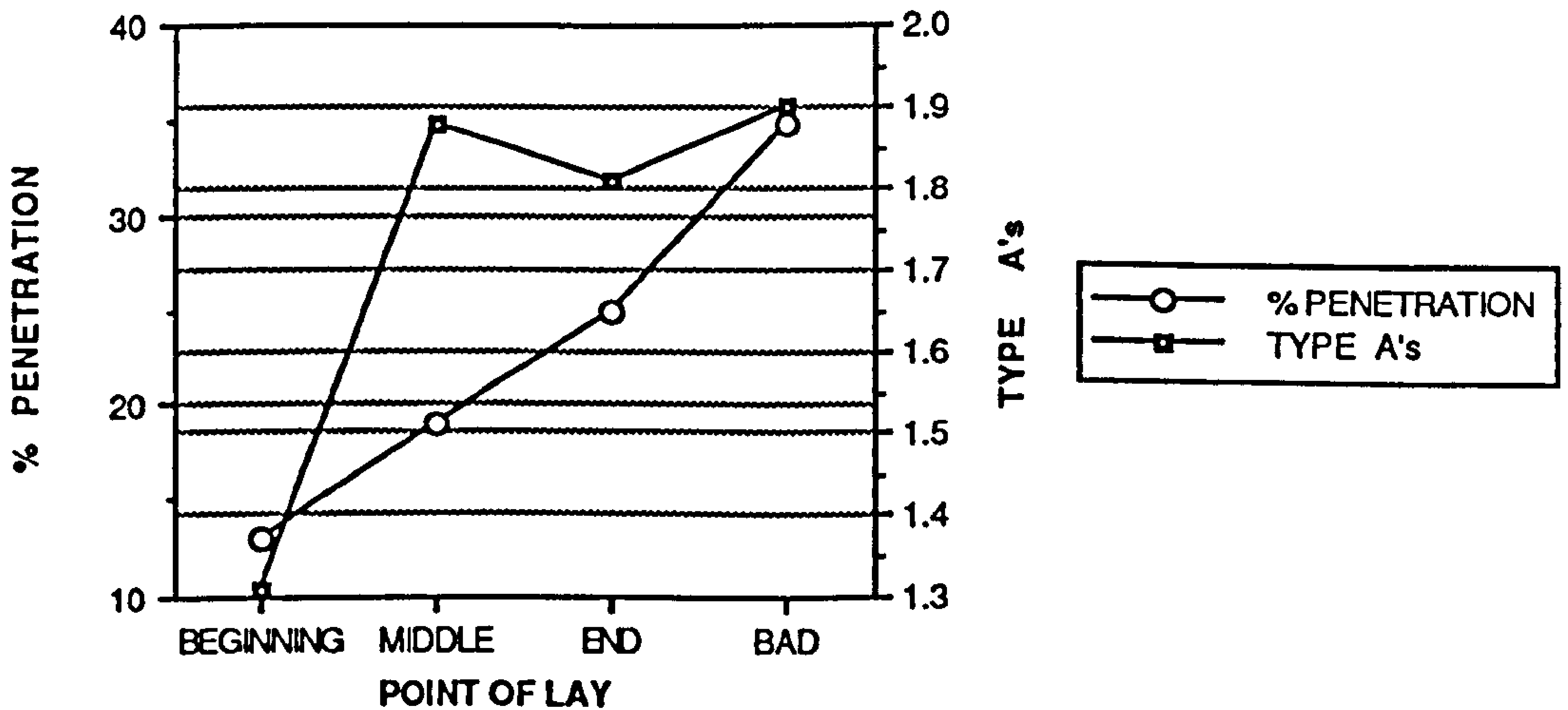
GRAPH 13: ARAGONITE X % PENETRATION IN EACH GROUP



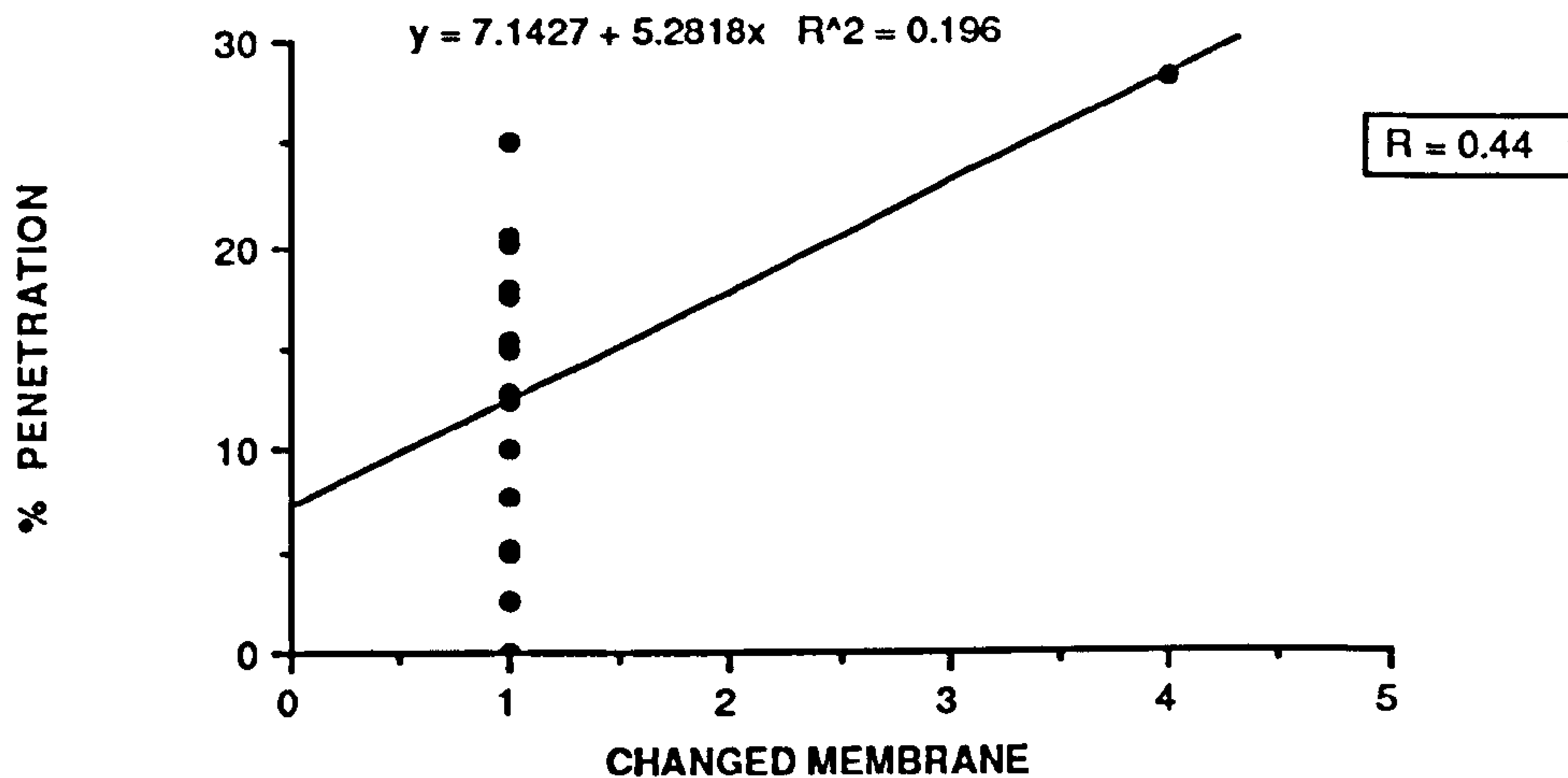
GRAPH 14: LINE OF BEST FIT (CORREL. % PENETR. X TYPE A's (TOTAL))



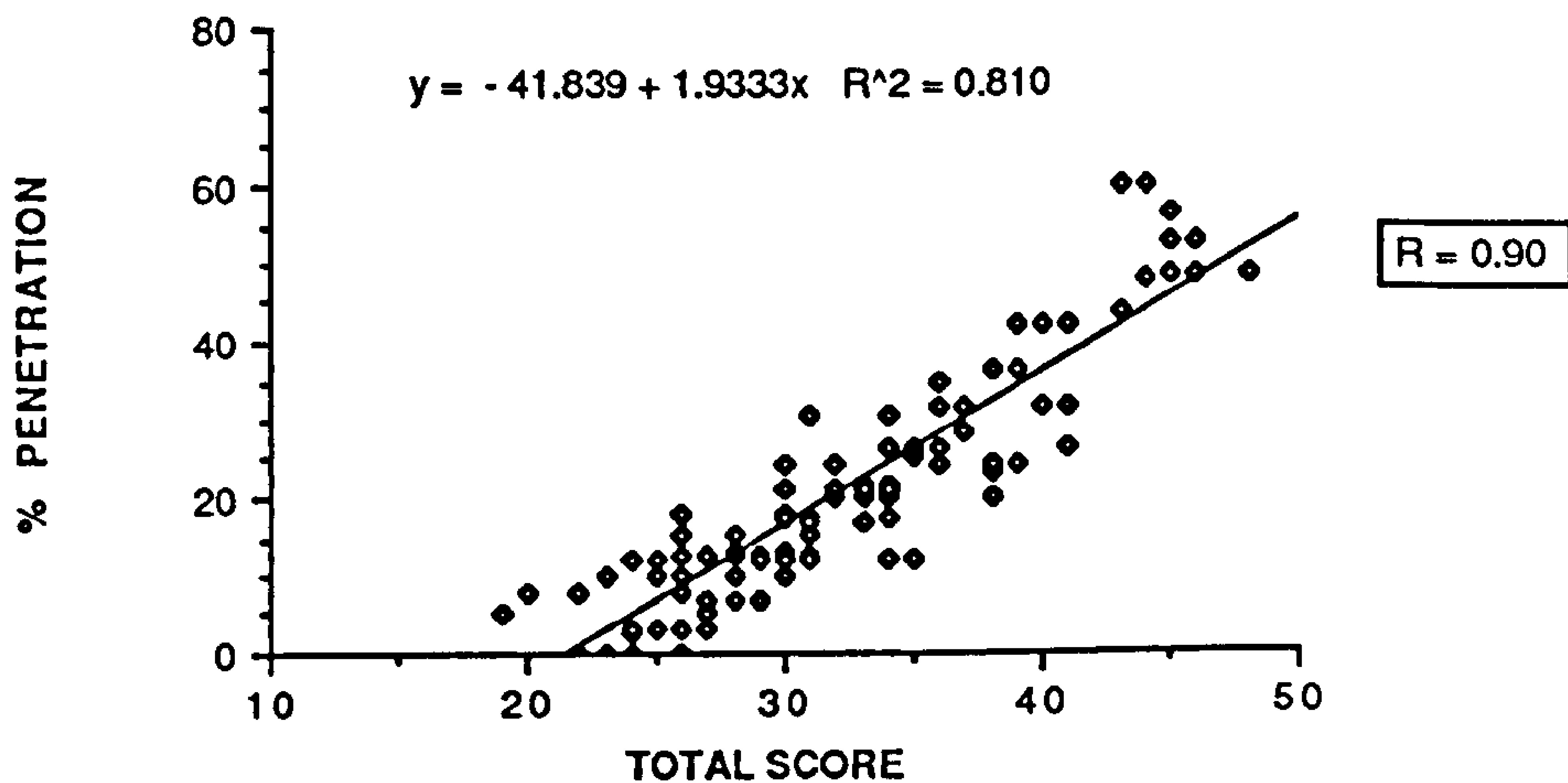
GRAPH 15: TYPE A's X % PENETRATION IN EACH GROUP



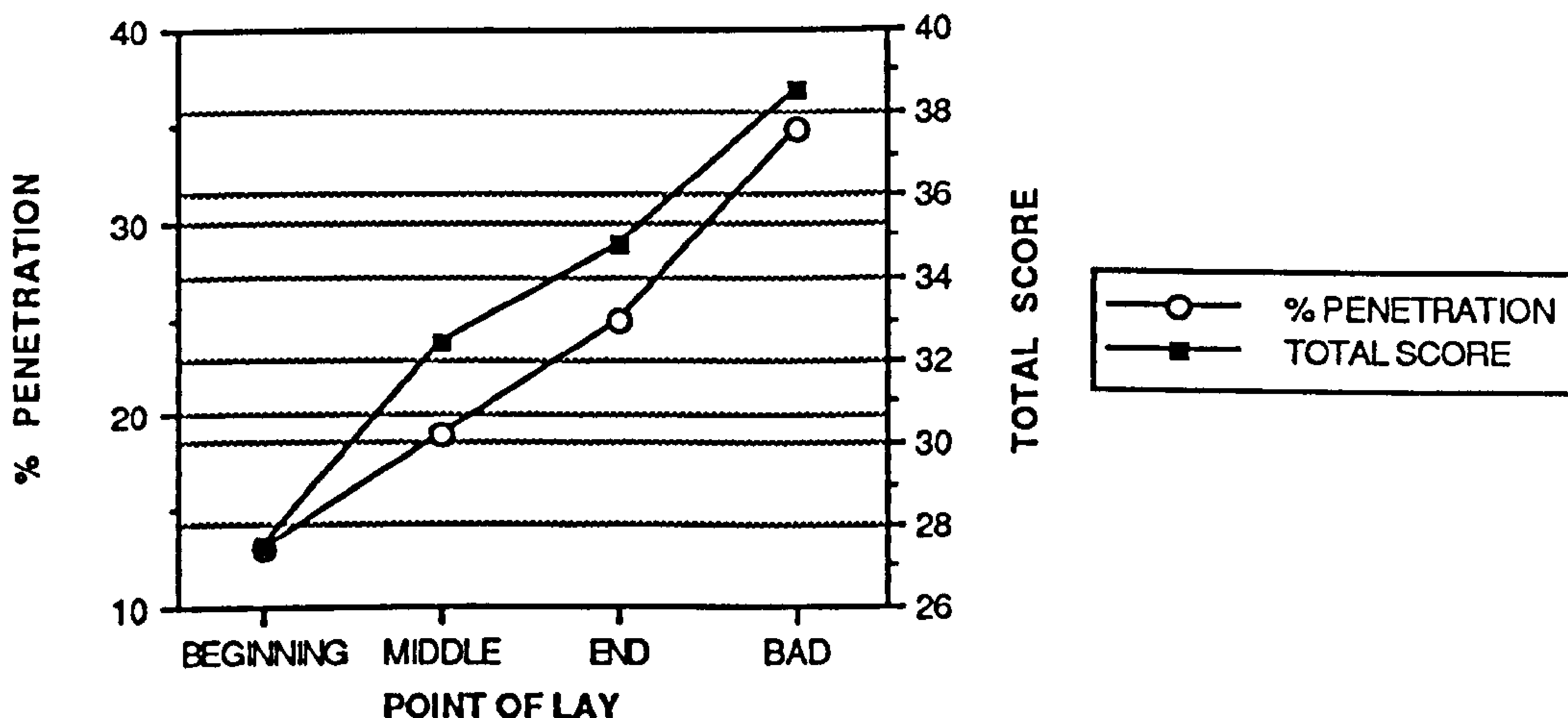
GRAPH 16: LINE OF BEST FIT (CORR. % PENET. X CHANG. MEMB. (BEG))



GRAPH 17: LINE OF BEST FIT (CORREL. % PENET. X TOTAL SCORE)



GRAPH 18: TOTAL SCORE X % PENETRATION IN EACH GROUP



CHAPTER 3

EGGSHELL QUALITY AND BACTERIAL PENETRATION AS AFFECTED BY THE STRAIN OF THE BIRD (STRAIN EFFECT)

3. EGGSHELL QUALITY AND BACTERIAL PENETRATION AS AFFECTED BY THE STRAIN OF THE BIRD (STRAIN EFFECT)

3.1. INTRODUCTION

There are strains of birds that have a history of poor shell quality and others that have good shell quality. Since this characteristic is inherited at a low degree (average 0.39 according to Poggenpoel (1982)), it makes genetic selection for improvement a more important and difficult step (Ewing, 1963). Belyavin *et al.* (1987) proved that impact cracks, *i.e.* star and straight, increased in Shaver flocks over the laying period, while in the ISA flock there was an initial decline, then an increase, before a final decline at the end of lay. Shaver birds were shown by Hughes and Dun (1982) to have a higher mortality rate than Warren birds on Range, and the opposite in cages, perhaps indicating that the Shaver bird is more sensitive.

According to Harrington and Hormaeche (1986), cited in Finlay and Falkow (1989), genetic selection and inbreeding in chickens may select animals with an increased susceptibility to *Salmonella*, as has happened in mice. Certain strains of birds have been shown to be more prone to infection by *S. gallinarum* than others (Buxton and Fraser, 1977). Considerable differences in spoilage of eggs from 3 different strains of birds were observed by Vadehra *et al.* (1970a). The breed of the chicken may also affect the level of the animal's antibody response (Garren and Hill, 1959). The detrimental effect of stress on eggshell quality has already been mentioned and it has been suggested by Solomon (1990b) that some strains of birds can withstand or adapt to stressors more readily than others, and by so doing, illustrate different patterns of eggshell quality deterioration as a consequence.

3.2. MATERIALS AND METHODS

3.2.1. GENERAL

The eggs used in this trial were collected at random from two strains of commercial brown egg layers (strains A and B), housed in a three tier battery system, fed a commercial layers diet and located in the same premises mentioned in section 2.2.1., except the eggs from the end (69 weeks old) of lay of the strain A, which were obtained from a similar strain held under similar conditions from Harper Adams Poultry Research Unit. The other eggs from strain A were collected at the beginning (birds 24 w.o.) and middle (46 w.o.) of lay, while eggs from strain B were collected at the beginning (23 w. o.), middle (42 w. o.) and end (60 w. o.) of lay. These details are summarised below:

	Beginning of lay	Middle of lay	End of lay
Strain A	WSAC (24 w.o.)	WSAC (46 w.o.)	Harper A. (69 w.o.)
Strain B	WSAC (23 w.o.)	WSAC (42 w.o.)	WSAC (60 w.o.)

A total of 186 eggs were used (32 from each age group in strain A and 30 from each age group in strain B).

The *S. enteritidis* PT 4 technical procedures and positioning of the eggshell on the agar plate were all done in the same way as described in sections 2.2.2. and 2.2.4.. The treatment of eggshells was done in the same manner as described in 2.2.3., except that only samples with no membranes attached were used in the analysis. The percentage of penetration values were obtained and analysed as described in 2.2.4. and 2.3.1..

3.3. RESULTS AND DISCUSSION

The tables and graphs are placed at the end of this section. Analysis of the results presented in table 18 showed that caps from the strain B group were consistently significantly better than those from strain A at beginning and middle of lay, although no single feature can be considered in isolation, cap quality could possibly be one of the reasons explaining why strain B eggshells showed less bacterial penetration at those periods (graph 19). Late fusion was also significantly lower in the strain B group in the same laying periods mentioned above (graph 20).

Mammillary alignment was highly significantly lower in the strain B group in an overall evaluation over the 3 laying periods, although at the end of lay both strains were similar (graph 21). Type B's were significantly lower in the strain B group over all periods (beginning, middle and end of lay), but especially at the end (graph 22).

Pitting levels were strikingly (although non-significantly) higher in strain B eggs. Again, other characteristics may have helped to compensate the effect of this occurrence in terms of bacterial penetration, and possibly the fact that pitting levels remained low in real terms (only a few isolated depressions detected) may have contributed to keep penetration levels low. On the other hand, cuffing levels showed a different picture, with higher levels (lower scores) present in strain A at the beginning and middle of lay, but with significantly higher levels (lower scores) in strain B at the end of lay (graph 23). Cuffing has a beneficial effect, and it may be speculated that this was one more factor that contributed to balance the amount of restrain each individual characteristic exerted on bacterial penetration.

The levels of Changed membrane, which were similar at the beginning and middle of lay, showed a sharp and very highly significant increase in occurrence at the end of lay in the strain B group. Normally considered as an

unwanted feature in terms of eggshell quality *in vivo*, these sulphur-rich changed membranes in this case may have helped to physically restrain bacterial penetration through the eggshell, since the remnants were retained attached to the shell even after the plasma etching. This anomaly could only happen *in vitro*, as *in vivo* these altered membranes weaken shell structure (graph 23).

The total score values did reflect the slightly improved shell structure showed by the strain B group at the beginning and middle of lay. The non-significant higher total score mean presented by the same group at the end of lay was not totally unexpected, due to higher levels of confluence (positive feature for penetration protection) present, and also the higher amounts of changed membrane. The fact that a trial run with another end of lay group from the same strain (results not shown) indicated a mean total score of 30 suggests that, despite being usually the best indicative of shell quality, sometimes this summation of individual values alone is not sufficient to explain an egg's capacity to restrain bacterial penetration, and makes it necessary to consider each structural variant independently (graph 24).

It has already been stated that pores are not the sole means of entry through the shell, nevertheless they must be considered as part of the jigsaw; thus patent unplugged pores must reduce resistance to bacterial penetration. The latter was consistently lower in strain B eggs, over all 3 periods of lay in comparison with strain A (graph 25). These results have to be interpreted with caution, and before extolling the virtues of one strain over another, it must be pointed out that although the birds were kept in similar premises, and although management routines "appeared" to be similar, there were site differences.

Strain differences with respect to feed conversion and egg production do of course exist, and the results of ultrastructural examination of the shells of various strains, housed under identical conditions show a similar variation (Solomon, pers. comm.). Since the shell is required to perform so many

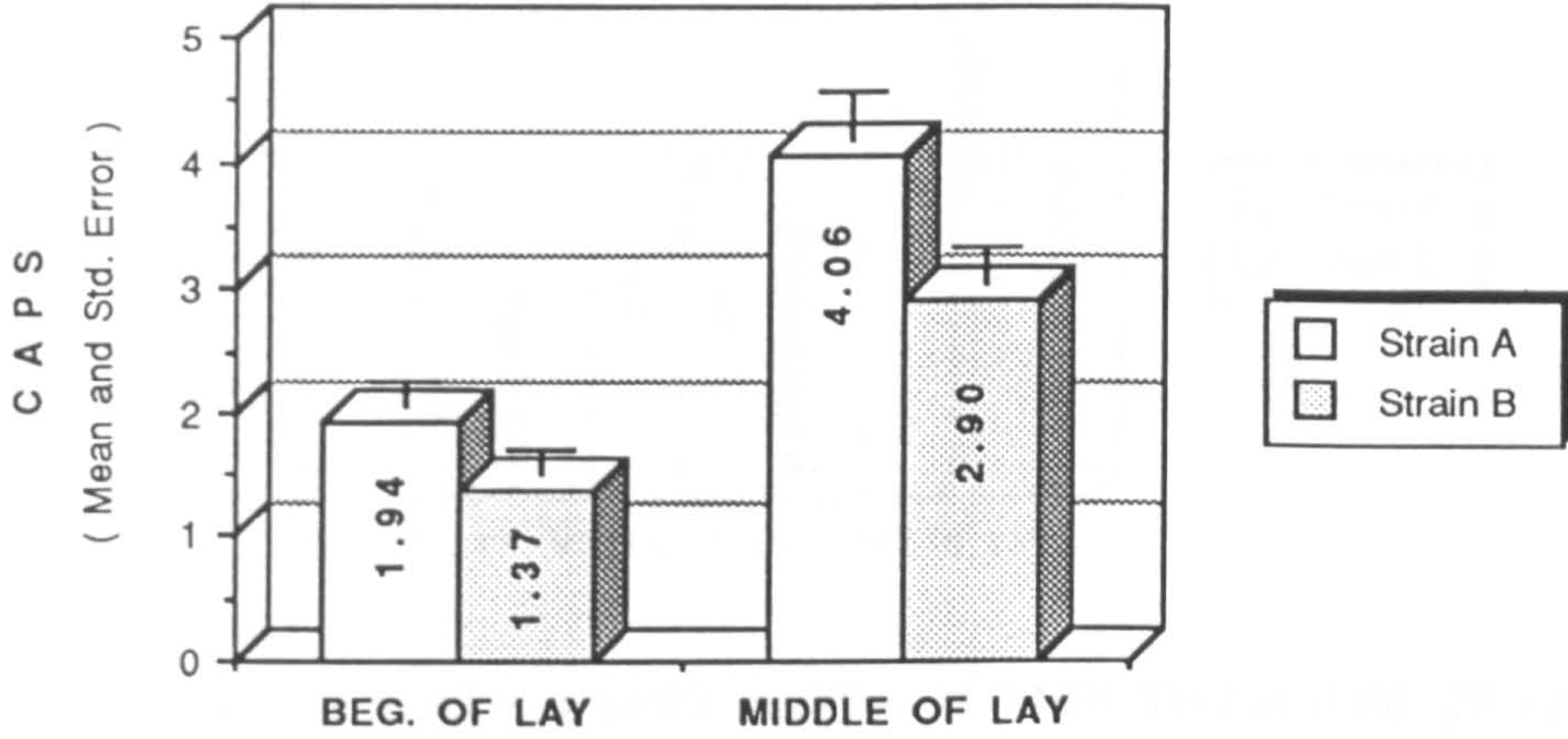
diverse functions, the ultimate goal for the poultry industry should be the compilation of these features.

	Overall (A x B)	Beginning of lay			Middle of lay			End of lay		
		Strain A	Strain B	(A x B)	Strain A	Strain B	(A x B)	Strain A	Strain B	(A x B)
CONFLUENCE	(NS)	3.94 ± 1.60	3.93 ± 1.39	(NS)	4.16 ± 0.99	3.87 ± 1.17	(NS)	3.66 ± 1.18	4.20 ± 1.42	(NS)
CAPS	(NS)	1.94 ± 1.01	1.37 ± 1.07	(*)	4.06 ± 2.17	2.90 ± 1.67	(*)	4.72 ± 1.71	5.63 ± 2.08	(NS)
EARLY FUSION	(NS)	1.97 ± 0.90	1.77 ± 0.63	(NS)	2.28 ± 0.89	2.37 ± 0.96	(NS)	1.97 ± 0.78	2.47 ± 1.07	(*)
LATE FUSION	(**)	4.34 ± 1.62	3.23 ± 1.76	(*)	4.72 ± 1.61	3.27 ± 1.87	(**)	4.59 ± 1.52	4.70 ± 1.51	(NS)
MAMM. ALIGNMENT	(**)	2.62 ± 1.04	1.63 ± 1.03	(***)	3.28 ± 1.73	2.53 ± 1.36	(NS)	2.59 ± 1.58	2.63 ± 1.35	(NS)
TYPE B's	(**)	2.50 ± 1.39	1.70 ± 1.21	(*)	2.81 ± 2.02	2.53 ± 1.87	(NS)	4.09 ± 2.13	2.63 ± 1.56	(**)
PITTING	(*)	1.00 ± 0.00	1.40 ± 1.22	(NS)	1.25 ± 0.98	1.67 ± 1.52	(NS)	1.81 ± 1.75	2.53 ± 2.08	(NS)
ARAGONITE	(NS)	1.03 ± 0.18	1.60 ± 0.50	(***)	1.22 ± 0.75	1.20 ± 0.41	(NS)	2.50 ± 1.65	1.97 ± 1.30	(NS)
TYPE A's	(NS)	1.31 ± 0.47	1.20 ± 0.41	(NS)	1.88 ± 0.34	1.57 ± 0.50	(**)	1.81 ± 0.40	1.83 ± 0.38	(NS)
CUBICS	(NS)	1.22 ± 0.42	1.07 ± 0.25	(NS)	1.59 ± 1.19	1.30 ± 0.47	(NS)	1.31 ± 0.47	1.40 ± 0.50	(NS)
CUFFING	(***)	4.44 ± 0.80	4.97 ± 0.18	(***)	4.06 ± 1.27	4.97 ± 0.18	(***)	4.88 ± 0.34	4.63 ± 0.49	(*)
CHANGED MEMBRANE	(**)	1.09 ± 0.53	1.00 ± 0.00	(NS)	1.19 ± 0.74	1.00 ± 0.00	(NS)	1.00 ± 0.00	3.13 ± 3.05	(***)
TOTAL SCORE	(NS)	27.44 ± 4.47	24.87 ± 5.61	(*)	32.44 ± 5.84	29.23 ± 9.13	(NS)	34.78 ± 6.66	37.97 ± 7.62	(NS)
MAMMILLARY DENSITY	(NS)	82.91 ± 13.70	71.99 ± 15.38	(**)	78.58 ± 15.80	75.47 ± 15.00	(NS)	75.93 ± 17.20	80.76 ± 15.09	(NS)
PORES (number)	(NS)	18.57 ± 16.30	60.13 ± 18.29	(***)	116.80 ± 70.70	99.93 ± 81.61	(NS)	174.97 ± 119.90	146.17 ± 90.13	(NS)
% PENETRATION	(***)	12.92 ± 6.33	7.70 ± 6.69	(**)	18.91 ± 14.14	8.43 ± 6.80	(***)	24.97 ± 14.00	9.22 ± 6.55	(***)

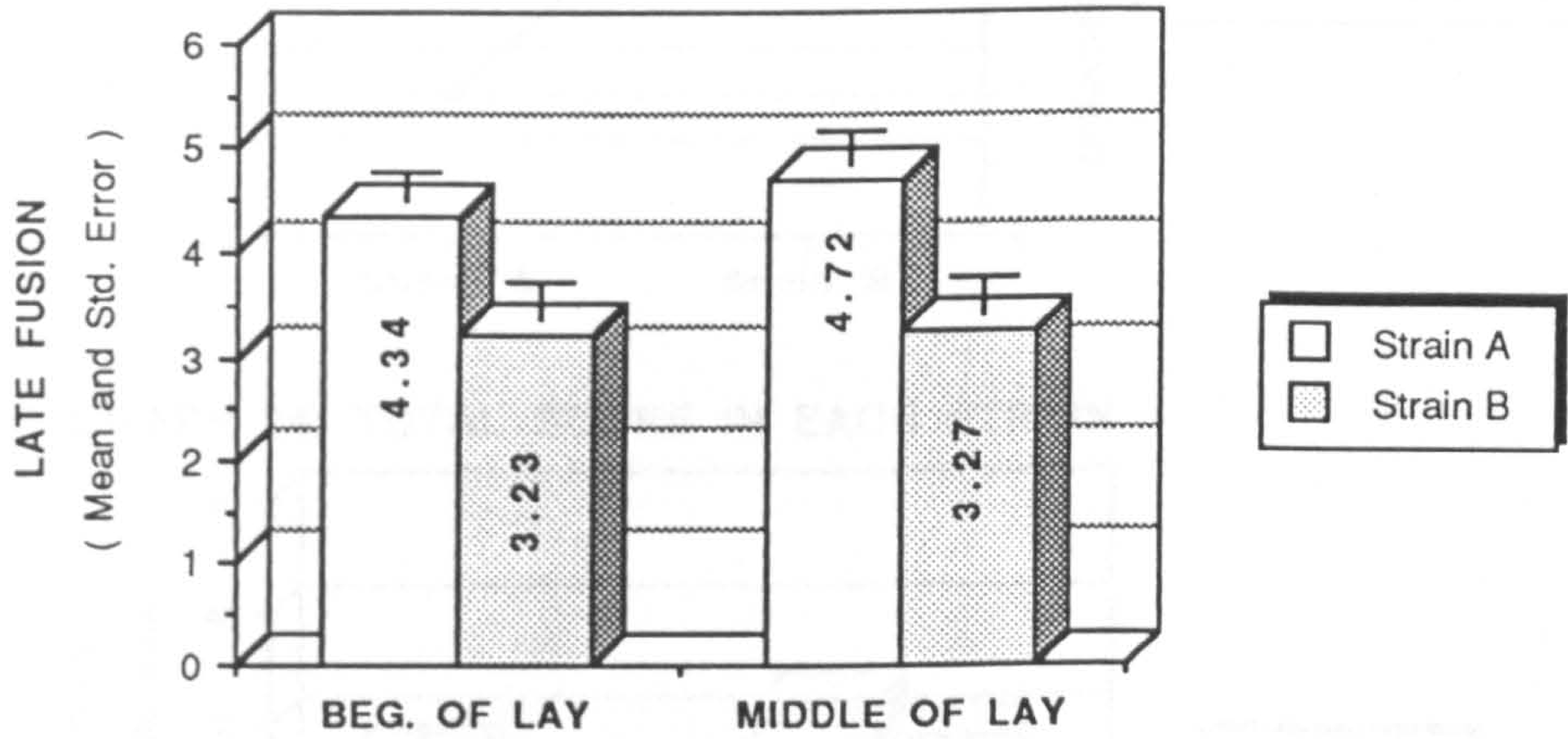
Table 18: Structural scores (mean ± s.d.) for each individual characteristic of the cone layer in eggs from strains A and B, and number of pores and percentage of penetration.

