A TYPING SCHEME FOR *Neisseria gonorrhoeae*

by

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DEDICATED TO MY WIFE.

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SUMMARY

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Growth of gonococci *in vitro* was studied during an attempt to find a typing scheme for epidemiological purposes. Many strains were found to be capable of remaining viable for several weeks in a liquid medium formulated particularly to prolong survival in laboratory cultures. Gonococcal strains were found to survive even longer when they were kept in liquid culture media at 30°C which is known to be their minimal growth temperature.

The feasibility of using gonococcal bacteriocins (the gonocins) in typing gonococci was investigated, and was found to be impracticable, because some inhibitory short-chain fatty acids were observed to interfere with, and to dominate, the inhibitory activity of gonocins. These fatty acids were found by gas-liquid chromatographic analysis mainly to be acetic acid and isovaleric acid. When these two acids were added to uninoculated media in concentrations equivalent to what was produced during the metabolic activity of gonococci, some gonococcal strains were inhibited on that media.

The colicins of *Shigella sonnei* were found to be possible substitutes for gonocins. Ten Colicin Type strains inhibited gonococci selectively. Thus it was possible to divide ninety two strains into groups on the basis of their sensitivity to the colicins. Certain limitations made it difficult to evaluate this typing scheme fully. Yet some indication was given that it could be a useful tool for epidemiological studies. In spite of the high stability of colicinogeny of *Shigella sonnei* strains, some inhibitory by-products might accumulate in the medium when the Colicin Type strains were incubated for four days. In this way the inhibitory activity of colicins against gonococci might increase. But by gas-liquid

chromatographic analysis it was found that the by-products, namely acetic and propionic acids, which were produced after incubating the Colicin Type strains for one day were not adequate to cause misinterpretation of the activity of colicins. These by-products however, might accumulate after prolonged incubation and reach an inhibitory concentration which interfered with the colicin activity. It was demonstrated that Colicin Type strains used in the typing scheme should be incubated for not longer than one day.

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INTRODUCTION

1. # INTRODUCTION

Neisseria gonorrhoeae, the causative organism of gonorrhoea, was the first member of the genus Neisseria to be described. It was given its name in 1885 by Trevisan in recognition of the fact that it had been originally observed by Dr. Albert Neisser in 1879 in the genital discharges of patients with the disease (Wilson & Miles, 1966; Bergey, 1974).

The disease was known to man long before the discovery of its cause. Venereal urethral discharge was first described by the Chinese Emperor Huang Ti as early as 2600 B.C. (Rein, 1977). The only natural host for N. gonorrhoeae is man (Wilson & Miles, 1966; Bergey, 1974; Cruickshank et al., 1975a; Rein, 1977). All attempts to induce the infection experimentally in animals had failed till it was discovered, comparatively recently, that it was possible to infect chimpanzees with the organism (Cruickshank et al., 1975a).

Lack of susceptible laboratory animals made it difficult to ascertain the basic facts about the gonococcus (N. gonorrhoeae). Kellogg et al., (1963, 1968) observed variation in the colonial morphology of gonococci which related to their virulence when inoculated into male volunteers. The disease could also be induced in man by injecting pure cultures of gonococci or gonorrhoeal pus into the urethra; symptoms appeared within 1 - 3 days (Wilson & Miles, 1966).

Apart from experimentally acquired gonorrhoea, direct sexual exposure to a person infected with the disease is the only mode of transmission, because the main portal by which the organism enters and leaves the body is the urogenital tract, and "the coccus is so

exceptionally susceptible to killing by the conditions of the extracorporeal environment, e.g. drying, cold, exposure to air, absence of nutrients, that it can only very rarely survive" indirect transmission (Cruickshank et al., 1975a). Moreover, epidemiological evidence has shown that an uninfected person does not invariably develop gonorrhoea after a single exposure to infection (Holmes et al., 1970). Many authors have reported that resistance to gonorrhoea might be a result of bacterial interaction (Volk & Kraus, 1973; Kraus & Ellison, 1974; Shtible, 1976). The possibility of acquiring gonococcal infection by non-venereal contact has been considered (Doyle, 1972). The survival of gonococci outside the body was reported to be at most 24h when urethral discharge from male patients was placed on a Petri dish at room temperature (Elmors & Larsson, 1972). There is, however, evidence to suggest that gonococci may survive rapid transfer of exudate on the fingers to the conjuctiva or on to damp towels as in gonococcal vaginitis, but this can happen only occasionally. (Cruickshank et al., 1975a).

1.1 Epidemiology

Epidemiological studies show a remarkable increase in the incidence of gonorrhoea during the past 25 years in most countries of the world (Cruickshank et al., 1975a; Kallings & Moberg, 1977; Rein, 1977). In Britain, a peak incidence immediately after the Second World War declined to a relatively low level during the early 1950's, probably as a result of effective chemotherapy. Since then the annual returns from venereal disease clinics in England and Wales have shown a steady increase in incidence (Cruickshank et al., 1975a). In the USA and many other countries the numbers

of reported cases of gonorrhoea have increased dramatically during the period 1960 - 1976 (Kallings & Moberg, 1977; Rein, 1977). In some other countries, however, the incidence of the disease was more or less stable. In Scotland and Norway, for example, the annual number of cases of gonorrhoea reported to the WHO* (World Health Organisation) has decreased in the period 1973 - 1975, and probably since then. In Sweden there was a remarkable and continuous decline in the incidence of the disease (Kallings & Moberg, 1977).

One possible reason for the decreased incidence of gonorrhoea is improvement in the treatment and management of the disease (Kallings & Moberg, 1977). On the other hand, increased resistance of the organism to antibiotics has tended to have the opposite effect. There are also factors related to the host. These are mainly promiscuity, increased population mobility, recidivism (Rein, 1977), and the spread of infection by asymptomatic carriers, which poses a special problem (Kallings & Moberg, 1977). Another factor which also contributes to the increased incidence of gonorrhoea is difficulty in diagnosing the disease. There are several critical aspects of laboratory diagnosis each of which has an influence on the accuracy of the final result (Kellogg, 1977), namely the collection and transport of specimens, the detection and isolation of gonococci, and confirmatory tests on the strains isolated. Without efficient procedures and media for the maintenance, culture, and identification of the organisms, there can be no certainty of diagnosis (Kellogg, 1977). In the first place, gonococci are fastidious organisms and the conditions they require for growth are

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* WHO figures for the U.K. from 1976 onwards not available.

very exacting although these are likely to be due more to their susceptibility to inhibitory substances *in vitro* than to their nutritional needs (Cruickshank *et al.*, 1975a). Antagonistic substances produced by other micro-organisms have also been reported to inhibit the growth of *Neisseria gonorrhoeae*. Hipp *et al.* (1974) reported that *Candida albicans* produced an inhibitor to the growth of gonococci when both were present in the same specimen, and they suggested that failure to culture *N. gonorrhoeae* from a suspected case of gonorrhoea in a female should be considered as a possible false negative if the patient was known to have candidal vaginitis. Shtible (1976) observed many bacterial species to cause inhibition of gonococcal growth and suggested that it was essential to use media that are known to be efficient in maintaining and protecting the gonococci.

In general, failure to diagnose gonorrhoea adversely affects the control of the disease. Hinman (1977) stated that (in the USA) a relatively stable incidence of gonorrhoea in males, with an increase in the incidence in females in recent years would suggest " that the prevalence of infection may be remaining more or less constant, with the increase in reported cases representing a result of discovery of previously undiagnosed cases". Many of the control programmes have relied heavily on the use of antibiotics, or have stressed the importance of contact tracing and other epidemiological studies. In fact, both treatment and contact tracing, as well as diagnosis, are important in the control of the disease (Catterall, 1977).

Control of gonorrhoea, like that of many other communicable diseases, is not possible without active follow-up of the

epidemiological process, i.e. identifying the source of infection, and tracing the transmission and spread of the pathogen (Meitert & Meitert, 1978).

1.2 Growth requirements and survival of gonococci

N. gonorrhoeae is the most difficult member of the genus Neisseria to cultivate (Wilson & Miles, 1966). Gonococci grow better on the surface of a solid medium than in an equivalent liquid medium (Cowan, 1975). "They are not always easy to maintain in cultures; even though they are subcultured regularly every 2 - 3 days, the organisms not infrequently die out, and strains are lost." (Wilson & Miles, 1966). The addition of blood, serum, ascitic or hydrocele fluids to solid media greatly enhances their growth (Wilson & Miles, 1966). Most strains require 3 - 10% carbon dioxide for growth, especially on solid media, and this can be achieved by incubation either in a carbon dioxide incubator or in a candle jar which supplies 3 - 4% CO_2. The latter was reported to provide the optimum requirement of CO_2 since it permitted the gonococcal cultures to mature within 24h, whereas in a carbon dioxide incubator which contained 10% CO_2 the cultures did not reach maturity before 48h, (James - Holmquest et al., 1973; Jones & Talley, 1977). Catlin (1973) reported that 55 out of 74 gonococcal strains grew in air on a defined medium, but their growth improved when incubated with additional CO_2. A significant reduction in the total number of organisms as well as in colonial size resulted when incubation in carbon dioxide was delayed for 2h or a little longer (Chapel et al., 1976). However, many strains lost their requirement for CO_2 after several subcultures (Platt, 1976).

Gonococci are aerobic and little, if any, growth occurs under strictly anaerobic conditions (Wilson & Miles, 1966; Bergey, 1957). But this statement was challenged by Williams & Wende (1972). James-Holmquest et al. (1973), who made a comparison between different atmospheric conditions for gonococcal growth, found that all 65 strains which were incubated anaerobically failed to grow on any of three suitable media. Morse et al. (1974, 1977), in an extensive radiorespirometric study, confirmed the inability of gonococci to grow anaerobically.

In general, gonococcal growth in liquid media is very poor, and appears in the form of a slight turbidity and a finely granular deposit, which hardly disintegrates on shaking (Wilson & Miles, 1966). Earlier studies of the nutritional requirements of gonococci enabled the workers to cultivate both stock and freshly isolated strains in vitro. Mueller & Hinton (1941) made a significant step forward in this field, when they showed that an essentially protein-free medium of casein hydrolysate, meat infusion, starch and agar gave excellent growth of gonococci. Their medium (Mueller Hinton Medium) is commercially available in solid and liquid form and is widely used in laboratories nowadays.

Later studies revealed that within the species N. gonorrhoeae there were strains of diverse nutritional requirements (Catlin, 1973). This is the phenomenon on which auxotyping was based (Carifo & Catlin, 1973). Essentially, there are common constituents of culture media which can be utilised by the vast majority of gonococcal strains, e.g. peptones, glucose, serum, starch, sodium chloride and sodium bicarbonate. The constituents which support the growth of only some strains are not necessarily antagonistic to

the growth of others. For example, glutathione is only required on primary isolation by about 20% of strains (Bergey, 1974) and there is no evidence that it is inhibitory, in the recommended concentrations, to any other strains. Apart from antibacterial agents, like antibiotics and bacteriocins, some other chemicals which are usually used in bacteriological media were reported to inhibit the growth of gonococci. Ley & Mueller (1946) isolated from agar an inhibitor for the growth of many gonococcal strains; the inhibition was probably due to long-chain unsaturated fatty acids present in the agar. They suggested that the inhibitory action could be neutralized by starch as the fatty acids were adsorbed by the linear component of starch. Since then starch has been used in all media formulated for the cultivation of gonococci. Gould et al. (1944) reported that when glucose and phosphate components were mixed in a medium and autoclaved at 121°C for 30 minutes the medium inhibited gonococcal growth. This was probably due to inactivation of the cystine present in peptone by degradation products of glucose (Wilson & Miles, 1966).

"Peptone is a chemically indefinite term used to describe the water-soluble products obtained after hydrolysis of (different) proteins" (Oxoid Manual, 1976). A wide range of peptones are commercially available. Their use in bacteriological media depends mainly on the particular requirements for amino-acids and peptides by different bacteria. Amino-acids are utilized as a source of energy (Oxoid Manual, 1976). Peptone in a concentration of 1-3% was reported to favour the growth of Neisseria species (Wilson & Miles, 1966). Gonococcal growth was best "when amino-acids were used as a source of nitrogen for the biosynthesis of other amino-acids,

proteins, or other nitrogen compounds." (Morse et al., 1977).

Cystine was reported to be a common requirement for all gonococcal strains (Catlin, 1978). Gould et al. (1944) found that 0.05% cystine stimulated the growth of gonococci, but inhibited the growth of those strains which were dependent on glutathione. A need for glutathione, however, is acquired only by some old laboratory strains (Gould et al., 1944; Bergey, 1974).

Glucose in a concentration of 0.2% increased the gonococcal colony size (Wilson & Miles, 1966). Increasing the concentration of glucose in the growth media stimulated the synthesis of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Holten, 1974a, 1974b).

Serum supports a luxuriant growth of gonococci when used in a concentration of about 10% in liquid media (Amies & Garabedian, 1967). It is usually added to the autoclaved media as a sterile supplement. A supplemental nutrient mixture which contained, among other constituents, a solution of lysed red blood cells and horse plasma was suggested for use in a medium for the isolation of both N. gonorrhoeae and Mycoplasma (Faur et al., 1973, 1974). Horse serum was used in a medium for the growth of gonococci (Gladston & Fildes, 1940). When the medium is to be used for fermentation tests, human or rabbit serum (10%) is suitable, but not horse, sheep or ox serum because these contain maltase which may cause a false positive maltose fermentation reaction (Wilson & Miles, 1966). Serum, and blood, may contain antibiotics (Cruickshank et al., 1975b) and therefore should be screened before use in gonococcal culture media.

As a substitute for gaseous carbon dioxide, sodium

bicarbonate can be used, especially in liquid and semi-solid media (Talley & Baugh, 1975). It was used in chemically-defined liquid media by many previous authors (Kenny et al., 1967; La Scolea & Young, 1974), but not without additional gaseous CO_2. Recently the role of the bicarbonate anion in gonococcal media has been studied, and $NaHCO_3$ has been recommended as a possible substitute for gaseous CO_2 (Jones & Talley, 1977). Earl et al. (1976) had previously suggested that it might be utilised even better than the latter.

The problem of the poor yield of gonococci in liquid media seemed to have been resolved, at least partially, when a concentrated culture of gonococci in an agar-liquid (biphasic) medium was obtained (Gerhardt & Heden, 1960). The count was stated to be as high as 5×10^9 cells per ml, and the turbidity was similar to that normally seen in cultures of staphylcocci. A dense culture of gonococci in a liquid medium was also achieved when the pH value was controlled in a continuous culture (Brookes & Sikyta, 1967). An efficient liquid medium was formulated by Hafiz & McEntegart (1976) and was successfully used for the primary isolation of gonococci as well as for stock cultures, for which it was particularly useful, as it prolonged the survival of gonococci for several weeks. This medium has recently been suggested for penicillin-sensitivity tests of gonococci because of its high efficiency in supporting the growth of very small inocula e.g. fewer than 10 cells per 10ml of medium (Eaves, 1978).

A major problem in laboratory studies of gonococci usually is difficulty in preserving gonococcal cultures for any length of time. A procedure for solving this problem suggested by Ward & Watt (1971)

was "snap" freezing the cultures in liquid nitrogen which resulted in a 100% recovery of the cells after thawing; moreover the colony types, T1-T4 (Kellogg et al., 1963, 1968), remained stable.

Alternatively strains can be preserved at 36°C in a sloppy agar broth containing 5% hydrocele fluid; subcultures are required at monthly intervals (Wilkinson, 1977). But the original colony types may not remain stable during subculture by this method, and hydrocele fluid is not always available on demand. Hafiz & McEntegart (1976) reported that the survival period of gonococci is much longer in liquid than on solid media. In the medium which they prepared they found that about 75% of strains survived for 2 weeks, and about 30% for 3 weeks. Four out of 106 strains survived for up to 56 days. This is the longest reported period, so far, for the survival of gonococci in a liquid medium at 36°C. Further studies on the colony type stability of gonococci in this medium revealed that 80-100% of strains of colony type T1 remained stable for longer than 3 weeks (Hafiz et al., 1977).