*** = Very highly significant at a 0.1 % level (P< 0.001)
* = Significant at a 5 % level (P< 0.05)
** = Highly significant at a 1 % level (P< 0.01)
NS = Not Significant (P> 0.05)

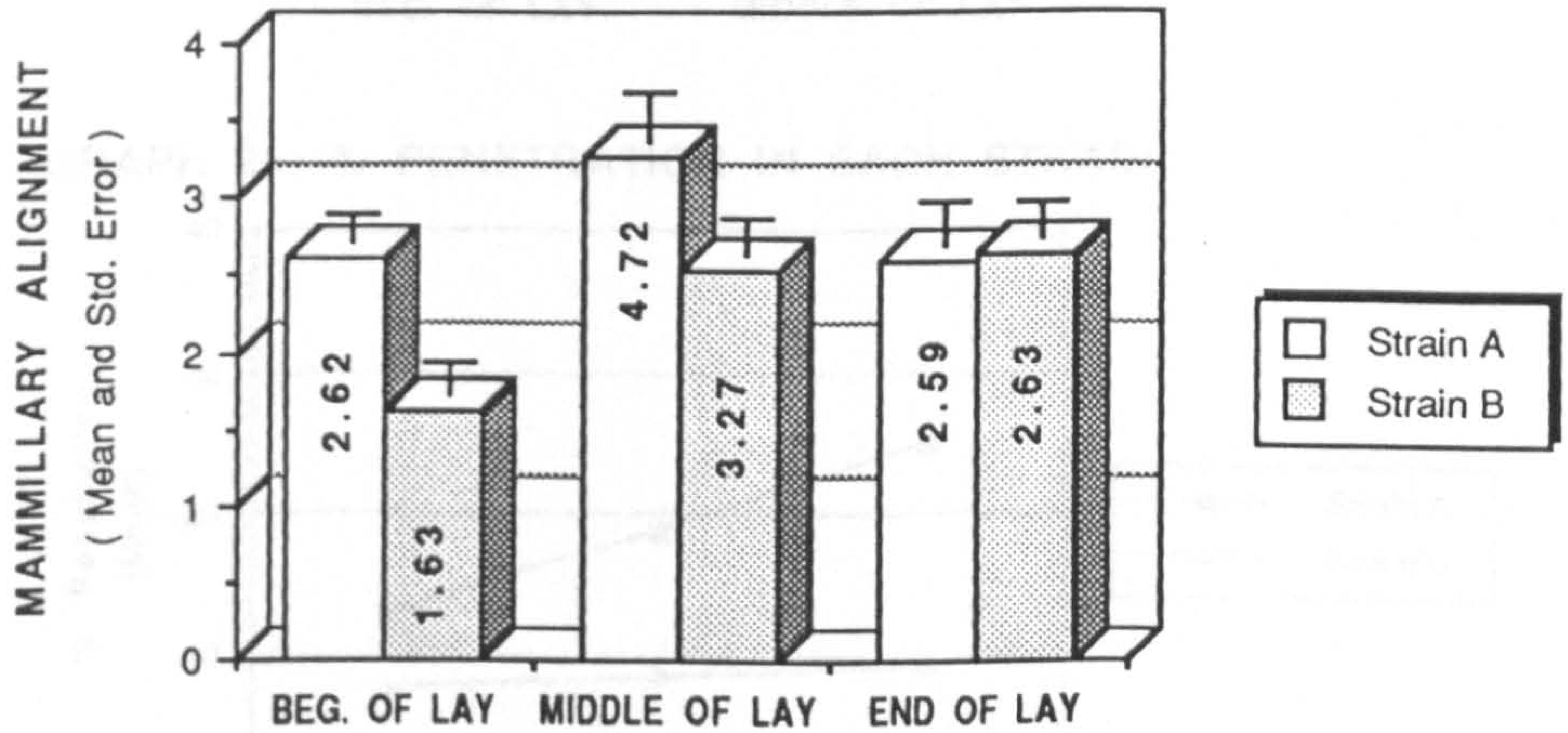
GRAPH 19: C A P S IN E A C H S T R A I N



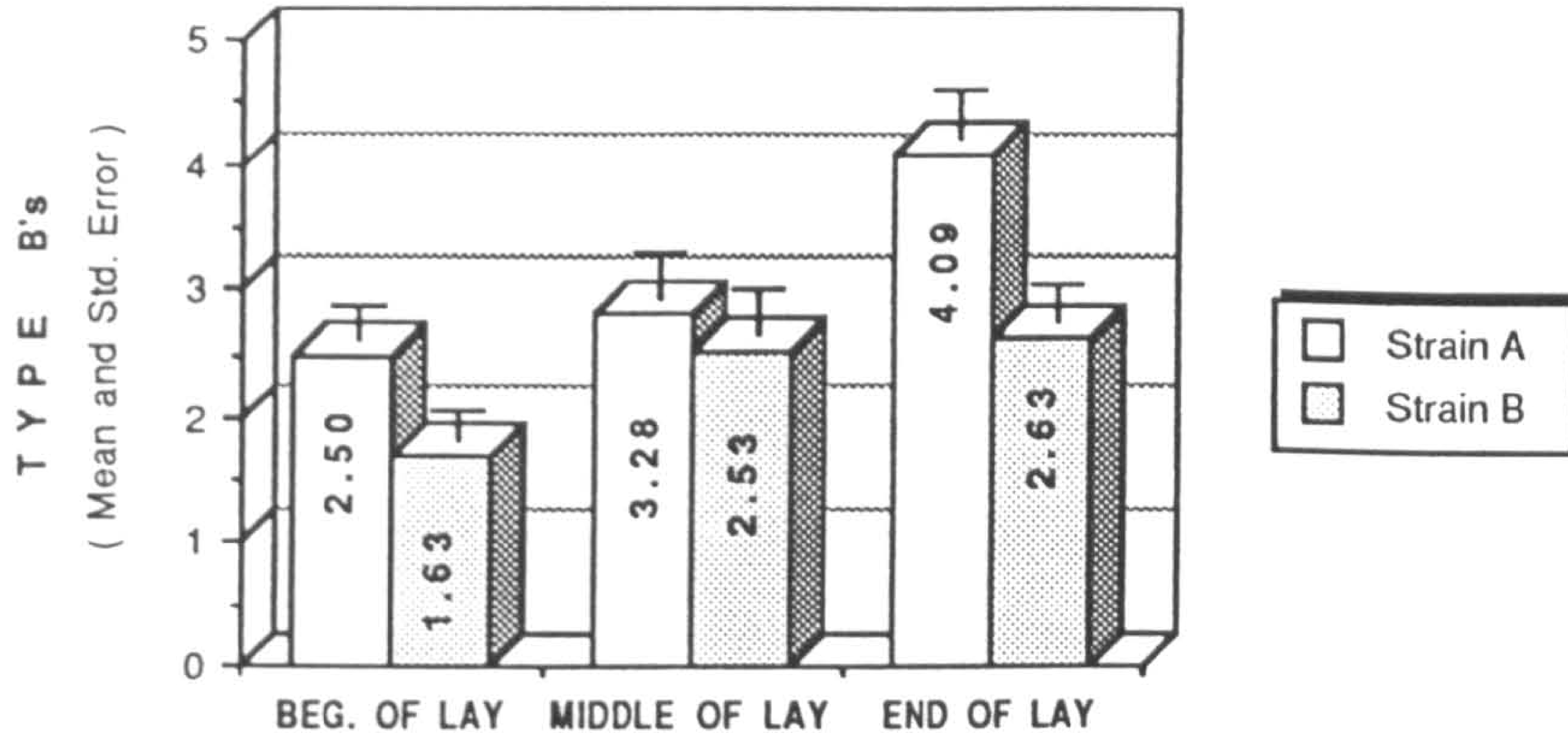
GRAPH 20: LATE FUSION IN EACH STRAIN



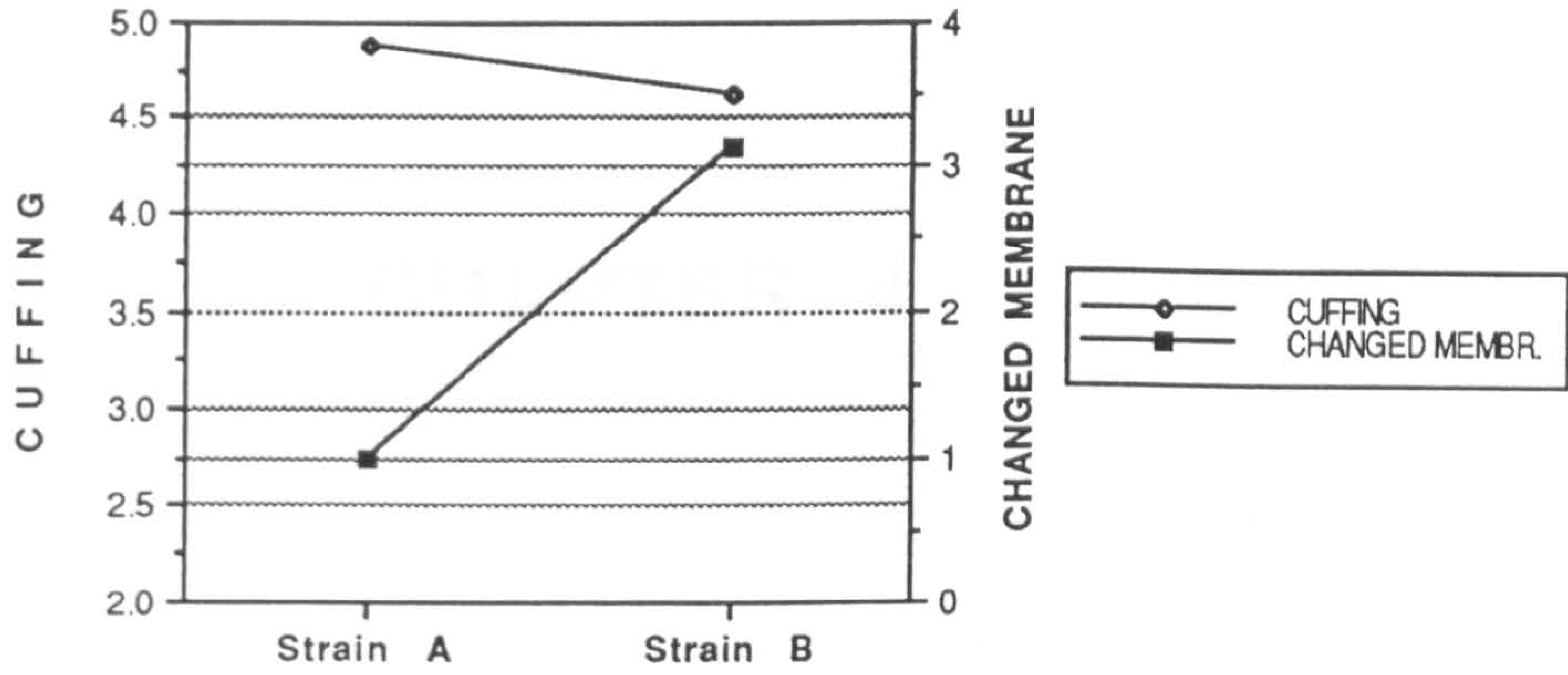
GRAPH 21: MAMMILLARY ALIGNMENT IN EACH STRAIN



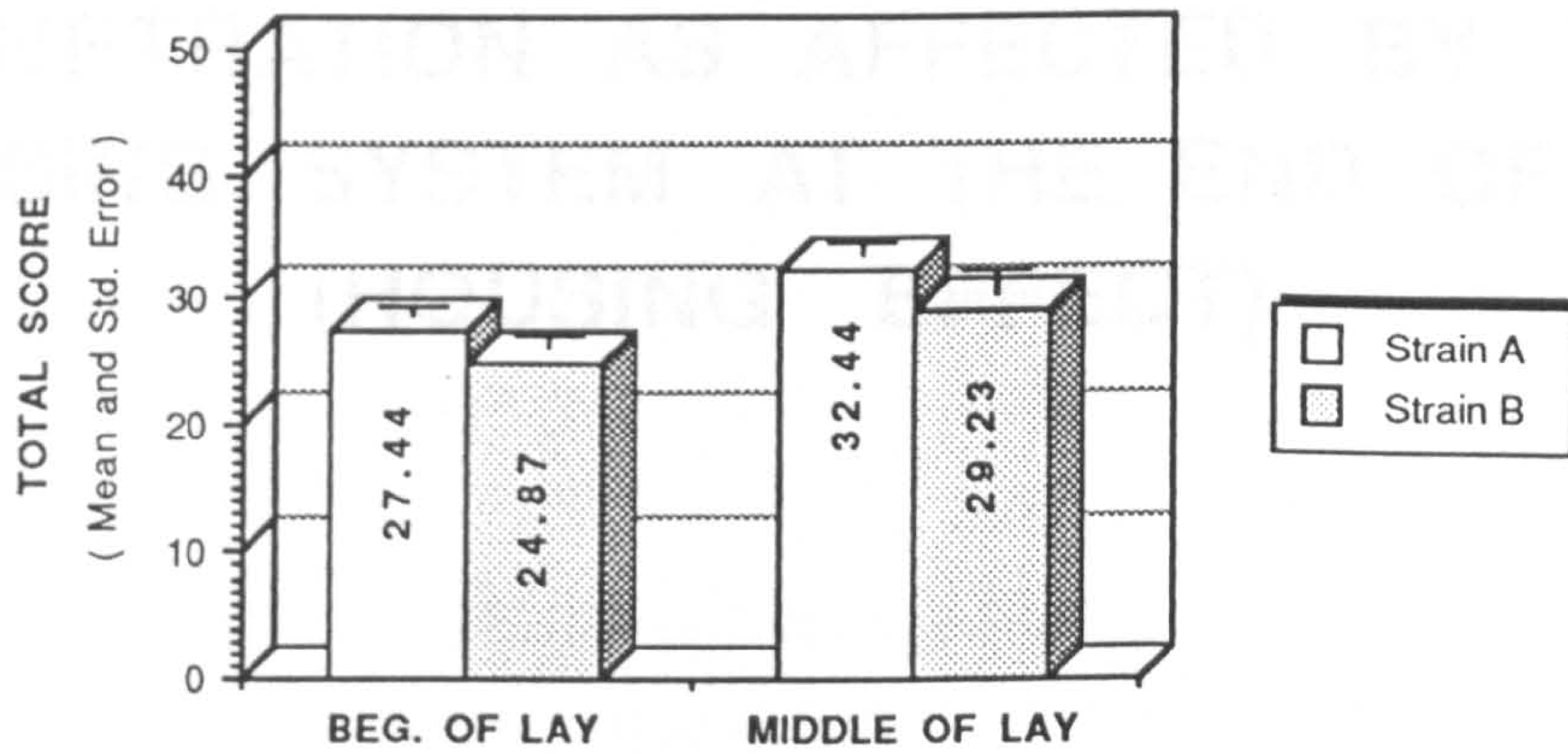
GRAPH 22: TYPE B's IN EACH STRAIN



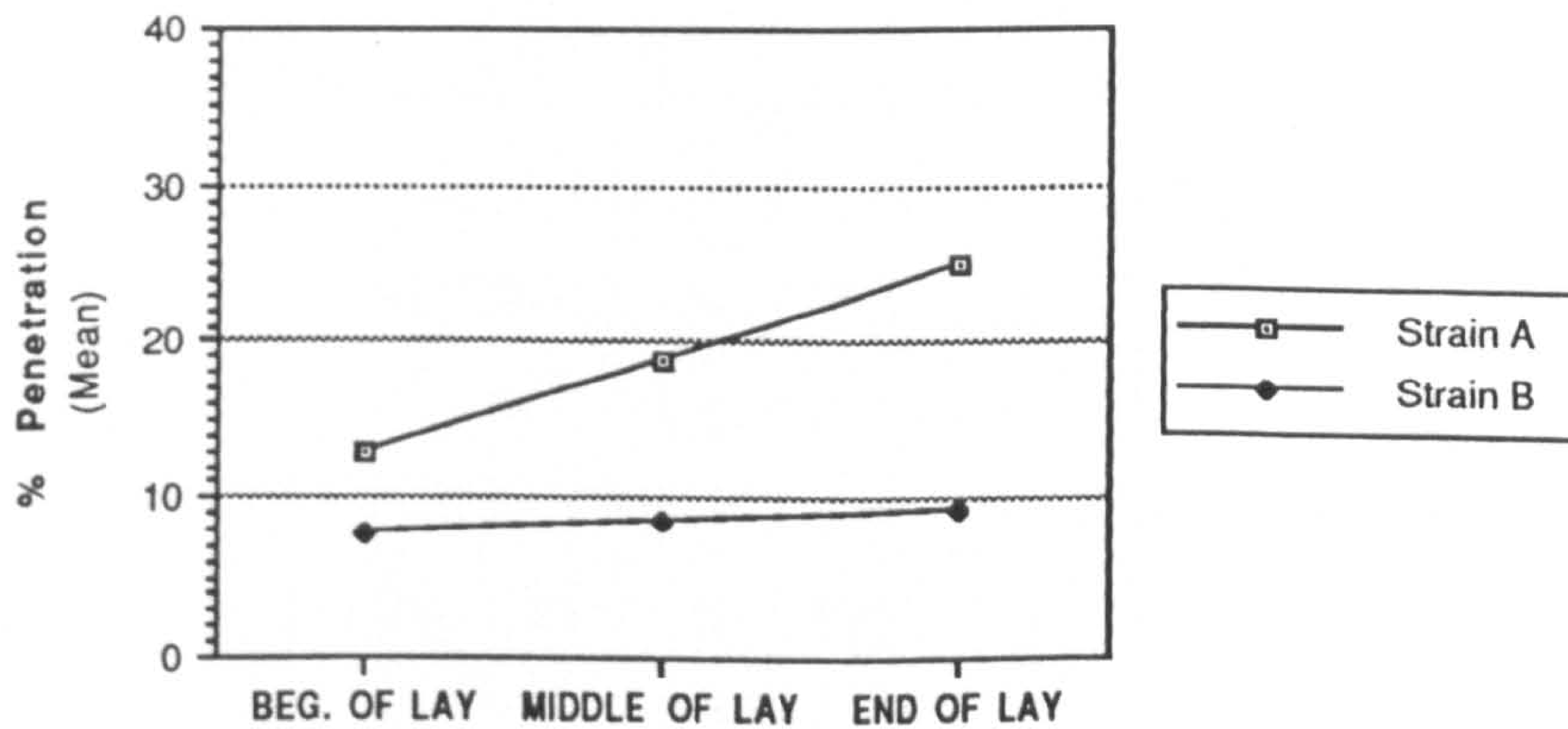
GRAPH 23: CUFFING and CHGD. MEMBR. IN EACH STRAIN (END OF LAY)



GRAPH 24: TOTAL SCORE IN EACH STRAIN



GRAPH 25: % PENETRATION IN EACH STRAIN



CHAPTER 4

EGGSHELL QUALITY AND BACTERIAL PENETRATION AS AFFECTED BY THE HOUSING SYSTEM AT THE END OF LAY (HOUSING EFFECT)

4. EGGSHELL QUALITY AND BACTERIAL PENETRATION AS AFFECTED BY THE HOUSING SYSTEM AT THE END OF LAY (HOUSING EFFECT)

4.1. INTRODUCTION

According to Hughes and Dun (1982), information concerning the relative performance and condition of hens kept in different management systems was lacking. The available data were often confused by the inclusion of too many variables, and inconsistency in experimental methods only served to confuse the issue even more. Thus, egg weight and food intake were found to be consistently higher on free-range, with proportionally less cracked eggs in comparison with caged birds (Hughes and Dun, 1982 and 1983 and Belyavin, 1988), and while having greater egg mass, sexual maturity was delayed on range compared to cages (Hughes and Dun, 1983).

Shell thickness was greater in eggs from range in comparison with eggs from cage. Such eggs also had 8.9 % more shell strength at 36 weeks and were 6.5 % stronger at 70 weeks (Hughes *et al.*, 1985), which contradicts the results of Funk *et al.* (1958), Timmons *et al.* (1961) and Johnson and Zindel (1962) (all cited in Wolford and Tanaka, 1970).

Feed efficiency, however, was better in the caged groups (Hughes and Dun, 1982 and 1983) and, as expected, there was a higher incidence of dirty eggs from the range group (Hughes and Dun, 1982 and Belyavin, 1988).

Conventional measures of quality (specific gravity, deformation) indicated that eggs produced by caged birds were poorer than those from birds on range, despite the former displaying ultrastructural superiority (Belyavin *et al.*, 1991). These findings are in agreement with the earlier work of Mohumed (1986), Solomon (1988a) and the recent findings of Bain *et al.* (1992). One of the reasons given was the more controlled environment and

nutrient availability in the cage system.

Free-range, strawyard and perchery held birds show a more rapid decline in shell structure quality associated with age than their battery-held counterparts (Bain *et al.*, 1992). This is in agreement with Mohumed (1986), although he also found battery eggs deformed more and were thinner.

These apparently contradictory results may be partially explained by the fact that eggs laid in cages are exposed to more damaging shocks and severe environmental insults than on range (Hughes *et al.*, 1985 and Belyavin, 1988), suggesting that even when the egg is structurally sound, a higher level of insult will ultimately cause damage. Range eggs, on the other hand, although structurally inferior, are not exposed to the same level of insult, and so crack less frequently. Hughes *et al.* (1985) observed that the proportion of cracked eggs from caged birds increased from about 1 % at the beginning of lay to about 10 % at the end.

Shapiro and Sarles (1949) claimed that there was no difference in the intestinal microflora of birds reared on range or wire, while Coates (1976) claimed that birds managed in deep litter or free-range have more opportunities than those on wire to benefit from the synthetic abilities of their gut microflora.

Controlling *Salmonella* in free-range birds with the viability of the latter on pasture, in faeces and in the soil for up to 7 months is very difficult, according to Blood *et al.* (1983). Humphrey *et al.* (1991a) reported no significant increase in the incidence of infection of the contents of eggs from birds on range with *S. enteritidis*, when compared with eggs from birds kept in batteries.

In general terms, cages are reported to be at present, the best housing system (Prof. C. Spedding, in Dun, 1992). Birds housed on the floor or on range have ample opportunity to spread infection and recontaminate their

environment, while those raised in cages have the cross-contamination between birds minimised, with lower residual contamination after cleaning and disinfection (Higgins *et al.*, 1982). Despite having this lower disease risk environment, the reduced exercise space available in cages is certainly a negative feature, according to Dun (1992). On the other hand, the author comments that the potential advantage of the alternative systems with their increased space can also be undermined by increasing stocking densities to make them more economically competitive with cages. No doubt a new system will be developed which will take into account not only the justified growing awareness of the welfare problem, but also the ever present economic aspect, an essential point in the highly competitive and industrialised food market.

4.2. MATERIALS AND METHODS

4.2.1. GENERAL

Eggs used were collected at random from one strain (strain B) of commercial brown egg layers, housed in Barn and on Range, but both located at Walesby Farm, England. Eggs were also collected from two different strains (A and B) housed in batteries and located in controlled environment houses, strain A at Harper Adams Poultry Research Unit and strain B at the W.S.A.C.. They were all collected from birds at the end of lay (60 weeks old for barn /range, 69 w. o. for battery strain A, and 60 w. o. for battery strain B).

A total of 122 eggs were used (30 from each barn, range and battery strain B, plus 32 from battery strain A).

The *S. enteritidis* PT 4 technical procedures and positioning of the eggshell on the agar plate were all done as described in sections 2.2.2. and 2.2.4.. The treatment of eggshells was done in the same manner as described in 2.2.3., except that only samples with no membranes attached were used in the analysis. The percentage of penetration values were obtained and analysed as described in 2.2.4. and 2.3.1..

4.3. RESULTS AND DISCUSSION

The tables and graphs are placed at the end of this section. Results shown in table 19 indicate that Late fusion levels were higher in the Range group, than in the Barn and Battery 2 (Bat. 2) groups, and although non-significant, these differences may have helped to improve the latters' capacity to resist bacterial challenge.

Mammillary alignment followed the same pattern, with Barn and Bat. 2 groups showing significantly less occurrence than in the Range group. Type B bodies were more prevalent in the Battery 1 (Bat. 1) group than in the Range group, but the latter in turn displayed more of this structural variant than the Barn or Bat. 2 groups. Since these findings are in line with the penetration results (graph 26), it may be hypothesised that, as in previous experiments, the level of type B's present helped in determining the level of resistance.

Both pitting and aragonite varied in the same way as bacterial penetration, *i.e.* as the incidence of these variations increased so too did bacterial transfer. Battery 1 proved to be the exception, insofar as it displayed lower levels of pitting (although non-significantly). With respect to the aragonite modification, the battery 1 group had significantly more aragonite than the others. The eggs from battery 1 also displayed significantly less cuffing than the 3 other groups. The results are displayed in graphs 27 to 28.

Changed membrane levels were lowest in the Bat. 1 group. The firm attachment between these membranes and the calcitic shell may have provided a greater physical restrain to bacteria (graph 29).

Total score values indicate that Range eggs are structurally inferior to their Barn and Battery counterparts (graph 30). As in chapter 2, pores cannot be cited as the sole portals of entry.

Stress is probably the most important single factor influencing eggshell structure and its capacity to offer resistance against microbial invasion. The diffuse origins of stressors affecting Range birds may be a factor determining a drop in shell quality, but above all, more specific and intense stressors sometimes suffered by caged birds can provoke deeper, long-lasting effects which will be detectable several days after the stressing agent has disappeared. This fact may provide an explanation to the wide differences in penetration levels observed between the two battery groups evaluated. Also of great importance is the fact that these groups were very heterogeneous in terms of other variables, *viz.* different management systems, locations, photoperiods, nutrition and strain.

Structural quality declines more markedly with bird age, as revealed by the results obtained for the battery housing. In this respect, the end of lay period is the most pertinent time at which selection for structural variation should be made. The analysis under discussion in this chapter indicate that housing exerts a considerable effect on the structural integrity of the shell, and that the alternative systems currently available fail to compensate for the observed age effects.

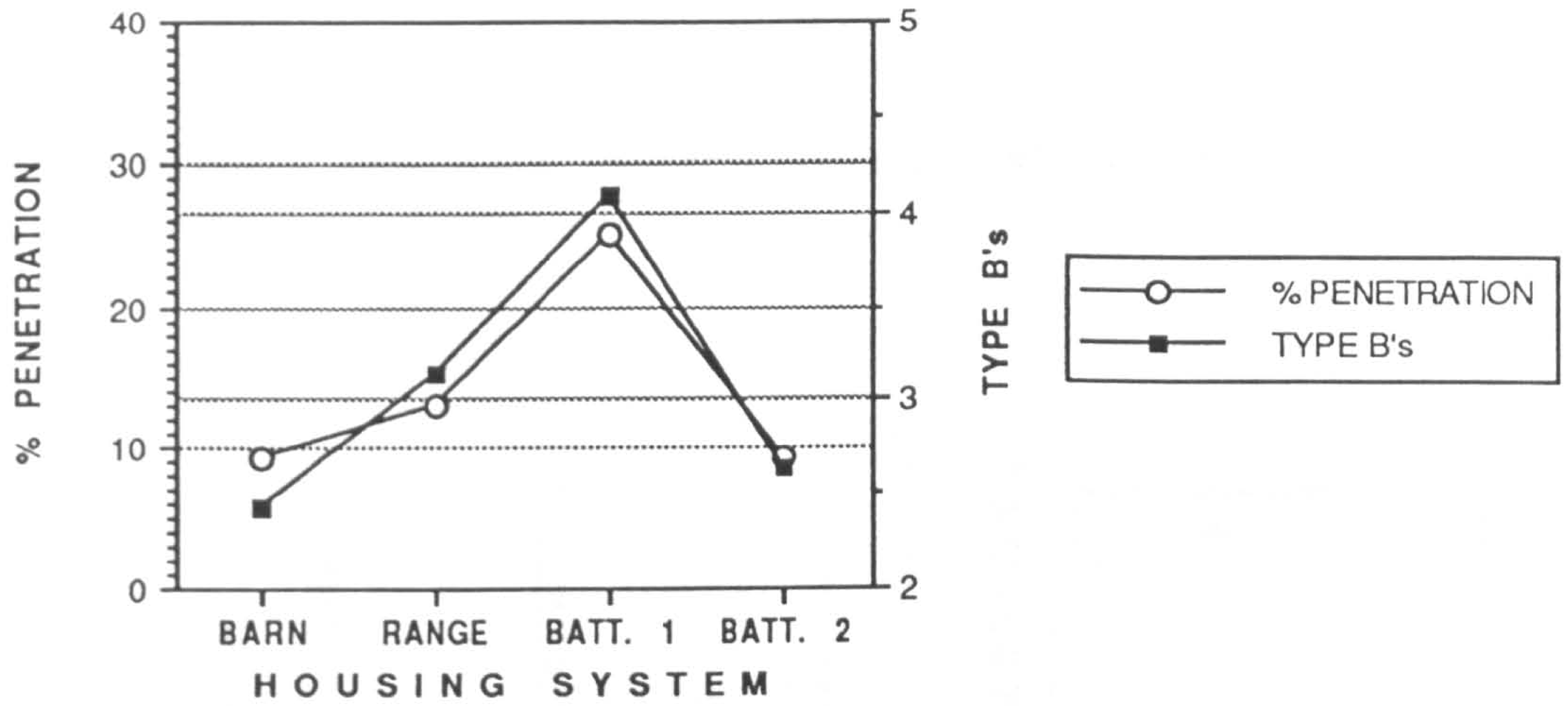
	Overall (4 groups)	Barn (B)	Range (R)	(B x R)	Battery 1st. (Bat.1)	(B x Bat.1)	Battery 2nd. (Bat.2)	(B x Bat.2)	(R x Bat.1)	(R x Bat.2)	(Bat 1 x Bat.2)
CONFLUENCE	NS	3.50 ± 1.46	4.17 ± 1.88	(NS)	3.66 ± 1.18	(NS)	4.20 ± 1.42	(NS)	(NS)	(NS)	(NS)
CAPS	NS	4.60 ± 2.82	5.73 ± 2.05	(NS)	4.72 ± 1.71	(NS)	5.63 ± 2.08	(NS)	(*)	(NS)	(NS)
EARLY FUSION	NS	2.20 ± 1.00	2.33 ± 0.99	(NS)	1.97 ± 0.78	(NS)	2.47 ± 1.07	(NS)	(NS)	(NS)	(*)
LATE FUSION	NS	4.47 ± 1.47	5.07 ± 1.64	(NS)	4.59 ± 1.52	(NS)	4.70 ± 1.51	(NS)	(NS)	(NS)	(NS)
MAMM. ALIGNMENT	**	2.03 ± 0.89	3.30 ± 1.64	(***)	2.59 ± 1.58	(NS)	2.63 ± 1.35	(*)	(NS)	(NS)	(NS)
TYPE B's	***	2.43 ± 1.57	3.13 ± 2.32	(NS)	4.09 ± 2.13	(***)	2.63 ± 1.56	(NS)	(NS)	(NS)	(**)
PITTING	NS	2.67 ± 2.11	3.03 ± 2.02	(NS)	1.81 ± 1.75	(NS)	2.53 ± 2.08	(NS)	(*)	(NS)	(NS)
ARAGONITE	*	1.53 ± 0.82	2.00 ± 0.91	(*)	2.50 ± 1.65	(**)	1.97 ± 1.30	(NS)	(NS)	(NS)	(NS)
TYPE A's	NS	1.67 ± 0.48	1.90 ± 0.31	(*)	1.81 ± 0.40	(NS)	1.83 ± 0.38	(NS)	(NS)	(NS)	(NS)
CUBICS	NS	1.27 ± 0.45	1.33 ± 0.48	(NS)	1.31 ± 0.47	(NS)	1.40 ± 0.50	(NS)	(NS)	(NS)	(NS)
CUFFING	*	4.43 ± 0.82	4.50 ± 0.51	(NS)	4.88 ± 0.34	(**)	4.63 ± 0.49	(NS)	(**)	(NS)	(*)
CHANGED MEMBRANE	***	2.40 ± 2.28	3.40 ± 2.70	(NS)	1.00 ± 0.00	(***)	3.13 ± 3.05	(NS)	(***)	(NS)	(***)
TOTAL SCORE	*	33.40 ±10.61	39.93 ± 11.77	(*)	34.78 ± 6.66	(NS)	37.97 ± 7.62	(NS)	(*)	(NS)	(NS)
MAMMILLARY DENSITY	**	88.90 ±19.40	71.53 ± 15.38	(***)	75.93 ± 17.20	(**)	80.76 ± 15.09	(NS)	(NS)	(*)	(NS)
CUTICLE	**	0.70 ± 0.47	0.83 ± 0.38	(NS)	n/a	n/a	1.07 ± 0.45	(**)	n/a	(*)	n/a
PORES (number)	NS	148.53 ± 103.21	170.30 ± 91.16	(NS)	174.97 ± 119.90	(NS)	146.17 ± 90.13	(NS)	(NS)	(NS)	(NS)
% PENETRATION	***	9.12 ± 6.37	13.03 ± 7.20	(*)	24.97 ± 14.00	(***)	9.22 ± 6.55	(NS)	(***)	(*)	(***)

Table 19: Structural scores (mean ± s.d.) for each individual characteristic of the cone layer in eggs from the housing experiment, number of pores and percentage of penetration. All values correspond to the end of lay.