The optimum temperature for the growth of gonococci was stated to be 37°C (Wilson & Miles, 1966) or 35-36°C (Bergey, 1974; Cruickshank et al., 1975a, 1975b). No growth at all occurs below 30°C (Wilson & Miles, 1966; Bergey, 1974; Cruickshank et al.,1975b), or at a temperature over 38.5°C (Bergey, 1974).

The optimum pH value is 7.4 ± 0.2 (Wilson & Miles, 1966; Cruickshank et al., 1975a; Catlin, 1978).

1.3 Variation in colonial morphology of gonococci

Kellogg et al. (1963, 1968) made detailed studies of the variations in colonial morphology of gonococcal isolates and their

association with virulence. Four characteristic colonial types (T1-T4) were recognised. T1 and T2 were isolated from patients with acute gonorrhoea, whereas T3 and T4 arose on subcultures of T1 and T2. On Difco G.C. medium base plus supplement B the colonial types could be characterized after 20-24h incubation at 36°C as follows: T1 and T2 colonies were 0.5mm diameter, dark gold, translucent and amorphous. T1 had an entire edge whereas T2 was defined or crenated. T3 and T4 were both entire colonies of 1mm diameter, but T3 was light brown, translucent and granular, whereas T4 was colourless, transparent and amorphous (Kellogg et al., 1963). Jephcott & Reyn (1971) and Reyn et al. (1971), in an extensive study, confirmed the 4 colony types described previously, and recognized a new type, which they named type 5. The new type was first isolated in old laboratory strains. Its colonies were very shiny like those of type 2, but as large as those of type 3, and granulated with a dark brown colour and an irregular edge. Sparling & Yobs (1967) confirmed the association of T1 and T2 with acute gonococcal infections. Thus, on subculture, the virulent T1 and T2 types change into the non-virulent types T3 and T4 (and T5). Ward et al. (1970) were able to show that virulent strains of gonococci possessed a protective factor responsible for their resistance to killing by natural antibodies. This factor was lost after even one subculture (Watt et al., 1972).

Jephcott (1972) stated that cultures which were inoculated with T1 or T2 gave rise to T3 or T4, whereas the reverse did not occur. Hafiz et al. (1977) in the same laboratory studied the stability of colony types in Hafiz & McEntegart Medium (1976) and found that T1 colonies could be derived from T5, and that T4 represented a final form of the organism till it died out. But in

the presence of ferric citrate, T1 colonies rapidly arose and predominated.

A method was suggested to simplify the differentiation between different colony types (Juni & Heym, 1977) by means of which the observation that T1 mutated to T4 and T2 to T3 was confirmed. In an attempt at clarifying the features of gonococcal colony types, coloured photographs of the five types (T1-T5) were displayed (Brown & Kraus, 1974).

1.4 Methods of typing gonococci

As already mentioned, control of any communicable disease is not possible without tracing the transmission and spread of the pathogen. It is therefore essential that an active typing method is applied. The typing methods applied today are mainly: biochemical typing, serotyping, phage typing, bacteriocin typing and resistotyping (i.e. typing by means of antibiograms and the identification of R factors). These methods are applied either separately or together when more detailed levels of differentiation are required (Meitert & Meitert, 1978).

The typing of N. gonorrhoeae seems to present a particularly difficult problem. Most of the methods applied to the typing of pathogenic bacteria have been tried on gonococci (Geizer, 1977), the three main systems used being: serological typing, auxotyping, and bacteriocin typing.

Serological studies of gonococci started as early as the beginning of this century. The objectives were chiefly to find markers for epidemiological studies, and to develop methods for laboratory diagnosis (Danielsson & Maeland, 1978). Many methods are applied currently in serological diagnosis of gonococcal

infections (White & Kellogg, 1965; Rodas & Ronald, 1974; Kellogg, 1977; Danielsson et al., 1977; Danielsson & Maeland,1978), "either by demonstrating anti-gonococcal antibodies in serum by, for example, complement fixation, haemagglutination, flocculation, precipitation, radio-immune assays etc., or by demonstrating the gonococcal organisms with the use of immunofluorescence and co-agglutination techniques. However despite the increasing use of these methods the occurrence of false positives in most tests remains a major problem". (Danielsson et al., 1977).

Serological typing, on the other hand, does not seem to be feasible. The antigenic structure of gonococci is complicated. "Recognized groups or international types do not exist". (Bergey, 1974). All attempts during the last 70 years to establish an acceptable pattern of gonococcal serotypes have failed. But some of the new immunological techniques and cell biology studies of the organism might open up more fruitful approaches (Danielsson & Maeland, 1978).

Auxotyping, a system for differentiating gonococci based on their growth responses on chemically defined media, has been suggested by Carifo & Catlin (1973) and Catlin (1973). A set of 11 chemically defined media was used to subdivide 74 strains of gonococci into 13 auxotypes depending on their varying nutritional requirements as reflected in growth or absence of growth on the defined media. The same workers also studied 251 more strains to improve and evaluate the system and these strains were divided into 20 auxotypes (Carifo & Catlin, 1973). The system has recently been expanded to distinguish 35 auxotypes by testing isolates on 13 media, viz one complete chemically-defined medium and its 12

modifications (Catlin, 1977, 1978). As there were 13 different media for each test, contamination presented a problem. Standardizing the incubation of all cultures is essential as there are genetic and physiological variations among gonococcal responses to environmental conditions (Catlin, 1973, 1978).

1.5 Bacteriocins of gonococci: Comments on their application in typing

Bacteriocins are "antibiotics" produced by certain strains of bacteria which inhibit some other strains, usually of the same or related species (Reeves, 1972). The various groups of bacteriocins have been named after the major genus or species producing them, e.g. colicins after Escherichia coli, staphylococcins after the genus Staphylococcus, meningocins after N. meningitidis and gonocins after N. gonorrhoeae.

Bacteriocinogeny and sensitivity to bacteriocins have been suggested by many authors as "epidemiological markers" for typing strains of various bacteria, e.g. Proteus (Al-Jumaily, 1975a,1975b, Senior, 1977), and Pseudomonas aeruginosa (Gillies & Govan, 1966; Tagg & Mushin, 1973), and there are bacteriocinogenic strains of almost every genus of bacteria (Nomura, 1967; Reeves, 1972; Tagg et al., 1976).

The production of bacteriocins by strains of Neisseria meningitidis is well recognised (Kingsbury, 1966; Counts et al., 1971). Attempts have been made to type N. gonorrhoeae by using their bacteriocins (Flynn & McEntegart, 1972; Luger, 1975), but these attempts were evidently not convincing. Other authors mentioned that Flynn & McEntegart (1972) had not, in fact, demonstrated the production of bacteriocins (gonocins) but other,

non-specific, inhibitory substances. They based their comments on the characterization of the inhibitory substances (Walstad et al., 1974; Knapp et al., 1975; Lawton et al., 1976).

It is a known fact that bacteriocinogenic cells are not usually susceptible to the lethal or biochemical action of their own bacteriocins (Nomura, 1967). This must be so because otherwise it would not be possible to obtain a culture of a bacteriocinogenic strain. Nevertheless Ryan et al. (1955) and Goodwin et al. (1972) did report the production of a bacteriocin by cells that were sensitive to it. This, however, must be a very rare phenomenon.

Published reports of gonocin typing of gonococci describe the use of a group of indicator strains that exhibited variable patterns of inhibition by the gonocins produced by the tested strains. In other words, the typing procedure was based on the bacteriocinogeny of the tested strains as demonstrated solely by their effect on a standard group of sensitive strains, which means that the reaction of the producer strains to their own inhibitory substances was not checked.

Of the 100 strains tested by Flynn & McEntegart (1972) 75 were producers. Luger (1975) tested 150 strains of which 135 (90%) were producers. On the other hand, Lawton et al. (1976) reported that only 17 out the 2123 strains they tested (i.e. less than 1%) were gonocinogenic.

The nature of the inhibitory substance was not investigated by Flynn & McEntegart (1972) or Luger (1975). Walstad et al.(1974) reported that separation of total gonococcal lipids by silica gel chromatography revealed inhibitory activity by both the free fatty acids and the phospholipid fraction.

In spite of the suggestion that gonocinogenic strains do exist (Lawton et al., 1976) gonocin typing of gonococci is still not practicable for the following reasons:

i. No one has yet managed to increase the yield of gonocins from producer strains by any of the usual methods of inducing bacteriocins like for example ultraviolet light or mitomycin C (Lawton et al., 1976). Thus, extraction of gonocin is very difficult and therefore typing can only be accomplished by using live cultures of gonocinogenic strains.

ii. Again, because living gonocin-producing cultures are used, long preservation of frequently-used cultures is required and this is difficult when working with such fastidious organisms. Furthermore, there is a risk of instability of the gonocinogenic factor during continuous subculturing.

iii. Lawton et al. (1976) suggested that the use of gonocins in typing gonococci was complicated by a number of factors. They observed that susceptibility to gonocin depended on colony type, and that all of 15 T1 isolates were susceptible to all of the 12 gonocins used. The 12 gonocin producer strains were in turn divided into 2 groups (A & B) according to their inhibitory activity, with the result that all of 60 T4 isolates tested were divided into 4 types; susceptible to both group A and group B, susceptible only to group A, susceptible only to group B, and resistant to both group A and group B. The conclusion is that all gonococcal strains belong to one or other of these four groups, a feature which severely limits the usefulness of gonocin typing in epidemiological studies.

1.6 Inhibition of gonococci by other bacteria

Many authors previously reported the inhibition of gonococci by antibacterial substances, mainly bacteriocins and bacteriocin-like substances, produced by a variety of bacterial species (Hsu & Wiseman, 1967; Geizer, 1968; Volk & Kraus, 1973; Shtible, 1976). Among these species was Staphylococcus aureus which produced staphylococcin as reported by Hsu & Wiseman (1967) and Shtible (1976) and confirmed by Moriss et al. (1978). Species of other genera such as Vibrio cholerae and NAG vibrios, Micrococcus, Aeromonas hydrophila, Escherichia coli, and Pseudononas aeruginosa were all found to produce antibacterial substances that inhibited gonococci (Geizer, 1968). Shtible (1976) reported that bacteriocins from the following bacterial species (listed in order of frequency) inhibited gonococcal growth: N. meningitidis, Staphylococcus epidermidis, Corynebacterium species, Staphylococcus aureus, Streptococcus pyogenes, and N. subflava. Morse et al. (1976) in a detailed study, found that pyocins from Ps. aeruginosa inhibited gonococci. Sidbery & Sadoff (1977) not only confirmed the inhibition of gonococci by pyocins, but suggested pyocin sensitivity of gonococci as a feasible epidemiological tool for typing them. They stated that by using 23 pyocin preparations, 106 strains of N. gonorrhoeae clearly showed 30 different sensitivity patterns. Very recently the sensitivity of gonococci to pyocins was investigated further (Blackwell et al., 1979). The results showed that 94.5% of the gonococcal isolates were inhibited selectively by 11 partially purified R-type pyocins, and it was suggested that "there was a relationship between the pyocin receptor sites in the lipopolysaccharide of Ps. aeruginosa and

N. gonorrhoeae".

As already mentioned, Hipp et al. (1974) reported the inhibition of gonococcal growth also by a lethal factor produced by Candida albicans, and tests of other strains under different conditions have confirmed this finding (unpublished observations, quoted by Catlin, 1978). Using five reference strains of C.albicans selected for potent inhibition activity, and also inhibitory Candida strains which were isolated together with gonococci from a given specimen, 27 strains of gonococci were divided into three groups on the basis of their sensitivity to the inhibitors (Hipp et al., 1975).

Apart from the species E. coli, none of the members of the family Enterobacteriaceae has been studied, so far, for the effect of their bacteriocins on N. gonorrhoeae, although there is evidence that the latter is susceptible to bacteriocins of Gram-negative rods.

1.7 Colicins of Shigella sonnei

Over the past 15 years epidemiological studies have shown that Sonne dysentery has become the most prevalent form of bacillary dysentery in the U.K. and the U.S.A. (Morris & Wells, 1974). Before the work of Abbott & Shannon (1958), typing of Sh. sonnei strains was almost impossible. They could not be typed by methods used for typing other species in the family Enterobacteriaceae. Serological typing was inapplicable because all strains of Sh. sonnei, unlike the other species in the genus, are serologically homogeneous (Gillies, 1964, 1978; Morris & Wells, 1974; Cruickshank et al., 1975b). Biochemical typing was of very limited use as it subdivided the isolates into only two groups,

according to their ability, or inability, to ferment xylose (Abbott & Shannon, 1958; Gillies, 1964). Finally, bacteriophage typing is of little practical value, because of type instability (Abbott & Shannon, 1958; Gillies, 1964; Meitert & Meitert, 1978).

Abbott & Shannon (1958) suggested a method for typing epidemic strains of Sh. sonnei using colicin production as a marker. Over 2100 strains were typed in England (Abbott & Graham, 1961; Barrow & Ellis, 1962) using the colicin typing method. Further modifications of the technique of Abbott & Shannon (1958) were suggested by Gillies (1964) who typed more than 5000 strains by the modified method. Evaluation of the method was performed by Morris & Wells (1974) who suggested that known colicin type strains should be used as controls for the typing procedure. Gillies (1978) stated that "the most significant bacteriocin typing technique of species within the Enterobacteriaceae concerns Sh. sonnei; this serologically homogeneous species is now the most common cause of bacillary dysentery in many parts of the world, both temperate and tropical, and this high prevalence undoubtedly attracted worldwide interest in the method of typing introduced by Abbott & Shannon (1958) and subsequently modified by Gillies (1964)". Authors who recommended the use of standardized media for colicin typing to protect colicin activity from the effect of minimal changes in the media stated that colicinogeny and sensitivity to colicins were found to be remarkably stable in Sh. sonnei strains which had been either freeze-dried or stored in Dorset medium at 4°C (Abbott & Graham, 1961).

The colicins of Sh. sonnei can be extracted and purified (Hinsdill & Goebel,1966; Matsugushi, 1975). The colicin

preparations can be sterilized by a variety of agents commonly used in the sterilization of liquid cultures, e.g. ultraviolet light, chloroform, etc. (Senior et al., 1970). Purification can be achieved only partially, but the stability of many bacteriocins was shown to decrease with increasing purity (Mayr-Harting et al.,1972). Sterilization of a crude colicin might also inactivate it (Senior et al., 1970). Purified colicins, however, can be used instead of colicin-producer cultures in testing the sensitivity of other strains, in which case the inhibition of the tested strains will only be due to colicin activity, as all other probable inhibitors are washed off.

1.8 Inhibition of gonococci by fatty acids

Many authors reported sensitivity of gonococci to free fatty acids of different chain-length (Ley & Mueller, 1946; Miller et al., 1977). When Walstad et al. (1974) studied the inhibition of gonococci by their own by-products, they found that phospholipids and free fatty acids had an inhibitory effect on gonococci. The major phospholipid, namely phosphatidylethanolamine, had no inhibitory activity of its own. Long-chain fatty acids, as well as a minor phospholipid (monoacyl phosphatidylethanolamine), which resulted from the degradation of the major phospholipid, were quite inhibitory.

1.9 Gas-liquid chromatography

Chromatography is a method of chemical analysis used to separate substances in a mixture on the basis of their absorption properties. Gas-liquid chromatography (GLC) is a system by means of which substances which can be volatilized are simultaneously separated. Molecules of substances to be analyzed will instantly partition between a moving gas phase and a stationary liquid phase

absorbed on to an inert supporting material (Downes, 1963). The latter is uniformly packed in a column which is maintained at a desired temperature in an oven. Samples injected into the column are evaporated by heat and carried by a stream of inert (carrier) gas which passes at a controlled flow rate along the column where the components of the sample are distributed between the two (gas and liquid) phases. Components which leave the column pass from its far end through a detection system which notes the arrival of a component, converts it into an electric signal and feeds it to a recording device, usually a chart-recorder (Drucker. 1976). Because of the differences in the affinity of the liquid phase for the various components, they move through the column at proportional rates and emerge with separate portions of the effluent of inert carrier gas (Downes, 1963).

" The technique can now be used to examine bacterial fatty acids, sugars, amino-acids, purine and pyrimidine bases. By scaling up the analytical technique, preparative GLC is practicable.

GLC offers the chemical microbiologist two advantages (i) extremely low concentrations for detection of most substances and (ii) rapid analysis." (Drucker, 1976). GLC-chemotaxonomy is an advanced system in bacterial taxonomy. Production of fatty acids during metabolic activities <u>in vitro</u> is of a particular value as a taxonomic tool (Carlsson, 1973). Volatile fatty acids can be extracted from cultures with ether, and non-volatile acids with chloroform after being methylated (Carlsson, 1973).

GLC is not only useful in the separation of compounds from a mixture, but it is also an efficient method of identification and quantitative determination of individual components (Mitruka,1975).

Fatty acids found both in whole culture extracts and as fermentation products of several species of Neisseria were identified by GLC (Brooks et al. 1971, 1972). Analysis of acids which interfere with bacteriocins or mimic their activity can be performed by GLC.

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 Organisms

2.1.1 N. gonorrhoeae

N. gonorrhoeae strains isolated from patients with active infections were obtained from The City Laboratory. The isolates had been identified and maintained on Columbia Blood Agar (Oxoid) at 37°C for 1-4 days. They were numbered in special serial numbers (appendix).

2.1.2 Sh. sonnei

Sh. sonnei indicator strains (I.S.) and colicin type (C.T.) strains were also obtained from The City Laboratory but they were maintained as lyophilized cultures. Initially they were the complete international set of both the I.S. and C.T.strains,namely I.S. 1-15 and C.T. 1A,1B, and 2-13. The number of strains in each set was reduced to ten, as follows:-

I.S.	C.T.
1	1B
2	2
4	3A
5	4
6	5
7	6
8	8
9	9
13	11
15	13

2.2 Checking of cultures for purity

The purity of cultures was frequently checked by subculturing on solid media and by Gram staining. On some occasions the oxidase test was performed on the cultures.

2.3 Culture media and growth conditions

2.3.1 Media used

The gonococcal strains were subcultured for different experimental purposes in the laboratory either (a) on solid media enriched with defibrinated horse blood (Oxoid) or (b) in liquid media sometimes enriched with serum (horse or rabbit).

a. Solid media

(i) Columbia blood agar

The most frequently used solid medium for maintenance and/or viability tests was Columbia blood agar (CBA) (Oxoid). It had the following formula (Oxoid Manual, 1976).

Special peptone	23g
Starch	1g
Sodium chloride	5g
Agar No.1	10g
Distilled water	1 litre
pH	7.2 (approx.)

When the base medium had cooled to about 55°C, 7 - 10% defibrinated horse blood (Oxoid) was added to it.

(ii) G.C. Medium

This medium (also known as Thayer-Martin medium) was used for detection of gonocin production. It consists of GC agar base plus GC supplement (Oxoid) to which 2% (w/v) autoclaved haemoglobin

solution was added. Their formulae (Oxoid Mannual, 1976) were as follows:

GC agar base

Special peptone	15g
Corn starch	1g
Sodium chloride	5g
Dipotassium hydrogen phosphate	4g
Potassium dihydrogen phosphate	1g
Agar No.1	10g
Distilled water	1 litre
pH	7.2 (approx)

The sterile supplement was available in vials containing the following ingredients.

Yeast fractions	5g
Glucose	0.75g
Sodium bicarbonate	0.075g

The contents of two vials were added to one litre of base medium with haemoglobin.

(iii) Brain heart infusion blood agar

For colicin sensitivity tests of both gonococcal strains and Shigella indicator strains Brain heart infusion blood agar (Oxoid) was used chiefly. It was prepared by adding approximately 10% defibrinated horse blood (Oxoid) aseptically to Brain heart infusion agar (Oxoid) which had been autoclaved and cooled to about 55^{O}C. The formula of the base medium was as follows (Oxoid Manual, 1976):

Calf brain infusion solids.....	12.5g
Beef heart infusion solids	5g
Proteose peptone	10g

Sodium chloride 5g

Glucose 2g

Disodium phosphate 2.5g

Agar No.1 ..... 10g

Distilled water ..... 1 litre

pH 7.4 (approx)

b. Liquid media

(i) Enriched medium for Neisseria (EMN)

All the gonococcal strains were subcultured in a liquid medium referred to as (EMN) which was formulated especially to prolong the survival of most of the gonococci. Basically it was a double strength Tryptone soya broth (Oxoid) to which serum, starch and bicarbonate were added. The ingredients were as follows:

Tryptone 34g

Soya peptone 6g

Dipotassium hydrogen phosphate 5g

Sodium chloride 10g

Sodium bicarbonate 0.1g

Dextrose 5g

Soluble starch 1g

Distilled water ..... 1 litre

pH 7.2 (approx.)

Horse or rabbit serum (10%) was added after autoclaving. The medium was usually prepared in screw capped bottles of 7ml capacity.

(ii) Mueller Hinton broth (MHB), Tryptone soya broth (TSB) and Eugonbroth (EB)

These three liquid media were used in some experiments, particularly to make a comparison between gonococcal growth and

survival in EMN and in other media. They had the following formulae (Oxoid Manual, 1976, BBL Manual, 1971).

MHB (Oxoid)

Beef, infusion from	300g
Casein hydrolysate	17.5g
Starch	1.5g
Distilled water	1 litre

pH 7.4 (approx.)

TSB (Oxoid)

Tryptone	17g
Soya peptone	3g
Dipotassium hydrogen phosphate ...	2.5g
Sodium chloride	5g
Dextrose	2.5g
Distilled water	1 litre

pH 7.3 (approx.)

EB (BBL)

Trypticase peptone	15g
Phytone peptone	5g
Sodium chloride	4g
L-Cystine	0.7g
Glucose	5.5g
Distilled water	1 litre

pH 7 (approx.)

c. Buffers and solutions for certain purposes

(i) Diluting Solutions

For Total Viable Count (TVC) tests peptone water and phosphate buffer saline (Dulbecco solution A) were used as diluents.

Because a significant reduction in TVC was observed when the phosphate saline was used, diluting procedure was performed with peptone water in the majority of tests. The two solutions were prepared as follows:

Phosphate buffer saline (Dulbecco solution A)

Sodium chloride	8g
Potassium chloride	0.2g
Disodium hydrogen phosphate	1.15g
Potassium dihydrogen phosphate	0.2g
Distilled water	1 litre

pH 7.3

The solution was distributed into small screw capped bottles containing 9ml each and autoclaved for 10 minutes at 110°C.

Peptone water

Special peptone (Oxoid)	10g
Sodium chloride	5g
Distilled water	1 litre

pH 7.2 (approx.)

The solution was distributed and autoclaved as for the phosphate saline.

(ii) Solution for preserving gonococci in liquid nitrogen

Living gonococcal cells in 0.5ml broth culture were inoculated in 1% Proteose or Special peptone (Oxoid) containing 8% glycerol as a protective agent.

(iii) Medium for maintaining Shigella sonnei

All I.S. and C.T. strains were stored in duplicates in Nutrient broth No.2 (Oxoid) at 4°C after being incubated for 1 day at 37°C. One culture of the duplicate was used for the various colicin tests

and the other was used for subculturing stock cultures at about 6 weekly intervals. The nutrient broth had the following formula (Oxoid Manual, 1976).

'Lab Lemco' powder	10g
Peptone.....	10g
Sodium chloride	5g
Distilled water	1 litre
pH	7.5 (approx.)

2.3.2 Conditions of incubation

a. Carbon dioxide supplement

Using McIntosh & Fildes' anaerobic jars, gonococcal strains cultured on solid media were incubated in candle-extinction jars containing 3-4% CO_2 (Ferguson,1945; James-Holmquest et al., 1973).

Broth cultures were incubated in a water bath or in an incubator at any desired temperature without added CO_2.

b. Incubation temperature

Unless otherwise stated, all gonococcal cultures were incubated at 37°C as the optimal temperature for growth (Wilson & Miles, 1966). Plates, broths and diluting fluids were all kept at 37°C for at least one hour prior to inoculation with gonococci. Equipment such as anaerobic jars was similarly pre-warmed.

2.3.3 Effect of various concentrations of sodium chloride and potassium phosphate on growth of gonococci.

The Enriched Medium for Neisseria (EMN) was prepared with the modifications in sodium chloride and dipotassium phosphate content shown in Table 2.1. The gonococcal strains were inoculated with aliquot inocula each in one set of EMN and its modifications. Viability tests were made after 1, 2, 3, 7, 14, 21 days

by subculturing one loopful from each broth culture on CBA plates (Oxoid) which were incubated in a candle extinction jar at 37°C. Results were obtained after 24hr.

Modifications	NaCl (g/l)	K_2HPO_4 (g/l)
EMN-0	0*	0
EMN-1	3	2.5
EMN-2	3	5
EMN-3	3	7
EMN-4	5	2.5
EMN-5	5	5
EMN-6	5	7
EMN-7	10	2.5
EMN	10	5
EMN-8	10	7

* NaCl present in peptones is not included

Table 2.1: The concentrations of sodium chloride and potassium phosphate in EMN and its modifications.

The maximum concentrations of NaCl tolerated by gonococci was studied in EB (BBL) and EMN . The total concentrations of NaCl in the two media is shown in Table 2.2.

Both media were distributed in 10ml amounts into 15ml screw capped bottles. Two gonococcal strains were inoculated with aliquot inocula each in duplicates of the 12 media set.

Medium	Added NaCl (g/l)	Original NaCl (g/l)	Total NaCl (g/l)
EB-0	0	4.3	4.3
EB-1	5	4.3	9.3
A-0*	0	10.3	10.3
EB-2	10	4.3	14.3
A-1	5	10.3	15.3
EB-3	15	4.3	19.3
A-2	10	10.3	20.3
EB-4	20	4.3	24.3
A-3	15	10.3	25.3
EB-5	25	4.3	29.3
A-4	20	10.3	30.3
A-5	25	10.3	35.3

* (A) in this experiment represents EMN

Table 2.2. Total concentrations of NaCl in EB and EMN prepared with additional NaCl.

Viability tests were made by subculturing one loopful from each broth culture on CBA (Oxoid) after 1 and 2 days.

2. 3.4 Methods of preserving gonococci

a. Preservation by Ward & Watt's Method

Ward & Watt's Method (1971) could only be applied with modifications. The procedure, after modification, was simply to add 0.5ml of a 2-day old gonococcal broth culture containing about 5×10^5 cells to 1.5ml peptone-glycerol solution. The latter was prepared and autoclaved in 30ml polypropylene bottles. The solution

after being inoculated with the cells was snap frozen by dipping the polypropylene bottles into liquid nitrogen for 55 seconds. They were then stored at -20°C. After one week they were transferred to a water bath at 37°C for thawing. 0.5ml of the thawed broth culture was spread on the surface of a CBA plate which was then incubated at 37°C for one day. All the growing colonies were counted, the count thus being one quarter of the total surviving cells.

A total of 25 cultures of gonococci were preserved by this method either by using Proteose or Special peptone (Oxoid) as shown below:

Peptone used	No. of gonococcal strains
Proteose (Oxoid)	7
Special (Oxoid)	18

b. Prolonging the survival of gonococci in culture media

In this study two methods of prolonging the survival of gonococcal cultures were used:

(i) By maintaining gonococci in EMN

All gonococcal strains which had been maintained on CBA were subcultured to EMN in firmly screwed bottles. A number of strains inoculated in EMN were kept at 37°C and were checked weekly for continued viability. A total of 165 strains were divided into 6 batches; all the strains in a batch were inoculated simultaneously. As most gonococcal cultures were required for 1 - 2 weeks, EMN provided satisfactory maintenance for about 75% of strains (Table 3.1).

(ii) By incubating gonococci at their minimum growth temperature (30°C)

As mentioned in the previous chapter the minimum growth temperature for gonococci is 30°C (Wilson & Miles, 1966; Bergey, 1974).

Incubating gonococci at 30°C was found to prolong their survival. This was shown practically in the following experiments.

Experiment No.1 Survival of gonococci in liquid media at 30 and 37°C.

Six gonococcal strains were inoculated in 4 replicates into three liquid media: MHB (Oxoid), EB (BBL), and TSB (Oxoid). The three media were prepared in small screw capped bottles which were firmly screwed after inoculation. Cultures were divided equally into two groups; one group was incubated at 37°C and the other was incubated for 24hr at 37°C then transferred to 30°C. All cultures were tested for viability after 3, 7, 10, 15, 20, 25, and 35 days by subculturing one loopful on CBA (Oxoid).

Experiment No.2 Survival of gonococci in EMN at 30°C as compared with their survival at 37°C.

Duplicates of EMN prepared as usual were inoculated with 22 strains, and incubated at 30 and 37°C as in experiment no.1. A viability test was made by subculturing one loopful from each culture on CBA (Oxoid) after 5, 10, 15, 20, 25, 30, 35, 40, 45, 60 and 70 days.

Experiment No.3 Total Viable Count of gonococci at 30 and 30°C. A Total Viable Count (TVC) test was made for 2 gonococcal strains in EMN. Thirty millilitres of EMN were prepared in a 50ml conical flask plugged with cotton wool and covered with aluminium foil. Each of the two strains was inoculated in aliquots in 2 flasks which were immediately incubated after inoculation as follows: one flask at 37°C and the other at 37°C for one day then transferred to 30°C. A TVC test was made for all the cultures at the time of inoculation (T_o) and after 1, 2, 3, 4, 5, 8, 16 and 35 days. The test was

performed each time by transferring 0.5ml from the culture with a graduated pipette to a screw capped bottle containing 4.5ml of special peptone water. The latter was shaken gently 20 times before 0.5ml was withdrawn from it and transferred to a new bottle containing 4.5ml peptone water, and so on to the desired number of diluent. When the diluent was to be count tested, 0.55ml was withdrawn, 0.05ml was inoculated on the surface of a CBA plate and the rest was transferred to the next diluting bottle. All these steps were performed rapidly so that the gonococci were left for no longer than 20 seconds in a diluting bottle before being inoculated on CBA. After completing the diluting series the inocula were spread on the surface of the medium and the plates were incubated immediately in a candle jar at 37°C. Results were obtained after 24hr.

Experiment No.4 Comparison between survival of gonococci in EMN and EB at different temperatures.

Six gonococcal strains heavily seeded on CBA plates were used for inoculation. Each strain was inoculated in 7 screw capped bottles of EMN and 7 others of EB. The resulting 84 cultures were divided into 7 identical groups; each group was incubated at a different temperature as follows:

Group No.	Incubation temperature
1	37°C
2	30°C
3	29°C
4	28°C
5	37°C (for 1 day) then 30°C
6	" " " 29°C
7	" " " 28°C

Viability tests were performed after 7 and 14 days by subculturing on CBA

2. 3.5 Colonial types of gonococci

Precise "Kellogg typing" of the strains was not attempted but, on the criterion of the colony size which could be seen with the unaided eye, it was possible to classify them broadly into two types, A and B. Type A, like T_1 and T_2, formed pinhead-sized colonies, while the colonies of Type B were larger in size resembling those of T_3, T_4 and T_5.

2. 4 Detection of gonocin production and self inhibition of gonococci

The method described by Abbott & Shannon (1958) for the detection of colicins was, at the start of the study, applied to the detection of gonocin production, but this was soon abandoned and the method of Kekessy & Piguet (1970) used instead.

The method is based on the fact that as diffusion of bacteriocins is "tridimensional" in solid media, they are detectable on the reverse side of the agar. The procedure was as follows: the producer strains were diametrically streaked on the surface of the solid medium. After incubation, the agar was detached from the circumference of the Petri dish with a sterile spatula. The Petri dish was then inverted and tapped gently on a sheet of cork, so that the agar fell into the lid of the Petri dish. The indicator strains were then streaked at a right angle to the diametric streaking of the producer strain but on the "new" surface of the medium (Kekessy & Piguet, 1970). The new surface of the medium (which was previously at the bottom of the dish) was very smooth, and therefore detection of inhibition of the indicator strains was easier than it was by Abbott & Shannon's method.