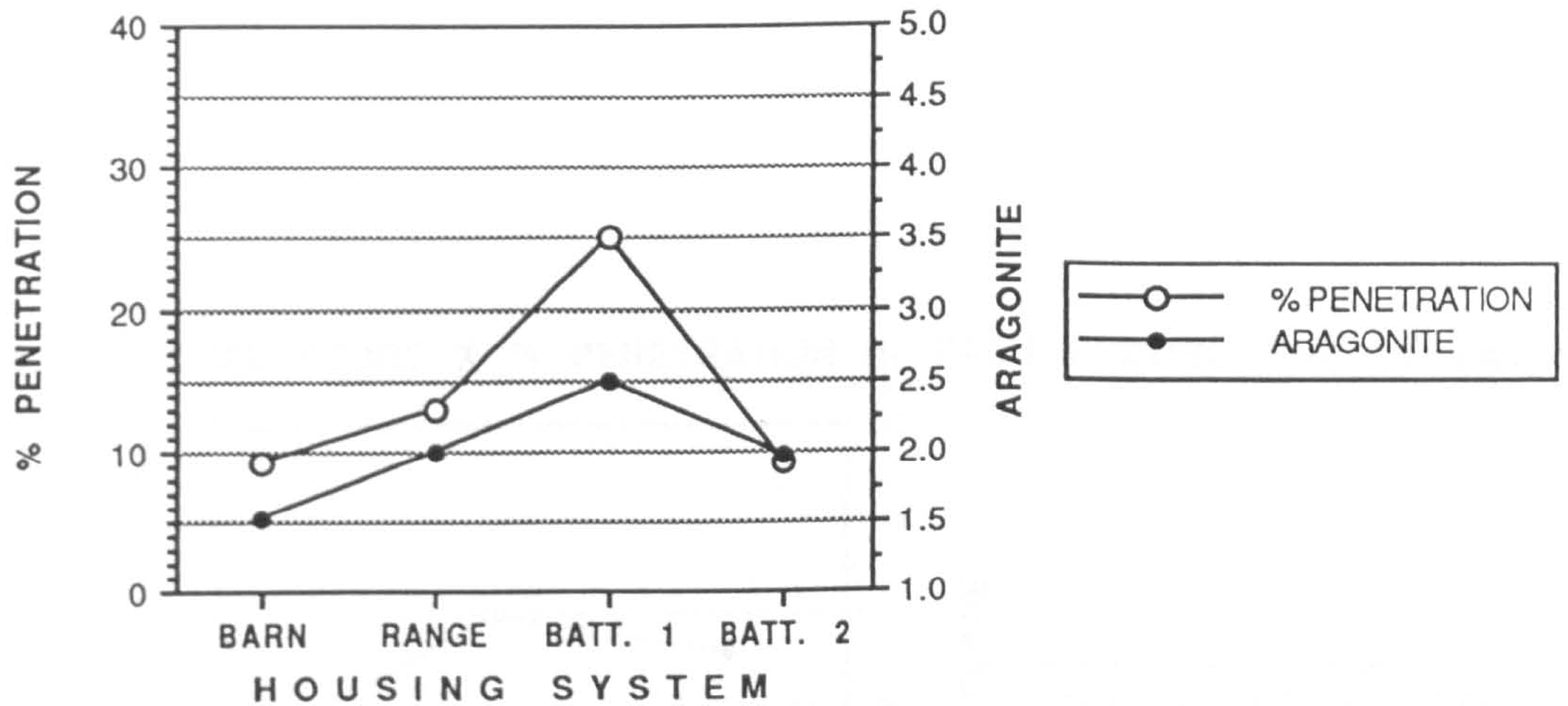
*** = Very highly significant at a 0.1 % level (P< 0.001)   ** = Highly significant at a 1 % level (P< 0.01)

* = Significant at a 5 % level (P< 0.05)   NS = Not Significant (P> 0.05)

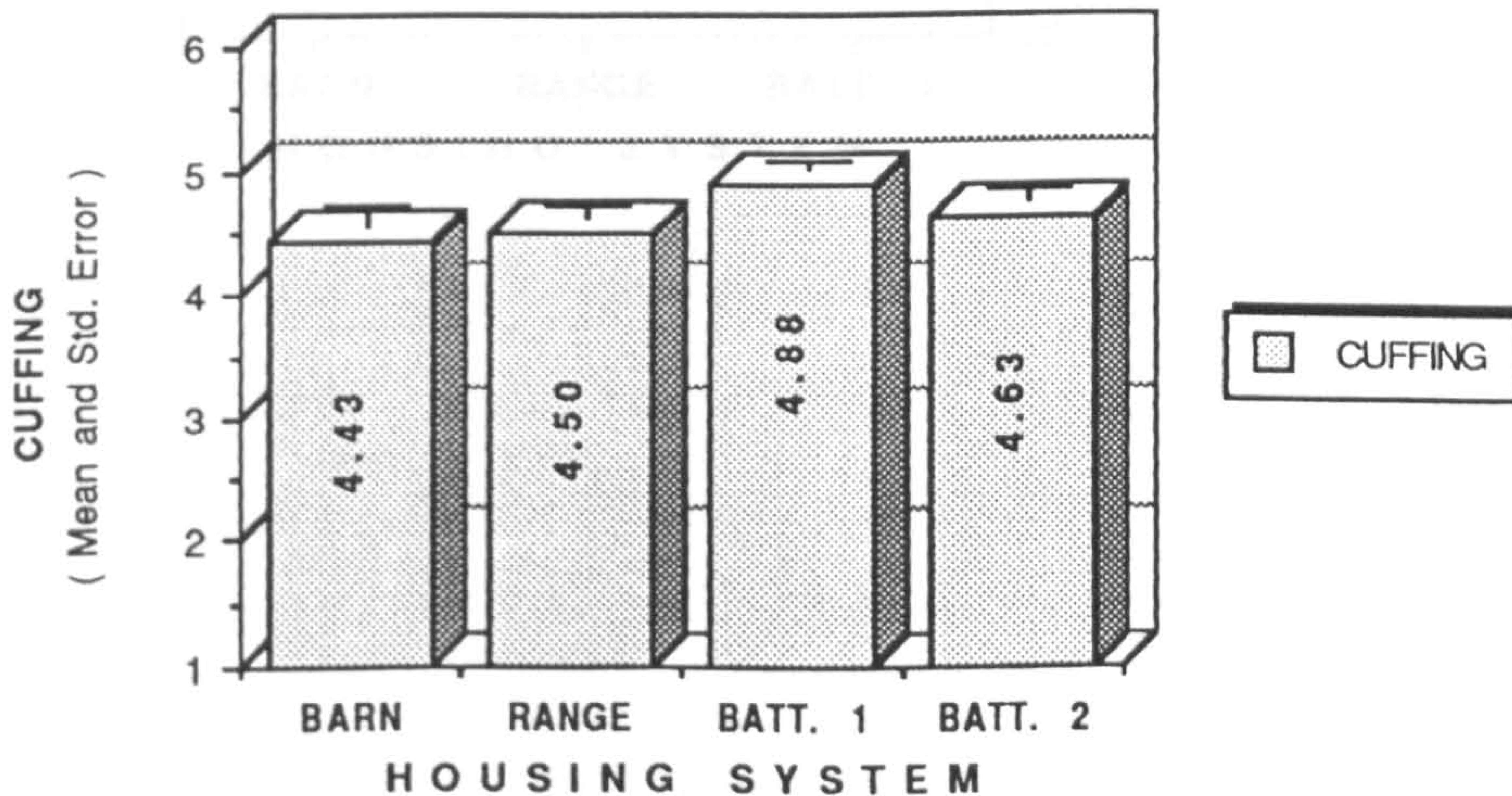
GRAPH 26: TYPE B's X % PENETRATION IN EACH SYSTEM (END OF LAY)



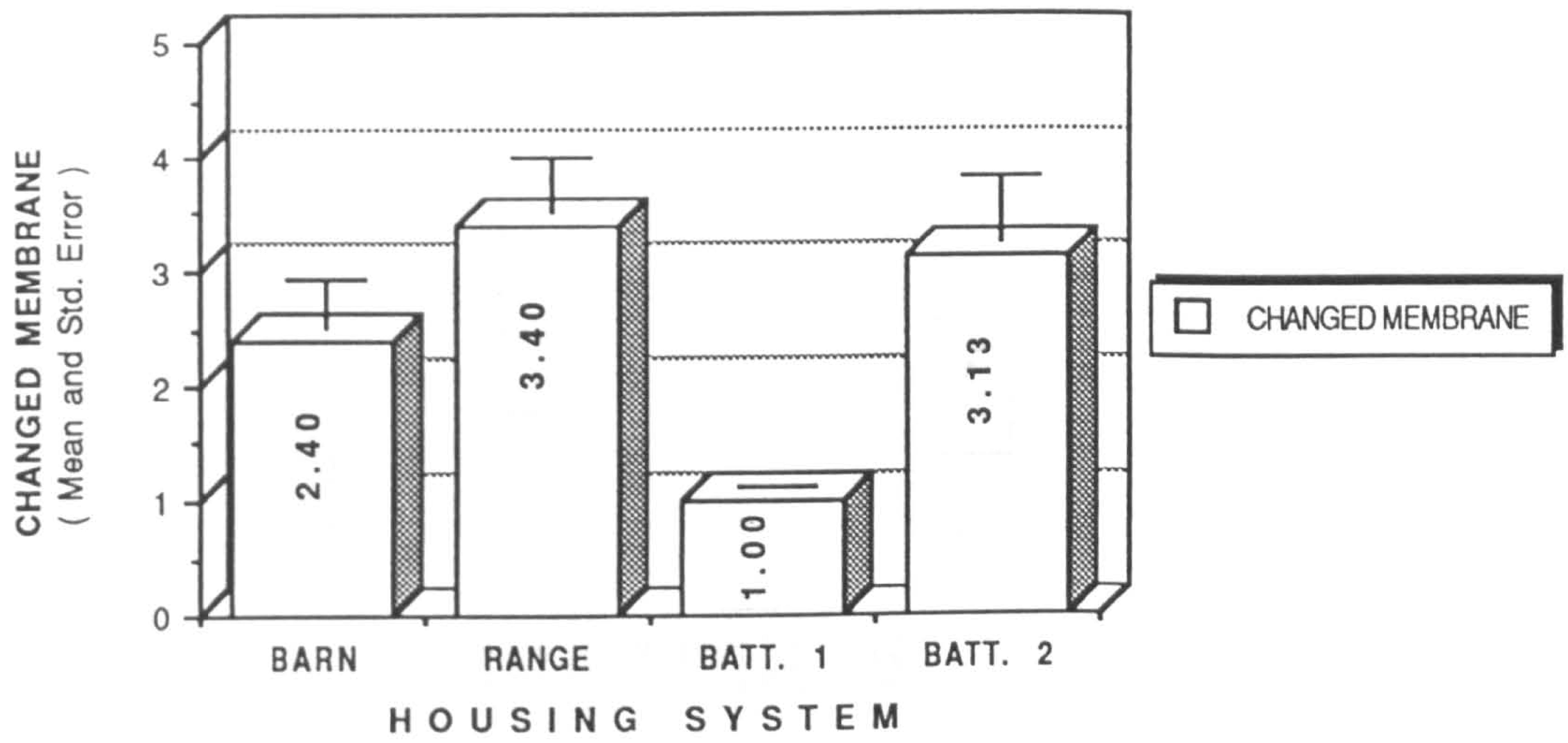
GRAPH 27: ARAGONITE X % PENETRATION IN EACH SYSTEM (END OF LAY)



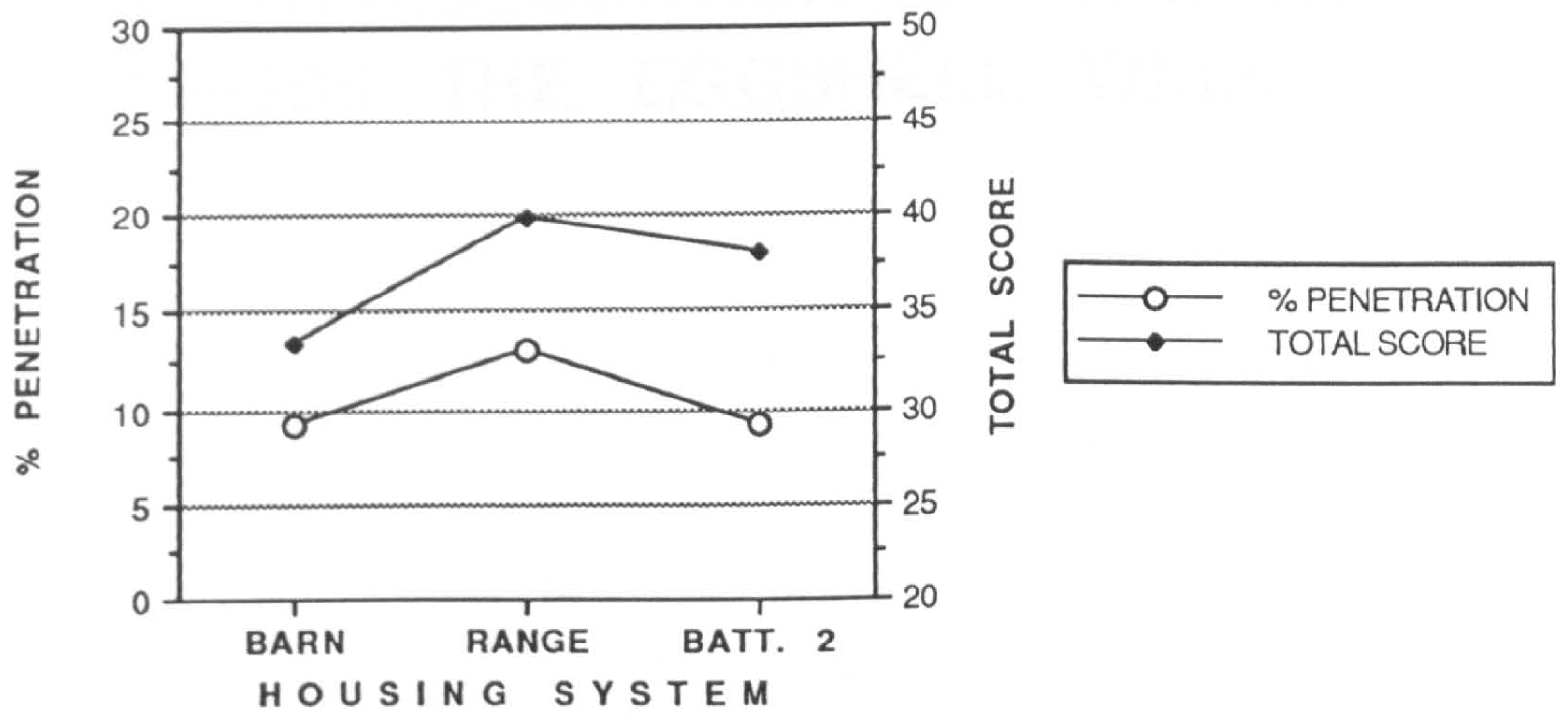
GRAPH 28: CUFFING IN EACH SYSTEM (END OF LAY)



GRAPH 29: CHANGED MEMBRANE IN EACH SYSTEM (END OF LAY)



GRAPH 30: TOTAL SCORE X % PENETRATION IN EACH SYSTEM (END OF LAY)



CHAPTER 5

IMMUNOCYTOCHEMICAL MONITORING OF THE TRANSLOCATION OF *Salmonella* ACROSS THE EGGSHELL WALL

5. IMMUNOCYTOCHEMICAL MONITORING OF THE TRANSLOCATION OF *Salmonella* ACROSS THE EGGSHELL WALL

5.1. INTRODUCTION

Immunocytochemistry is defined by Polak and Van Noorden (1987) as the use of labelled antibodies (Ab) as specific reagents for the detection of tissue constituents or antigens (Ag) *in situ*. Immunogold staining is a highly sensitive immunocytochemical method for the demonstration of Ag using both S.E.M. and T.E.M.. The method utilises the immunogold reagent (immunoglobulin (Ig) adsorbed on to colloidal gold particles), which will then attach to antigenic sites and be revealed under electron microscopy.

The marker should be of a size and shape that can be readily visualised against the possibly complex topographical background, and also be small enough to allow good target site localisation, apart from being chemically stable and demonstrate an absence of natural binding affinity for biological surfaces (Hodges *et al.*, 1985).

Gold markers of reproducible and constant size can be produced by the controlled reduction of tetrachloroauric acid ($HAuCl_4$), by using reducing agents like sodium citrate (which make the reduction slow, thus forming few nuclei and relatively large gold spheres – size range of 15-150 nm mean diameter) or by using sodium ascorbate, or sodium or potassium thiocyanate, or white phosphorus, which will result in a fast rate of nucleation of $HAuCl_4$, with the formation of many small (up to 13 nm) gold particles (Hodges *et al.*, 1985 and Beesley, 1989a).

These gold particles are then adsorbed with proteins, to form a protein-gold complex. Proteins most commonly complexed with gold spheres are protein A and antibodies (mainly secondary, although primary Ab are

sometimes used) (Beesley, 1989a). The process of protein adsorption to gold particles is a highly complex and incompletely understood phenomenon, with a number of physicochemical factors influencing the adsorption process in which the pH value of the colloidal gold is of paramount importance. The use of high quality and specific Ab is also crucial to the success of the technique. Today's commercially available conjugates make it easier to perform the technique. It is only necessary to produce the conjugate in the laboratory when a very unusual secondary Ab or a very large gold probe is needed (>30 nm).

The advantage of using this technique is because the high electron backscattering coefficient of gold (the scattering of primary high energy electrons, whose collection and display will give the backscattered electron (BSE) signal, is one of the products of the electron beam-specimen interaction (Abraham and Denee, 1974)). This provides superior visualisation in the S.E.M., when used with a dedicated BSE detector, where gold particles appear as bright, well defined spheres on cells, instead of the electron-dense deposits. Backscattered electron imaging (BEI) used in normal or reversed polarity mode, allows the unambiguous identification of gold particles distributed over the bacterial cell surface. In BEI, the contrast is generated by the atomic number (Z) differences in the specimen, rather than topography (based on the low Z of the elemental constituents of biological material (C, H, O, N) compared with the gold's high Z (= 79)).

The indirect labelling procedure (which was used in this experiment) has been the approach most favoured in S.E.M. immunocytochemistry, according to Hodges *et al.* (1985). In this technique, specimens are first exposed to an unmodified primary specific ligand, and the latter is then detected in one or more additional steps, by a second ligand-marker conjugate, directed against the first.

5.2. MATERIALS AND METHODS

5.2.1. GENERAL

The eggshells used in this experiment were selected at random from the control eggs used in the experiment described in chapter 6.

Small pieces of shell of *ca.* 0.5 cm^2 were cut from the equatorial region of the egg, and plasma etched for 4 h., as described in section 2.2.3.. After that, they were surface sterilised with 70 % ethanol and allowed to dry. Other pieces of shell from adjacent areas were plated as controls, and none showed any previous contamination.

Two *Salmonella* serotypes were used in this experiment. *S. typhimurium* PT 49 for the trials and *S. enteritidis* PT 4 for the experiments. They were both isolated from chickens and identified by the National Collection of Type Cultures (NCTC). The strains were kept on Dorset's egg slopes and in Nutrient Agar (Oxoid Ltd.) tubes, from which one wire loop was used to transfer the microorganisms to a bottle containing 25 ml. of Nutrient Broth no. 2 (Oxoid Ltd.). An overnight shaken (140 rpm) culture of the microorganism at 37 °C was then harvested by centrifuging it at 4,000 g for 20 minutes at 4 °C, washed with sterile saline, re-centrifuged (20 min.) and resuspended in sterile distilled water, from which an appropriate dilution was made in order to achieve a challenge solution which would contain approximately 5×10^7 organisms per ml.. From this dilution an 0.01 ml. (10 µl) inoculum containing 5×10^4 organisms was used on the eggshell. The inoculum was heavy to provide every opportunity for maximum penetration, under the conditions used. Colony counts, after spreading of 0.1 ml.of the different dilutions were made in the same way as described in section 2.2.2., and after several trials a dilution of 10^{-1} was decided upon.

The identity of the colonies formed on the plates following incubation was confirmed by using the same methods described in section 2.2.2., except that for *S. typhimurium* serology was done by slide agglutination using the same *Salmonella* polyvalent somatic ("O") agglutinating serum, but subsequent testing was done by using the *Salmonella* "O" group B agglutinating serum (Ag 4) (Wellcome), apart from the *Salmonella* flagellar ("H") antisera.

5.2.2. IMMUNOGOLD STAINING PROCEDURES

Before the definitive experimental procedure was performed, preliminary trials were carried out to test:

a) The effect of the fixatives on the antigenicity of the *Salmonella* organisms;

b) the affinity between the Ag and the primary Ab, and between this and the secondary immunogold conjugate, by means of the positive controls;

c) the most appropriate times for fixation, washes, incubation with the primary Ab and with the secondary Ab-immunogold conjugate, and the secondary (post) fixation;

d) the effect of different coatings on the visualisation of the gold probe and the eggshell's structural features and

e) the effect of freeze-drying and critical point drying on the antigen /primary Ab /secondary Ab complex.

5.2.2.1. Primary Antibodies

Two types of primary Ab were used in this experiment. The preliminary trials used polyclonal Ab directed against *S. typhimurium* PT 49 lipopolysaccharide (LPS) from the bacterial cell, generated in rabbit (hyperimmune serum). This Ab was tested before by Enzyme Linked Immunosorbant Assay (ELISA) and proved effective in another experiment (Dadrast, 1989). The experimental Ab was also polyclonal directed against *S. enteritidis* PT 4 LPS, generated in rabbit. This Ab was also tested before in immunofluorescence tests, and equally proven specific. Both antibodies were further tested in the actual experimental situation as known positive controls, which confirmed their specificity in immunocytochemical labelling.

As many fixation methods are recognised to traditionally destroy flagellar Ag (Hassan, 1991), it was decided to use the Ab available against LPS, in case the use of formaldehyde caused a significant loss in flagellar antigenicity. LPS was also shown by immunoblotting and ELISA to be the

major *S. enteritidis* Ag recognised by chicken serum Ab (Chart *et al.*, 1990b), which conversely reinforced its suitability for this experiment.

S. typhimurium antiserum was diluted 1:8 in phosphate buffered saline (PBS) pH 7.3 (buffer recipe in the annexes), while *S. enteritidis* antiserum was diluted 1:4 in PBS. Normal rabbit serum (preimmune) used as controls was diluted 1:8. They were both kept frozen in small aliquots at -20 °C in sealed containers (Eppendorfs), and were thawed just prior to use.

5.2.2.2. Secondary Antibodies

The secondary Ab /gold complex used was the commercial Goat anti-rabbit IgG (heavy + light chain – H+L) conjugated to 30 nm gold particles (BioCell Research Laboratories, Cardiff, Wales) with a sensitivity capable of detecting at least (less than) 10 picograms of protein (rabbit IgG). The conjugate was kept at 4 °C, and was diluted to 1:10 just before use, as recommended by the supplier, by mixing 0.05 ml. (50 µl) with 0.45 ml. (450 µl) PBS.

5.2.2.3. Fixatives

The fixatives used were 40 % formaldehyde vapour, freshly prepared from 97 % paraformaldehyde powder (BDH Ltd., Poole, England) diluted in PBS pH 7.3 and 25 % glutaraldehyde vapour (Bio-Rad, Watford, England).

5.2.2.4. Experimental Protocol

This protocol is an adaptation of that recommended by Hodges *et al.* (1985) and Beesley (1989a,b) and on personal communications from both these senior authors. Previously cut pieces of shell were placed cuticle side uppermost inside a 90 mm plastic Petri dish carpeted with 8 x 5 cm Parafilm "M" (American Can Company, Greenwich, U.S.A.). A 0.01 ml. drop of the challenge suspension diluted to 10^{-1} was put on the cuticular side (since the area was small and flat, a grease ring was not required to contain the bacteria) and allowed to penetrate for 15 min.. The known positive controls from the above were then exposed on the mammillary side. This process was used to demonstrate the validity of the primary Ab and of the method in general. Successful labelling requires that non-specific binding of the gold marker to surfaces, as measured by control experiments, should be at least one order of magnitude less than that observed in actual test experiments, according to Molday (1983). The shells were then carefully transferred to another Petri dish containing a filter paper no. 1 (Whatman, England), damped with 40 % formaldehyde, and placed on a glass slide sitting on the filter paper, and left for 30 min. to be fixed. The shells were then transferred to another dish, where a 0.1 ml. drop of 0.02 M glycine (Sigma Chemical Co., St. Louis, U.S.A.) was added, to obviate free aldehyde groups which could cause non-specific binding. After that, shells were washed in 0.1 ml. drops of PBS + 0.5 % pH 7.3 Bovine Serum Albumin (BSA) (Sigma) 3 times for 1 minute (which prevents attachment of Ab to non-specific "sticky" sites). The shells were then transferred to a closed humid chamber, previously covered with Parafilm, where the experimental shells were exposed to a 0.05 ml. drop of diluted primary Ab on their mammillary side, while one group of controls received a 0.05 ml. drop of PBS and another received a 0.05 ml. drop of rabbit preimmune serum. They were left to incubate for 50 min., after which time they were washed twice for 2 minutes, in 0.1 ml. of PBS; then the shells were transferred once more to the humid chamber, where they received a 0.05 ml. of already diluted 1:10 Goat anti-rabbit IgG 30 nm gold conjugate (pH 7.3), and left to incubate overnight

at 4 °C.

After the incubation period, samples were washed 3 x 1 min. in PBS + BSA, and were fixed for 30 min. in 25 % glutaraldehyde vapour as previously described, and then left in bidistilled sterile water (pH 7.3) at 4 °C and taken to the freeze-drier (High Vacuum - Low Temperature Freeze Drier, Zoology Department, University of Glasgow). The freeze-drying procedures take some 26-28 hours to dehydrate the samples (frozen and at high vacuum, at -75 °C and 10^{-4} Pascals, respectively) and to slowly bring them to room temperature (*ca.* 30 °C). The transfer of the biological material into a clean solvent (preferably distilled water) is an essential step, to avoid the formation of salt crystals. This method is preferred instead of the critical point drying method, as the structures to be preserved are very delicate and fragile. Samples were then kept in a glass dessicator in the presence of $CaSO_4$ until viewed by the S.E.M..

5.2.3. ELECTRON MICROSCOPY PROCEDURES

The samples were mounted on 10 mm diameter aluminium cylinder stubs (Agar Scientific Co., Stansted, Essex, England), covered with 12 mm diameter carbon tabs (Agar Scientific Co.), with the inner surface uppermost.

The samples were examined in a JEOL JSM-6400 Scanning Electron Microscope (Japan Electron Opticals, Japan) equipped with a dedicated Si pn junction (divided annular type) paired semiconductor element backscattered detector, with a JEOL BEI preamplifier set to 50 times. They were viewed using the compositional image mode, which shows the atomic number contrast of the specimen, with an aperture of 50 μm diameter (stage 4, the smallest possible in that equipment), in normal and reversed polarities. The most common beam current used for backscattered was 1×10^{-9} A, with a spot size of 9 μm, and an accelerating voltage of 14 KV. If the intensity of the BSE signal from the gold particles appeared too low, a slightly increased probe current or a higher accelerating voltage was used (up to 30 KV, but for very short periods, since the beam inciding over a small spot size will quickly destroy the structure of an uncoated sample). Whenever possible, the lowest accelerating voltage was used (as low as 3.5, but usually from 8 to 14 KV), in order to preserve the sample. A very short working distance (W. D.) of less than 10 mm (usually 8 mm) had to be used in order to maximise the collection of the primary electrons emission from the specimen and also the signal-noise ratio of the image, as the BS detector was placed directly below the objective pole piece. A beam current range of 1×10^{-10} A to 6×10^{-10} A was used in SEI mode, while a slightly higher beam current of 6×10^{-10} A to 6×10^{-9} A was preferred for BEI. Samples were viewed at magnifications ranging from 160 X up to 45,000 X.

5.3. RESULTS AND DISCUSSION

5.3.1. DEVELOPMENT OF THE TECHNIQUE

According to Kellenberger and Hayat (1991), higher labelling is generally attributed to smaller gold particles (up to 8 nm) than that obtained with relatively larger ones (10-40 nm), as the former occupy more available binding sites and produce a more even and dense labelling, with less steric hindrance (which can also be avoided by using indirect procedures). In this series of experiments, the resolution capacity of the microscope used and its kind (S.E.M. or T.E.M. or L.M.) was the decisive factor in choosing the particle size. This necessitated monitoring the conjugate concentration, since the larger the gold particles, the more concentrated it has to be, in order to achieve the maximal detecting sensitivity.

The reasons for choosing polyclonal antibodies in this experiment, apart from the fact that they were readily available, was that they would probably recognise more different epitopes (usually a small area of about 15 aminoacids forms one single epitope). Polyclonal antibodies contain several populations of different antibodies directed to various portions of the antigen molecule, increasing the chances of finding a non-cross-linked Ag portion (Beesley, 1989a), and yet being of high specificity and affinity, and when produced with pure Ag, very rarely cross-reacting with other Ag.

By definition, specificity determines the ability of the IgG to distinguish between several possible epitopes (the lower the specificity, the smaller the area of the epitope – fewer aminoacids – involved), while affinity is the strength of binding between Ab and Ag (the affinity will be stronger, the stronger the the specificity), and this guarantees the label will not be washed off during the various steps of the procedure (Kellenberger and Hayat, 1991). The primary Ab concentrations tested here were based on previous optimised concentrations used for immunofluorescence, which according to

De Waele (1989) are also suitable for immunogold-staining.

Primary Ab directed against bacterial lipopolysaccharide (LPS) was used instead of anti-flagella, because the visualisation of the very thin flagella in the S.E.M. is rather difficult, while the marked LPS is easier to localise, since it is on the surface of the bacterial cell. The use of the T.E.M. offers a good image of the flagella, as demonstrated by Robinson *et al.* (1987). Bovine serum albumin (BSA) was added to the buffer to reduce non-specific binding of certain ligands or conjugates (by competing with the specific Ab for miscellaneous non-specific "sticky" sites) and for better maintenance of specimens during the labelling process (J. Beesley and G. Hodges, pers. communications).

Specimen preparation is probably one of the major problems encountered in immunocytochemistry, according to Hodges *et al.* (1985), with no single method available that will simultaneously preserve the specimen's ultrastructure and maintain the antigenic integrity of the target sites. For instance, glutaraldehyde will adequately preserve the cellular fine structure, but can impose losses in antigenicity by destruction of epitopes, while formaldehyde will achieve minimal loss of immunoreactivity, but with sometimes less than adequate maintenance of ultrastructural integrity. Vapour fixation was used because of the nature of the eggshell (hard tissue) and also the necessity to retain antigenicity (Beesley, pers. comm.).

Pre-fixation of specimens with aldehydes (especially glutaraldehyde) can also cause non-specific attachment between the free aldehyde groups with ligand ξ-amino groups (non-specifically "fixing" Ab to the tissue). The problem was minimised by blocking any cell-bound free aldehydes with reactive amino groups (0.02 M glycine) prior to labelling.

Post-fixation with OsO_4 was not used for two reasons: first, because it is usually not desired for immunocytochemical studies since it may denature the epitopes, and second because in order to visualise with accuracy the

particles in the S.E.M., the elimination of any other high atomic number (Z) element, like Osmium (Z= 76) is necessary, since it would obliterate the signal from the gold marker.

The use of ruthenium tetraoxide as a post-fixation treatment specifically enhances the contrast of the cell surface, so that the distribution of gold particles as well as the topography are enhanced (Takata and Hirano, 1989; M. Huxham, pers. comm.). This approach was tried, but possibly because of the unique characteristics of the tissue in question, no difference was found between the samples exposed to ruthenium and those which were not.

Incubation times had to be empirically established, by testing the suggestions of Hodges *et al.* (1985) of 30 minutes at ambient temperature or 60 min. to several hours at 4 °C, adapting it to the experimental system under study. The degree of staining is known to be related to the square root of the time (if the concentration of gold is diluted twice, the staining time has to be extended fourfold longer, according to Park *et al.* (1989)). So, if the concentration of gold markers is low or the staining time is short, then probably not all the target epitopes will be stained. Incubation of the samples with the secondary Ab-gold conjugate for 50 min. at ambient temperature gave labelling, but incubation at 4 °C overnight was found to be far superior, as it gave lower nonspecific background labelling and a considerable improvement in the uniformity and intensity of the specific labelling, by preventing the formation of patches or caps on the bacterial surface. It also allowed more individual particles to be visualised, which is in agreement with the results shown by Davis and Brlansky (1991).

The literature suggests the samples can be coated before viewing with the S.E.M. by evaporation of 5-20 nm of Carbon (De Harven and Soligo, 1986; Hodges *et al.*, 1987 and Horisberger, 1989). This procedure was tested in this experiment, but the results showed that despite the fact that coating might be necessary to avoid sample deterioration when a higher KV,

a short working distance (w. d.) or small spot size are used, the highly grainy Carbon coat usually masked the eggshell's mammillary surface, thus rendering it practically useless for quality evaluation. It is very difficult to get an even, thin, non-grainy Carbon coating because of the highly empirical way of achieving the film thickness. As a result, samples were used uncoated whenever possible, taking extra care with the examination time and beam intensity.

Finally, the necessity for a S.E.M. with a dedicated backscattered (BSE) detector proved to be indispensable for the visualisation of the immunogold particles. Trials were run, firstly trying to use the EDAX P501 (Edax International, Illinois, U.S.A.) to collect the X-ray emissions from the gold particles, which proved to be too insensitive to detect such small amounts, and also by tilting the specimen stage in a conventional S.E.M. to maximise the collection of backscattered electrons by the detector, which also proved to be inefficient.

5.3.2. THE LOCALISATION OF THE Ag IN THE EGGSHELL

As far as the visualisation of the immunogold-labelled bacteria on the eggshell mammillary surface is concerned, diverse images were obtained. In many samples, interconnected bacteria forming a mat of microorganisms were observed (figures 30 and 31). This phenomenon has been previously demonstrated by Soerjadi *et al.* (1982). Antibody cross-linking was also found to cause organisms to aggregate, which is common with a group of bacteria or protozoa (Beesley, 1989b). This would then prevent the gold probe penetrating into the centre of the clump and false negative results may occur. This was experienced in some of the earlier trials in this experiment.

Another phenomenon to be observed in the spatial localisation of the Ag in the tissue is the fact that when the surface of the bacteria is labelled, the Ab is not maintained in an upright position on the antigenic sites, and during drying it "falls" to the side in a random direction as a consequence of surface tension. Occasionally it will be flat on the surface, sometimes at a distance of 12-18 nm from the epitope, hence some of the micrographs display some accumulation (clustering) of particles in marginal areas. The superimposing of one particle on another gives an impression of variability in size (figures 32, 33, 34 and 35).

The use of the immunogold labelling allowed the confirmation of some structural characteristics on the mammillary layer of eggshells to be related to the ease of translocation and subsequent localisation of *Salmonella*. It demonstrated, for example, that the cone layer may be implicated in the process of penetration. The cone layer demonstrates variable porosity (vesicular holes), and as such could harbour bacteria temporarily and encourage their growth. Wolk *et al.* (1950) proposed such a mechanism, in which temperature and water permitting, bacteria could even multiply in such a situation.

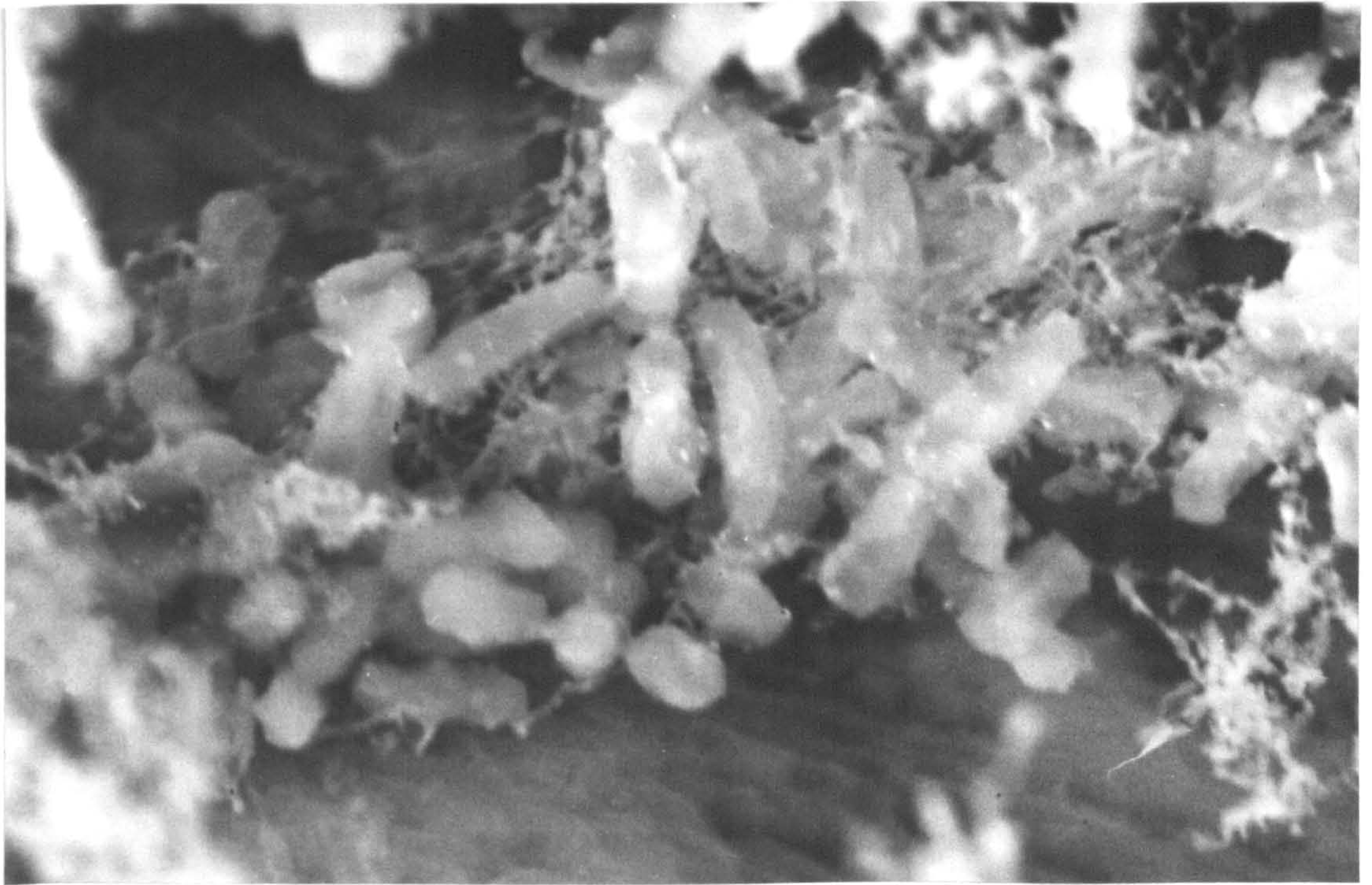


Figure 30: Secondary electron image (SEI) of interconnected tagged *Salmonella* forming a mat of microorganisms on the mammillary layer (29,250 X).

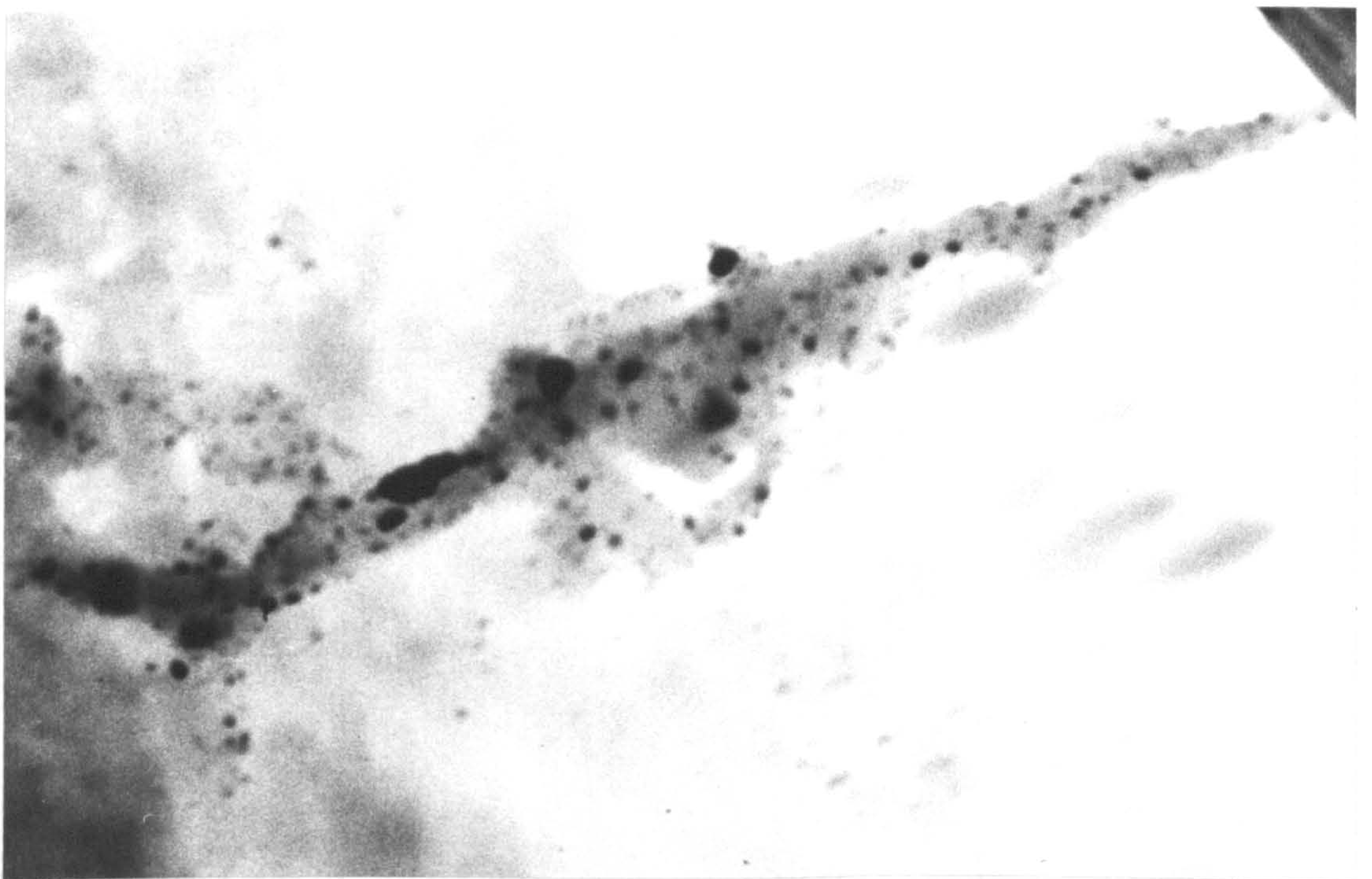


Figure 31: Backscattered electron image (BEI) with reversed polarity of interconnected tagged *Salmonella* on the mamm. layer (15,750 X).

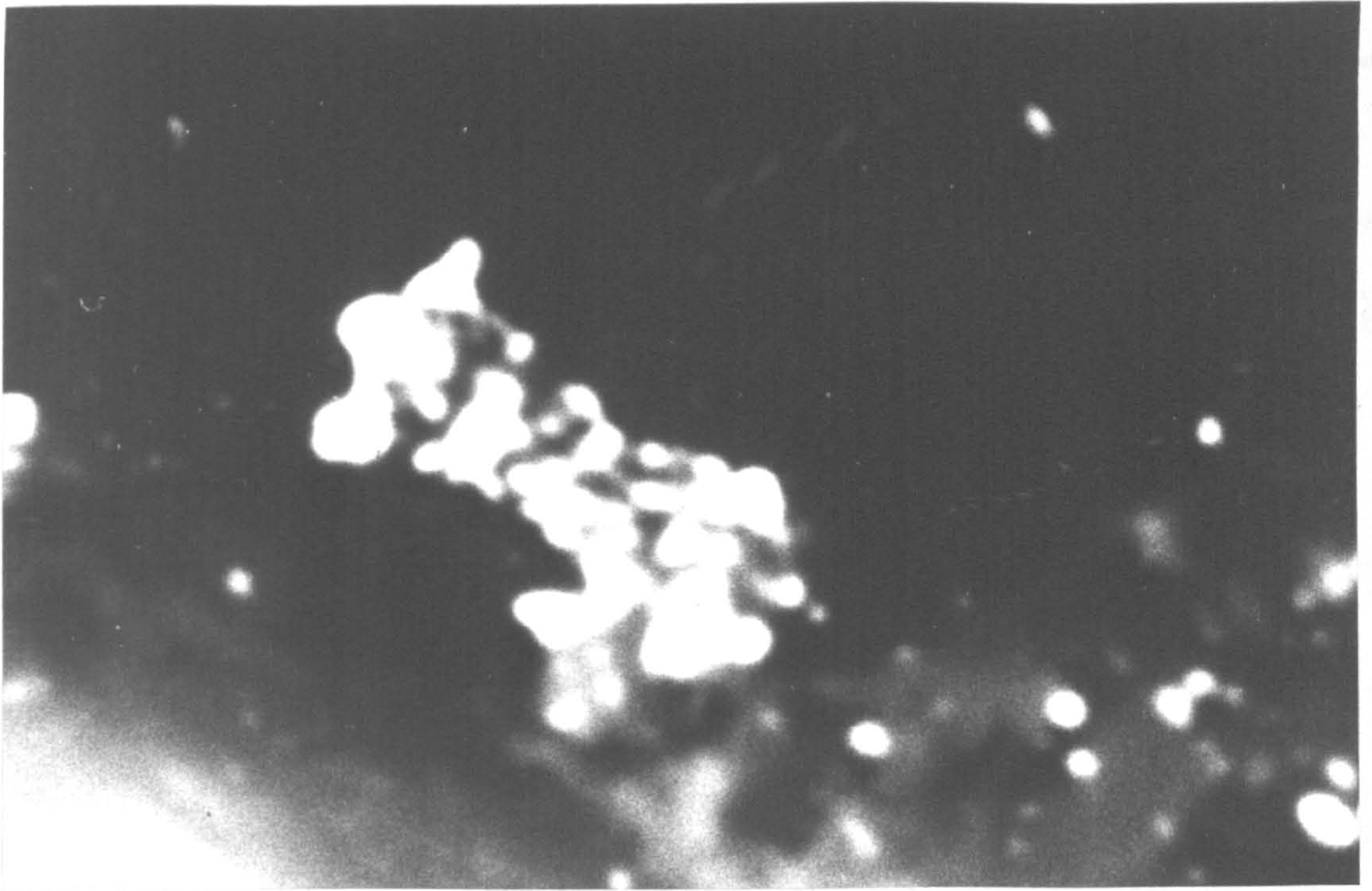


Figure 32: BEI with normal polarity of *Salmonella* tagged with immunogold markers, showing the superimposition of one particle on another (clustering) (arrow) (56,250 X).

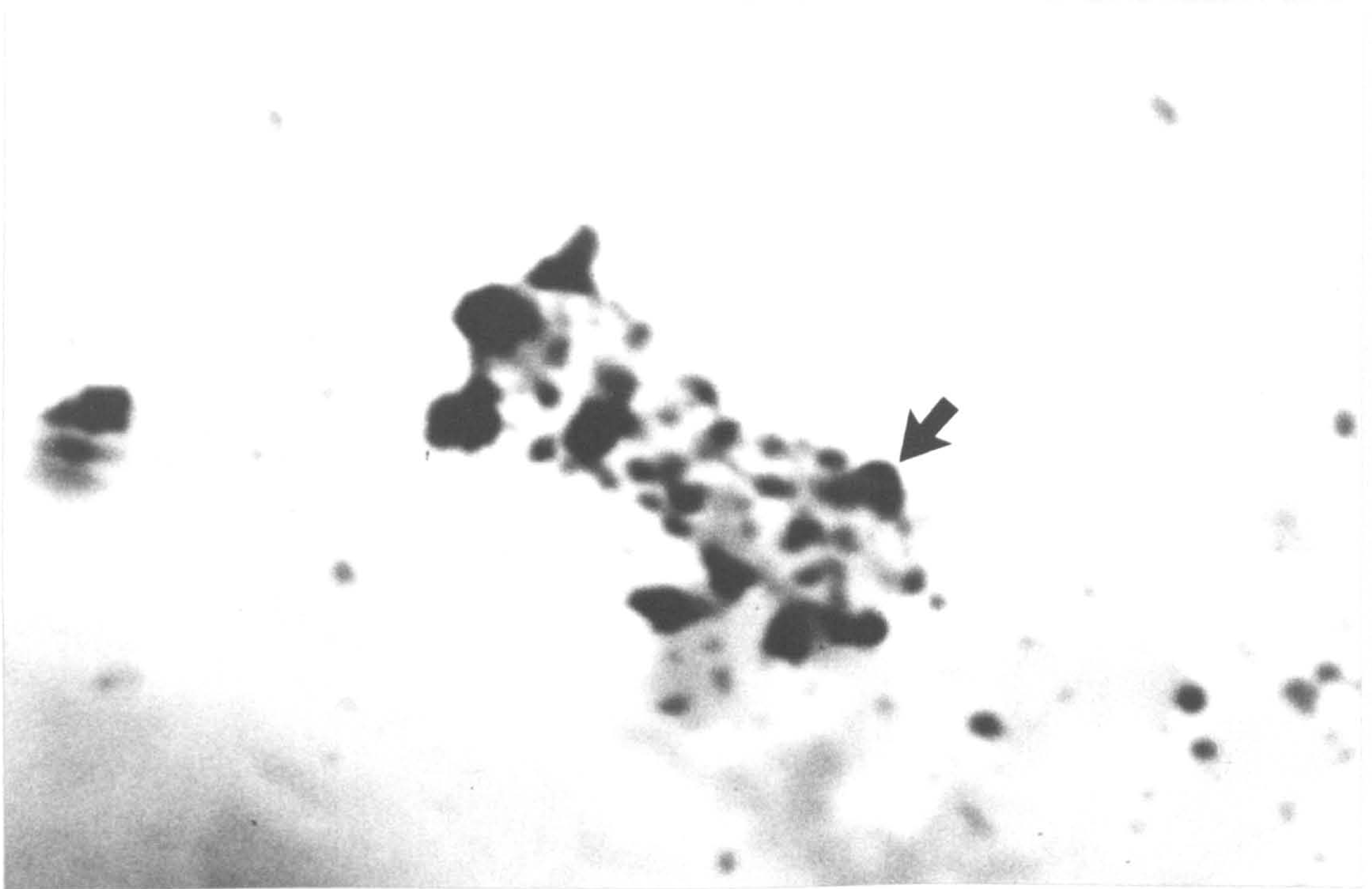


Figure 33: Same as above, with reversed polarity (56,250 X).

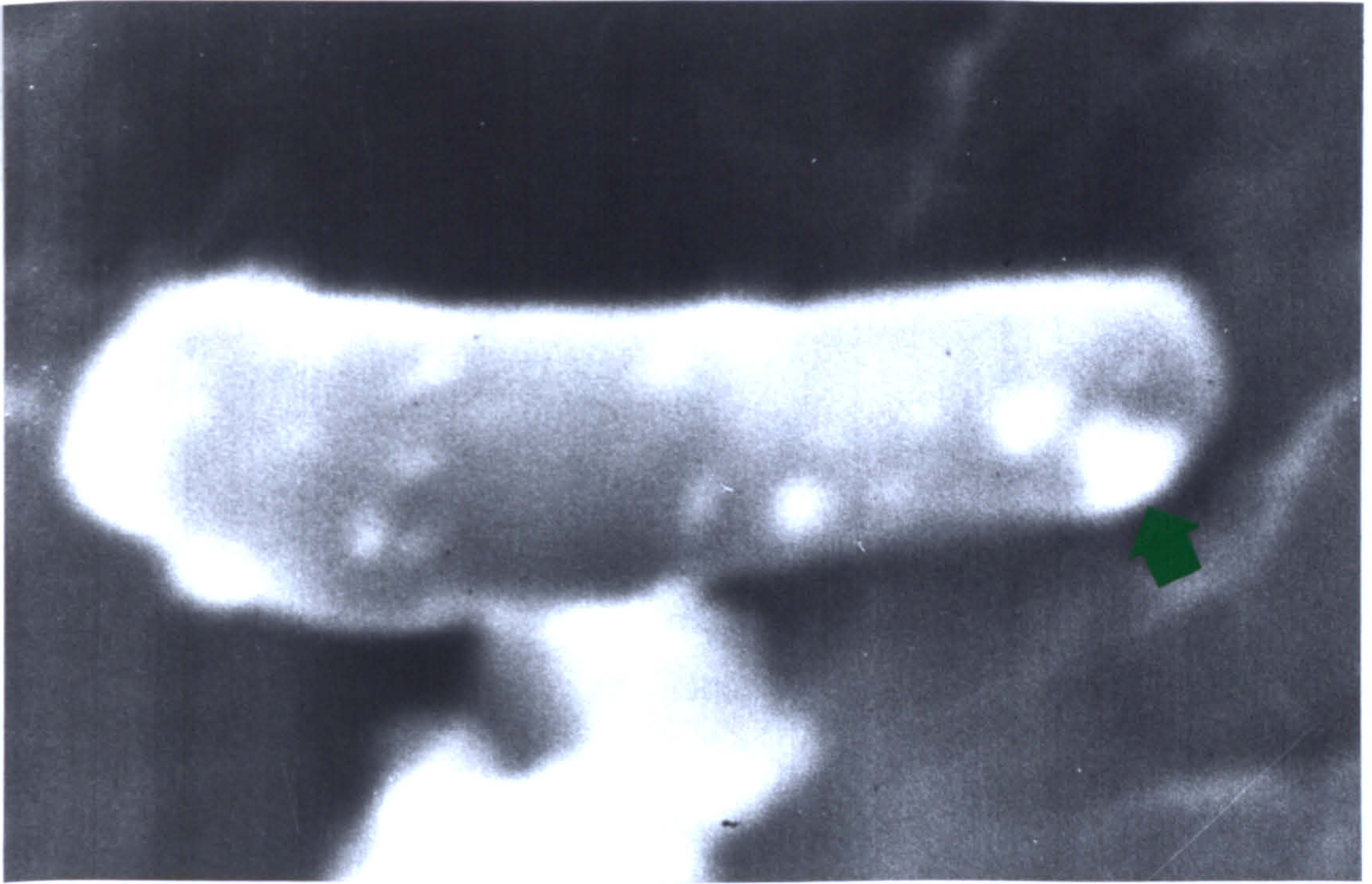


Figure 34: Same as in figure 32, but now a SEI (129,000 X).

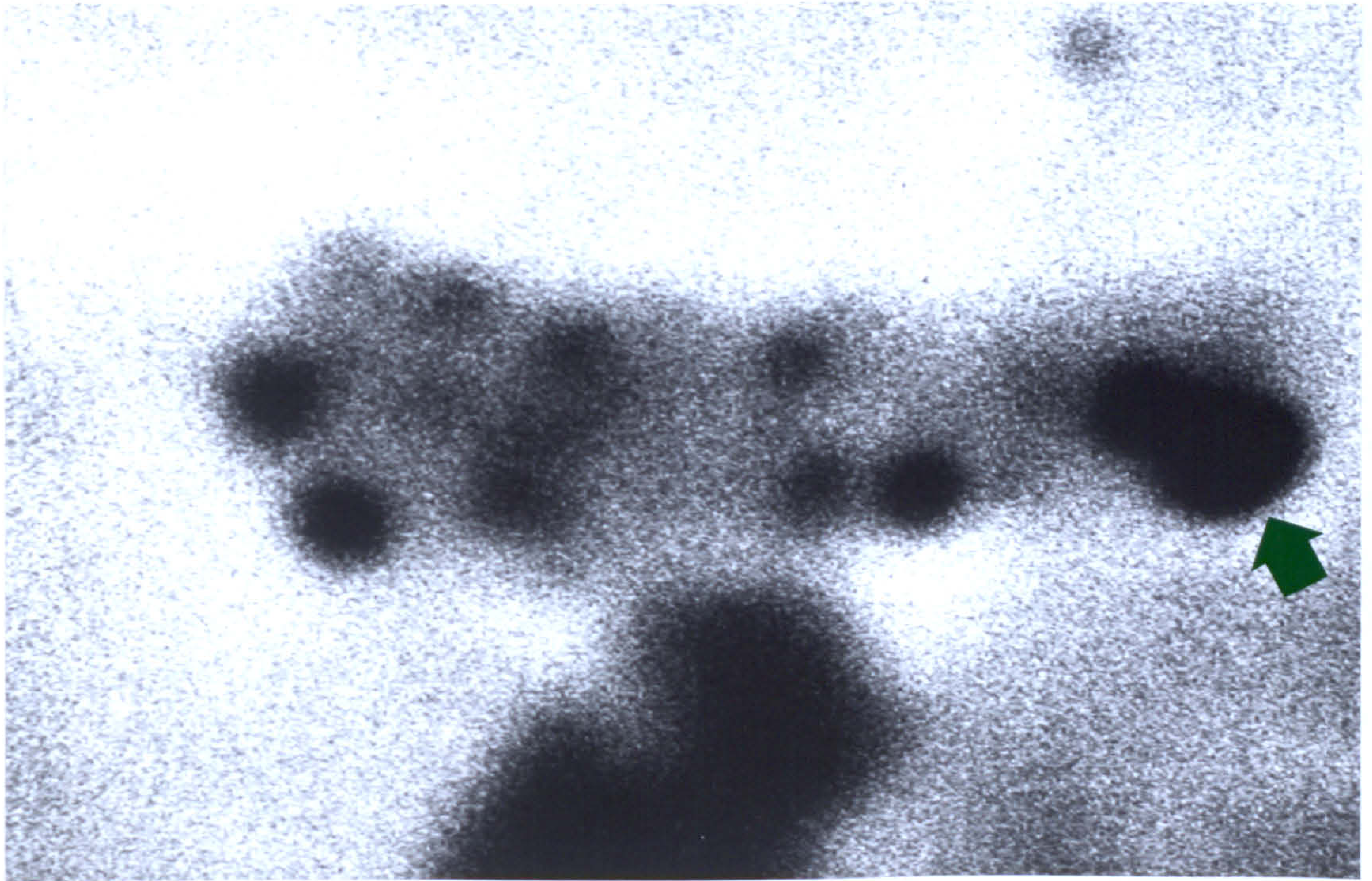


Figure 35: Same as in figure 32 with reversed polarity (129,000 X).

Late fusion and bacterial accumulation (figures 36 and 37) appear to go hand in hand, as the former renders the shell thinner, thus allowing bacteria to be closer to the suggested 15-20 μm distance of the membranes (Board, 1977), where the level of available water is then favourable for growth. This is also in line with the results shown in previous chapters, where the presence of increasing levels of late fusion was positively correlated with bacterial penetration (figure 22).

Labelled bacteria also aggregated near aligned mammillae. To demonstrate the latter one would routinely use a magnification of 160 X. To visualise labelled bacteria, a magnification of at least 1,000 X is required. In such instances, a compromise has to be made (figures 23 and 38). Alignment correlated positively with bacterial penetration in previous experiments in this thesis.

Pitting (erosion, depression, pin holes), by its very nature creates a large break in the continuity of the mammillary layer. The positive correlation between the presence of faults of this type and bacterial movement has been demonstrated and the immunogold results only serve to substantiate their negative effect on shell quality. As with the alignment phenomena, it is difficult to display both the structural defect and the labelled bacteria at the same magnification. In many instances, the magnitude of the fault was such that bacteria, having flooded through them, relocated marginally.

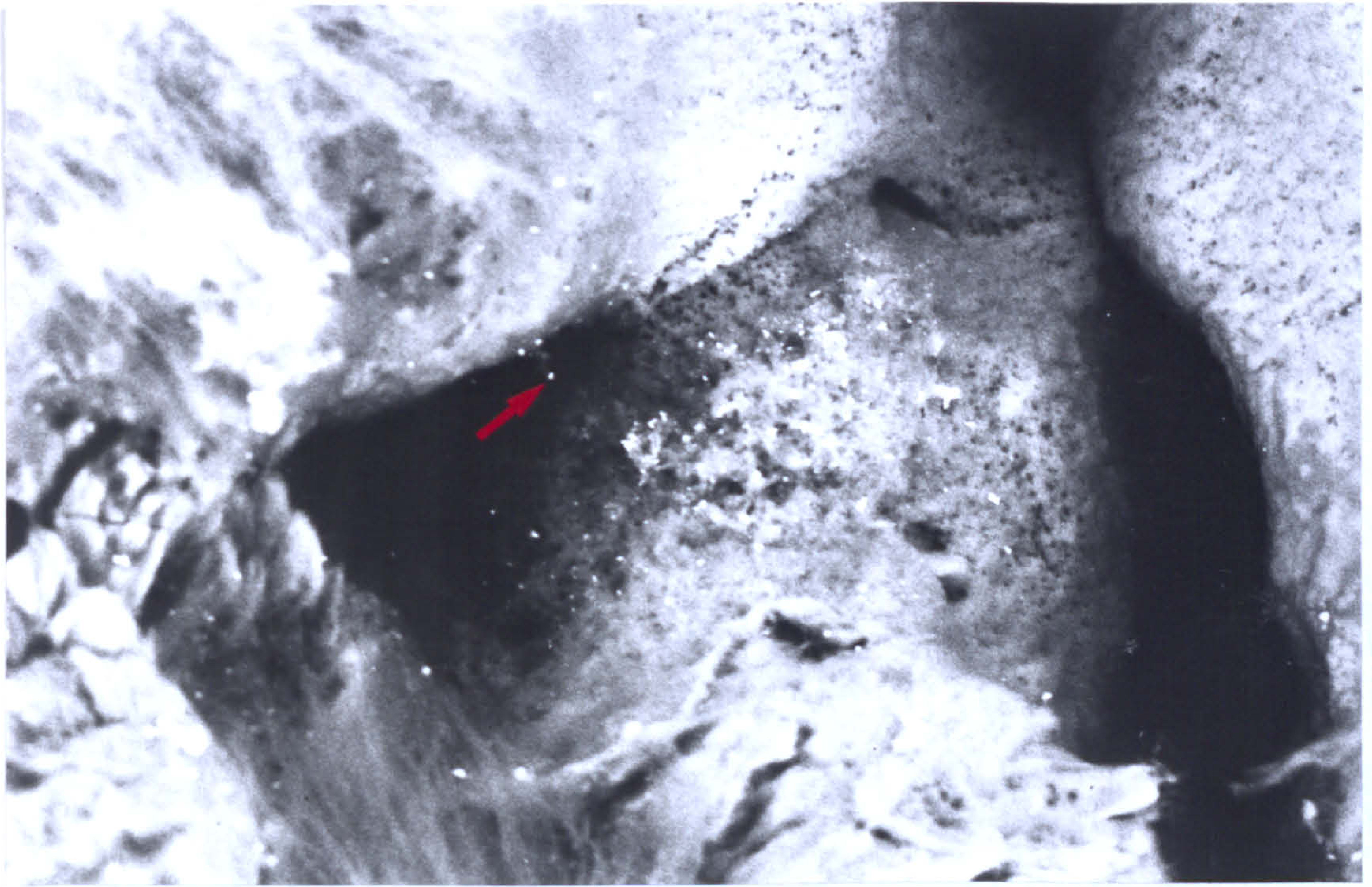


Figure 36: BEI micrograph in normal polarity showing bacteria (tagged *Salmonella*) (arrow) in large numbers, near to a site displaying late fusion of the mammillae. Note the vesicularity of the palisade columns (3,375 X).

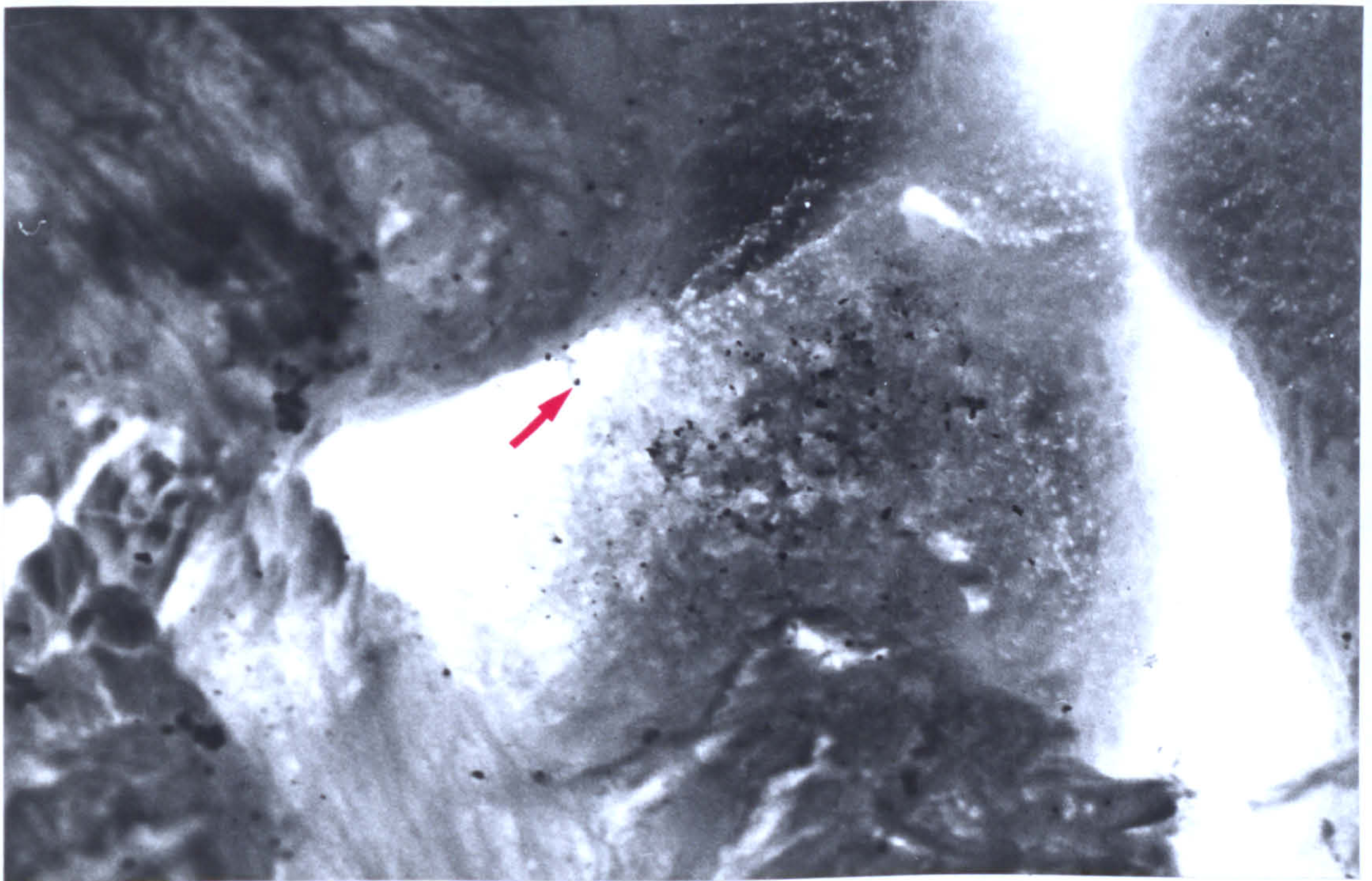


Figure 37: Same as above with reversed polarity (3,375 X).