Inhibition of the indicator strains was detected by thinning or complete disappearance of growth at the central region of streaking which was referred to as the zone of inhibition. Thus the indicator strain which was sensitive to the inhibitor of the producer strain appeared, after incubation, as a line of growth interrupted by a central gap, whereas a resistant indicator strain grew in a continuous line. Three or four indicator strains were streaked on each plate.

Fifty gonococcal strains were tested for gonocin production on GC agar plus supplement (Oxoid) using the Kekessy & Piguet method. Each strain was tested against itself and 5 - 7 other strains. Six of the producers were tested against themselves and six others by the same method on CBA (Oxoid).

2. 5 Colicins of *Shigella sonnei*

2. 5. 1 Inhibition of gonococci by colicins

Using the method of Kekessy & Piguet, 92 strains of gonococci were tested for their sensitivity to 10 colicin types of *Shigella sonnei*. The C.T. strains were chosen at random. The medium used was Brain heart infusion blood agar (Oxoid). The incubation period of the C.T. strains was not allowed to exceed 24hr at 37°C. Gonococci, on the other hand were incubated for 24hr or, when necessary, 48hr at 37°C in a candle jar.

2. 5. 2 Stability of colicinogeny in colicin type strains

Ten indicator strains of *Sh. sonnei* were used to check the colicinogeny of the 10 C.T. strains at intervals of approximately 6 weeks. The 10 I.S. were chosen in such a way that for each C.T. strain there were some resistant and some susceptible indicator strains.

2.5.3 Effect of extra incubation of colicin type strains on the inhibition of gonococci and indicator strains

It was noted that the inhibition pattern of some gonococcal strains as well as some indicator strains altered when the C.T. strains were incubated for more than 3 days at 37°C.

A comparison was made between the patterns of inhibition of all indicator strains and 17 gonococcal strains when the C.T. strains were incubated for 1 day and when they were incubated for 4 days, at 37°C.

2.5.4 Extraction and sterilization of colicins

The colicins of C.T. 2, 3A and 9 were extracted from the cells to test the sensitivity of I.S. and gonococci to the extracted colicins. The method used for extraction was the one suggested by Hinsdill & Goebel (1966) which was briefly as follows:

The C.T. strain was grown in 15 litres of Nutrient broth No.2 (Oxoid) distributed into 5 conical flasks (of 5 litre capacity). After incubation for 48h at 37°C, the cultures were killed by adding phenol to a final concentration of 0.5% after which they were left at 4°C for 6h. They were then "spun" in a refrigerated centrifuge (MSE Mistral 4L) at 3800 rpm and 4°C for 40 min. The sediment "consisting primarily of whole cells" was resuspended in a small amount of distilled water and lyophilized in a Vacuum Freeze Drying Unit, Model 30 P. 2 T.S. (Edwards High Vacuum). The dried cells were extracted twice with 0.85% NaCl at 65°C for 10 min, "a step which removed appreciable quantities of nucleic acids, and, but little, colicin". The wet cells were then suspended in distilled water and left for 30min at 65°C. After centrifugation, the supernatant was collected and concentrated

in a rotary vacuum evaporator. The solution was then lyophilized again. The crude colicin was dissolved in distilled water and sterilized either by filtration or by chloroform added to a final concentration of 5% w/v for 5min (Senior et al., 1970).

2.5.5 Test of the activity of crude colicins

The extracted colicins were tested for their activity on the 10 I.S. and some gonococcal strains which had been tested by the Kekessy & Piguet method. Colicin activity was tested by spreading 0.2ml of the sterile colicin diametrically on the surface of blood agar. It was then kept at 37°C for 1 - 2h to dry. The strains which were to be tested were streaked across the colicin zone. Sensitive strains were inhibited at the colicin zone and grew only at its two edges while resistant ones grew across the colicin zone.

2.6 Gas-liquid chromatography (GLC)

2.6.1 Gas-Liquid chromatographic conditions

Detection of the volatile and non-volatile fatty acids was performed on a Pye Unicam 104 series Chromatograph equipped with a flame ionization detector (FID) and a 5ft coil glass column packed with diatomite C that had been treated with phosphoric acid and impregnated with 10% polyethylene glycol. The instrument was operated at 125°C using oxygen-free nitrogen as the carrier gas at a flow rate of 100 ml/min, hydrogen at 55 ml/min and compressed air at 450 ml/min. The attenuator was adjusted at 1×10^2. A chart recorder (Kipp & Zonon, model BD8, Holland) was used; its speed was set at 5 mm/min with an input signal of 1 mV. A 1 µl aliquot sample for analysis was injected on to the column by a 1 µl syringe (Hamilton, Model 7001- N).

2. 6. 2 Chemicals and media

The reagents used in preparing the standard solution and media extractions for the analysis are listed in Table 2.3.

Media which were used to demonstrate gonocin production and colicin sensitivity were G.C. medium and Brain heart infusion blood agar (Oxoid) respectively. The main objective of GLC analysis in this study was to trace the fatty acids which might be produced in quantities inhibitory to gonococcal strains first during the detection of gonocin production, as the fatty acids mimic bacteriocin activity, and then during colicin sensitivity testing of gonococci as the acids possibly interfere with colicin activity.
Therefore, G.C. medium was extracted and prepared for GLC analysis after it had been utilised by gonococci, and Brain heart infusion blood agar was extracted after the C.T. strains had grown on it for 1, 2, 3 and 4 days.

Table 2.3 Chemicals used for GLC analysis

Reagent	Supplier
Acetic Acid	BDH, Poole, England
Propionic acid	" " "
Isobutyric acid	" " "
Butyric acid	" " "
Isovaleric acid	" " "
Valeric acid	" " "
Isocaproic acid	" " "
Caproic acid	" " "
Heptanoic acid	" " "

Lactic acid	Sigma, London
Oxalic acid	BDH, Poole, England
Methyl malonic acid	Sigma, London
Malonic acid	BDH, Poole, England
Fumaric acid	" " "
Succinic acid	" " "
Maleic acid	" " "
Glutaric acid	" " "
Malic acid	" " "
Boron trifluoride methanol BF_3 14%	" " "
Magnesium sulphate	" " "
Diethyl ether	" " "
Chloroform	" " "

2. 6. 3 Procedure for preparation of standard solutions and specimens

a. Volatile acid standard

To 100ml distilled water the following reagents were added (The amounts represent approximately 1 meq of the acids, depending on their purity).

Reagent	Amount
Acetic acid	0.057 ml
Propionic acid	0.075 ml
Isobutyric acid	0.092 ml
Butyric acid	0.091 ml
Isovaleric acid	0.127 ml
Valeric acid	0.125 ml
Isocaproic acid	0.126 ml

Caproic acid	0.126 ml
Heptanoic acid	0.126 ml

To prepare the volatile acid standard solution (as an external standard) for injection on the GLC, a 4ml sample was acidified with 50% H_2SO_4 and treated as for culture extraction (see section d below.)

b. Non-volatile acid standard No.1

To 100ml of distilled water the following reagents were added (The amounts represent approximately 1 meq of acids, depending on their purity).

Reagent	Amount
Lactic acid	0.084 ml
Oxalic acid	0.060 g
Methyl malonic acid	0.060 g
Malonic acid	0.050 g
Fumaric acid	0.06 g
Succinic acid	0.060 g

To prepare the non-volatile acid standard solution No.1 (as an external standard) for injection on the GLC, a sample of 1ml was acidified with 50% H_2SO_4, methylated with BF_3-methanol and treated as for the culture extraction (see Section d below).

c. Non-volatile acid standard No.2

As some peaks appeared beyond the peak of succinic acid, which was the last in the series of acids in solution No.1, the following acids were prepared and treated in exactly the same way as for solution No.1. The amounts were added to 100ml distilled water.

Reagent	Amount
Maleic acid	0.060 g
Malic acid	0.067 g
Glutaric acid	0.066 g

d. Procedure

The steps of preparation for GLC analysis are shown in Fig. 2.1.

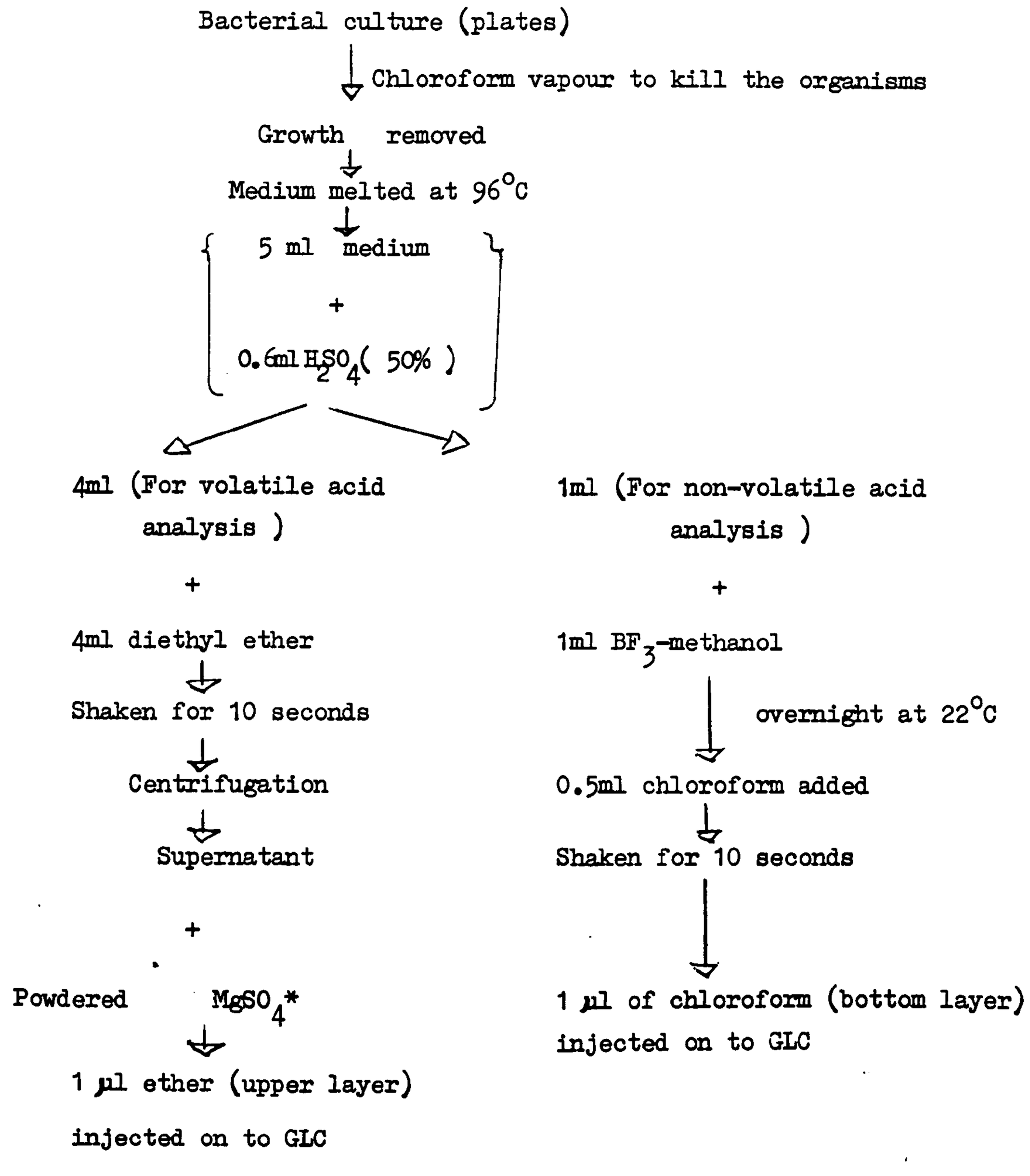
Heavy growths of gonococci and C.T. strains on G.C. medium and Brain heart infusion blood agar (Oxoid) respectively, were killed by exposure to chloroform vapour, and the growth was removed with a razor blade. A sample of the medium underneath the growth was melted in a water-bath at 96°C, and 5ml of that sample acidified with 0.6ml of H_2SO_4 solution (50% w/v). As this adjusted the pH to 2.0 thus ensuring that the agar did not re-solidify, the fatty acids could be extracted.

i. Preparation for GLC analysis of volatile acids

Four ml of the acidified sample were extracted with an equal volume of diethylether as follows: the sample was pipetted into a small bottle, 4ml ether were added and shaken for 10 seconds. It was then centrifuged briefly to break the ether-medium emulsion. The ether layer (supernatant) was pipetted off with a Pasteur pipette into a new bottle where it was dried by adding a suitable amount of $MgSO_4$ which removed dissolved water from the ether. The bottle was allowed to stand for about 5min to ensure the settlement of all the salt particles. The ether was once again transferred with a Pasteur pipette to another bottle ready for injection on to the GLC column.

External standard solution containing measured amounts of volatile fatty acids, prepared (as in Section a) in order to allow

Fig. 2.1 Preparations for GLC analysis



* Sufficient to ensure complete dehydration of the sample

calibration of peaks due to bacterial metabolism, was extracted in the same way.

ii. Preparations for GLC analysis of non-volatile acids

1ml of the original acidified medium was placed in a small bottle and an equal volume of BF_3-methanol added and the mixture was left overnight at room temperature to allow methylation (derivitization) of the non-volatile acids. Alternatively, it could be heated in a boiling water bath for 5min. Half its volume of chloroform was then added and mixed by shaking for 10 secs, and the chloroform layer (bottom) was transferred with a Pasteur pipette to another bottle. One µl was injected on to the column.

External standardization for the non-volatile acids was made by preparing a solution containing measured amounts of the acids and treating it in the same way.

2.6.4 Separation of the peaks of propionic and isobutyric acids

"The extent of separation of two peaks is expressed by the resolution" (Mitruka, 1975). Because the resolution of the GLC column was sub-optimal, there was less than 2mm difference between the "retention volumes" of propionic and isobutyric acids. The difference was measurable when the two acids were injected separately on to the column. But when they were injected together, a complete overlapping of their peaks occurred. As some of the culture extract samples produced a peak at the propionic-isobutyric peak, that peak had to be identified by a more efficient column, i.e. with a higher separation factor. This can be achieved either by repacking the column, by using a longer column, or by using a different stationary phase (Drucker, 1976). The two latter factors were available on

another GLC unit on which some of the culture extract samples were analysed. The conditions of the new GLC were as follows:
It was a Perkin Elmer GLC Unit, Model F33, provided with a coiled glass column (0.3cm internal diameter by 2m length).
The latter was packed with Chromosorb G 80/100 mesh acid washed, treated with dimethyldichlorosilane (AW-DMCS), and coated with 5% free fatty acid phase (FFAP). The instrument was operated isothermally at 140°C. Nitrogen flow rate was 20ml/min. The recorder was operated with an input signal of 1mV, and a chart speed of 5mm/min. The electrometer attenuation was 4 x 10.

2. 6. 5 Qualitative analysis of chromatograms

There is a specific interval for every compound, under constant GLC conditions, between the time of injection and the time at which that compound leaves the GLC column. This interval is known as the retention time (R.T.). Compounds can thus be identified by their R.T. values.

The R.T. of each acid is represented in the chromatogram by the distance between the solvent front (or time of injection) and the maximum of the peak of that acid. It was possible, therefore to identify peaks in chromatograms of samples by comparing their R.T. with those of peaks of known acids in the standard solutions.

The peak of each acid in the standard solution was, in turn, identified either by preparing the acid alone in a standard solution, or by enlarging its peak. The latter can be achieved by doubling the amount of the acid during the preparation of the complete standard solution and comparing the chromatogram with one in which that particular acid was prepared in its ordinary amount.

2. 6. 6 Quantitative analysis of chromatograms

The area under a peak obtained for a component is directly related to its amount. Many methods can be applied to measure peak areas; some of them are listed below (Mitruka, 1975).

a. Automated integration

b. Multiplying the peak height by its width at half height

c. Cutting out the peak and weighing the paper

d. Triangulation, i.e. by drawing a triangle from two tangents through the inflection point of the peak and the base line.