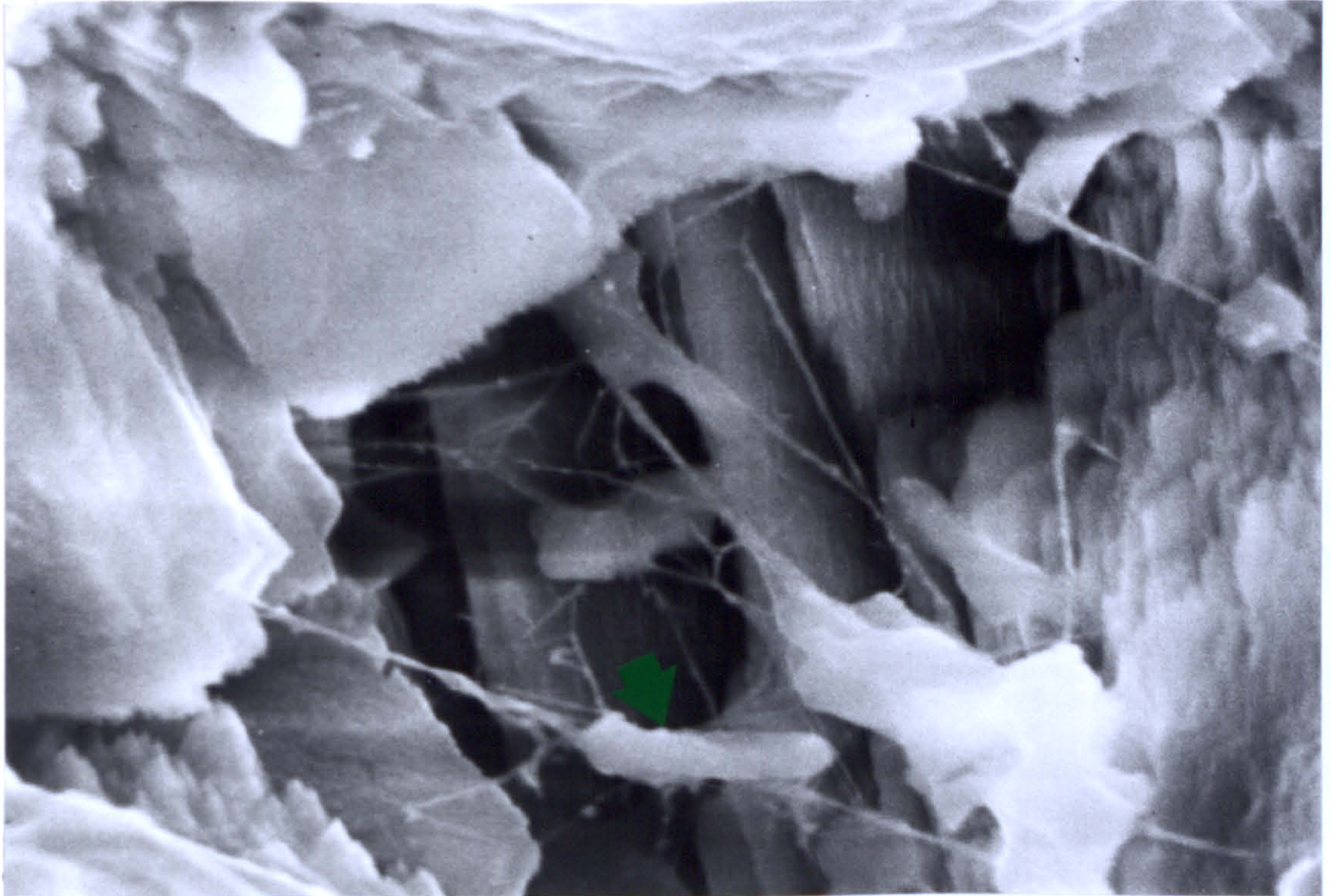


Figure 38: SEI micrograph displaying *Salmonella* (arrow) located near to mammillary alignment (21,375 X).

CHAPTER 6

EGGSHELL QUALITY AND BACTERIAL PENETRATION AS AFFECTED BY THE USE OF THE GROWTH PROMOTER VIRGINIAMYCIN IN THE DIET OF THE BIRD (ADDITIVE EFFECT)

6. EGGSHELL QUALITY AND BACTERIAL PENETRATION AS AFFECTED BY THE USE OF THE GROWTH PROMOTER VIRGINIAMYCIN IN THE DIET OF THE BIRD (ADDITIVE EFFECT)

6.1. INTRODUCTION

6.1.1. GENERAL AND MECHANISM OF ACTION

Growth promoters are a general group of antibiotics characterised by Gram + activity and poor absorption from the gut (Gustafson, 1986), and their use is recommended in intensive conditions of animal production, where animals are stressed by overcrowding, temperature variations, subclinical disease, sub-optimal nutrition, mixing and transport (Walton, 1983).

The use of antibiotics in animal production has been estimated to have saved US$ 3.5 billion a year in food costs to the U.S. consuming public (Hays, 1986), while the feed antibiotics market is estimated to be US$ 1 billion per year in the U.S. and almost as much in Europe (Burg, 1986).

Under conditions of dense bacterial colonisation in the gut (like the filamentous segmented bacterium showed by Erlandsen and Chase (1974) to colonise the chicken's small intestine), the alterations in the surface of the host cell may become so severe that it is no longer possible to discern any recognisable features of the microvillous border, rendering them unlikely to perform their normal functions of nutrient transport. On the other hand, the intestines of germ-free bird are thinner-walled (by a reduction in connective tissue, especially the lamina propria), the villi are more evenly shaped and the rate of renewal of epithelial cells is slower (Cook and Bird, 1973 and Rolls *et al.*, 1975 cited in Coates, 1976). Fat absorption is also greater in germ-free and monocontaminated chicks than in conventional ones,

suggesting that the usual enteric flora actually exerts an inhibiting effect (Cole and Boyd, 1967).

The growth promoting attributes of antibiotics in feeds reside in their capacity to reduce the already mentioned negative effects and to stimulate the positive ones by having an antibacterial activity in the intestine. This can be proved by their non-effect in germ-free chicks reared in a clean environment and fed antibiotic supplemented feed, likewise the controls, while animals reared in "dirty" premises show a marked growth response to antibiotics in comparison with their controls, according to Coates *et al.* (1952 and 1954) cited in Ewing (1963).

The growth promoters' mode of action over the bacterial cell has been described by Branen (1983) to include: a) reaction with the bacterial cell membrane, causing increased permeability and loss of cellular constituents; b) inactivation of essential enzymes and c) destruction or functional inactivation of genetic material. The use of antibiotics also suppresses mild but unrecognised infections, reduces microbial destruction of essential nutrients, vitamins and microbial toxins (Shahani and Whalen, 1986). It also minimises stress-related pathologies brought about by highly intensive rearing conditions (Katz, 1983 and Dafwang *et al.*, 1987). Growth depression has been reported at high stocking densities by various workers cited by Dafwang *et al.* (1987), due to non-specific stress resulting in adrenal hypertrophy (and significant decline in the Bursa of Fabricius and thymus weights, suggesting also immunocompromised birds).

Feeding antibiotics leads, in some cases, to an increased absorption of nutrients by the small gut, with an increased metabolic activity of the gut wall (Hudson *et al.*, 1971). The amount of growth depression is thought to be variable and dependent on various factors, including the genetic strain (breed) of the animal (Ewing, 1963 and Portsmouth, 1987). There are strains which are more resistant to a dirty environment, while others are more sensitive, with a consequent decline in the level and quality of production. In

any case, the "dirtier" the farm conditions, the greater will be the response, although Coates (1962) claims that growth-depressing conditions appear to establish themselves even in the best managed flocks. Sex is another factor, with males giving a more marked growth response than females (Portsmouth, 1987, Fraser, 1989 and Salmon and Stevens, 1990).

6.1.1.1. Virginiamycin

Virginiamycin is an antibiotic produced by the mutant of the bacterium *Streptomyces virginae* (Veltmann and Weideman, 1987). It has one of the two basic primary structures or groups A and B, *viz.* virginiamycin M_1 and M_2 part of the group A and virginiamycin S_1, S_2, S_3 and S_4 part of group B. Both structures are classified as macrocyclic lactone peptolides, although having different formulae, *viz.* group A specifically a macrolide and group B a depsipeptide (Tanaka, 1975; Vazquez, 1975 and Cocito, 1979). They exhibit a strong synergistic (A+B) effect with respect to their activity against Gram + bacteria, so causing a more pronounced inhibition of bacterial growth than they do separately (Bycroft, 1977 and Cocito, 1979). The product "Eskalin", used in this experiment, is a combination of the A and B structures. Despite being active against Gram +, this antibiotic is also active against two genera of Gram -: *Neisseria* and *Haemophilus* (Van Dijck, 1969), and active *in vitro* but not *in vivo* against *Mycoplasma* strains (*in vitro* growth could have helped it by inducing some ribosomal conformational changes, which render them more sensitive to the inhibitory action of the antibiotic). It requires a higher dose for protection than other antibiotics (Cocito, 1979).

The precise mechanism of action is not yet fully understood, but it appears that the primary site of action is at the bacterial ribosome, where the formation of the 50S ribosomal subunits is blocked (and thus protein biosynthesis) in bacteria treated with virginiamycin (Bycroft, 1977) (this site is normally sensitive to the action of most antibiotics). When one of the virginiamycin groups is used individually, its removal allows the formation of ribosomal subunits to resume immediately, which does not occur when they are used together (A+B), as permanent damage is then done to protein synthesis. Bacterial resistance to virginiamycin can occur by the modification of the 50S ribosomal subunit target, by means of chromosomal modification. Inactivation of the drug (especially by plasmids) and alterations in bacterial

permeability can also cause the same effect.

Virginiamycin is reported by Cocito (1979) to have extremely low toxicity, even if administered for prolonged periods at high doses, having also a very low intestinal absorption (thus not interfering with other systemic infections) and a very rapid excretion, through the urine, bile and faeces.

The same author reported that this antibiotic has been used for human therapeutics in the past, but now its use has been abandoned in favour of its role in animal feeds, since it meets all the requirements demanded from a growth promoter, while in humans it displayed some problems with regard to its solubility and resorption.

Hedde (1984) and Veltmann and Weideman (1987) report enhanced energy, increased utilisation of sulphur amino-acids, phosphorus and manganese and a reduction in nutrient competition and energy uptake (by fermentation of glucose) by small intestinal bacteria. There have also been reports of improved intestinal absorption and stimulated growth in broilers treated with virginiamycin (Eyssen and DeSomer, 1963; Fraser, 1989), with these authors and also March *et al.* (1978) claiming that the improvement was greater during the early period of growth.

Virginiamycin also showed better results in feed efficiency in broiler birds (Combs and Bossard, 1963; Bird, 1968), when compared with flavomycin and Zinc bacitracin (Hakim *et al.*, 1989). Although the literature registers plenty of publications dealing with the effect of virginiamycin in broilers, there is a paucity of information concerning its effect in layers. Miles *et al.* (1985) found that supplementation with 10 ppm of virginiamycin resulted in increased egg production, feed efficiency, weight gain and higher egg specific gravity (ESG) (although producing smaller and lighter eggs) in 36 weeks old HyLine hens. In contrast, Harms *et al.* (1990) reported that when the ESG was corrected for egg weight (EW), it was shown that the majority of the improvement in shell quality was due to a reduction in EW, and

not to more Ca being placed on the egg.

Veltmann and Weideman (1987) showed a significant strain (DeKalb x HyLine) effect for body weight, feed consumption and feed conversion for pullets, with the DeKalb group showing a two-fold decrease in mortality and the HyLine showing no improvement in growth, when fed virginiamycin. Inherent differences in terms of the action of the antibiotic in the gastrointestinal tract were offered as explanation. On the other hand, Arbor Acres birds may be really inherently unresponsive to this antibiotic, according to Proudfoot *et al.* (1990). Significant benefits from virginiamycin supplementation were found by Miles *et al.* (1984), with low dietary protein when fed after 8 weeks of age.

Virginiamycin supplementation increased the cost per ton of feed by approximately US$ 3 (Veltmann and Weideman, 1987) with the economic return, especially among DeKalb birds, being a saving of two weeks of feed cost, with 16 w.o. birds weighing the equivalent of 18 w.o. pullets.

Virginiamycin added at 30 ppm to the layer diet has been demonstrated to increase the incidence of positive structural characteristics in eggshells (Belyavin *et al.*, 1991), particularly at the beginning of lay (Solomon, 1990a). Carson and Eaton (1954) found that eggshell thickness, in birds with Chronic Respiratory Disease infection, exhibited a downward trend when no antibiotic was used, whereas birds treated with terramycin maintained shell thickness during the course of the experiment.

Virginiamycin and bacitracin were found to produce a synergistic effect when used coupled with competitive exclusion treatment, according to Humbert *et al.* (1991). They believed that the antibiotics possibly promoted the most protective bacteria which had been administered as part of the competitive exclusion treatment.

6.1.1.2. Calcium metabolism

Since it is generally accepted that the major direct effect of antibiotics on eggshell quality is related to an improvement in Ca utilisation by the laying hen, it is considered relevant to review the mechanism of Ca absorption and metabolism.

A 55 g egg will contain about 2.2 g of Ca, which must ultimately be supplied by the diet. If Ca supplements are excluded from the diet, thinner eggshells will result, but even if Ca is practically absent from the diet, the bird will not produce shell-less eggs. The shell becomes progressively thinner until it comprises only 60 % of its normal weight (within 10 days). Laying then usually stops, with the hen having removed about 35 % of the mineral matter from its skeleton (Taylor and Moore, 1954; Balnave, 1988).

After sexual maturity (about 20 weeks of age), a minimum of 30 g of Ca per kg of diet is needed by the mature laying hen to maximise egg production and to meet the specific needs of eggshell formation. Normally, at least 35 g Ca/kg is included in diets to optimise eggshell quality, since its Ca requirement is higher than for maximum egg production (Balnave, 1988). Despite that, increasing the dietary Ca concentration above the recognised requirement is claimed by Hurwitz and Griminger (1961) to have little effect on shell quality, since Ca retention declines as the dietary Ca concentration increases. A 2.25 % Ca level in the diet will result in three times more eggs of inferior shell quality than a 3.75 % level, at the end of the laying season, according to Ewing (1963).

Petersen (1965) disagrees, stating that it appears that hens can retain 50 % or more Ca up to 4 g intake per day. Further increases in Ca intake, although not retained, still improve shell quality. Conversely, it is accepted that Ca is absorbed more efficiently, mediated by a mechanism which acts when animals are adapting to low-Ca diets (Hurwitz and Bar, 1966). This

mechanism can be explained by the fact that Ca absorption in the duodenum and jejunum is normally a passive process with birds maintained on Ca-sufficient diets, but when low Ca diets are fed, or at the onset of reproductive activity (Simkiss, 1975), an active transport of Ca is put in action (Hurwitz, 1976).

Food intake in general and Ca in particular is thought to be influenced by the fact that in egg-forming days the Ca requirements are higher (Hughes and Wood-Gush, 1971), and it was suggested by Hughes (1972) that maximum Ca intake occurs just prior to the start of shell formation. Although accepting that Ca absorption during eggshell formation is almost twice that occurring when no shell is forming, Hurwitz (1976) claims that this mechanism is independent of dietary Ca intake, being instead the result of the greater permeability of the intestine to Ca during this time. Even though intestinal Ca absorption appears to be limited, under times of Ca demand and when the dietary source is inadequate, skeletal Ca will be utilised. The main source in these circumstances is a highly labile store of non-structural bone (capable of being formed and resorbed at higher rates than any other bone, in the marrow cavities of the skeleton (medullary bone), which only exists in the female bird at the time of reproductive activity and whose dynamic state is controlled by the parathyroid hormone (PTH) and calcitonin. According to the same author, the parathyroid glands chiefly control Ca metabolism, responding directly to the Ca ion levels of the plasma, by inducing osteoclastic resorption of the bone (possibly by stimulating the enzyme carbonic anhydrase to release protons from osteoclasts, according to Waite and Kenny (1970)), excretion of phosphate and the resorption of Ca from the urine, and enhancing the intestinal absorption of Ca. Hypercalcaemia, on the other hand, is reduced by the secretion of calcitonin, a hormone secreted by the ultimobranchial gland (base of the neck, posterior to thyroid and parathyroid glands), which then inhibits bone resorption, causing plasma Ca levels to be reduced, so antagonising the PTH in a feedback control system (Simkiss, 1975).

Part of the mechanism of Ca absorption and transportation is possibly due to the action of the calcium-binding protein (CaBP), which occurs in the goblet cells and glycocalyx or brush border of the intestinal epithelial cells (Taylor and Wasserman, 1970). The precise physiological role of CaBP remains obscure (Levin, 1984) except that it binds one Ca atom to one protein molecule, and that its occurrence shows an almost perfect correlation with Ca absorption.

Cholecalciferol or vitamin D_3 stimulates the formation of CaBP in the mucosa (Wasserman and Taylor, 1966) and also increases the permeability of the intestine to Ca ions (Harrison and Harrison, 1965). In the absence of vit. D, the mineralisation of the skeleton is deficient. It can be supplied either from the diet or it can be synthesised in the body from the natural precursor 7-dehydroxycholesterol, under ultraviolet irradiation (Hurwitz, 1976).

According to Simkiss (1975) cholecalciferol is hydroxylated in the liver to 25-hydroxycholecalciferol, which circulates in the plasma, and it is further hydroxylated in the kidneys (the enzyme catalysing the synthesis is regulated by PTH (Hurwitz, 1987)), to form a dihydroxycholecalciferol (DHCC), either in the 1 or 24 position, forming 1,25-DHCC or 24,25-DHCC. They appear to act as steroid hormones controlling Ca metabolism.

The shell gland is also acknowledged to contain CaBP, which would not be affected by low Ca diets. Its occurrence is, however, associated with the onset of egg production and decreases as egg production ceases (Bar and Hurwitz, 1973), but unlike the intestinal CaBP, there is no correlation between the rate of Ca transport by the shell gland and its CaBP content, suggesting that shell formation can occur in its absence (Simkiss, 1975). Bar *et al.* (1984) are not in complete accordance with the above. They claim that a significant correlation exists between CaBP and eggshell Ca, and similarly between the concentration of 1,25-DHCC in the shell gland and plasma, but not between the vit. D-hormone content in the shell gland and CaBP or shell Ca.

Zinc is involved in shell formation, as it is a component of the enzyme carbonic anhydrase, which is an important factor in the conversion of Ca in the diet into Ca in the eggshell, according to Ewing (1963). The same author also reported that ascorbic acid or vit. C seems to be associated with shell deposition, particularly at temperatures above 21 ^{o}C, with higher levels of Ca, and with protein levels lower than commonly used in commercial rations.

In terms of the influence of the microbial status of the gut in the absorption of Ca, Palmer and Rolls (1981) suggested that the ability of the enterocyte to absorb Ca was unaffected by microbial presence or absence, but in its absence the removal of Ca was reduced. In an earlier paper, Jayne-Williams and Fuller (1971) agreed, citing several reports of increased tibial deposition of Ca, improved utilisation of Ca and P, enhanced bone calcification, improved retention of Ca and increased blood Ca in laying hens given dietary antibiotics. Penicillin and aureomycin were specifically said to enhance the absorption of Ca (which is reported by Edwards and Boyd (1963) to be higher in germ-free birds) and also to be egg promoters (Coates *et al.*, 1955 and Portsmouth, 1987). Price and Zolli (1959) also reported that downgrading the Ca contents of feeds increases antibiotic absorption through intestinal villi.

6.1.2. GROWTH PROMOTERS IN LAYING HENS

The ability of hens to extract Ca from nutrients and to transform it into the eggshell is thought to be materially influenced by health status, where differences induced by the use of feed additives may be minute, according to Hunton (1989), requiring therefore comparatively large numbers of eggs and precise measurement techniques to assess them.

The literature, although dealing with the effects of antibiotics in laying hens, puts most of the emphasis on the production aspects, with only a few references relating to the eggshell quality aspect.

Thus, feeding chlortetracycline (Eoff *et al.*, 1962), penicillin (Gabuten and Shaffner, 1954) or oxytetracycline (Casey *et al.*, 1963) were reported to increase both eggshell percentage and specific gravity, and also the shell breaking strength and Ca blood level. Shell quality in hens fed furazolidone and nitrofurazone, as measured by specific gravity, was reported to be slightly, although not significantly, improved, according to Francis and Shaffner (1956).

The addition of antibiotics in general was reported to have significantly increased body weight and improved feed efficiency in turkeys (Jiraphocakul *et al.*, 1990) and in chickens (Petersen *et al.*, 1958; Miles *et al.*, 1984 and Dafwang *et al.*, 1987) and also improved egg production and product quality (Boorman, 1987), in terms of lower numbers of shell checks and cracks, although sometimes the effect was statistically non-significant (Eoff *et al.*, 1962).

Gabuten and Shaffner (1952 (cited in Ewing, 1963) and 1954) and Bogdoroff and Shaffner (1954) fed procaine penicillin at 15 and 30 ppm to laying hens and observed an improvement in eggshell quality during the summer months, as measured by specific gravity. As the season progressed,

the quality of the eggs from the control hens declined, but the eggs from the experimental hens kept their original quality (Ewing, 1963). Petersen *et al.* (1958) however were unable to find any improvement in eggs from White Leghorn pullets fed penicillin and kept in a high temperature environment, perhaps reflecting their natural ability to produce high quality shells.

Virginiamycin has been specifically reported to increase egg production, feed efficiency, weight gains and egg specific gravity in HyLine 36 w.o. hens at a 10 ppm dose (Miles *et al.*, 1985), improving the performance of the poor producing hens while maintaining the production of the best producers. The same has also been demonstrated to increase the incidence of positive structural characteristics of the eggshells when fed at a 30 ppm level (Solomon, 1990a and Belyavin *et al.*, 1991).

Finally, there are a few reports of sulpha drugs such as sulphanilamide and sulphapyridine having an adverse effect on shell quality (causing hens to lay eggs with thin or even no shells), because of their inhibition of the enzyme carbonic anhydrase (Hinshaw and McNeil, 1943 and Ewing, 1963).

6.1.3. DEVELOPMENT OF RESISTANCE AND INCREASE IN *Salmonella* SHEDDING

The Swann Report, published in 1969 in the U.K., and which came into force two years later as legislation, recommended that feed additives used for growth promotion and feed efficiency continue to be permitted for free sale, but that antibiotics used for prevention or control of disease be used only on veterinary prescription (Gustafson, 1986).

Before 1969, therapeutic antibiotics, particularly penicillin and tetracycline were extensively used in the animal industry, for growth promotion as well as for treatment in veterinary and human medicine (Smith *et al.*, 1985). Strangely enough, tetracyclines were still permitted as growth promoters in the U.S.A. in 1984, despite their ban in the European Community where they were used as therapeutics in human and veterinary medicine (Armstrong, 1984 cited in Armstrong, 1986). Threlfall and Rowe (1984) raised an important issue, when they reported that almost all gentamycin-resistant *Salmonellae* isolated from humans in Britain were confined to persons who had either acquired their infections abroad, or had had contact with persons from other countries where these strains had caused extensive outbreaks.

Forty-five to 55 % of all the antibiotics produced each year in the U.S. are administered to animals in feed (Hays, 1986). In 1950 essentially all antibiotics were used as medicinals in humans and animals (Katz, 1983). With these facts it is estimated that over 2/3 of U.S. multiple-drug-resistant *Salmonella* infections that had a defined source in the period 1971-1983, came from food animal populations (Holmberg *et al.*, 1984). It is also important to point out that, according to Lacey (1984), where animal use of antibiotics is large and medical use is small, then resistant animal organisms might pose a significant contribution to the overall amount of resistance in human pathogens, but where human use exceeds animal use by a large

amount, then the contribution of animal use must be small.

Prior ingestion of antibiotics reduces the dose of *Salmonellae* necessary to initiate infection in mice and humans pretreated with streptomycin (Blaser and Newman, 1982). An experiment by Bonhoff *et al.* (1954) cited in Clegg (1962) demonstrated that is possible to infect mice with less than 3 *S. enteritidis* organisms in pretreated animals, while about 100,000 were necessary for the controls.

Within the family *Enterobacteriaceae*, multiple drug resistance may be transferred by conjugation from one bacterium to another, by means of the episomes known as R (resistance) factors in association with resistance transfer factors (RTF), in the intestinal tracts of animals and humans as well as *in vitro*, under laboratory conditions. These episomes consist of DNA, which occurs in the cytoplasm of the donor bacterium and multiplies independently of the chromosomal DNA. Thus, a bacterium with an R factor only, is a cell that is resistant to one or more antibacterial drugs, but is not able to transfer this resistance to another susceptible cell, while a bacterium with both an R factor and an RTF can transfer resistance (Buxton and Fraser, 1977). The problem is the administration of subtherapeutic doses of antibiotics to livestock for prophylactic or nutritional purposes and the propensity of *Salmonella* for intra- and inter-generic exchange of this cytoplasmic DNA (R plasmid) (D'Aoust, 1989). Plasmids are extrachromosomal self-replicating genetic elements of a cell. They are circular DNA molecules that reproduce themselves apart from the chromosome; they include the F (Fertility) factor and the R (Resistance) factor, and may also become integrated into the chromosome, being then called episomes (Blood and Studdert, 1990). This transference can take place not only in the normal bacterial flora of the intestines of animals, but also even in the absence of antibiotics in the environment, with the frequency being higher when colonisation of the bacteria occurs in the intestines (Buxton and Fraser, 1977 and Frappaolo, 1986). These transfers can occur between bacteria belonging to the same genus (*e.g. S. enteritidis* to *S. enteritidis*) or

even via non-pathogenic organisms, such as *E. coli* , which can be selected for resistance and act as reservoirs of resistant plasmids, which can then be transferred to other pathogenic and non-pathogenic bacteria, (*e.g. E. coli* to *S. enteritidis*) (Shahani and Whalen, 1986 and Falkow *et al.*, 1987). The pathogenicity and drug resistance factors have also been shown to be linked on the same plasmid, and therefore can be transferred simultaneously to other organisms (Frappaolo, 1986).

Walton (1966) and Smith (1970) demonstrated specifically the transfer of R-factors from R^+ *E. coli* to *S. typhimurium* in the digestive tract in chicks and adult chickens. Smith (1977) even found that in chickens in which a high rate of transfer had occurred, it was common to find R^+ *S. typhimurium* organisms in the liver in addition to their inclusion in the faeces, indicating that these organisms, after acquiring the R factors (and maybe invasiveness factors as well) from *E. coli* in the alimentary tract, had then invaded the body.

According to Hays (1986), if the conditions are right for bacterial growth, the transference of resistance could take place even in prepared food, while Levy (1984) suggests that daily ingestion of these resistant organisms present in the food will propagate antibiotic-resistant bacteria and their plasmid pools in man and animals. Rampling *et al.* (1990) found that 95 % of the isolates of *S. enteritidis* PT 4 infecting broiler chickens were resistant to nitrofurantoin, with also some cross-resistance between nitrofurantoin and and furazolidone. This can be dangerous because nitrofurans are used for poultry treatment of *Eimeria* sp. infections, and furazolidone is important for the prevention and treatment of *Salmonella* infections in poultry. Apart from that, nitrofurantoin is employed in human medicine, although limited to treatment of infections of the lower urinary tract.

Walton (1977) reported that with the use of the growth promoter zinc bacitracin (which is supposed to be active only against Gram + organisms), the overall susceptibility of enteric (Gram -) bacteria can be increased by

weakening the bacterial cell (non-lethally), and will lead to a decrease in antibiotic resistance of enteric bacteria in the environment. The problem is that this cell wall effect could be produced by a specific antibiotic (used for treatment) as well, and so the additional (non-lethal) effect provided by the growth promoter could in fact result in the bacteria developing some kind of resistance against this specific action, and subsequently no other antibiotic would be able to use this mechanism as part of its strategy of action.

Similarly, one of the main recommendations proposed by Linton (1987), that growth promoters should be a distinct group of agents not used for therapy in man and animals seems to be disregarded in Canada, where despite the fact that the maximum level of virginiamycin permitted in poultry feeds is 11 ppm for nutritional (growth promoting) uses, the same antibiotic is also allowed to be used for medicinal (treatment) purposes, especially for Necrotic Enteritis, and in this case the level allowed is 22 ppm (Blair, 1990). The risks of development of resistance will be much higher in this situation. In pigs, 11 ppm is allowed for nutrition, and 55-110 ppm for treatment purposes (mostly for Synovitis), increasing the chance of transference of resistance in the farm. The danger is real, as Van Leeuwen *et al.* (1979) reported, the use of therapeutic antibiotics as feed additives (at 30 ppm) has selected resistant strains of *Salmonella* with a single R determinant only (*e.g.* for tetracycline).

Heffernan (1991) suggests the extremely low frequency of resistance among *Salmonellae* from non-human sources maybe due to the fact that animals in New Zealand were not intensively reared and the use of antibiotics for growth promotion is controlled by legislation.

Medicated feeds contribute substantially not only to the problem of bacterial antibiotic resistance, but also increases the potential for cross-infection of animals through prolonged faecal shedding of *Salmonella*. Indirect use of drugs on the farm maintains a selective environment through animal excretion of unabsorbed drugs (Bird, 1968 and D'Aoust, 1989).

The period of excretion of *Salmonella* by an infected bird is recognised to be extended by feeding antibiotics (Jordan, 1990), especially avoparcin (Barrow *et al.*, 1987 and Humbert *et al.*, 1991). Growth promoting antibiotics (having no significant Gram - activity, like nitrovin, flavomycin and tylosin) may indirectly affect the excretion rate (increasing shedding) of *Salmonella* by poultry, by disturbing the complex ecology of the intestinal tract involving many types of bacteria which are inhibitory to *Salmonella* in the caeca (Smith *et al.*, 1985). It was reported by Hinton (1988) that there is evidence that certain growth promoting antibiotics have an undesirable side-effect of favouring the persistence of *Salmonella* infections in chicks.

Nevertheless, in their turn, virginiamycin and bacitracin have been recognised to have little effect on excretion levels (Gustafson, 1984; Frappaolo, 1986 and Humbert *et al.*, 1991). These observations may explain why Abou-Youssef *et al.* (1983) suggested that recognition of growth promoters should be limited to virginiamycin, and other antibiotics that primarily inhibit Gram + microorganisms. It should be pointed out that although virginiamycin is not active against enterobacteria, it is active against many of the other bacteria that inhabit the alimentary tract, and so may disturb the ecological balance in favour of the enterobacteria, particularly the *Salmonella* (Smith, 1977).

In previous sections of this thesis, attention has been directed towards illustrating the diversity of structure which exists within the "true shell" and the diverse factors which can influence the same. Using the gold probe technique, specific morphological variations can now with confidence be correlated with bacterial transfer. This chapter records the results of a series of experiments designed to test whether shell structure can be improved by the use of virginiamycin in the diet and if so, to assess whether the improvement enhances the role of the shell as a "resistance network" (Sparks, 1985).

6.2. MATERIALS AND METHODS

6.2.1. GENERAL

The eggs used in this trial were collected at random from a strain of commercial brown egg layers (strain B), housed in a three tier battery system, fed *ad libitum* two different diets, with the control group receiving Basal diet + no additive and the experimental group receiving Basal diet + 30 ppm (mg/kg) of virginiamycin by the use of the commercial product "Eskalin" (SmithKline Beecham Animal Health, Surrey, England) and located in the same premises as mentioned in section 2.2.1.. The house was thoroughly cleaned using an industrial vacuum cleaner and washed down with a steam cleaner before being disinfected and fumigated with formaldehyde. Eggs were collected from the beginning (23 w. o.), middle (42 w. o.) and end (60 w. o.) of lay.

A total of 180 eggs were used (30 from each age group for each of the two different dietary treatments).

The *S. enteritidis* PT 4 technical procedures and positioning of the eggshell on the agar plate were all carried out as described in sections 2.2.2. and 2.2.4.. The treatment of eggshells followed the procedure given in section 2.2.3., except that only samples with no membranes attached were used in the analysis. The percentage of penetration values were obtained and analysed as described in 2.2.4. and 2.3.1..

6.3. RESULTS AND DISCUSSION

The relevant tables and graphs appear at the end of this section. The baseline for the evaluation of the effects of the use of the growth promoter virginiamycin (at a 30 ppm dose in the feed administered to layer hens during one laying year) was the results from the control group, which are presented in table 20. The experimental results are, in turn, reported in table 21. Both tables show the statistical significance of the differences observed in each group over the three periods sampled (beginning, middle and end of lay).

Subsequently, in table 22, the results from the control and experimental groups are compared against each other over the same three periods. In table 23, the percentage penetration results are crossed against the various eggshell parameters, thus giving the correlation coefficients. The size of the latter indicates approximately the degree of relationship existent between each of the parameters evaluated and the observed variation in bacterial penetration.

In general, eggshell defects increased significantly as the birds aged, in the control group, following a similar pattern already demonstrated in chapter 2. Graphs 31, 32 and 33 illustrate the same.

The number of *Salmonella* organisms which managed to penetrate the shell also increased, although non-significantly, showing a steady, upward trend throughout the laying year.

The first interesting variation in the now anticipated behaviour of the eggshell's quality patterns was revealed when an analysis of the experimental data (table 21) was performed. Starting with percentage penetration, this increased significantly from the beginning to the middle of lay, but at the end of lay, it reduced to levels even slightly lower than at the beginning of lay (graph 34). Although the percentage of penetration values

at the beginning of lay corresponded numerically to those at the end, the lower standard deviation (s.d.) observed at the end of lay (5.29) when compared against the same parameter from the beginning of lay (10.83) illustrates that the former group had less variation around the mean, *i.e.* greater uniformity.

On the other hand, the total score mean values significantly increased from the beginning (24.60) to middle (29.30) and then remained practically the same at the end of lay (29.60). When individual structural characteristics are examined, however, they do differ in the two groups, *viz.* the levels of early fusion were slightly lower in the middle of lay group than at the end, and this coupled with higher levels of mammillary alignment would offer a more open framework. The pitting levels were also higher in the mid lay group, and also less cuffing (higher score). Finally, mid lay eggs had significantly less changed membrane, which were mentioned in previous chapters 3 and 4 to be possibly helping to protect the eggshell physically against bacterial penetration (graphs 35 and 36), under experimental conditions.

In terms of eggshell quality, results (table 22) indicate that there was almost no significant difference between the two groups at the beginning of lay in terms of the majority of individual features, including total score means (24.87 controls against 24.60 experimental) and bacterial penetration (7.70 % and 7.61 % respectively). This may indicate that the improvement usually proportioned by the use of growth promoters was not great enough in this particular situation to be detected by the methods used, or it may reflect the fact that under experimental conditions, the closely controlled environment leaves little room for improvement in sanitary conditions. Ewing (1963) suggested that a reduction in the "germ load" or disease potential may occur in the environment as a result of long term use of antibiotics.

At mid lay both the control and experimental groups displayed similar total score mean values (29.23 controls against 29.30 experimental), although the individual characteristics making up these total scores were

different. It is interesting to note that bacterial penetration did not increase significantly in the experimental group at this time. At this stage, it is difficult to isolate any one single factor responsible for the increased penetration in the experimental group. Undoubtedly, pore numbers and distribution may be involved as too can the vesicular nature of the palisade layer, which according to the nature of the structural defects present in the mammillary layer can provide a more open framework for the localisation of bacteria.

At the end of lay, shell quality was significantly better in the experimental group, and the bacterial penetration was significantly reduced (graph 37). This improvement in quality correlated well with the reduction in those undesirable features such as late fusion, mammillary alignment, pitting, aragonite and cubics (graphs 38, 39 and 40). The results are in accordance with the findings of Solomon (1990a), who in a pilot study demonstrated the beneficial effect of virginiamycin at 30 ppm on eggshell structure at the beginning of lay.

The present results corroborate the data presented in chapter 2, *viz.* eggshell ultrastructure and bacterial penetration are positively correlated (table 23) (graphs 41, 42, 43, 44 and 45). Correlation values give an insight into potential relationships between traits. For instance, the correlation coefficient (R) between the total structural score and bacterial penetration was found to be 0.70 in an overall analysis of the eggs from the experimental group over the 3 laying periods, indicating statistically that variations in total score levels accounted for about 50 % ($(0.70)^2 = 0.49$) of the variation in bacterial penetration. The total score data provides a good idea of the shell's structural soundness. Also, an analysis of the coefficient of determination (R^2) values, when plotted in a matrix containing most of the relevant variables involved in the study reveals that an interesting shift of importance occurred between beginning, middle and end of lay and especially between the control and the experimental groups (summarised at table 24).

The use of the coefficient of determination is necessary to verify the role

played by individual characteristics. In chapter 2 the trends in structural deterioration were marked and followed an acknowledged pattern with increased bird age (Bain, 1990). In this particular series of experiments, the decline was less obvious, thereby necessitating the use of more sophisticated statistical analysis. Specific structural traits (caps, mammillary alignment, aragonite and cuffing) were shown to be possibly determining changes in penetration values.

Finally, at the end of lay, a similar picture is observed, where again none of the individual parameters, when regressed collectively over penetration was capable of causing alone a statistically significant change in the behaviour of the latter dependent variable. Similarly, in the experimental group, several characteristics *viz.* caps, early fusion, type B's, cuffing and changed membrane (table 24) were significantly connected with variations in the pattern of penetration, when simultaneously regressed against the latter.

It would appear that certain individual features were in some way more closely involved with variations in the capacity of the shell to block or ease bacterial penetration in the experimental group than in the controls, and it can be further hypothesised that it will then be a matter of these parameters being either detrimentally or positively present, to reflect detectable differences in shell quality.

Obviously a clear image of a causal relationship will be one where the cause is necessary and sufficient for the effect to occur. However, there are very few examples of such infallible, deterministic relationships in science, and the more common picture is that most phenomena of interest are only probabilistically related to the causes which can be accessed, and the fact that there is only a small subset of the determinants of the scores on the dependent variable (Maxwell and Delaney, 1990).

Some hypothesis can be put forward in an attempt to supply a theoretical basement to the phenomena observed, starting with the claims by

White-Stevens *et al.* (1955), who reported an increase in egg production that spanned 6 months, as an effect of the inclusion of aureomycin in the feed. This supports the present results which show a late effect of the antibiotic. The present experiment was dealing with a naturally superior performance strain of birds, which will only allow very restricted room for improvement in the results. Also, it is probable that changes in microbial populations as a result of antibiotic administration are not constant, but change with time and/or age (Ewing, 1963), with diet, environmental temperature and humidity all playing a role in the antimicrobial growth response (O'Connor, 1980).

Antibiotics can indeed help to suppress stress, *e.g.* when housing conditions change or during vaccination periods, and more especially the ongoing stress of sustained egg production (Ewing, 1963 and Coates, 1980), which could be exacerbated at the end of lay.

Visek (1964), Quarles and Kling (1974) and Fraser (1989) maintain that toxic substances such as ammonia can adversely affect poultry either by direct action or by contaminating the environment. It is tempting to speculate that the growth promoter exerted a counter acting effect in the face of these increased levels or at least maintained the *status quo.*

Bar and Hurwitz (1987) claim that in older hens the reduction in shell quality results from altered Vit. D metabolism, so that these birds are unable to respond properly to an inadequate Ca supply in the diet. Older birds also have a decreased ability to absorb Ca from the intestine and to mobilise skeletal Ca (Solomon, 1987 and Tullett, 1987).

Belyavin (pers. comm.) suggested that in terms of efficiency of conversion of dietary Ca to shell Ca, the bird becomes less efficient as the daily Ca intake increases. As a relatively high level of Ca was used in this experiment (3.8 %), virginiamycin could have improved the absorption efficiency of the birds at the end of lay.

Virginiamycin has been shown to reduce the microbial burden in the gut and provide an increased surface area for absorption in birds and pigs (Solomon and Tullett, 1989 and Solomon *et al.*, 1991). Likewise, as the growth rate of germ-free chicks is faster than that of their conventional counterparts (Coates *et al.*, 1963) and since there is a close correlation between growth rate and Ca uptake (Adams and Norman, 1970), the germ-free chick's requirement for Ca might be expected to be greater, as well as its uptake of Ca and its incorporation into bone (Edwards and Boyd, 1963). It is feasible that growth promoters make the bird more prepared for earlier laying, and this could establish an improvement in the general well-being and quality of resources that will reflect in a slight compensation of the negative effects caused by increasing age.

It has to be taken into consideration that the experimental situation is highly controlled with respect to husbandry, and the magnitude of the animal's response to antibiotics is, according to Hays (1986), smaller in the experimental environment than in commercial production unit environments, probably because: a) animals are selected for uniformity and any non-thriving or unhealthy animals are usually not used. These animals will usually give the greatest response to antibiotics, b) the environment is less conducive to stress conditions, in comparison with commercial operations, c) sanitation is usually better, in that buildings are usually emptied, cleaned and disinfected between experiments and d) ration balancing and feeding procedures are generally more precise and closely monitored. As in the commercial structure such control is very difficult to maintain, it may be that only under these field conditions can the true impact of the growth promoter be estimated.

The claim by Harms *et al.* (1990) that the majority of the improvement in eggshell quality following virginiamycin treatment could be caused by a reduction in egg weight were not substantiated by these results, as the eggs from the experimental groups were shown to have a mean of 2 grams more than the controls.

The relationship between Ca absorption and antibiotic action in the gut is intricate, according to Ewing (1963). In the presence of a diet containing a high Ca content, Taylor (1972) observed depressed food consumption. In the present experiments, if this theory is correct, then both the controls and the experimental birds experienced a decrease in Ca uptake. However, the experimental birds were also ingesting the growth promoter, which is acknowledged to increase Ca absorption in the gut. The effect was most marked at the end of lay when shell quality characteristically declines.

In these days of increasing public awareness, growth promoters receive adverse publicity. Branen (1983) suggested that if the benefits of the use of antimicrobials significantly outweigh the risks, then the antimicrobial should be permitted. This view is in contradistinction to that voiced by Coates (1962). The latter author maintained that antibiotics should only be used as a last resort. This thesis was not designed as a sales pitch for growth promoters, but in carrying out the experiments, some interesting observations have been made on the influence of virginiamycin on shell quality. It remains to be seen whether the risk of the use of the latter is greater than the risk of *Salmonella* contamination.

	Overall	Beginning	Middle	(B x M)	End	(B x E)	(M x E)
CONFLUENCE	NS	3.93 ± 1.39	3.87 ± 1.17	(NS)	4.20 ± 1.42	(NS)	(NS)
CAPS	***	1.37 ± 1.07	2.90 ± 1.67	(***)	5.63 ± 2.08	(***)	(***)
EARLY FUSION	**	1.77 ± 0.63	2.37 ± 0.96	(**)	2.47 ± 1.07	(**)	(NS)
LATE FUSION	**	3.23 ± 1.76	3.27 ± 1.87	(NS)	4.70 ± 1.51	(***)	(**)
MAMM. ALIGNMENT	**	1.63 ± 1.03	2.53 ± 1.36	(**)	2.63 ± 1.35	(**)	(NS)
TYPE B's	*	1.70 ± 1.21	2.53 ± 1.87	(*)	2.63 ± 1.56	(*)	(NS)
PITTING	*	1.40 ± 1.22	1.67 ± 1.52	(NS)	2.53 ± 2.08	(*)	(NS)
ARAGONITE	**	1.60 ± 0.50	1.20 ± 0.41	(**)	1.97 ± 1.30	(NS)	(**)
TYPE A's	***	1.20 ± 0.41	1.57 ± 0.50	(**)	1.83 ± 0.38	(***)	(*)
CUBICS	**	1.07 ± 0.25	1.30 ± 0.47	(*)	1.40 ± 0.50	(**)	(NS)
CUFFING	***	4.97 ± 0.18	4.97 ± 0.18	(NS)	4.63 ± 0.49	(***)	(***)
CHANGED MEMBR.	***	1.00 ± 0.00	1.00 ± 0.00	(NS)	3.13 ± 3.05	(***)	(***)
TOTAL SCORE	***	24.87 ± 5.61	29.23 ± 9.13	(*)	37.97 ± 7.62	(***)	(***)
MAMM. DENSITY	NS	71.99 ± 15.38	75.47 ± 15.00	(NS)	80.76 ± 15.09	(*)	(NS)
CUTICLE	NS	0.83 ± 0.46	0.90 ± 0.31	(NS)	1.07 ± 0.45	(NS)	(NS)
PORES (number)	***	60.13 ± 18.29	99.93 ± 81.61	(*)	146.17 ± 90.13	(***)	(*)
% PENETRATION	NS	7.70 ± 6.69	8.43 ± 6.80	(NS)	9.22 ± 6.55	(NS)	(NS)

Table 20: Structural scores (mean ± s.d.) for each individual characteristic of the cone layer in eggs from the control group, number of pores and percentage of penetration. Values correspond to beginning, middle, and end of lay.
*** = Very highly significant at a 0.1 % level ($P < 0.001$) ** = Highly significant at a 1 % level ($P < 0.01$)
* = Significant at a 5 % level ($P < 0.05$) NS = Not Significant ($P > 0.05$)

	Overall	Beginning	Middle	(B x M)	End	(B x E)	(M x E)
CONFLUENCE	***	3.73 ± 1.28	5.73 ± 0.83	(***)	3.33 ± 1.65	(NS)	(***)
CAPS	***	1.53 ± 0.90	2.40 ± 1.38	(**)	3.83 ± 1.90	(***)	(**)
EARLY FUSION	NS	1.83 ± 0.87	2.07 ± 0.74	(NS)	1.93 ± 0.83	(NS)	(NS)
LATE FUSION	**	2.47 ± 1.36	2.93 ± 1.48	(NS)	3.90 ± 1.73	(***)	(*)
MAMM. ALIGNMENT	NS	1.93 ± 1.31	2.20 ± 1.42	(NS)	2.00 ± 1.02	(NS)	(NS)
TYPE B's	***	1.37 ± 0.81	2.07 ± 1.41	(*)	2.47 ± 1.68	(**)	(NS)
PITTING	NS	1.27 ± 1.01	1.93 ± 1.72	(NS)	1.67 ± 1.52	(NS)	(NS)
ARAGONITE	***	2.00 ± 0.00	1.20 ± 0.41	(***)	1.33 ± 0.80	(***)	(NS)
TYPE A's	*	1.33 ± 0.48	1.57 ± 0.50	(NS)	1.67 ± 0.48	(**)	(NS)
CUBICS	NS	1.17 ± 0.38	1.20 ± 0.41	(NS)	1.13 ± 0.35	(NS)	(NS)
CUFFING	***	4.80 ± 0.41	4.97 ± 0.18	(*)	4.23 ± 1.01	(**)	(***)
CHANGED MEMBR.	**	1.00 ± 0.00	1.00 ± 0.00	(NS)	2.00 ± 2.23	(*)	(*)
TOTAL SCORE	*	24.60 ± 5.44	29.30 ± 6.89	(**)	29.60 ± 8.51	(**)	(NS)
MAMM. DENSITY	***	63.43 ± 8.61	79.84 ± 16.06	(***)	87.84 ± 23.28	(***)	(NS)
CUTICLE	**	0.93 ± 0.25	0.70 ± 0.47	(*)	0.97 ± 0.32	(NS)	(*)
PORES (number)	*	63.00 ± 30.15	108.50 ± 89.04	(*)	115.30 ± 79.57	(**)	(NS)
% PENETRATION	*	7.61 ± 10.83	11.27 ± 7.54	(NS)	6.07 ± 5.29	(NS)	(**)

Table 21: Structural scores (mean ± s.d.) for each individual characteristic of the cone layer in eggs from the experimental group, number of pores and percentage of penetration. Values correspond to beginning, middle, and end of lay.
*** = Very highly significant at a 0.1 % level ($P < 0.001$) ** = Highly significant at a 1 % level ($P < 0.01$)
* = Significant at a 5 % level ($P < 0.05$) NS = Not Significant ($P > 0.05$)

	Overall (A x B)	Beginning of lay			Middle of lay			End of lay		
		Controls	Experimental	(Co x Ex)	Controls	Experimental	(Co x Ex)	Controls	Experimental	(Co x Ex)
CONFLUENCE	(NS)	3.93 ± 1.39	3.73 ± 1.28	(NS)	3.87 ± 1.17	5.73 ± 0.83	(***)	4.20 ± 1.42	3.33 ± 1.65	(*)
CAPS	(*)	1.37 ± 1.07	1.53 ± 0.90	(NS)	2.90 ± 1.67	2.40 ± 1.38	(NS)	5.63 ± 2.08	3.83 ± 1.90	(***)
EARLY FUSION	(NS)	1.77 ± 0.63	1.83 ± 0.87	(NS)	2.37 ± 0.96	2.07 ± 0.74	(NS)	2.47 ± 1.07	1.93 ± 0.83	(*)
LATE FUSION	(*)	3.23 ± 1.76	2.47 ± 1.36	(NS)	3.27 ± 1.87	2.93 ± 1.48	(NS)	4.70 ± 1.51	3.90 ± 1.73	(*)
MAMM. ALIGNMENT	(NS)	1.63 ± 1.03	1.93 ± 1.31	(NS)	2.53 ± 1.36	2.20 ± 1.42	(NS)	2.63 ± 1.35	2.00 ± 1.02	(*)
TYPE B's	(NS)	1.70 ± 1.21	1.37 ± 0.81	(NS)	2.53 ± 1.87	2.07 ± 1.41	(NS)	2.63 ± 1.56	2.47 ± 1.68	(NS)
PITTING	(NS)	1.40 ± 1.22	1.27 ± 1.01	(NS)	1.67 ± 1.52	1.93 ± 1.72	(NS)	2.53 ± 2.08	1.67 ± 1.52	(*)
ARAGONITE	(NS)	1.60 ± 0.50	2.00 ± 0.00	(***)	1.20 ± 0.41	1.20 ± 0.41	(NS)	1.97 ± 1.30	1.33 ± 0.80	(*)
TYPE A's	(NS)	1.20 ± 0.41	1.33 ± 0.48	(NS)	1.57 ± 0.50	1.57 ± 0.50	(NS)	1.83 ± 0.38	1.67 ± 0.48	(NS)
CUBICS	(NS)	1.07 ± 0.25	1.17 ± 0.38	(NS)	1.30 ± 0.47	1.20 ± 0.41	(NS)	1.40 ± 0.50	1.13 ± 0.35	(*)
CUFFING	(*)	4.97 ± 0.18	4.80 ± 0.41	(*)	4.97 ± 0.18	4.97 ± 0.18	(NS)	4.63 ± 0.49	4.23 ± 1.01	(NS)
CHANGED MEMBRANE	(NS)	1.00 ± 0.00	1.00 ± 0.00	(NS)	1.00 ± 0.00	1.00 ± 0.00	(NS)	3.13 ± 3.05	2.00 ± 2.23	(NS)
TOTAL SCORE	(*)	24.87 ± 5.61	24.60 ± 5.44	(NS)	29.23 ± 9.13	29.30 ± 6.89	(NS)	37.97 ± 7.62	29.60 ± 8.51	(***)
MAMMILLARY DENSITY	(NS)	71.99 ± 15.38	63.43 ± 8.61	(**)	75.47 ± 15.00	79.84 ± 16.06	(NS)	80.76 ± 15.09	87.84 ± 23.28	(NS)
CUTICLE	(NS)	0.83 ± 0.46	0.93 ± 0.25	(NS)	0.90 ± 0.31	0.70 ± 0.47	(NS)	1.07 ± 0.45	0.97 ± 0.32	(NS)
PORES (number)	(NS)	60.13 ± 18.29	63.00 ± 30.15	(NS)	99.93 ± 81.61	108.50 ± 89.04	(NS)	146.17 ± 90.13	115.30 ± 79.57	(NS)
% PENETRATION	(NS)	7.70 ± 6.69	7.61 ± 10.83	(NS)	8.43 ± 6.80	11.27 ± 7.54	(NS)	9.22 ± 6.55	6.07 ± 5.29	(*)
EGG WEIGHT	(NS)	49.07 ± 5.31	50.49 ± 2.92	(NS)	63.10 ± 4.88	65.41 ± 5.34	(NS)	68.87 ± 5.72	70.62 ± 4.57	(NS)

Table 22: Structural scores (mean ± s.d.) for each individual characteristic of the cone layer in eggs from control and experimental groups and number of pores and percentage of penetration.
*** = Very highly significant at a 0.1 % level ($P < 0.001$) ** = Highly significant at a 1 % level ($P < 0.01$) * = Significant at a 5 % level ($P < 0.05$) NS = Not Significant ($P > 0.05$)

	CONTROLS				EXPERIMENTAL			
% Penetration against...	Overall	Beginning	Middle	End	Overall	Beginning	Middle	End
CONFLUENCE	0.45 [***]	0.49 [**]	0.49 [**]	0.38 [*]	0.51 [***]	0.66 [***]	0.19 [NS]	0.53 [**]
CAPS	0.52 [***]	0.59 [***]	0.72 [***]	0.74 [***]	0.45 [***]	0.84 [***]	0.66 [***]	0.75 [***]
EARLY FUSION	0.49 [***]	-0.05 [NS]	0.63 [***]	0.71 [***]	0.60 [***]	0.61 [***]	0.55 [**]	0.76 [***]
LATE FUSION	0.67 [***]	0.67 [***]	0.76 [***]	0.65 [***]	0.49 [***]	0.69 [***]	0.48 [**]	0.78 [***]
MAMM. ALIGNMENT	0.74 [***]	0.70 [***]	0.80 [***]	0.79 [***]	0.56 [***]	0.47 [**]	0.65 [***]	0.72 [***]
TYPE B's	0.42 [***]	0.21 [NS]	0.77 [***]	0.15 [NS]	0.33 [**]	0.47 [**]	0.42 [*]	0.43 [*]
PITTING	0.33 [**]	0.37 [*]	0.53 [**]	0.14 [NS]	0.26 [*]	0.20 [NS]	0.51 [**]	-0.05 [NS]
ARAGONITE	0.18 [NS]	0.44 [*]	0.55 [**]	-0.01 [NS]	0.04 [NS]	0.01 [NS]	0.71 [***]	-0.17 [NS]
TYPE A's	0.50 [***]	0.56 [***]	0.62 [***]	0.40 [*]	0.42 [***]	0.56 [***]	0.39 [*]	0.41 [*]
CUBICS	0.39 [***]	0.50 [**]	0.44 [*]	0.29 [NS]	0.34 [***]	0.15 [NS]	0.71 [***]	0.18 [NS]
CUFFING	0.16 [NS]	0.08 [NS]	0.23 [NS]	0.33 [NS]	0.12 [NS]	0.26 [NS]	0.13 [NS]	0.57 [***]
CHANGED MEMBRANE	0.02 [NS]	0.01 [NS]	0.02 [NS]	-0.04 [NS]	0.07 [NS]	0.02 [NS]	0.03 [NS]	0.41 [*]
TOTAL SCORE	0.69 [***]	0.78 [***]	0.86 [***]	0.77 [***]	0.70 [***]	0.84 [***]	0.79 [***]	0.85 [***]
MAMMILLARY DENSITY	0.21 [*]	-0.08 [NS]	0.34 [NS]	0.32 [NS]	0.13 [NS]	0.22 [NS]	0.10 [NS]	0.32 [NS]
PORES (number)	-0.04 [NS]	0.39 [*]	-0.20 [NS]	-0.11 [NS]	-0.08 [NS]	-0.01 [NS]	-0.07 [NS]	-0.28 [NS]
CUTICLE	-0.07 [NS]	-0.07 [NS]	-0.22 [NS]	-0.02 [NS]	-0.06 [NS]	0.04 [NS]	0.10 [NS]	-0.14 [NS]

Table 23: Correlation values and t-test significance results between % of penetration and each structural characteristic score and number of pores, for the three sampling periods combined (overall), and for beginning, middle and end of lay for the control and experimental groups in the virginiamycin experiment.

[***] = Very highly significant at a 0.1 % level ($P < 0.001$)
[**] = Highly significant at a 1 % level ($P < 0.01$)
[*] = Significant at a 5 % level ($P < 0.05$)
[NS] = Not Significant ($P > 0.05$)

Cuticle assessment score = 2 (absent) / 1 (patchy) / 0 (complete)

Beginning of lay

CONTROLS: Caps (very highly significant (***)).

EXPERIMENTAL: Caps (very highly significant (***)).

Middle of lay

CONTROLS: None significant.

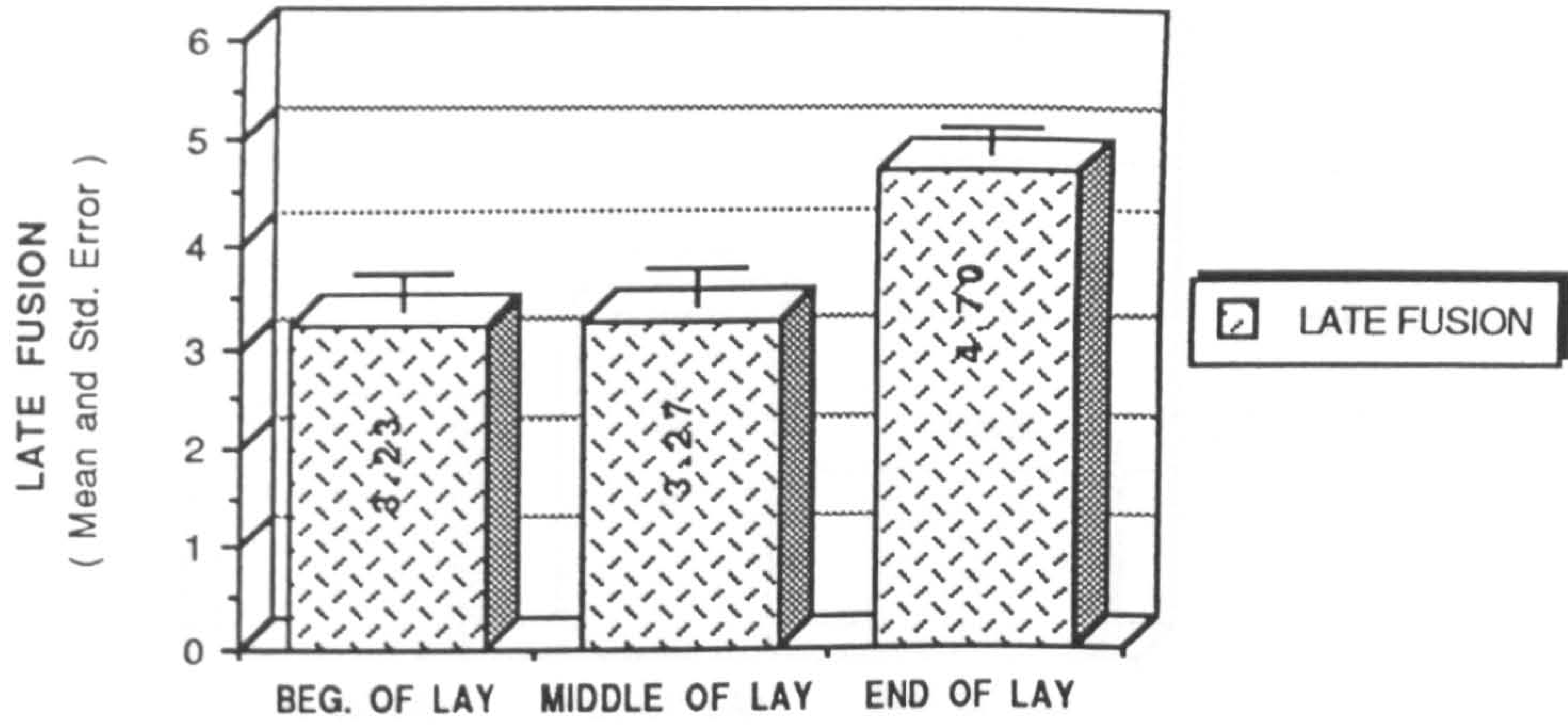
EXPERIMENTAL: Caps, Mammillary Alignment, Aragonite and Cuffing (all significant (*)).

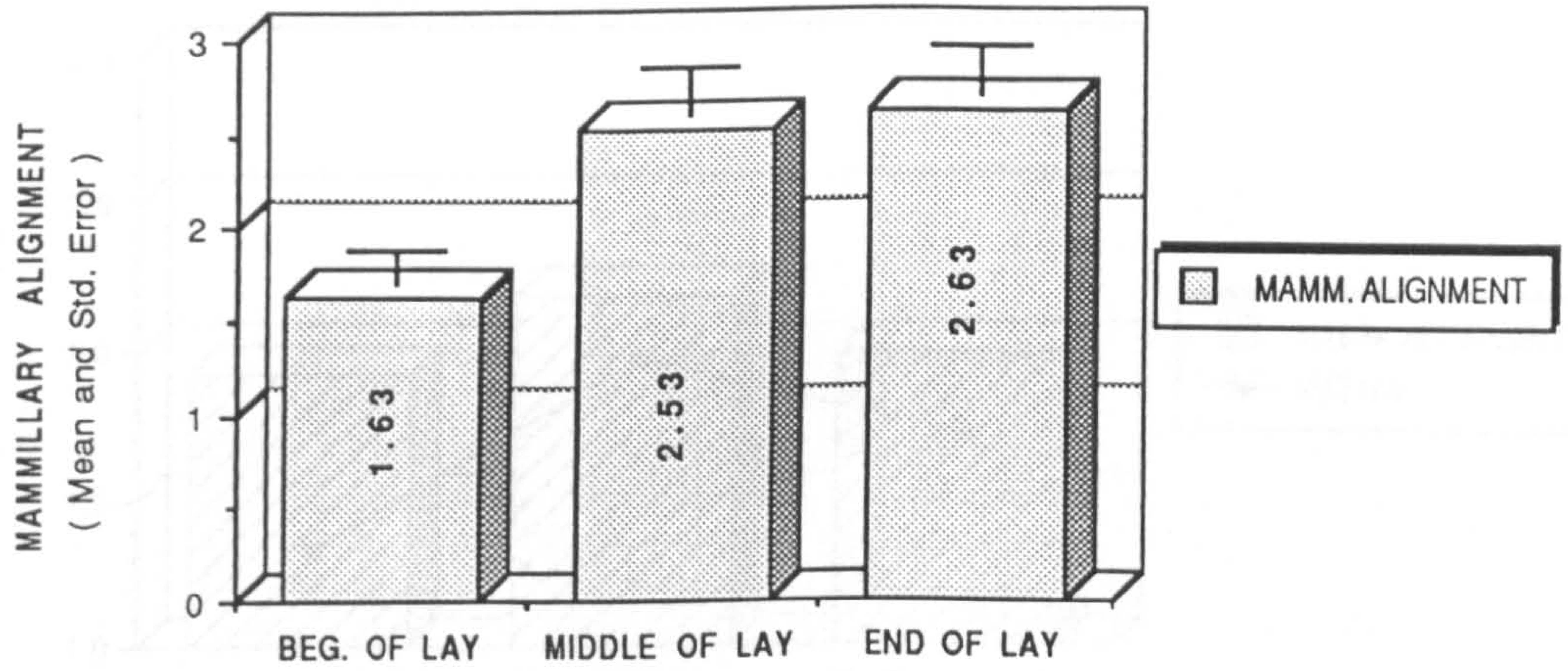
End of lay

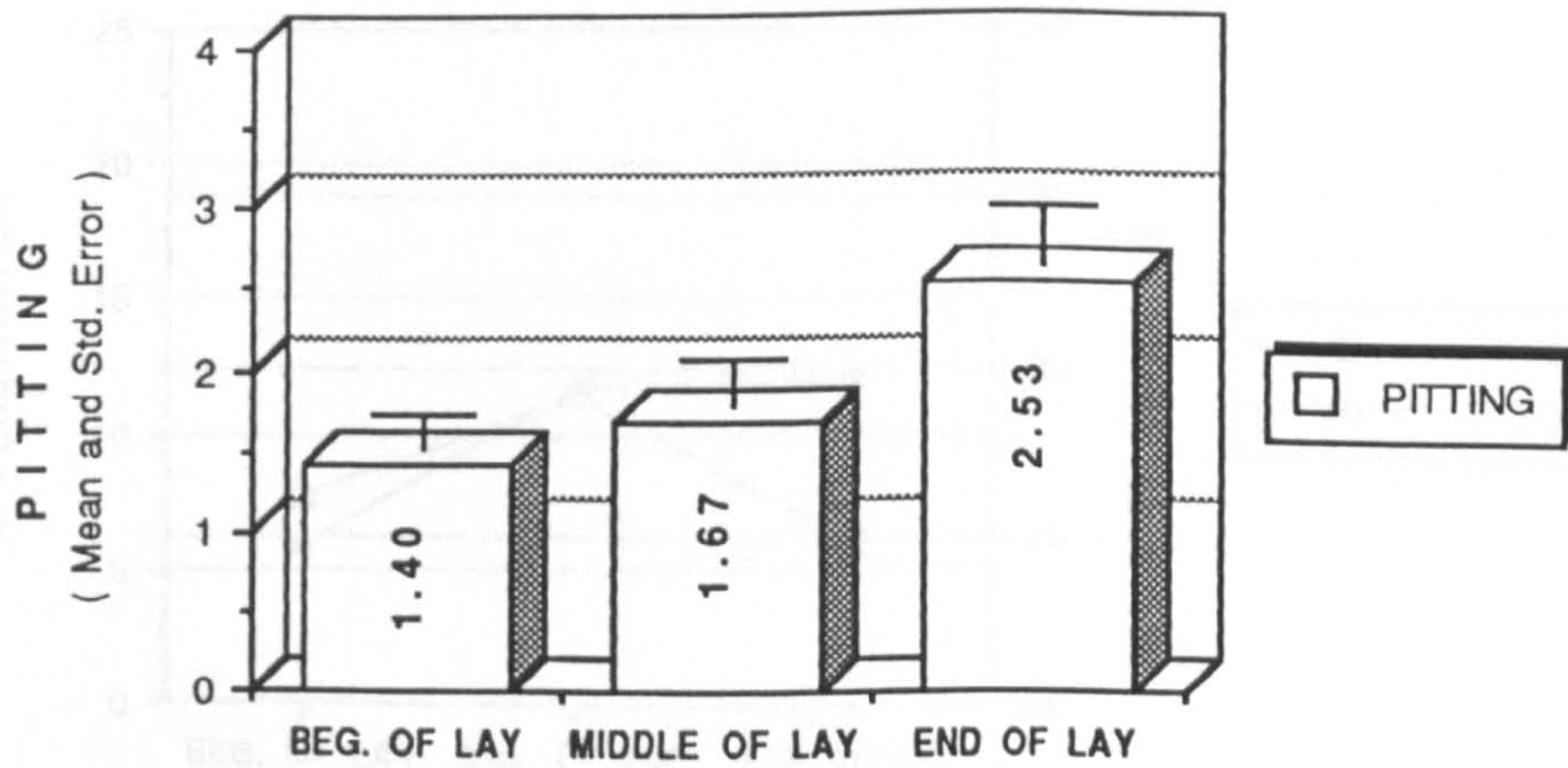
CONTROLS: None significant.