The area of the triangle is then calculated.

One of the most commonly used methods, when the GLC is not provided with an integrating device, is calculation of the peak height x peak width at half height. This method was used in quantitation of the chromatograms in this study.

2. 7 Inhibition of gonococci by fatty acids

The fatty acids which were present in appreciable amounts in the samples of utilized media, as revealed by the GLC analysis, but were absent in the samples of blank media, have been tested for their inhibitory activity to gonococci. The acids were sterilized by filtration and added to sets of G.C. medium and Brain heart infusion agar (Oxoid) in amounts equivalent to their highest concentrations as they appeared in the analysed samples. The media were poured in plates and inoculated with a few gonococcal strains to see whether those amounts of acids produced during metabolism were inhibitory or not.

RESULTS

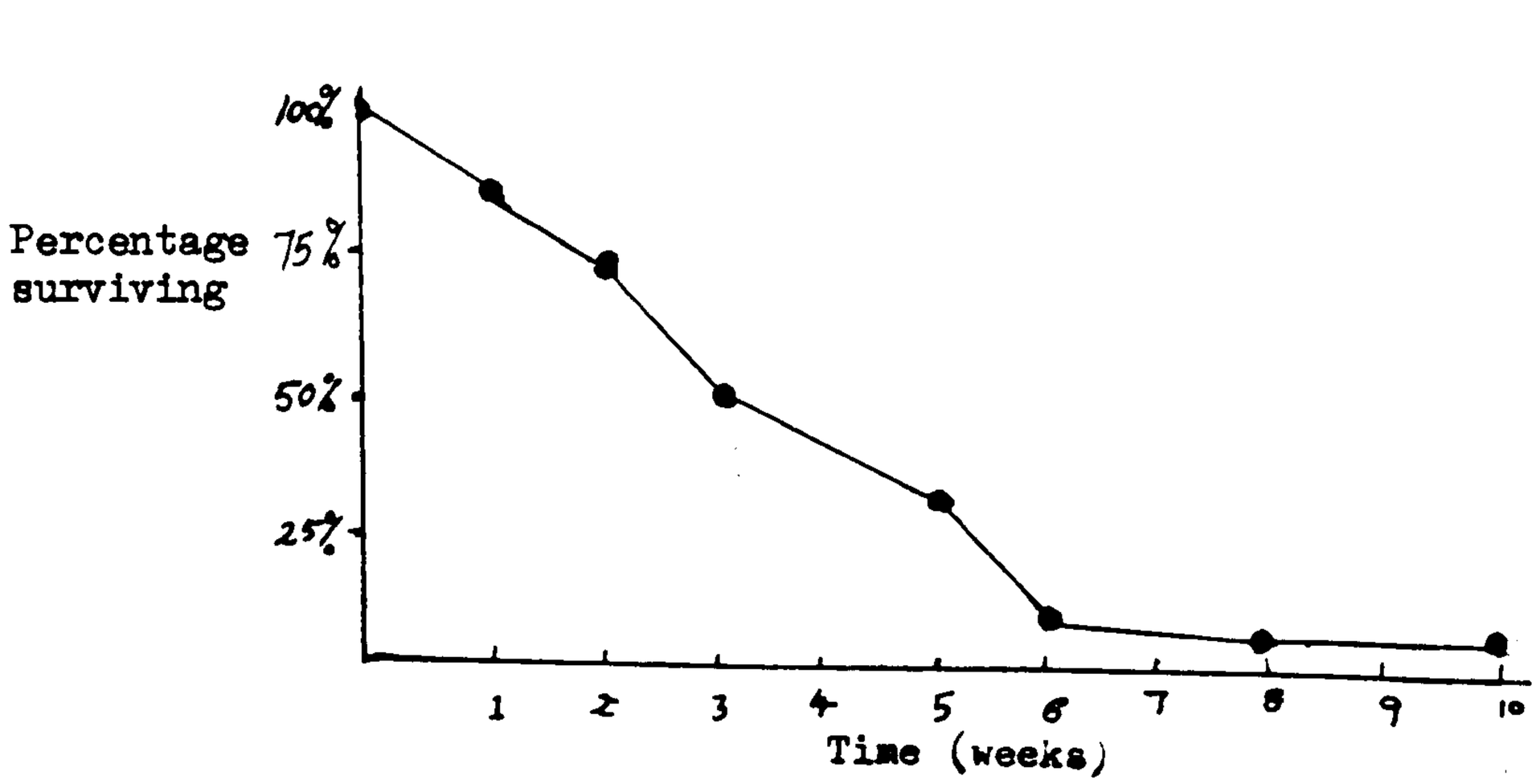
3. RESULTS

3. 1 Growth of gonococci in culture media

3. 1. 1 Growth and survival of gonococci in EMN and its modifications at 37°C

A total of 165 strains of gonococci were inoculated in Enriched Medium for Neisseria (EMN) and kept in an incubator at 37°C without added CO_2. The strains were divided into batches and examined for viability at weekly intervals. The numbers of surviving strains over the test period are listed separately for each batch in Table 3.1. The overall percentage survival figures are presented in graphical form in Fig. 3.1.

Fig. 3.1. Percentage survival of gonococcal strains (judged by growth on subculture) over a number of weeks in EMN



It can be seen that there is a fairly rapid fall in percentage surviving to a figure of 50% within the first 3 weeks, and to 30% in a further 2 weeks. This rate of survival is substantial when the organism concerned is N. gonorrhoeae.

TABLE 3. 1 Survival of gonococci in EMN at 37°C

Total number of surviving strains as checked at weekly intervals

Batch No.	No. of strains	1 wk.	2wks.	3wks.	5wks.	6wks.	8wks.	10wks.	Over 10 wks.
1	20	17	N.T.*	9	5	1	N.T.	0	0
2	50	43	N.T.	19	16	3	0	0	0
3	8	8	N.T.	2	2	2	N.T.	2	0
4	25	24	20	17	10	N.T.	3	1	1 (100 days)
5	12	10	8	7	N.T.	1	1	0	0
6	50	42	36	29	13	5	4	4	2 (78 days)
Total	165	144	64	83	46	12	8	7	3
Percentage		87.2%	73.5%	50.3%	30%	8.5%	5.8%	4.2%	1.8%

* N.T. = Not tested

In many other liquid media, as will be seen later, gonococcal strains can hardly survive for 3 weeks. Hafiz & McEntegart (1976), in a medium formulated by them, showed that after 2 weeks the percentage surviving was 73.58. This was almost the same as that showed here in EMN. The proportion of survivors dropped down more rapidly thereafter (31.13% in 3 weeks, 15.09% in 5 weeks, 3.77% in 7 weeks and 0% in 8 weeks).

All these figures are lower than those obtained for EMN. Yet, at the time of publication, the figures obtained by them were considered as the longest survival periods for gonococci in a liquid medium.

It was possible that a fall in pH value might be one of the main reasons for the reduction in numbers of surviving gonococci and therefore pH values were monitored at various time intervals. Comparative figures were obtained for eugonbroth (BBL) and the results are given in Table 3.2.

Table 3. 2 Changes in pH values for EMN and EB (BBL) inoculated with gonococci

Time		pH in EMN	pH in EB
Before inoculation		7.2	7
Days after incubation	3	7.2*	6.6
	5	7.1*	6.1
	35	6.5 x	

*: Average for 3 cultures x: Average for 2 cultures

It can be seen that the pH value in EMN dropped quite slowly in comparison with that for EB. The indication is that EMN is a well buffered medium, and this probably contributes to the favourable survival rate.

3. 1. 2 Effect of various concentrations of sodium chloride and potassium phosphate on growth and survival of gonococci

The effect of various concentrations of NaCl and K_2HPO_4 on growth and survival of gonococci was studied. Three strains of gonococci were inoculated each into one set of EMN and 9 modifications, namely EMN-0, EMN-1 ... EMN-8. Cultures were kept at 37°C and checked for viability after 1, 2, 3, 7, 14 and 21 days. Table 3. 3 shows the results of viability tests for the 30 cultures. The medium which was lacking phosphate salt (EMN-0) provided the shortest survival period for the organisms. During this short period (in the first 3 days) the growth rate was at its maximum in contrast with phosphate-containing media. The media which contained the highest phosphate concentration provided the longest survival period but the slowest growth rate for the organisms. The inference may be that potassium phosphate adversely affects growth rate but favours survival rate.

Sodium chloride did not seem to have an influence on the survival of gonococci. This can be seen in Fig. 3.3. During the first 14 days the highest concentration of NaCl (1%) gave the lowest figures (number of cultures that grew on subculture), the lowest concentration (0.3%) gave higher figures, and the medium concentration gave the highest figures. Fig. 3.2 shows that the effect of sodium chloride was probably on growth yield of the organism.

Table 3.3 Effect of various concentrations of NaCl and K_2HPO_4 on growth and survival of gonococci in EMN and its modifications

Strain No. / Time in days	Medium:	EMN-0	EMN-1	EMN-2	EMN-3	EMN-4	EMN-5	EMN-6	EMN-7	EMN	EMN-8
	NaCl %	0*	0.3	0.3	0.3	0.5	0.5	0.5	1	1	1
	K_2HPO_4 %	0	0.25	0.5	0.7	0.25	0.5	0.7	0.25	0.5	0.7
N 21	1	++	++	++	+++	+++	++	+	+	+	±
	2	++	++	++	+++	+++	++	+++	+++	+	+
	3	+++	+++	+++	+++	+++	++	+++	+++	+++	±
	7	±	++	+++	+++	+++	+++	+++	+++	+++	++
	14	-	-	+++	+++	++	+++	+++	C	++	+++
	21	-	-	++	±	±	+++	++	C	±	+++
N 22	1	+	++	+++	+	++	++	±	+	+++	±
	2	+	+++	+++	++	+++	++	++	+	+++	±
	3	++	+++	+++	+++	+++	++	+++	+	++	+
	7	-	+++	C	+++	+++	±	+++	C	+++	+++
	14	-	+	C	+	++	-	+++	C	++	++
	21	-	-	C	+	±	-	+++	C	++	++
N 20	1	+	++	+++	++	++	+++	++	±	++	±
	2	++	+++	+++	++	++	+++	++	++	++	±
	3	+++	+++	+	++	++	+++	+++	+++	+++	+
	7	±	+++	+++	++	+++	+++	+++	-	+++	+++
	14	-	+	+++	++	+	+	++	-	+++	+++
	21	-	±	-	-	-	-	+	-	++	++

+++: Heavy growth ++: Growth +: Scanty growth ±: Few colonies

-: No growth C: Contaminated culture *: NaCl present in peptone is not included

Table 3. 4 Effect of various concentrations of NaCl on gonococcal growth in EMN and EB (BBL)

Medium	Conc. of NaCl (w/v)	Strain No.N28		Strain No. N33	
		1 day	2 days	1 day	2 days
EB	0.43 %	++	+++	+++	+++
EB-1	0.93 %	+	++	+++	+++
A*	1.03 %	+++	+++	+++	+++
EB-2	1.43 %	-	-	+++	+++
A-1	1.53 %	C	C	+++	+++
EB-3	1.93%	-	-	++	+++
A-2	2.03 %	-	-	+	+++
EB-4	2.43 %	-	-	+	±
A-3	2.53 %	-	-	±	±
EB-5	2.93 %	-	-	±	±
A-4	3.03 %	-	-	±	-
A-5	3.53 %	-	-	-	-

* : (A) in this experiment represents EMN

+++ : Heavy growth

++ : Growth

+ : Scanty growth

± : Few colonies

- : No growth

C : Contaminated culture

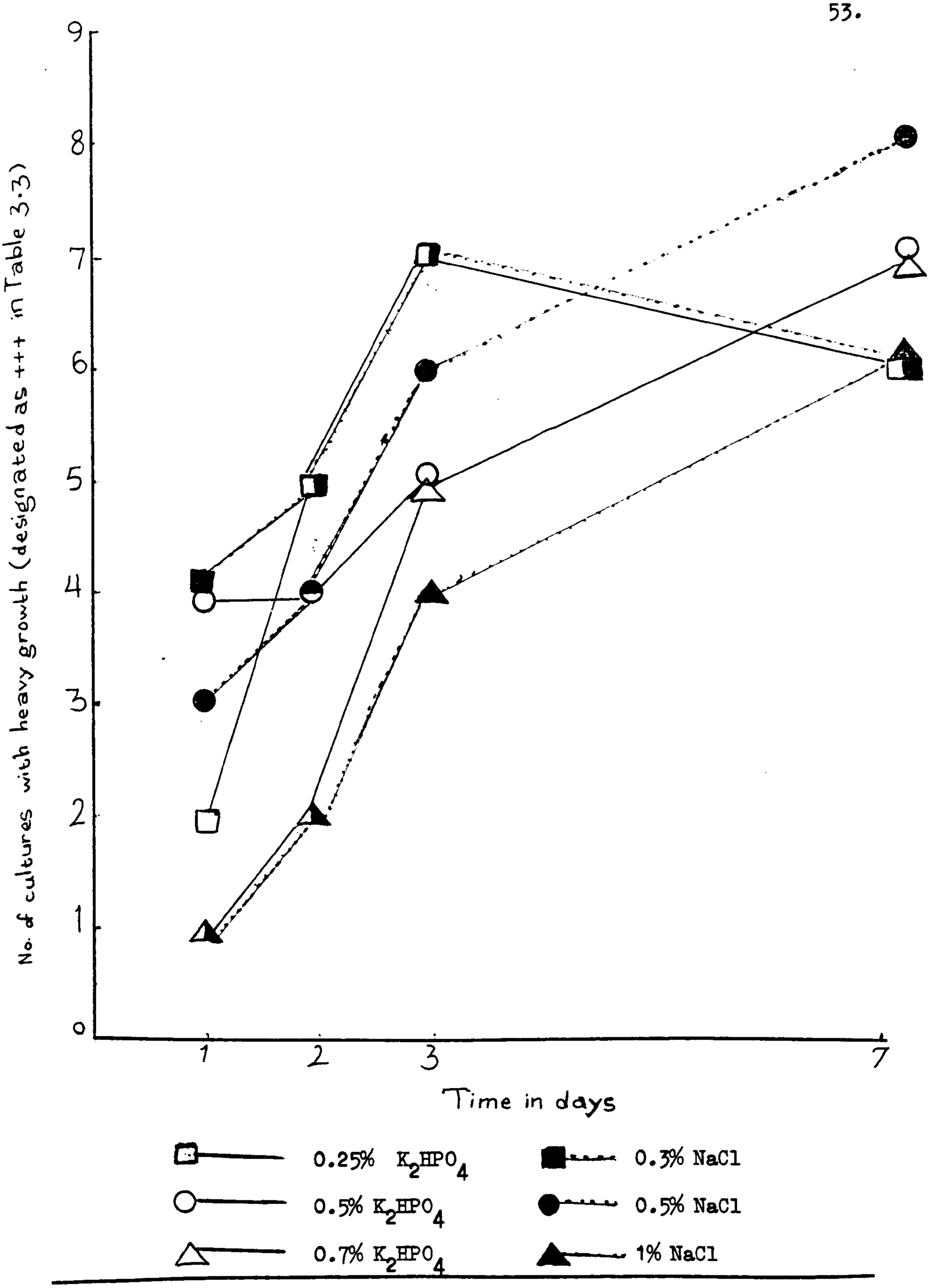
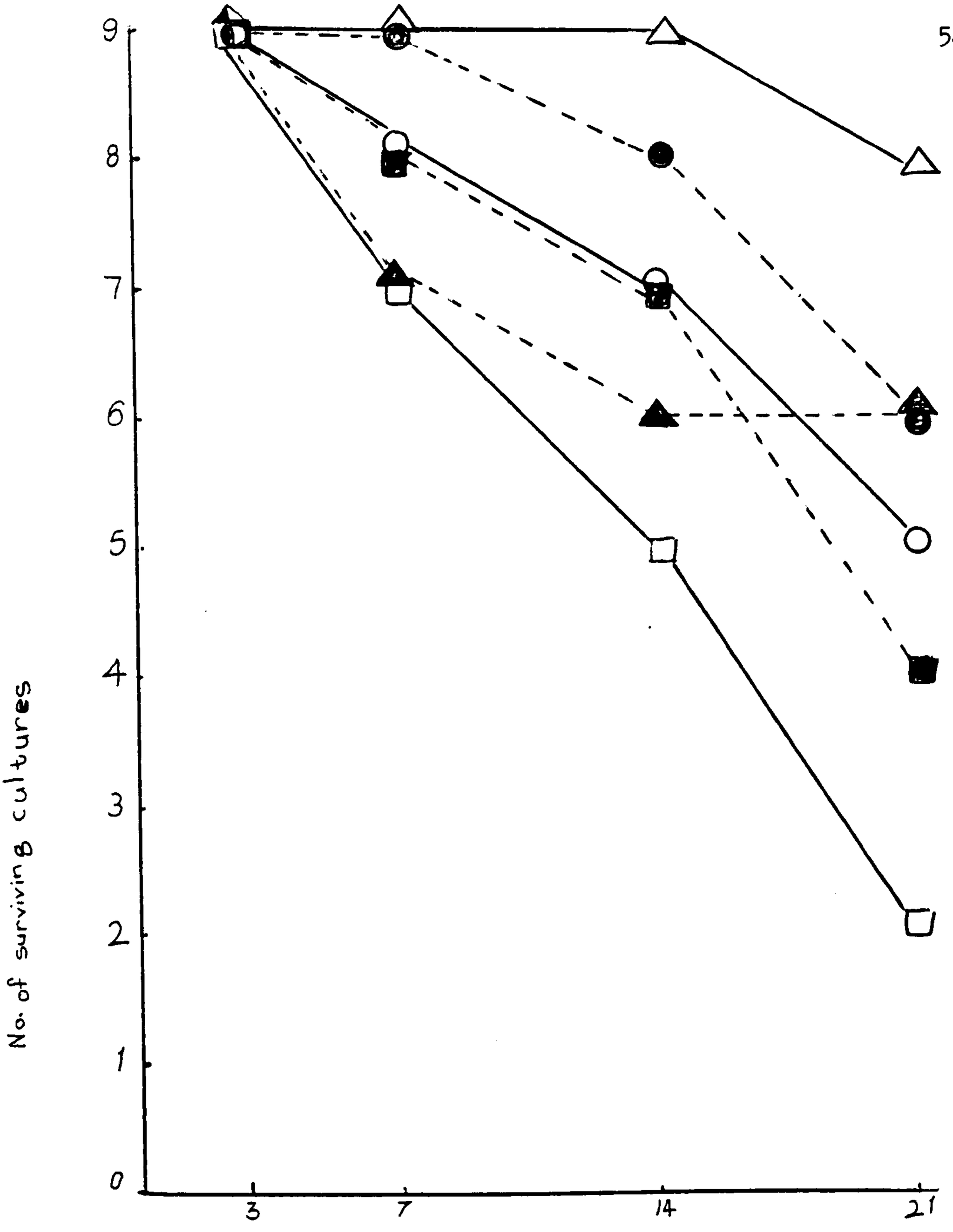


Fig. 3. 2. Effect of various concentrations of potassium phosphate and sodium chloride on growth of gonococci in EMN and its modifications.



□—	0.25%	K_2HPO_4	■---· 0.3% NaCl
○—	0.5%	K_2HPO_4	●- - - - 0.5% NaCl
△—	0.7%	K_2HPO_4	▲- - - - 1% NaCl

Fig. 3. 3 Effect of various concentrations of potassium phosphate and sodium chloride on survival of gonococci in EMN and its modifications.

During the first 3 days the growth yield increased with the decrease of the salt concentration. The inference from the two figures might be that the concentration of 0.5% represents an optimum because it gave the highest growth yield after 7 days and the highest number of surviving cultures for the period between 7 - 14 days.

The maximum concentration of NaCl tolerated by gonococci was studied in EB medium (BBL) and EMN. Twelve modifications of the 2 media were prepared containing varying concentrations of NaCl. Aliquot inocula were prepared for each of two strains and inoculated into the appropriate medium. Cultures were checked for viability after 1 and 2 days by subculturing on CBA (Oxoid). The viability test after two days confirmed whether the growth on first subculture represented progressing in growth or simply ability of the organism to survive. Table 3.4 shows that 3.03% NaCl is the maximum concentration of NaCl which can be tolerated by gonococci. In the range 2.53 - 2.93% it seems that very little, if any growth occurs. In medium EB4 and A4 the observed decrease in growth yield of strain N33 on subculture after 2 days might indicate that the cells did not grow but survived.

3. 1. 3 Preservation of gonococci

a. Preserving by Ward & Watt's method

The method of Ward & Watt (1971) was tried with modifications. A total of 25 strains were preserved in peptone water containing 8% glycerol, using Proteose or Special peptones (Oxoid).

Table 3. 5 Survival of gonococcal strains preserved by Ward & Watt's method

Peptone used	No. of strains preserved	No.of surviving strains	Total viable count (cfu/ml)
Proteose (Oxoid)	7	2	14 22
Special (Oxoid)	18	1	6

The cultures were 'snap' frozen in liquid nitrogen then kept at -20°C for one week. After thawing, 0.5ml of each culture was spread on CBA (Oxoid) and incubated at 37°C for 24hr. Poor results were obtained (Table 3. 5). The dramatic reduction in the number of surviving strains and in cfu/ml in the 3 surviving cultures necessitated thinking of other alternatives for this procedure.

b. Prolonging the survival of gonococci in liquid culture media

(i) By maintaining gonococci in EMN

It has already been shown in Table 3.1 that a high percentage of gonococcal strains survived in EMN for longer periods than usually observed in other available media. One of the cultures in batch No.4 (Table 3.1) survived for 100 days. Fig. 3.4 shows the 48hr growth on a CBA subculture which had been inoculated with 0.05ml of an 85 day old EMN culture. Colonies are off-white in colour. This is an artifact due to reflected light from the flash. Their actual feature is shiny with greyish to light brown colour.

Fig. 3. 4 A 48hr growth of 0.05ml subcultured from an 85 day old EMN culture.

(ii) <u>By incubating gonococci at their minimum growth temperature (30°C)</u>

During this study it was found that, in practice, incubating gonococci at 30°C in liquid media had the benefit of prolonging the survival of the cells. The following experiments support this observation.

Experiment No.1 <u>Survival of gonococci in liquid media at 30°C</u>

The survival of gonococci was studied at 30°C in Mueller Hinton broth (Oxoid), Eugonbroth (BBL), and Tryptone soya broth (Oxoid) by inoculating 6 strains of gonococci each in the three media.

Table 3. 6 A comparison between gonococcal survival at 30 and 37°C in MHB (Oxoid)

Strain No.	Temperature (°C)	Time in days						
		3	7	10	15	20	25	35
N 15a	30	++	++	++	++	++	+	-
	37	++	++	++	±	-	-	-
N 15b	30	++	++	++	++	++	+	-
	37	++	++	++	++	-	-	-
N 20a	30	++	++	++	++	++	++	-
	37	++	++	++	++	-	-	-
N 20b	30	++	++	C				
	37	++	++	++	++	-	-	-
N 22a	30	+	++	++	++	++	+	±
	37	±	-	-	-	-	-	-
N 22b	30	++	++	++	++	++	++	±
	37	++	++	-	-	-	-	-
N 24a	30	±	++	++	++	++	+	+
	37	±	-	-	-	-	-	-
N 24b	30	±	±	±	+	+	+	+
	37	+	+	±	-	-	-	-
N 28a	30	±	-	-	-	-	-	-
	37	±	-	-	-	-	-	-
N 28b	30	-	-	-	-	-	-	-
	37	±	-	-	-	-	-	-
N 29a	30	±	-	-	-	-	-	-
	37	±	-	-	-	-	-	-
N 29b	30	±	-	-	-	-	-	-
	37	±	-	-	-	-	-	-

++: Heavy growth +: Growth ±: Scanty growth -: No growth
C: Contamination (a or b) after the strain numbers represent duplicates

Table 3. 7 A comparison between gonococcal survival at 30°C and 37°C in EB (BBL).

Strain No.	Temperature (°C)	Time in days						
		3	7	10	15	20	25	35
N 15a	30	++	++	++	++	++	+	-
	37	++	?	C				
N 15b	30	++	++	++	++	++	-	-
	37	++	++	++	+	±	-	-
N 20a	30	++	++	++	++	++	-	-
	37	++	++	++	-	-	-	-
N 20b	30	++	++	++	++	++	+	-
	37	++	±	±	-	-	-	-
N 22a	30	+	+	+	-	-	-	-
	37	+	-	-	-	-	-	-
N 22b	30	+	±	-	-	-	-	-
	37	-	-	-	-	-	-	-
N 24a	30	±	±	±	-	-	-	-
	37	+	±	-	-	-	-	-
N 24b	30	±	±	±	-	-	-	-
	37	+	-	-	-	-	-	-
N 28a	30	±	++	++	++	++	±	-
	37	++	-	-	-	-	-	-
N 28b	30	+	++	++	++	++	+	-
	37	++	++	+	-	-	-	-
N 29a	30	+	++	++	++	++	-	-
	37	+	+	-	-	-	-	-
N 29b	30	+	++	++	++	++	-	-
	37	±	-	-	-	-	-	-

++: Heavy growth +: Growth ±: Scanty growth -: No growth
C: Contamination (a or b) after the strain numbers represent duplicates
?: Doubtful result

Table 3. 8 A comparison between gonococcal survival at 30°C and 37°C in TSB (Oxoid)

Strain No.	Temperature (°C)	Time in days 3	7	10	15	20	25	35
N 15a	30	++	++	++	++	++	++	+
	37	++	C					
N 15b	30	++	++	++	++	++	++	±
	37	++	++	++	++	-	-	-
N 20a	30	++	++	++	++	++	++	-
	37	++	++	++	±	-	-	-
N 20b	30	++	++	++	++	++	++	-
	37	++	++	±	±	-	-	-
N 22a	30	++	++	++	++	++	-	-
	37	++	+	-	-	-	-	-
N 22b	30	++	++	++	++	++	-	-
	37	++	-	-	-	-	-	-
N 24a	30	±	++	++	++	-	-	-
	37	++	++	++	±	-	-	-
N 24b	30	+	++	++	-	-	-	-
	37	++	++	++	+	-	-	-
N 28a	30	±	-	-	-	-	-	-
	37	+	+	±	-	-	-	-
N 28b	30	+	C					
	37	+	+	+	-	-	-	-
N 29a	30	+	++	++	++	++	++	++
	37	++	++	++	-	-	-	-
N 29b	30	++	++	++	++	++	-	-
	37	++	+	++	++	-	-	-

++: Heavy growth +: Growth ±: Scanty growth -: No growth

C: Contamination (a or b) after the strain numbers represent duplications.

Each strain was inoculated in 4 replicates of each medium. The four replicates were incubated at 37°C for 24hr after which two of them were transferred to 30°C. The viability of all the cultures was checked after 3, 7, 10, 15, 20, 25 and 35 days by transferring a loopful of subculture to a CBA (Oxoid) plate and reporting the intensity of growth. Table 3. 6, 3.7, and 3.8 show results in MHB, EB and TSB respectively. Excluding cultures designated as contaminated (C), 75% of cultures in all three media survived at 30°C much longer than did their duplicates at 37°C. Only 9.3% survived longer at the latter temperature.

It should be pointed out that the procedure of this experiment is at a satisfactory standard of accuracy only because the inoculation for viability testing was standardized by using the same wire loop for all cultures. Because the subculture is performed from the original cultures and no diluting series is required three advantages follow, namely (a) contamination is less frequent, (b) the time needed for the procedure is much less, and (c) less equipment is needed.

Experiment No.2 <u>Survival of gonococci in EMN at 30°C as compared with their survival at 37°C</u>.

The efficiency of EMN in prolonging the survival of gonococci was increased, as with other liquid media, when the gonococcal cultures were incubated at 37°C for 24hr only, then transferred to 30°C. A comparison was made between the survival of cultures at 37°C and the survival of identical cultures (cultures of the same strains containing equal numbers of cells) at 30°C.

Table 3. 9 shows that the survival of 22 strains of gonococci at 30°C was longer than it was at 37°C.

Table 3.9 A comparison between the gonococcal growth at 30°C and 37°C in EMN (after growth for 1day at 37°C)

Strain No.	Temperature *	Time in days 5	10	15	20	25	30	35	40	45	60	70
N 15	30	++	++	++	++	++	++	++	++	++	++	++
	37	++	++	++	++	++	++	++	+	+	+	+
N 20	30	±	++	++	++	++	++	++	±	-	-	-
	37	++	++	++	++	-	-	-	-	-	-	-
N 22	30	+	++	++	++	++	++	++	+	+	-	-
	37	++	++	++	++	++	++	++	-	-	-	-
N 24	30	++	++	++	+	+	+	+	+	±	±	-
	37	++	++	++	++	+	±	±	+	±	-	-
N 28	30	++	++	++	++	++	++	C				
	37	++	++	++	++	++	+	+	-	-	-	-
N 29	30	++	++	++	++	++	++	++	++	++	++	+
	37	++	++	++	++	++	++	+	+	+	+	+
N 4	30	+	+	+	±	-	-	-	-	-	-	-
	37	+	+	+	-	-	-	-	-	-	-	-
N 7	30	+	+	++	++	++	++	++	++	++	-	-
	37	++	++	++	++	+	+	+	±	±	-	-
N 9	30	±	++	++	-	-	-	-	-	-	-	-
	37	++	++	±	-	-	-	-	-	-	-	-
N10	30	++	++	++	++	++	++	++	±	-	-	-
	37	++	++	++	++	++	++	++	+	-	-	-
N11	30	+	+	+	+	+	+	+	-	-	-	-
	37	+	+	+	+	C						
N13	30	±	±	±	+	-	-	-	-	-	-	-
	37	+	+	±	-	-	-	-	-	-	-	-

contd.

Table 3.9 contd.