EXPERIMENTAL: Caps, Early Fusion, Type B's, Cuffing and Changed Membrane (all significant (*)).

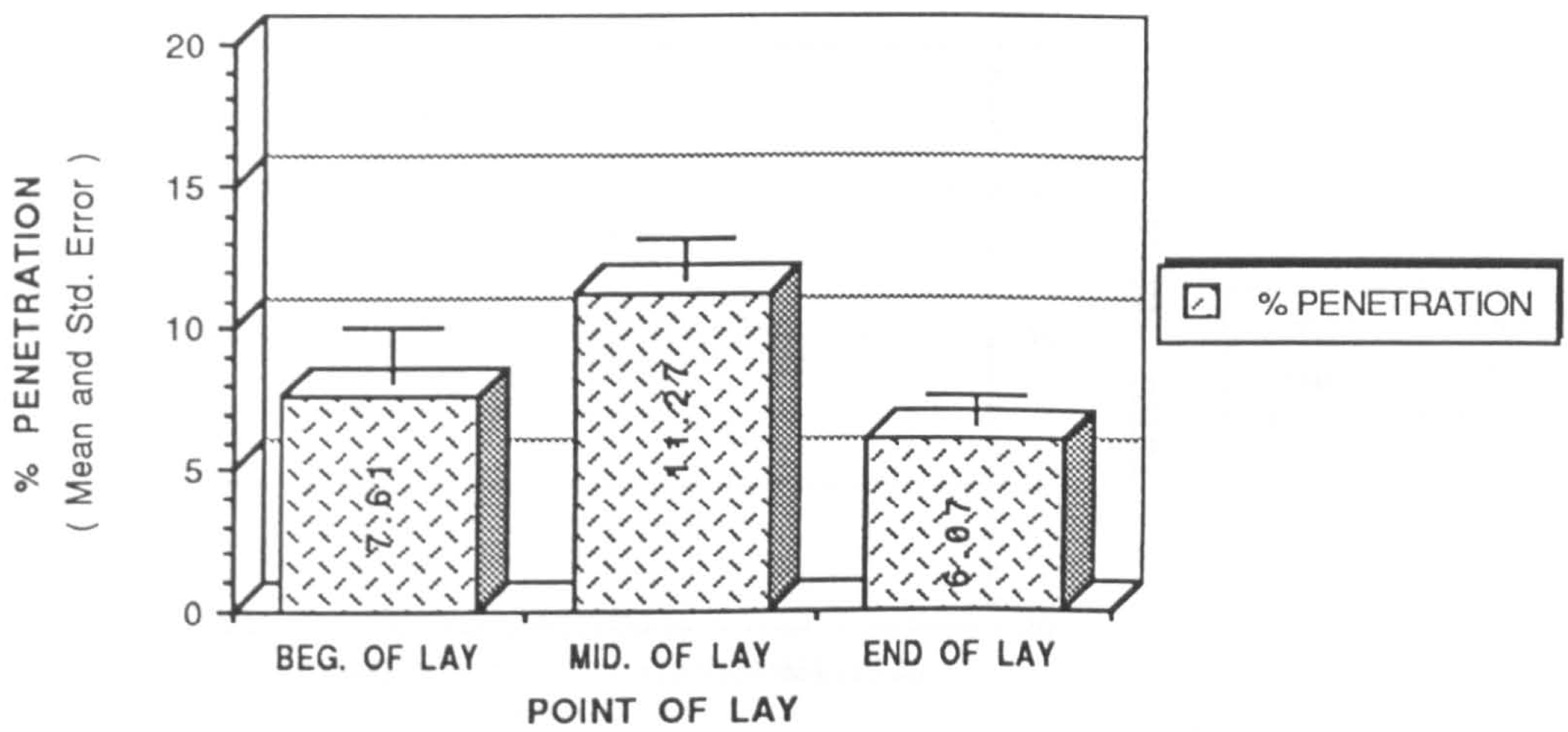
Table 24: Extract of the coefficient of determination's matrix, showing variables which were significantly indicated to be determining variation in the dependent variable (% penetration).


GRAPH 31: LATE FUSION IN THE CONTROL GROUP
LATE FUSION
(Mean and Std. Error)
0
1
2
3
4
5
6
3.23
3.27
4.70
LATE FUSION
BEG. OF LAY
MIDDLE OF LAY
END OF LAY

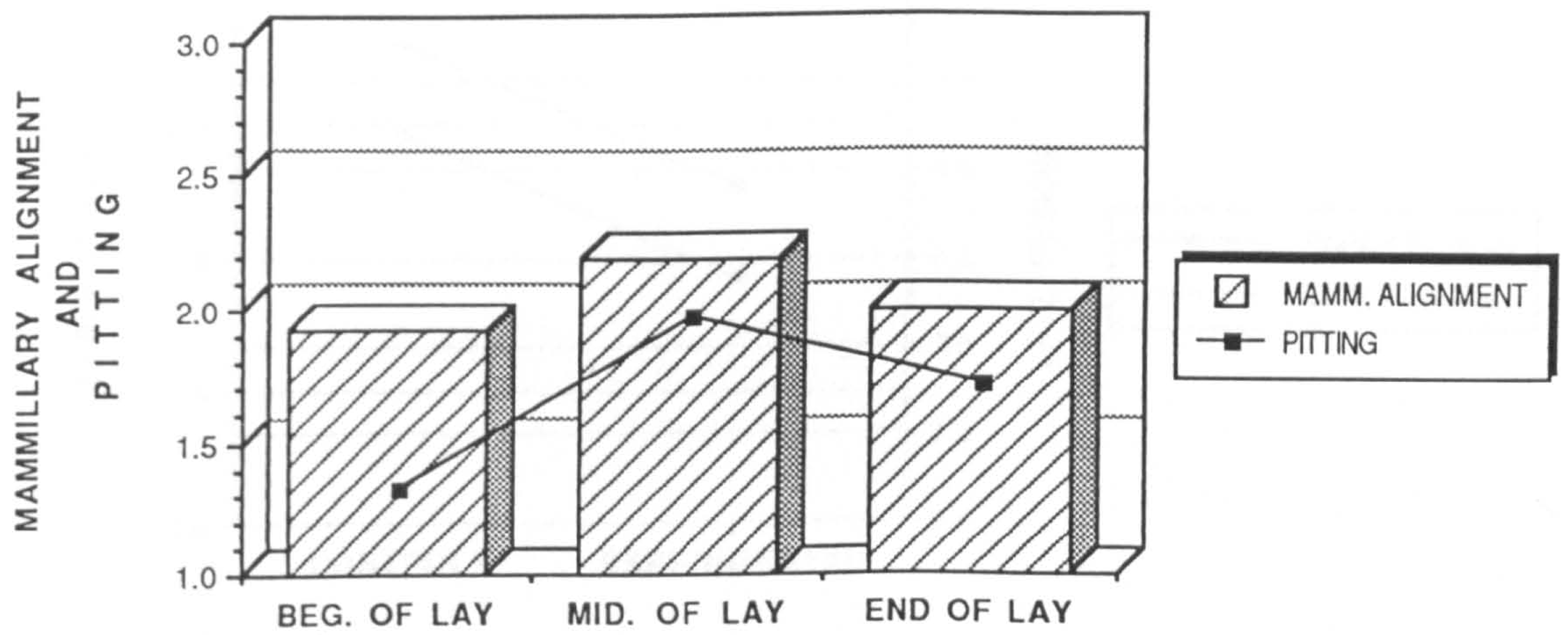

GRAPH 32: MAMM. ALIGNMENT IN THE CONTROL GROUP
MAMMILLARY ALIGNMENT
(Mean and Std. Error)
0
1
2
3
1.63
2.53
2.63
MAMM. ALIGNMENT
BEG. OF LAY
MIDDLE OF LAY
END OF LAY


GRAPH 33: PITTING IN THE CONTROL GROUP
P I T T I N G
(Mean and Std. Error)
0
1
2
3
4
1.40
1.67
2.53
PITTING
BEG. OF LAY
MIDDLE OF LAY
END OF LAY

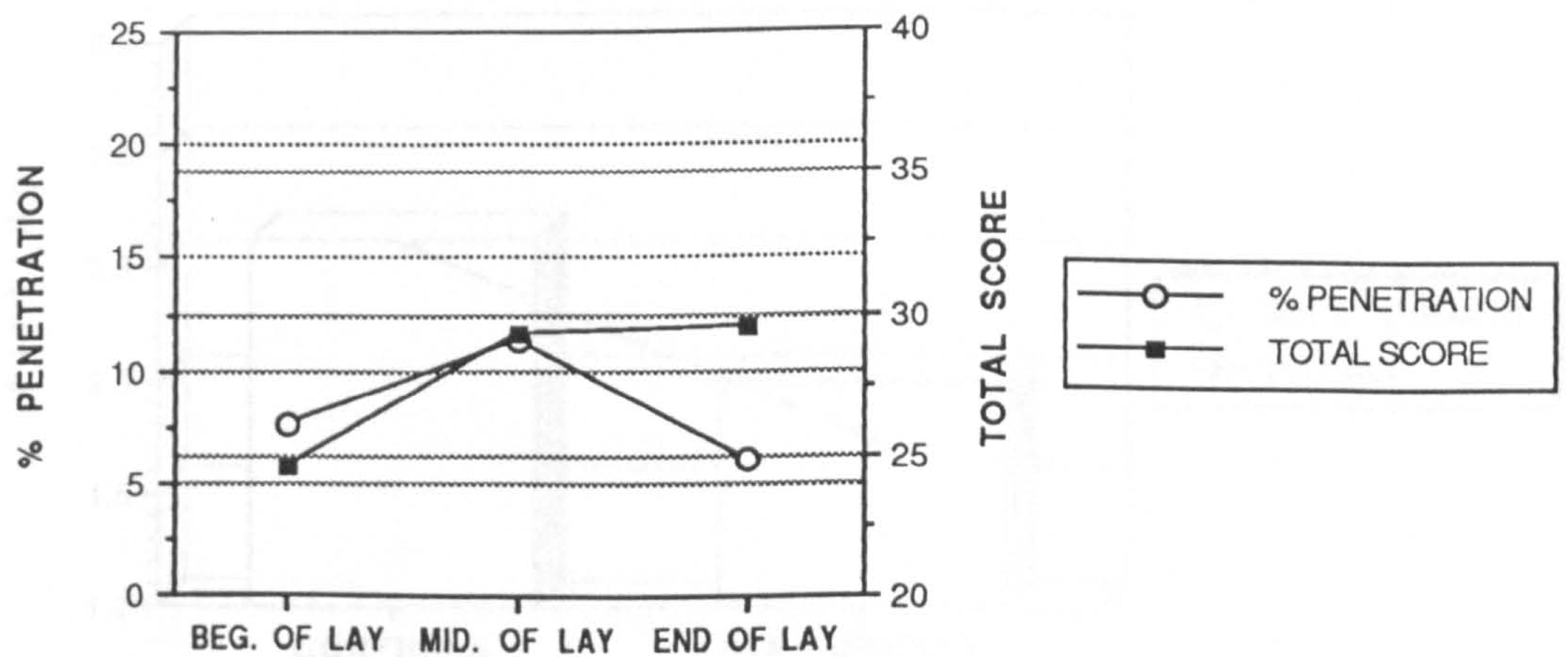
GRAPH 34: % OF PENETRATION IN THE EXPERIMENTAL GROUP



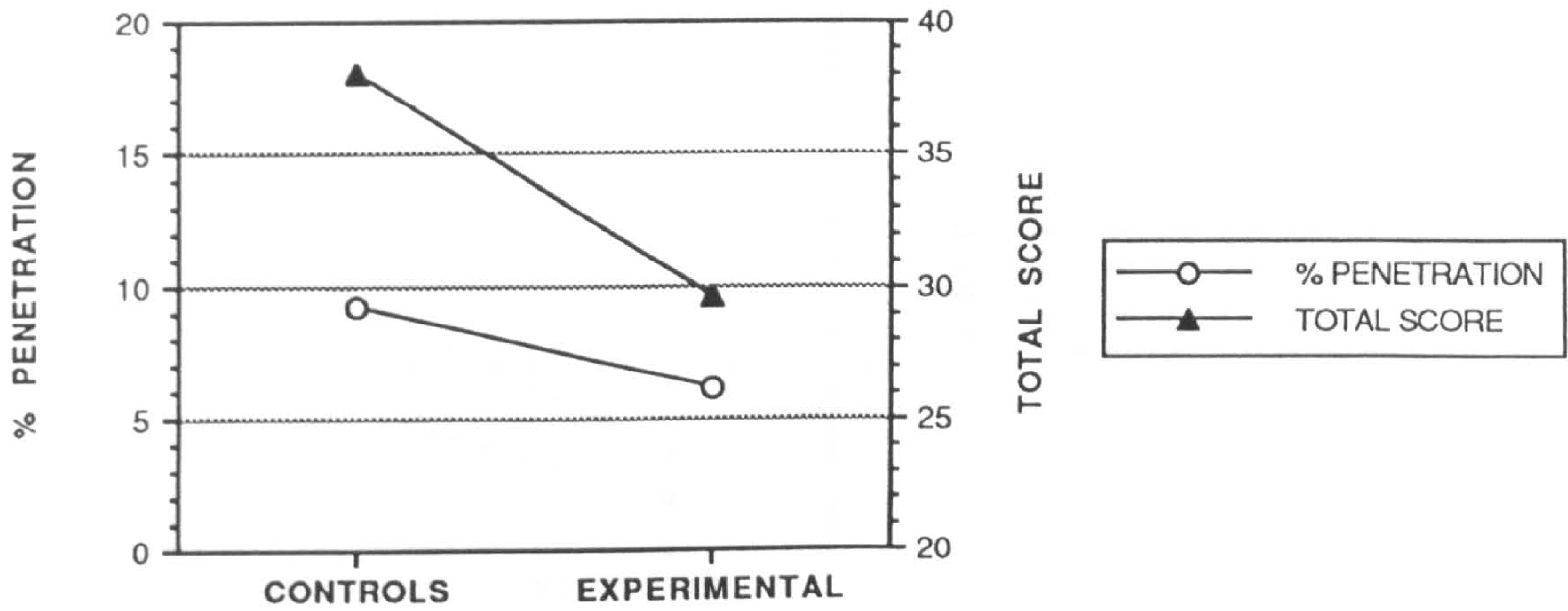
GRAPH 35: MAMM. ALIGNMENT AND PITTING IN THE EXPERIMENTAL GROUP



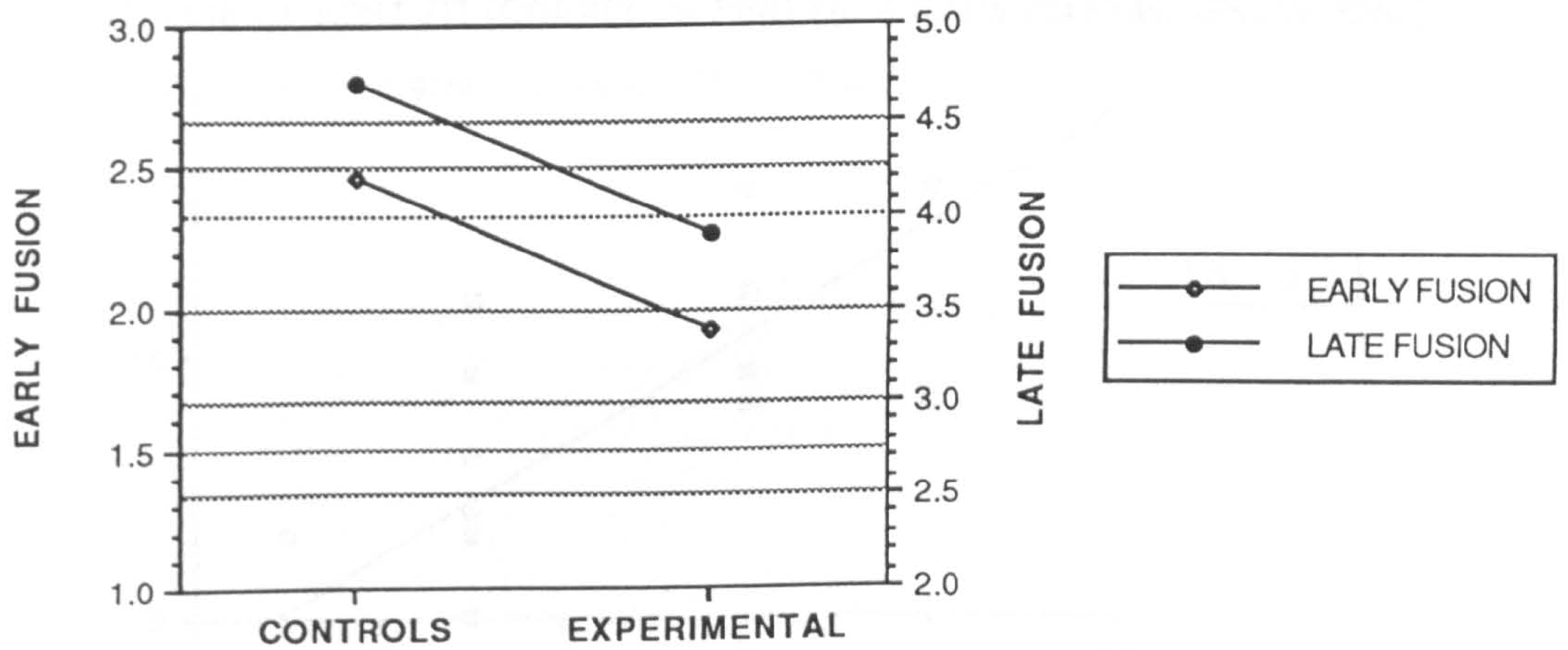
GRAPH 36: TOTAL SCORE X % PENETRATION IN THE EXPERIMENTAL GROUP



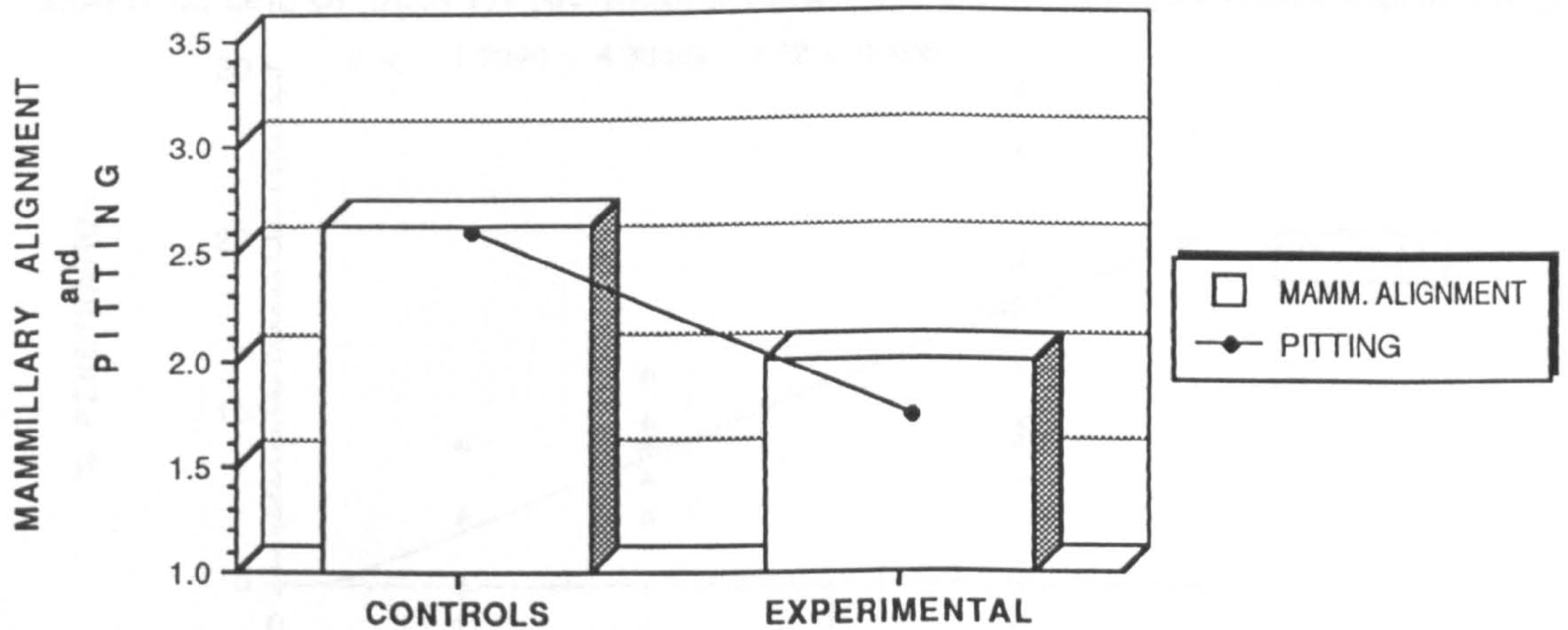
GRAPH 37: TOTAL SCORE X % PENETRATION AT THE END OF LAY IN BOTH GROUPS



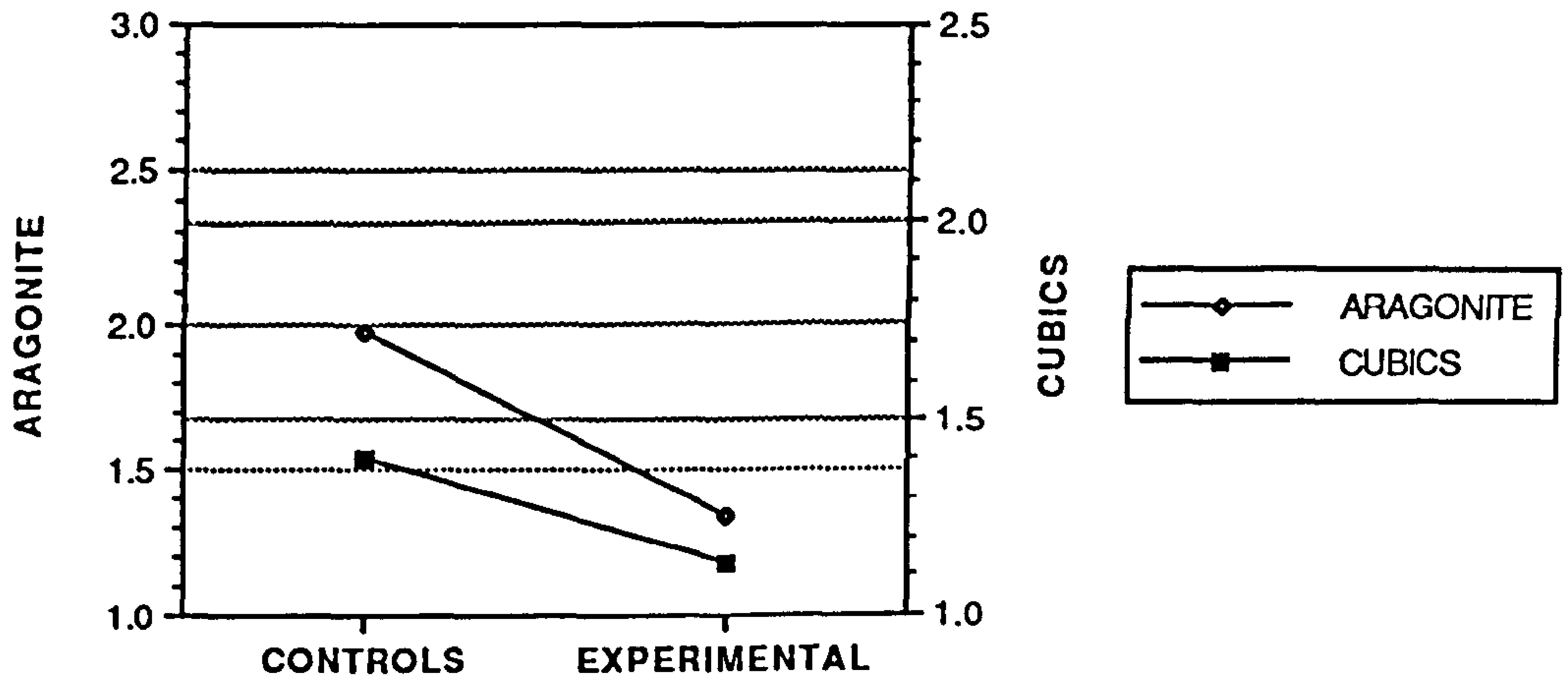
GRAPH 38: EARLY AND LATE FUSIONS AT THE END OF LAY IN BOTH GROUPS



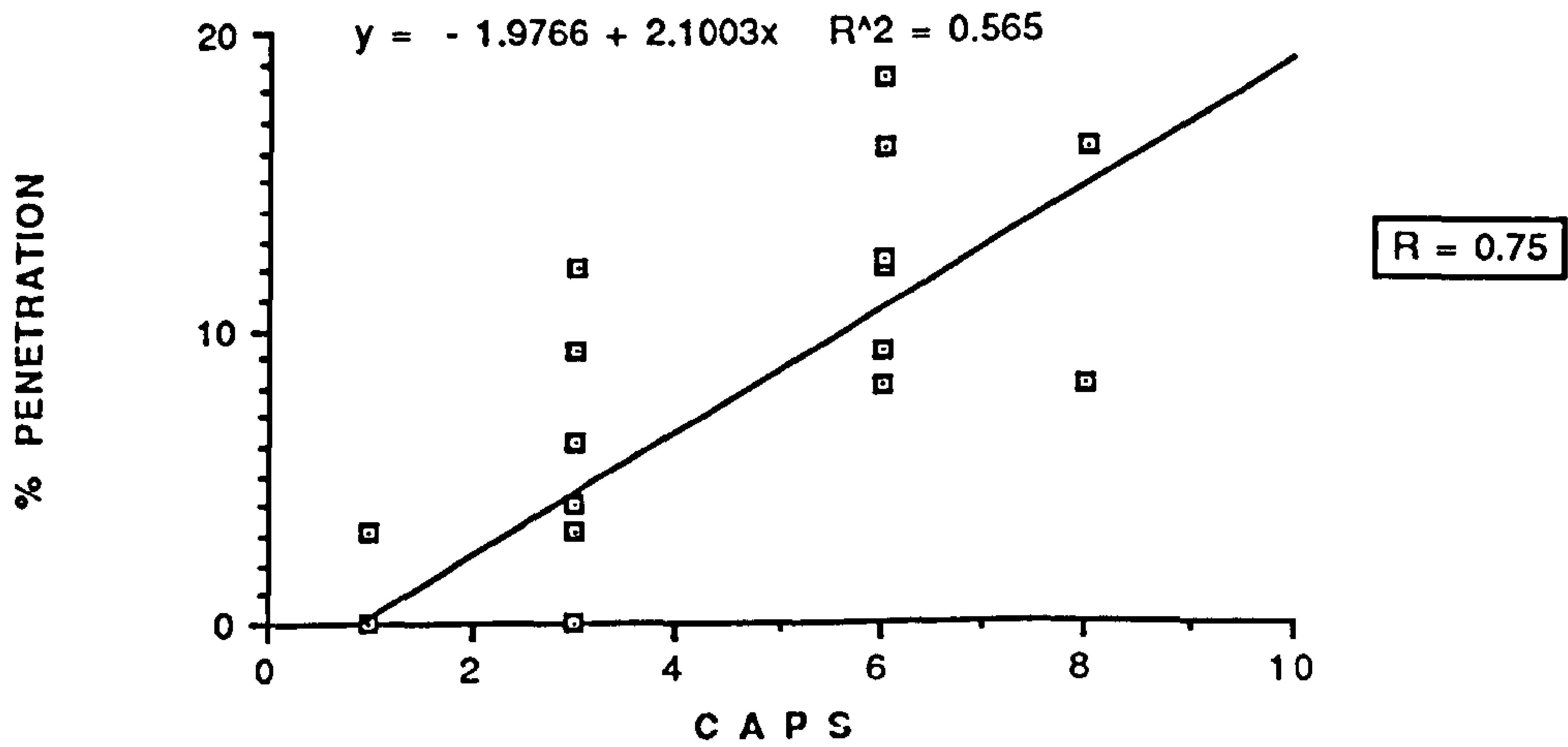
GRAPH 39: MAMM. ALIGN. AND PITTING AT THE END OF LAY IN BOTH GROUPS



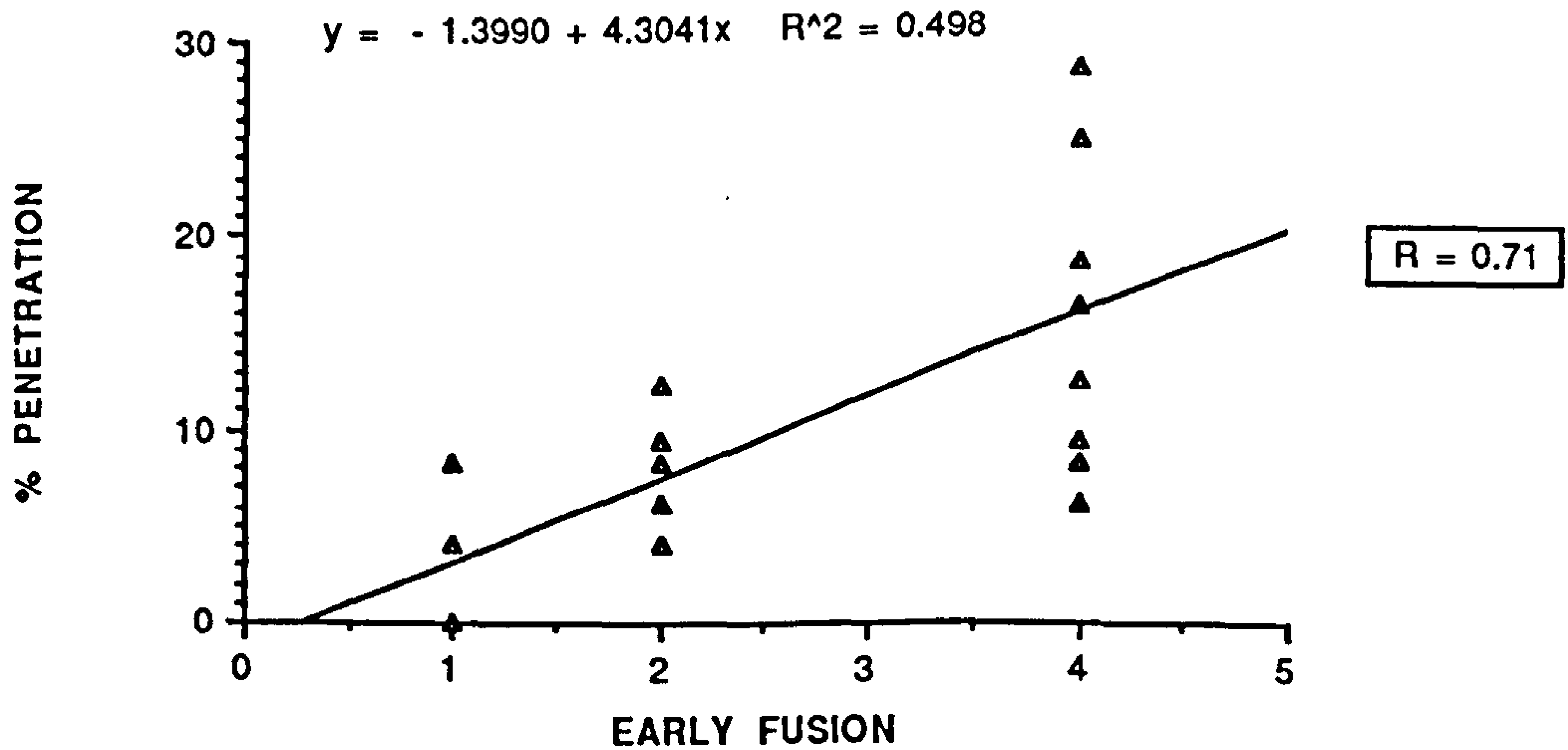
GRAPH 40: ARAGONITE AND CUBICS AT THE END OF LAY IN BOTH GROUPS



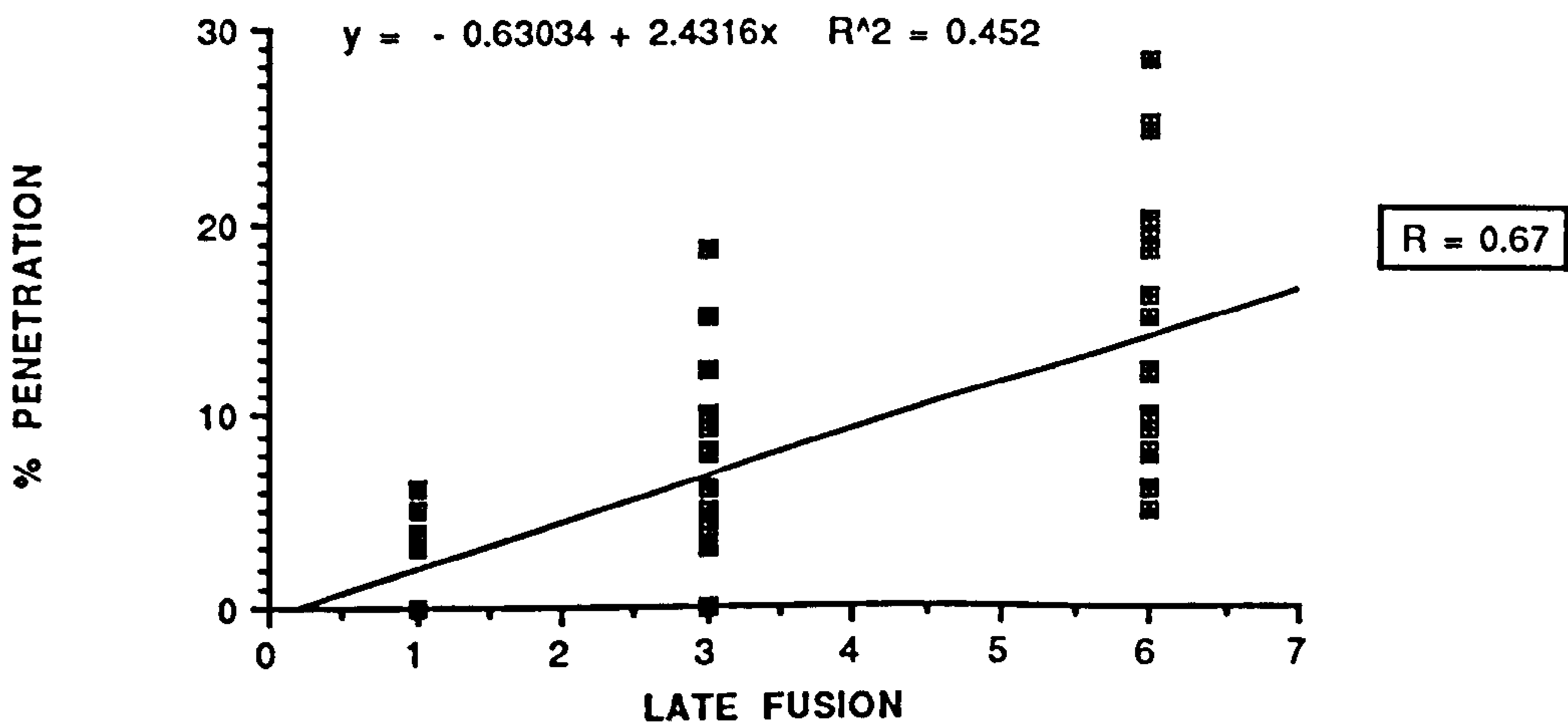
GRAPH 41: LINE OF BEST FIT (CORREL. % PENETR. X CAPS (EXPTAL. GROUP/END))