Strain No.	Temperature *	Time in days										
		5	10	15	20	25	30	35	40	45	60	70
N14	30	±	++	++	++	++	++	++	++	±	-	-
	37	+	+	+	+	C						
N16	30	++	++	+	+	+	-	-	-	-	-	-
	37	++	+	±	-	-	-	-	-	-	-	-
N17	30	±	-	-	-	-	-	-	-	-	-	-
	37	+	+	+	±	-	-	-	-	-	-	-
N19	30	++	++	++	++	++	++	+	+	+	+	-
	37	++	++	++	++	+	+	±	-	-	-	-
N23	30	++	++	++	++	++	++	++	++	+	+	+
	37	++	++	++	++	++	++	+	-	-	-	-
N25	30	+	+	++	++	++	++	±	-	-	-	-
	37	++	++	++	++	++	+	+	+	-	-	-
N26	30	+	++	++	++	++	++	++	±	±	-	-
	37	++	++	++	++	+	+	-	-	-	-	-
N27	30	++	++	++	++	++	++	+	-	-	-	-
	37	++	++	++	+	+	-	-	-	-	-	-
N30	30	++	++	++	±	-	-	-	-	-	-	-
	37	++	++	C								
N31	30	+	-	-	-	-	-	-	-	-	-	-
	37	+	+	±	-	-	-	-	-	-	-	-

++: Heavy growth +: Growth

±: Scanty growth -: No growth C: Contamination

*: Temperature is in degrees centigrade

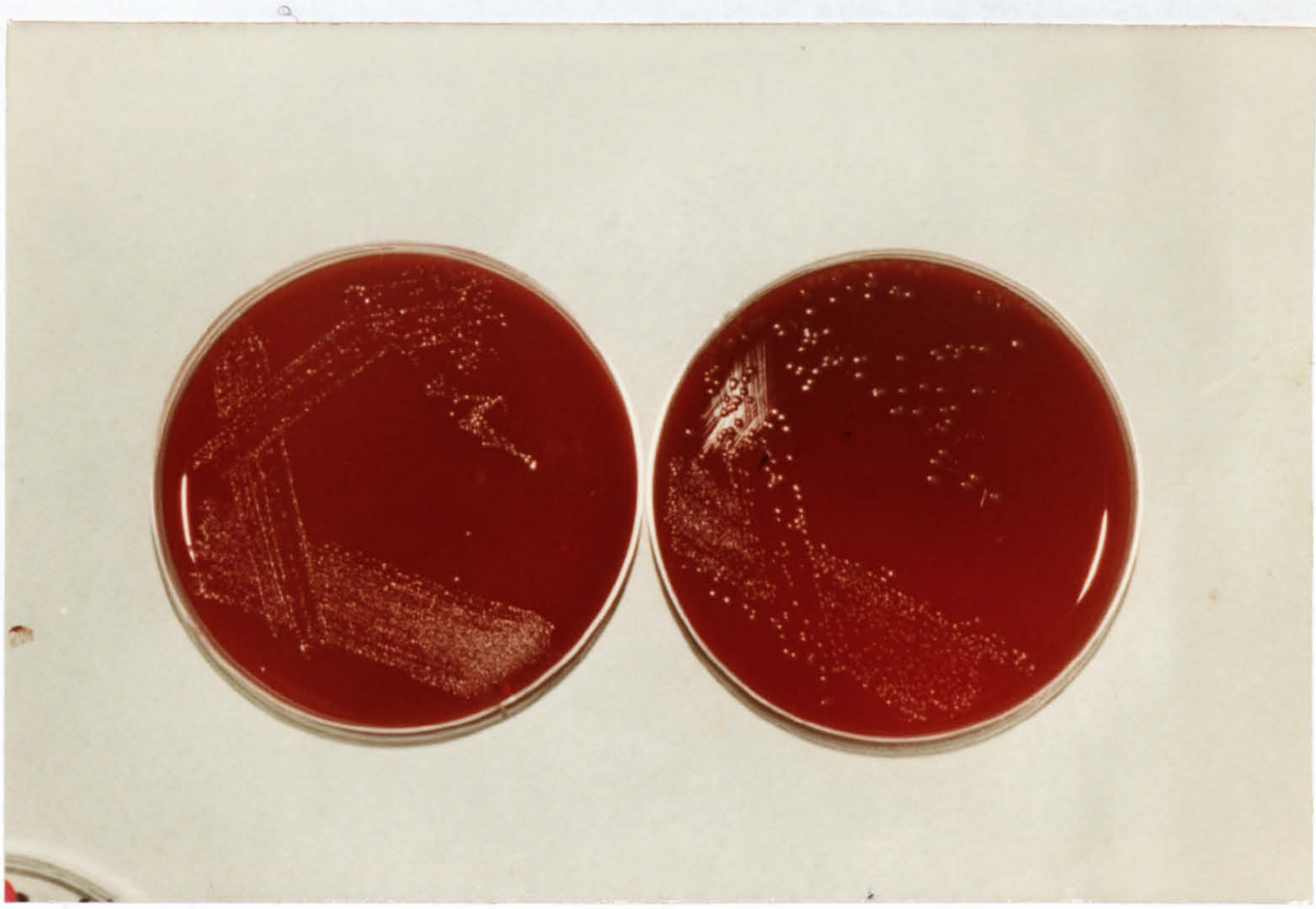


Fig. 3.5 A 24hr growth (on CBA plates) of 1 loopful subcultured from EMN cultures of 2 gonococcal strains which had been incubated at 30°C for 35 days.

■ EMN at 30°C

● EMN at 37°C

□ MHB at 30°C

○ MHB at 37°C

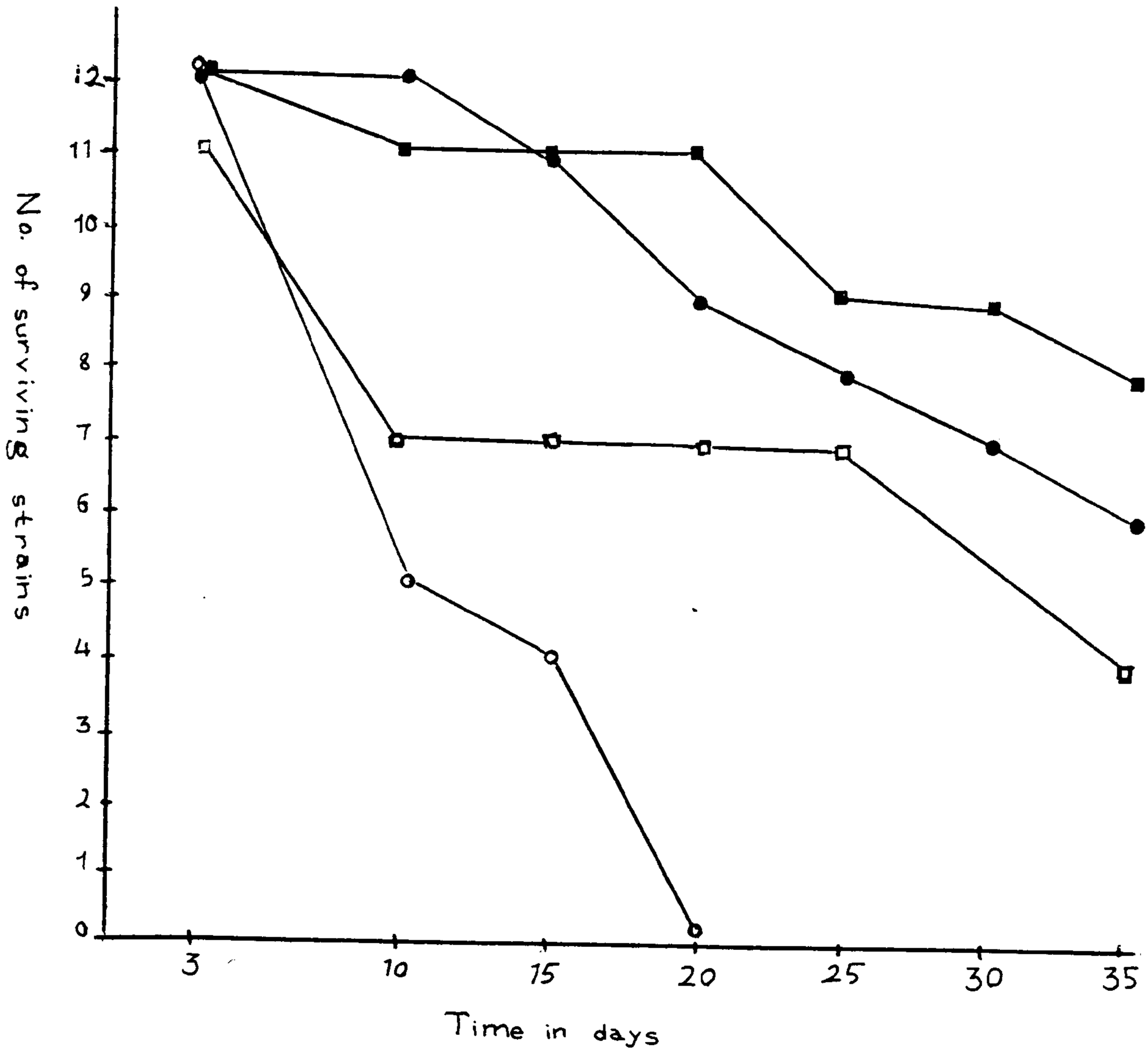


Fig. 3. 6 A comparison between survival of gonococci at 30 and 37°C in MHB (Oxoid) and EMN.

■ EMN at 30°C

● EMN at 37°C

□ EB at 30°C

○ EB at 37°C

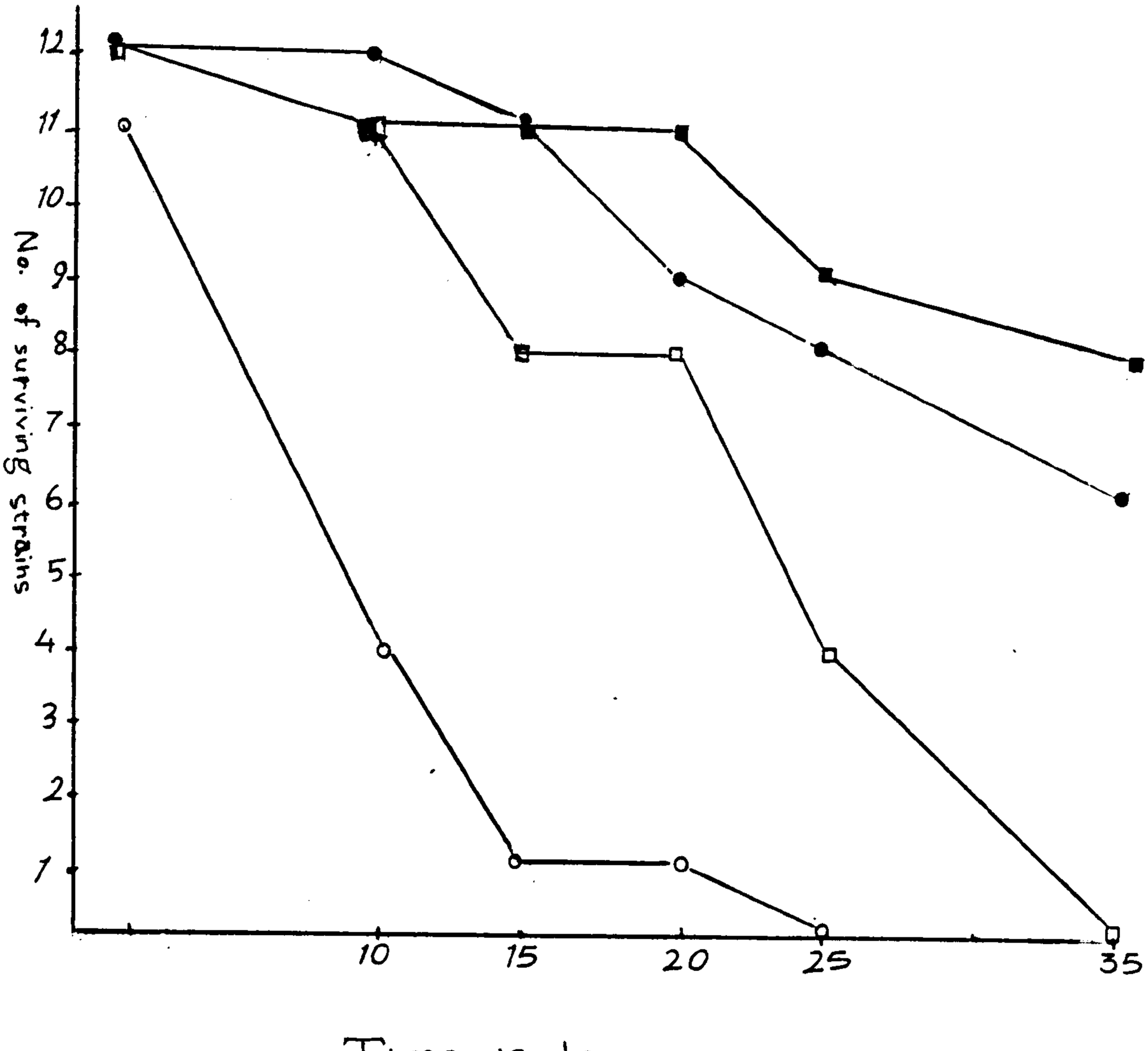


Fig. 3. 7 A comparison between survival of gonococci at 30 and 37°C in EB (BBL) and EMN

■ EMN at 30°C

● EMN at 37°C

□ TSB at 30°C

○ TSB at 37°C

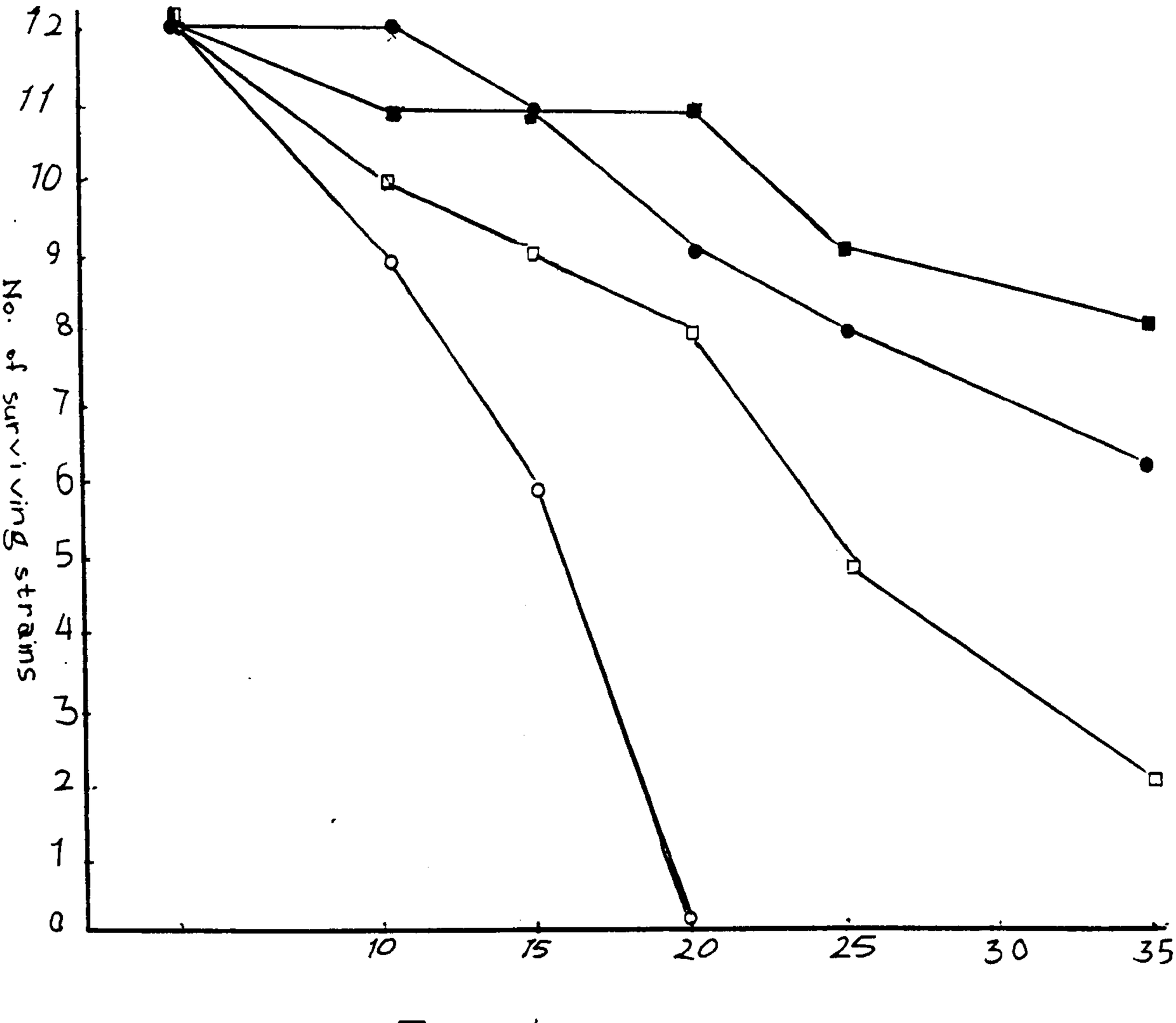


Fig. 3. 8 A comparison between survival of gonococci at 30 and 37°C in TSB (Oxoid) and EMN

Figure 3. 5 shows a 24hr growth on CBA incubated in EMN at 30°C for 35 days. Comparisons are made in graphical form in Figures 3.6, 3.7 and 3.8 between the survival of gonococci in EMN with that in MHB (Oxoid), EB (BBL), and TSB (Oxoid) at 37 and 30°C. The information has been abstracted from Tables 3.6, 3.7 and 3.8 as well as Table 3.9.

The combination of medium and temperature which are best for prolonging survival are (in decreasing order of efficiency) 1. EMN at 30°C, 2. EMN at 37°C, 3. the compared medium (MHB, EB or TSB) at 30°C and 4. the compared medium at 37°C.