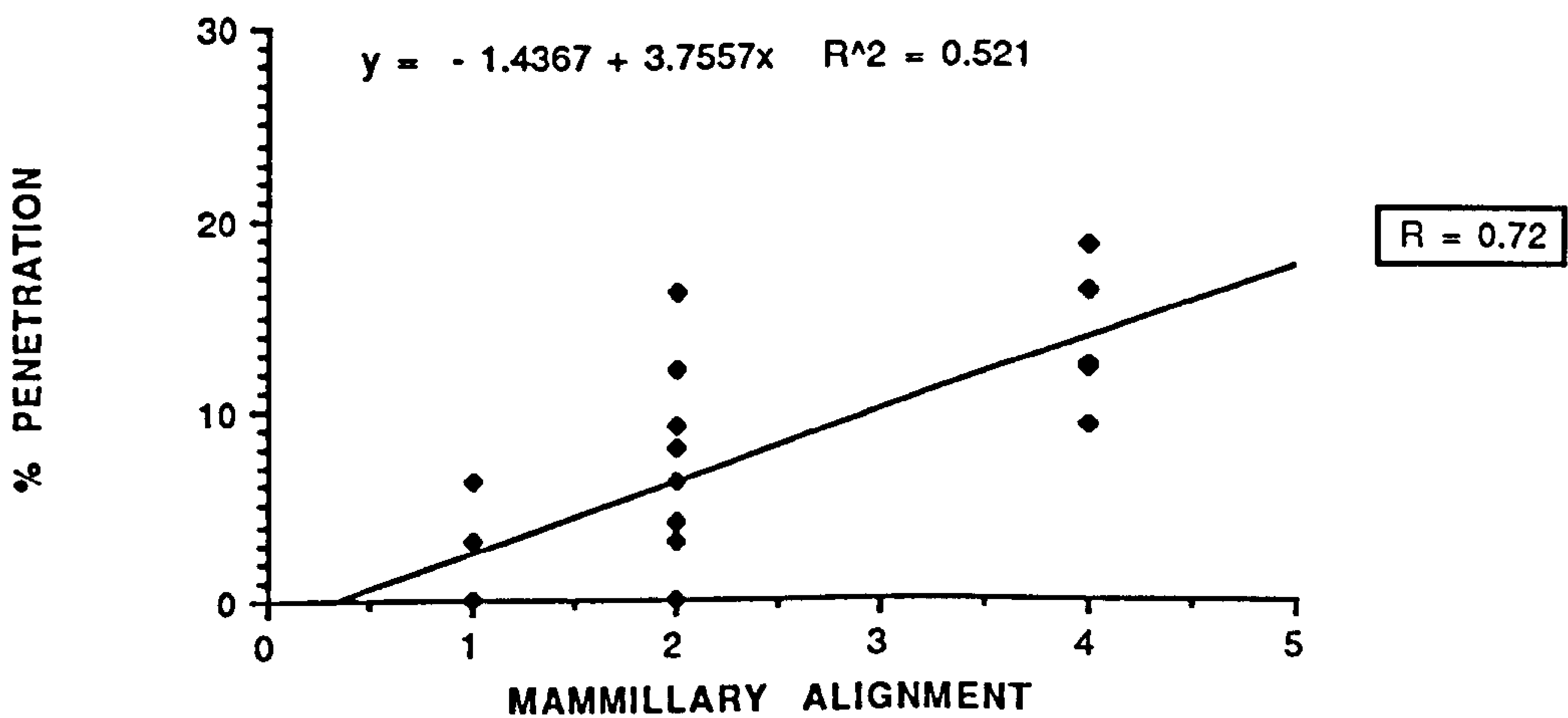
GRAPH 42: LINE OF BEST FIT (CORREL. % PENETR. X EARLY FUS. (CONTROL GROUP/END))



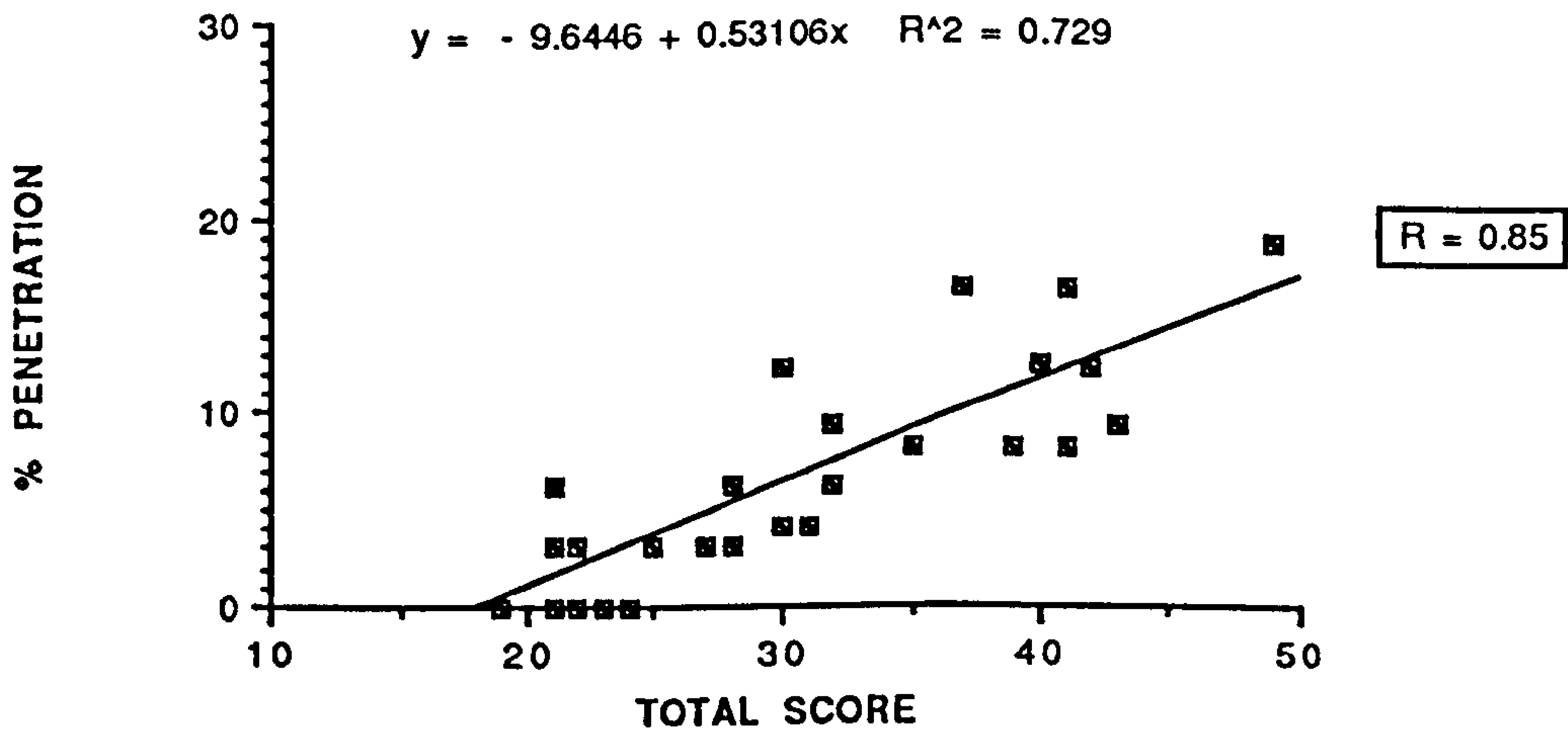
GRAPH 43: LINE OF BEST FIT (CORR. % PENET. X LATE FUS. (CONTROL GROUP/TOTAL))



GRAPH 44: LINE OF BEST FIT (CORR. % PENET. X MAMM. ALIGN.(EXPTAL.GROUP/END))



GRAPH 45: LINE OF BEST FIT (CORR. %PENET. X TOTAL SCORE (EXPTAL.GROUP/END))



CHAPTER 7

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7. GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

This thesis has served to highlight and corroborate the observations made by a number of workers; thus the cuticular layer declines in quality and volume with age. Solomon and Watt (1990) drew attention to the influence of environmental stress on the elaboration of this protein /carbohydrate complex. The paucity of covering over the eggs in this study may well be an indication of a stress response. Pore numbers show a significant increase with bird age, nevertheless there is a poor correlation between porosity and bacterial penetration, a feature previously agreed upon by Kraft *et al.* (1958b), Hartung and Stadelman (1963), Reinke and Baker (1966) and Sparks (1985). Patent pores do of course represent a portal of entry, but they represent only a fraction of the total number. Bacteria may penetrate patent pores, but whether they will ultimately reach the egg contents is a matter of debate.

The true shell, in terms of its total thickness, should in theory create an effective barrier with the attached shell membranes serving as the ultimate physical deterrent. The continuous barrier observed in the literature is rarely observed at ultrastructural level, pitting of the membranes is the norm, yet despite this impairment they did effect some control under the experimental conditions described, whether in the face of a high challenge this would be as effective has still to be answered, indeed are they but a staging post en route to the egg contents?

Bain (1990) succeeded in correlating many of the structural modifications of the shell with performance under load and in her dissertation highlighted those mammillary layer variations which increase /decrease shell quality, *i.e.* not all the variations at this level are detrimental. It is of interest and perhaps not surprising that many of the defects which the former regards as detrimental are now, through the results of this thesis, correlated with

bacterial penetration. As the bird ages, shell quality declines (Solomon, 1987 and Tullett, 1987) and the egg becomes more susceptible to breakage and the ingress of microorganisms (Lifshitz, 1963; Bruce and Johnson, 1978 and Bruce and Drysdale, 1991).

In 1991, the latter suggested that the increased incidence of *Enterobacteriaceae* in older birds was either the result of increased oviducal contamination, the environment becoming more unacceptable, or the egg *per se* being less capable of resisting the hazards of the environment. They concluded that the environment was clearly implicated and cited nest laying as a causative agent. These age effects have been further underlined by the work of Humphrey *et al.* (1991c), who observed that older SPF birds (52 w.o.) showed a severe response to challenge with *S. enteritidis* PT 4, with prolonged faecal carriage, contrasting with younger SPF birds (20 w.o.), which appeared unaffected by the organism. The authors proposed that the older birds were exhibiting immunodepression due to fatigue associated with intensive laying and Ca depletion. Such observations only serve to underline the need for greater care in the husbandry of older flocks.

While the environment exerts considerable influence on bird behaviour and shell quality, genetics too has a role to play. The results of this investigation support the latter and highlight the role played by specific structural traits in inhibiting bacterial transfer. While selection of these traits is desirable, they cannot be considered in isolation. Engineering an eggshell suitable for the table market in terms of its structure and resistance network may reduce the efficacy of the same shell as an embryonic chamber.

The debate over the battery system continues with pressure being exerted on the producer by the consumer, via the supermarkets to provide alternative housing. Tiered battery housing does not provide a ubiquitous environment. Temperature, lighting and dust levels vary from top to bottom and results are herein presented to suggest that birds housed on the bottom tier produce structurally superior eggs. Sparks (1991) demonstrated a

similar effect. When compared with eggs laid on Range, the Barn and the battery egg (irrespective of its provenance within the system) are structurally superior. The latter is in accordance with the works of Mohumed (1986), Solomon (1988a), Belyavin *et al.* (1991) and Bain *et al.* (1992). In designing a housing system, a fair balance between economic, welfare and health factors is often difficult to achieve.

The use of the growth promoter virginiamycin in the feed at 30 ppm appeared to improve the structural quality of the shell, particularly at the end of lay. Thus, those features classified as non-desirable and demonstrated by gold tagging to be correlated with the transfer of bacteria were significantly reduced. It is suggested that an improvement in nutrient absorption (and consequently a healthier bird) due to a reduced microbial intestinal burden is one of the main causes behind the observed improvement.

While the debate over the transovarian route of contamination will undoubtedly continue, the concept that the egg is extra-genitally contaminated by the microbial load present in the environment is more feasible. The moist, immature cuticle and the cooling of the egg after lay, sucking in any possible faecal material present on its surface no doubt provides the ideal mechanism by which an egg is invaded by bacteria (Barrow and Lovell, 1991).

Shivaprasad *et al.* (1990) however claimed that in their experiments shell contamination did not correlate well with the presence of the organism in the yolk or albumen, dismissing the shell as an important source of the organism for the interior of the egg. It should be pointed out that the authors were probably dealing with high quality eggshells, structurally capable of withstanding the challenge.

Although a rare occurrence (it has been estimated by Hunton (1991) as only 1 in 10,000), a contaminated egg, if mixed with other food or ingredients could cause food poisoning. Also, this contaminated product, if unproperly

processed, washed, stored, transported and commercialised, will certainly become a liability and a hazardous food.

St. Louis *et al.* (1988) raised another controversial issue, *i.e.* egg washing. These authors concluded that external contamination of Grade A eggs by *Salmonella* was not feasible, ignoring the fact that the washing process could in itself increase the vulnerability of the shell to bacterial transfer (Cranstoun, 1992). It is recommended that the water temperature should be 32 °C or higher (43 °C in the summer – USDA recommendation (Denton, 1991)), and at least 11 °C higher than that of the egg, to avoid bacteria being sucked through the shell (Haines, 1938; Haines and Moran, 1940 and Brant and Starr, 1962). The concentration of bacterial spoilage organisms in the wash water is critical, as also is the above mentioned temperature differential between the eggs and wash water; if it is more than 10 °C, it can increase the number of cracks due to the expansion of the contents of the egg (Stadelman, 1977 and Baker *et al.*, 1980 b).

Iron (Fe^{++}) is also known to encourage bacterial growth and contamination of the albumen (Tranter *et al.*, 1983), and can be retained by the shell membranes, apart from facilitating bacterial activity in general (Garibaldi and Bayne, 1962 and Sauter and Petersen, 1969).

Temperature is restrictive on *Salmonella* penetration and it has been recommended by Stokes *et al.* (1956), Ayres and Taylor (1956) and Simmons *et al.* (1970) that eggs must be stored at a temperature below 10 °C (4 °C, according to Clay and Board (1991)) at which *Salmonella* can neither penetrate nor grow. As maximum bacterial activity was claimed to occur at 25 °C, according to Wolk *et al.* (1950), and as this is the approximate temperature at which eggs are held in retail outlets, this should be changed, with eggs receiving the same treatment as any other perishable commodities and as such being kept under refrigeration, at least below 10 °C, with relative humidity at 70-80 % optimum (not below 60 % nor above 85 %) (Stadelman,

1977). Any increase in temperature should be avoided, even for a short period, because it has been shown by Stokes *et al.* (1956) that a period of storage in which the temperature is allowed to go up to 35 °C can lead to a rapid multiplication of *Salmonella*, even overcoming any previous repressed growth due to storage at low temperatures (as low as 1 °C). Three to four gatherings of eggs a day would also help in keeping eggs as clean as possible.

A new bill signed in the U.S.A. (proposed by the egg industry) will, twelve months after being regulated, require that table eggs be kept at 45 °F (around 7 °C) during packing, transporting and handling (Anonymous, 1992b). This is in agreement with the recommendations of Ayres and Taylor (1956), Stokes *et al.* (1956), Taylor (1956), Simmons *et al.* (1970) and Clay and Board (1991). Storage at higher temperatures would otherwise boost the chemical activities which will cause bacteria to multiply rapidly, ultimately causing the deterioration of the egg contents. *S. enteritidis* PT 4 was shown by Humphrey *et al.* (1990) to be more resistant to heat than some other common egg-associated *Salmonellae*, although storage of cells at 4 °C or 8 °C made it more heat sensitive (Humphrey, 1990).

The position in which the egg is stored has been reported, by Board (1964) and Brown *et al.* (1970), to influence spoilage, chiefly through the yolk being moved from its original central position and being allowed to contact the shell membranes, and therefore serving as a culture broth to any microorganism present. In the same way, as the egg ages, its chalazae may disrupt and the yolk cease to occupy its mid position, therefore putting it in direct contact with the shell membranes.

According to Stokes *et al.* (1956) and Williams *et al.* (1968), the relative susceptibility of the shell surface was more important than the number of microorganisms exposed, reinforcing the importance of eggshell quality in restraining bacterial penetration. However, Rosser (1942), Hartung and

Stadelman (1963), Brown *et al.* (1966), Board and Fuller (1974) disagree, stressing that the greater the challenge, the heavier the contamination. The present results support both opinions, *i.e.* shell structure and the level of challenge are both determining factors in egg spoilage.

Eggs infected with *Salmonella* are usually odourless and, as such, could easily pass inspection. In the bulk processing of egg contents, therefore, it may only take one egg to spoil the batch (Stokes *et al.*, 1956; Williams *et al.*, 1968 and Doyle, 1984).

In terms of control mechanisms, a number of proposals have been put forward, thus Lacey (1989) and Hunter and Izsák (1990) advocate that greater genetic heterogeneity should be introduced into battery hens, as the latter have been "accused" of being of a nearly uniform genetic stock. This would cost the industry an enormous amount of money, and will not by itself ensure complete safety. Since all modern laying strains are selected for high and profitable production, a much more sensible procedure would be to detect and modify those individuals which are more susceptible to contamination, instead of applying a blanket measure that could compromise years of genetic improvement. Treatment of feedstuffs with organic acids may also be of value (Humphrey, 1991b).

In terms of vaccination, bacterins (vaccines containing killed bacteria) are said to reduce intestinal shedding by 100 times, but should not be used preventively, and only in places where there was or there is a *Salmonella* problem. According to Dr. S. Naqi (Cornell University, U.S.A. – pers. comm.), bacterins in general do not induce good cellular immunity, and also do not produce good levels of IgA. The main objective in using them is to induce immunity as quickly as possible, as an emergency procedure. At least it would allow the hens to pass some antibodies against *S. enteritidis* to the progeny, apart from reducing shedding. If vaccination is to be recommended, it will have to be autogenous and done orally, as it will more probably generate a higher intestinal IgA response.

There is an urgent need to control or if possible eliminate *S. enteritidis* infection in breeder flocks, thus stopping the possible vertical transmission to the progeny, and also to prevent its reintroduction from contaminated feeds, vermin, *etc.*.

Current E.C. proposals are designed to control once and for all Salmonellosis in birds, among other zoonoses, by compulsory testing of hatcheries, broiler flocks, commercial laying flocks and breeders flocks. It is proposed to test pooled faecal samples from all Rearing flocks, from breeding and/or commercial egg laying flocks at least at one day old, at 4 weeks old and two weeks prior to entering the laying phase. The breeding laying flocks (for production of hatching eggs) will be sampled at least every 2 weeks during the laying period and the laying flocks producing eggs for human consumption will be sampled at least every 12 weeks during the laying period (Official Journal of the European Communities, 1991). Where infection with *S. enteritidis* and *S. typhimurium* is confirmed officially in a flock by liver, ovary and bone marrow culture of individual birds, no movement of live fowl or eggs may be made from that flock until it can be satisfactorily established by the Veterinary authority that infection is no longer present. With regard to breeder laying or commercial egg-laying flocks, if confirmed as above, no movement of live fowl other than licensed by the Veterinary authority direct to slaughter, and also no movement of eggs other than direct for processing of egg products may be made, until normality is re-established.

The slaughter of a laying breeder or commercial laying flock proved to be *S. enteritidis* carrier is still the most responsible attitude. Antibiotic treatment will probably increase faecal shedding, and by prolonging the carrier state of birds, will perpetuate the infection on the premises. The induction of resistance is also feasible. It is possible that *E. coli* could be sharing /transferring R-plasmids to *S. enteritidis* and in time the indiscriminate use of antibiotics could induce *E. coli* resistance, which in turn

may be passed to *S. enteritidis*. Gentamycin has also been proved to cause immunosuppression in birds as measured by cellular monitoring (Dr. S. Naqi, pers. comm.).

The use of the competitive exclusion (Nurmi concept) treatment offers a valid option in terms of preventing *Salmonella* infection in birds. By inhibiting the establishment of pathogenic bacteria in the intestine by occupying the available spaces for attachment at the earliest possible opportunity after hatch, the adult normal flora given in this treatment will, if improved, be capable of naturally controlling the occurrence of this disease. The problem is to define exactly which microorganisms should be included in the filtrate, in which proportions, and which ones are dispensable.

A number of new techniques are currently being developed which may prove of value in localising the site of *Salmonella* infection, amongst these nanometre particle video ultramicroscopy (Nanovid ultramicroscopy) is already providing good results, according to Beesley (1989a). This process allows visualisation *in vivo* of bacterial forms. It is feasible that the latter technology will also provide insight into the problem of intermittent shedding of *S. enteritidis*.

According to Solomon (pers. comm.), the eggshell as produced by today's commercial laying flocks is structurally different from its wild counterpart. The reasons for this change are diverse, but undoubtedly the pressures of intensive rearing have exerted considerable effect on the oviducal environment. To redress the balance will be both time consuming and costly, and within the U.K. it is doubtful whether an industry still recoiling from the effect of the 1988 debacle will be willing to meet such expenditure.

It is assumed that eggs or egg products will always find a place at consumer level. The pressures inflicted by the media, however, ultimately determine the size of the market, thus the nutritional status of the egg and its cholesterol content constantly do battle in the mind of the consumer.

If the egg is to resume its "rightful place on the breakfast table" (Solomon, 1991), then as a foodstuff it must pass current quality control measures, and as such prove to be capable of withstanding both rough handling and the entry of microorganisms from the various environments in which it finds itself en route from bird to the kitchen. To achieve this peak of perfection, industry is faced with a number of alternatives, *viz.* genetic selection for specific beneficial traits within the eggshell or the use of feed additives to improve calcium absorption, and by so doing, regulate the process of mineralisation.

The general public is lead to believe by supermarket pressure via the media that alternative systems will provide the answer. At this point in the debate, the latter assumption does not appear to have any validity, and in these days of ecological orthodoxy, it is unlikely that growth promoters will find favour with a nervous public. The choice is therefore reduced to genetic selection, a long, tortuous process fraught with many difficulties – but perhaps the corrective measures are justifiably lengthy – it has taken many years to radically alter the structure of what was ultimately intended to be an embryonic chamber.

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APPENDICES

APPENDIX 1: (RECIPES)

PHOSPHATE BUFFERED SALINE (PBS):

Sodium Chloride (NaCl)... 40 g
Potassium Chloride (KCl).. 1 g
Di-Sodium Hydrogen Orthophosphate (Na_2HPO_4)............... 5.75 g
Sodium Di-Hydrogen Orthophosphate (NaH_2PO_4)............... 1 g
Sterilised double-distilled water.............................. 5 litres

Set the pH to 7.2-7.4 with 0.1 N HCl (to lower it) or 0.2 M K_2CO_3 or 0.1 N NaOH (to raise it). Filter through with filter paper no. 1 and store at 4 °C.

PBS + 0.5 % BOVINE SERUM ALBUMIN (BSA):

BSA.. 5 g
PBS.. 1 litre

Set the pH to 7.2-7.4, filter through filter paper no. 1, and store at 4 °C. Must be used fresh. Aliquots can be frozen and thawed when needed. Cloudy solutions must be discarded.

0.02 M GLYCINE:

1 M Glycine.. 1.52 g
PBS.. 1 litre

Set the pH to 7.2-7.4, filter through filter paper no. 1, and store at 4 °C.

APPENDIX 2

The following hypothesis were tested using the chi-squared (X^2) formula:

$$\frac{(\text{Observed number} - \text{Expected number})^2}{\text{Expected number}}$$

The chi-squared tests were only performed on contingency tables where none of the cells had expected frequencies below four, as recommended by a statistician (Arnott, 1989, personnal communication).

X^2 (1):
Null Hypothesis: There is no difference in the occurrence of "confluence" characteristic between beginning, middle and end of lay and the "bad" group.

	BEGINNING	MIDDLE	END+ BAD	TOTALS
NONE	3	7	20	30
ISOLATE	16	19	12	47
MODER.+ EXT.	13	6	10	29
TOTALS	32	32	42	106

X^2 value = 17.39 (4 d.f.)
Significant at a 0.1 % level (P< 0.001)
Null hypothesis is false.

X^2 (2):
Null Hypothesis: There is no difference in the occurrence of "good" and "poor" quality caps characteristic between beginning, middle and end of lay and the "bad" group.

	BEGINNING	MIDDLE	END+ BAD	TOTALS
GOOD	17	6	0	23
GOOD -	15	12	18	45
POOR+/POOR/P-	0	14	24	38
TOTALS	32	32	42	106

X^2 value = 41.48 (4 d.f.)
Significant at a 0.1 % level (P< 0.001)
Null hypothesis is false.

X^2 (3):
Null Hypothesis: There is no difference in the occurrence of type "B" abnormality between beginning, middle and end of lay and the "bad" group.

	BEGINNING	MIDDLE	END+ BAD	TOTALS
NONE + ISOLATED	25	25	18	68
MODER.+ EXTENS.	7	7	24	38
TOTALS	32	32	42	106

X^2 value = 13.72 (4 d.f.)
Significant at a 1 % level (P< 0.01)
Null hypothesis is false.

X^2 (4):
Null Hypothesis: There is no difference in the occurrence of pitting abnormality between beginning, middle and end of lay and the "bad" group.

	BEGINNING	MIDDLE	END+ BAD	TOTALS
NONE	32	30	33	95
DEP. + EROS.+ HOL.	0	2	9	11
TOTALS	32	32	42	106

X^2 value = 9.81 (2 d.f.)
Significant at a 1 % level (P< 0.01)
Null hypothesis is false.

X^2 (5):
Null Hypothesis: There is no difference in the occurrence of aragonite abnormality between beginning, middle and end of lay and the "bad" group.

	BEGINNING	MIDDLE	END+ BAD	TOTALS
NONE	31	28	13	72
ISOLATED	1	3	14	18
MODERATE	0	1	15	16
TOTALS	32	32	42	106

X^2 value = 45.23 (4 d.f.)
Significant at a 0.1 % level (P< 0.001)
Null hypothesis is false.

X^2 (6):
Null Hypothesis: There is no difference in the occurrence of type "A" abnormality between beginning, middle and end of lay and the "bad" group.

	BEGINNING	MIDDLE	END+ BAD	TOTALS
NONE	22	4	7	33
ISOLATED	10	28	35	73
TOTALS	32	32	42	106

X^2 value = 30.40 (2 d.f.)
Significant at a 0.1 % level ($P < 0.001$)
Null hypothesis is false.

X^2 (7):
Null Hypothesis: There is no difference in the occurrence of cubics abnormality between beginning, middle and end of lay and the "bad" group.

	BEGINNING	MIDDLE	END+ BAD	TOTALS
NONE	25	22	27	74
ISOLAT. + MODER.	7	10	15	32
TOTALS	32	32	42	106

X^2 value = 1.68 (2 d.f.)
Not Significant ($P > 0.05$)
Null hypothesis is true.

X^2 (8):
Null Hypothesis: There is no difference in the occurrence of cuffing characteristic between beginning, middle and end of lay and the "bad" group.

	BEGINNING	MIDDLE	END+ BAD	TOTALS
NONE	17	14	34	65
ISOLAT. + MODER.	15	18	8	41
TOTALS	32	32	42	106

X^2 value = 11.90 (2 d.f.)
Significant at a 1 % level ($P < 0.01$)
Null hypothesis is false.

X^2 (9):
Null Hypothesis: There is no difference in the occurrence of changed membrane abnormality between beginning, middle and end of lay and the "bad" group.

	BEGINNING	MIDDLE	END+ BAD	TOTALS
NONE	31	30	42	103
ISOL.+ MOD.+ EXT.	1	2	0	3
TOTALS	32	32	42	106

X^2 value = 2.59 (2 d.f.)
Not Significant (P> 0.05)
Null hypothesis is true.

X^2 (10):
Null Hypothesis: There is no difference in the occurrence of total structural score categories between beginning, middle and end of lay and the "bad" group.

	BEGINNING	MIDDLE	END+ BAD	TOTALS
EXCEL./ GOOD	21	11	6	38
GOOD -	9	11	12	32
POOR +	2	6	13	21
POOR / POOR -	0	4	11	15
TOTALS	32	32	42	106

X^2 value = 28.16 (6 d.f.)
Significant at a 1 % level (P< 0.01)
Null hypothesis is false.

X^2 (11):
Null Hypothesis: There is no difference in the occurrence of early fusion characteristic between beginning, middle and end of lay and the "bad" group.

	BEGINNING	MIDDLE	END+ BAD	TOTALS
ISOL.+ MODER.	23	29	32	84
EXTENSIVE	9	3	10	22
TOTALS	32	32	42	106

X^2 value = 3.81 (2 d.f.)
Not Significant (P> 0.05)
Null hypothesis is true.

X^2 (12):

Null Hypothesis: There is no difference in the occurrence of late fusion abnormality between beginning, middle and end of lay and the "bad" group.

	BEGINNING	MIDDLE	END+ BAD	TOTALS
ISOL.+ MODER.	17	13	17	47
EXTENSIVE	15	19	25	59
TOTALS	32	32	42	106

X^2 value = 3.66 (3 d.f.)
Not Significant ($P > 0.05$)
Null hypothesis is true.

X^2 (13):

Null Hypothesis: There is no difference in the occurrence of mammillary alignment abnormality between beginning, middle and end of lay and the "bad" group.

	BEGINNING	MIDDLE	END+ BAD	TOTALS
NONE + ISOLATED	21	17	31	69
MODER.+ EXTENS.	11	15	11	37
TOTALS	32	32	42	106

X^2 value = 3.43 (2 d.f.)
Not Significant ($P > 0.05$)
Null hypothesis is true.

X^2 (14):

Null Hypothesis: There is no difference in the occurrence of abnormal cuticle between beginning, middle and end of lay and the "bad" group.

	BEGINNING	MIDDLE	END+ BAD	TOTALS
NORMAL	1	0	1	2
ABNORMAL	63	64	78	205
TOTALS	64	64	79	207

X^2 value = 0.94 (2 d.f.)
Not Significant ($P > 0.05$)
Null hypothesis is true.
(Normal = Complete / Abnormal = Patchy or Absent)

X^2 (15):
Null Hypothesis: There is no difference in the occurrence of pitted O.S.M. or I.S.M. between beginning, middle and end of lay and the "bad" group

	BEGINNING	MIDDLE	END+ BAD	TOTALS
NORMAL	2	1	3	6
ABNORMAL	30	31	34	95
TOTALS	32	32	37	101

X^2 value = 0.77 (2 d.f.)
Not Significant ($P > 0.05$)
Null hypothesis is true.
(Normal = Intact / Abormal = Pitted)

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