Experiment No.3 Total viable count (TVC) of gonococci in EMN at 30°C and 37°C

Standard volumes of EMN broth cultures of 2 gonococcal strains (nos. N60 and N63) were inoculated in duplicate into EMN, contained in 50ml conical flasks, and incubated at 37°C. After 1 day one culture of each duplicate was transferred to 30°C. TVC's were performed at the time of inoculation (T_0) as well as after 1, 2, 3, 4, 5, 8, 16 and 35 days. Table 3. 10 shows the results of the counts. It can be seen that at 37°C the numbers of cells increased more rapidly and that there was a higher growth yield than at 30°C throughout the first 8 days. After 16 days counts at 37°C progressively decreased while counts at 30°C remained more or less steady or even increased. After 35 days the number of cells at 37°C was very much reduced and approached zero, whereas at 30°C the reduction in the number of cells was much slower. The inference is that the cells, growing more slowly at 30°C than at their optimum temperature (37°C), utilize the medium more sparingly.

Table 3. 10 TVC's of gonococci incubated at 30 and 37°C in EMN

Strain No.	Temperature (°C)	Time in days								
		T_0	1	2	3	4	5	8	16	35
N 60	37	$1x10^5$	$7.1x10^6$	$1.24x10^7$	$1.6x10^7$	$2.6x10^8$	$6x10^8$	$7.1x10^8$	$1.2x10^7$	0
	30*	$1x10^5$	$3.4x10^6$	$4.1\ x10^6$	$1x10^7$	$1.6x10^7$	$2.3x10^7$	?	$2x10^7$	$1.6x10^7$
N 63	37	$1.3x10^5$	$4x10^6$	$6.8\ x10^6$	$8.4x10^6$	$7.8x10^7$	$2x10^8$	$9x10^8$	$1.8x10^7$	$1.5x10^2$
	30*	$1.3x10^5$	$5.9x10^6$	$6.5x10^6$	$7.6x10^6$	$1.7x10^7$	$2.4x10^7$	$2.3x10^7$	$2.8x10^7$	$2.2x10^4$

* : First 24hr incubated at 37°C

?: No results obtained.

Thus, not only are nutrients conserved but the accumulation of toxic metabolic waste-products is also retarded, the effect of both these processes being to prolong the survival of the organisms.

Experiment No.4 Comparison between survival of gonococci in EB (BBL) and EMN at various temperatures

The survival of 6 strains of gonococci in both EMN and Eugonbroth (BBL) at temperatures 30, 29 and 28°C as well as 37°C was compared. The effect of incubation at 37°C for 24hr prior to incubation at 30, 29 or 28°C was also investigated. All the cultures were checked for viability after 1 and 2 weeks.

Table 3. 11 shows details of the results.

There were 12 cultures at each temperature and each culture was checked twice for viability so that survival at each of the 7 various temperatures of incubation could be compared through 168 viability tests. From the results given in Table 3.11 the following observations can be made.

1. At all temperatures of incubation, except at 37/29°C, the number of cultures surviving in EMN was higher than in EB (at 37/29°C the survival rate was the same in both media). This again shows that EMN favours the survival of gonococci more than EB medium.

2. Five cultures survived for 2 weeks at each of the temperatures 37, 30 and 28°C. Of these survivors the number of cultures showing a heavy growth were 1, 4 and 5 respectively. Again this shows that gonococci survive longer at 30°C than at 37°C and that they might survive even longer at 28°C.

3. Six cultures survived for 2 weeks at 37/30°C, 4 of them showing a heavy growth and none of the remaining cultures was contaminated. Six other cultures survived, in one or other of the

Table 3.11 Comparison between survival of gonococci at various temperatures in EB (BBL) and EMN

Temperature:		37°C		30°C		29°C		28°C		37-30°C*		37-29°C*		37-28°C*	
Medium:		EB	EMN	EB	EMN	EB	EMN	EB	EMN	EB	EMN	EB	EMN	EB	EMN
Strain No.	Time														
N 201	1wk	+	+	±	+	C	C	-	++	++	++	C	C	-	-
	2wk	+	+	-	++	C	C	-	++	-	+	C	C	-	-
N 211	1wk	-	-	-	++	±	++	-	±	-	++	++	++	-	-
	2wk	-	-	-	++	-	++	-	-	-	-	++	++	-	-
N 215	1wk	+	++	++	++	C	++	-	++	++	++	-	++	-	++
	2wk	-	-	++	-	C	C	-	++	++	++	-	++	-	++
N 216	1wk	-	±	+	++	++	C	-	++	-	++	C	C	-	++
	2wk	-	+	+	-	C	C	-	++	-	++	C	C	-	-
N 222	1wk	+	++	-	++	?	++	±	-	-	+	+	C	++	C
	2wk	-	-	-	-	-	C	-	-	-	-	+	C	-	C
N 224	1wk	++	++	++	++	-	++	++	++	++	++	++	+	+	-
	2wk	++	+	-	++	-	+	++	++	+	++	++	++	-	-

++: Heavy growth +: Growth ±: Scanty growth

-: No growth C: Contamination

*: Incubation at 37°C for 1 day.

two media, for 2 weeks at 37/29°C, 5 of them showing a heavy growth, although 5 of the rest of cultures were contaminated and survival could not be tested. Evidently incubation at 37°C for 1 day prior to incubation at 30°C, or a little below, provides optimum conditions for the survival of gonococci in a suitable liquid medium.

4. Gonococci can survive at 28°C for 2 weeks or more in liquid media. It still needs more investigation to confirm whether or not growth occurs at a temperature below 30°C.

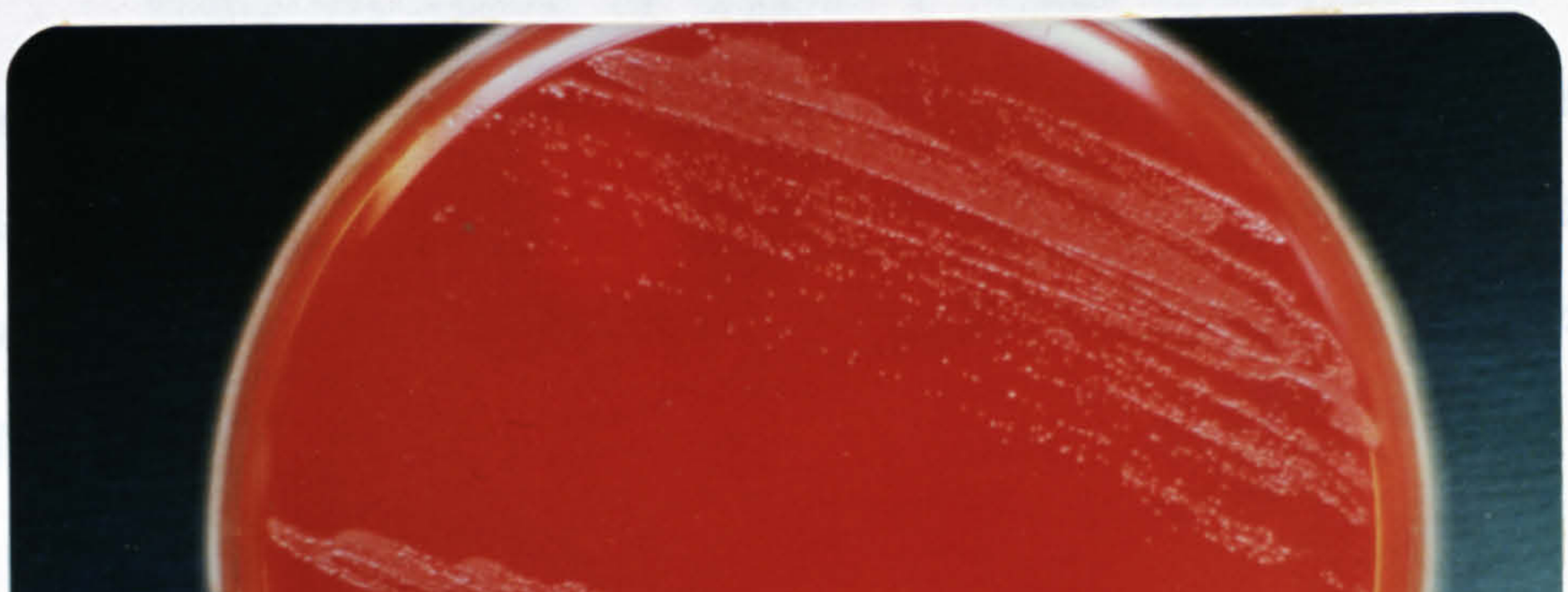


Fig. 3.9 24hr growth, on CBA (Oxoid), of 1 loopful taken from EB culture (above) and EMN culture (below). Both cultures had been incubated at 27°C for 1 day.

One strain (No. N211) was inoculated in EB (BBL) and EMN and incubated immediately in a water bath at 27°C ± 0.5°C. The two cultures were daily tested for viability. The strain survived for 4 days in EMN and 2 days in EB. Fig. 3.9 shows a 24hr growth of one loopful subcultured on CBA (Oxoid) from the 2 cultures after 1 day at 27°C; the growth from the EMN culture is clearly heavier than that from EB culture. One possible explanation is that there may be some factor present in EMN which slows down the rapid loss of gonococci seen in EB medium.

3. 2 Production of inhibitory substances by gonococci

Using the method of Kekessy & Piguet (1970) for the detection of bacteriocins, 50 strains of gonococci were tested for their ability to produce gonocins. Each was tested against itself as well as 5 - 7 other indicator strains. The results are given in Table 3.12.

Table 3.12 The effect of inhibitory substances produced by gonococci using gonococcal strains as indicators

Strain tested as a producer	Producer tested against itself (as an indicator)	Number of other indicator strains tested			
		Susceptible (+)	Partially resistant (±)	Resistant (-)	Total
N133	-	0	0	7	7
N134	+	4	0	3	7
N135	+	7	0	0	7
N136	+	3	2	0	5
N137	+	0	1	5	6
N138	-	0	0	7	7

contd..

contd.

Strain tested as a producer	Producer tested against itself (as an indicator)	Number of other indicator strains tested			
		Susceptible (+)	Partially resistant (±)	Resistant (-)	Total
N139	-	0	2	5	7
N140	-	0	0	5	5
N141	-	0	0	7	7
N142	-	0	0	7	7
N144	-	0	0	7	7
N145	+	4	1	2	7
N146	+	6	0	0	6
N147	±	0	2	4	6
N148	-	0	0	7	7
N149	+	0	1	6	7
N150	-	0	2	5	7
N151	+	6	1	0	7
N152	+	4	1	0	5
N153	-	0	0	6	6
N154	+	4	1	1	6
N155	+	4	0	3	7
N156	+	1	4	0	5
N157	+	6	1	0	7
N158	+	6	0	0	6
N159	+	5	1	0	6
N160	+	4	1	0	5
N162	-	0	0	5	5
N163	-	0	0	5	5
N166	-	0	0	5	5
N167	+	2	0	3	5

contd...

contd.

Strain tested as producer	Producer tested against itself (as an indicator)	Number of other indicator strains tested			
		Susceptible (+)	Partially resistant (±)	Resistant (-)	Total
N168	+	5	0	0	5
N169	+	5	0	0	5
N170	+	4	1	1	6
N171	+	0	0	6	6
N172	-	0	0	6	6
N173	+	3	1	3	7
N174	+	3	2	1	6
N176	-	0	0	5	5
N177	±	4	2	0	6
N180	+	2	3	1	7
N181	+	3	2	2	7
N182	+	3	2	0	5
N183	+	3	1	3	7
N184	-	0	0	6	6
N185	+	4	1	2	7
N187	+	5	2	0	7
N190	-	0	0	7	7
N191	-	0	0	6	6
N192	-	0	0	5	5

+ : Susceptible ± : Partially resistant - : Resistant

It can be seen that 32 strains (64%) produced inhibitory substances to which some or all of the tested strains were susceptible. But, unlike bacteriocin-producing bacteria, most of the producer strains were themselves susceptible to their own inhibitors. Furthermore, one producer (N171), which was susceptible to its own inhibitor, had no effect on 7 other strains against which it was tested.

Only 2 strains (N139 and N150) which produced inhibitors to other strains were resistant to their inhibitors. The inhibitors produced by these two strains could be true bacteriocins but, even if they were, they would be only of limited value for typing purposes because

1. They would have to be extracted to make sure that no other inhibitors interfered with their action.
2. Only 2 out of 7 strains were inhibited by each of these two producers - and even they were only partially inhibited; the rest were resistant.
3. The inhibitor was not produced by either of the two strains when tested on CBA (Oxoid) (Table 3.13).

Table 3.13 Test of production of inhibitory substances by gonococci on CBA (Oxoid)

Producer strain	Indicator strain						
	Producer against itself	N140	N163	N164	N165	N166	N167
N137	-*	-	-	-	-	-	-
N139	-	-*	-	-	-	-	-
N150	-	-*	-	-	-	-	-
N156	-*	-*	-*	-	-	-	-

contd...

contd.

Producer strain	Producer against itself	Indicator strain N140	N163	N164	N165	N166	N167
N159	-*	-*	-	-*	-	-	-
N168	-*	-	-	-*	-	-	-

- : No inhibition * : Complete or partial inhibition occurred on GC medium

An attempt was made to ascertain whether strains which had been found to produce inhibitors when growing on GC medium (Oxoid) also produced them on CBA (Oxoid). Table 3.13 shows that no such inhibitory substances were produced by any of six strains tested. Perhaps the medium lacks some essential ingredient, probably glucose, without which inhibitors cannot be produced.

From the observations made thus far a gonococcal typing scheme based on gonocin inhibition patterns did not seem practicable.

To investigate the nature of the inhibitory substances, it was necessary to screen for the presence of other possible inhibitors, mainly fatty acids. Gas-liquid chromatography made it possible to resolve this matter. (See Section 3.4.3).

3. 3 Colicins of Sh. sonnei

3.3.1 Inhibition of gonococci by colicins

As already mentioned in the review of the literature, bacteriocins have been successfully applied to the typing of bacteria. Yet the work so far described shows that gonocins are not satisfactory for this purpose. But gonococci are susceptible to a wide range of bacteriocins. Therefore it was considered worthwhile attempting to

find some other suitable class of bacteriocins that could be used for typing these organisms.

The following requirements govern the choice of bacteriocins for this purpose:-

1. The bacteriocins should inhibit gonococcal strains selectively.
2. It should be possible to choose a set of bacteriocin producer strains in such a way that gonococcal strains could be sub-divided according to their sensitivity to the bacteriocins.
3. The bacteriocins should be produced by strains which are (a) easily maintained in the laboratory, (b) strong enough to survive frequent subculture, and (c) highly stable in their productive ability.
4. The producer strains should preferably belong to a set already used in bacteriocin typing.

Colicin Type (C.T.) strains of Sh. sonnei seemed to meet the last two of these requirements. Whether they met the first two remained to be demonstrated.

Ninety two strains of gonococci were tested for their sensitivity to 10 C.T. strains using the Kekessy & Piguet method. Table 3.14 shows details of the patterns of inhibition of each strain by the 10 C.T. strains. Strains which show inhibition or partial inhibition are designated as (+) or (±) respectively. Those which show no inhibition are designated as (-).

It was thus possible, by means of colicin-typing, to classify the gonococcal strains into 32 fairly well-defined groups. These groups, however, could not be correlated with colonial morphology; there is an observed reason for this.

Table 3.14 Patterns of inhibition of gonococcal (G.C.) strains by Colicin Type (C.T.) strains of Sh. sonnei

G.C strain	Pt.Sex/ G.C. Source	C.T. strain 1B	2	3A	4	5	6	8	9	11	13	Inhibition pattern
N15	XY/Psy.	-	+	±	±	+	+	+	-	+	-	A
N20	XY/Abn.	+	+	+	-	+	+	+	-	-	-	B
N22	XY/L/BC	-	+	-	±	+	+	-	-	+	±	C
N29	XY/L/SH	+	+	-	-	+	+	+	-	-	-	D
N30	XY/L/SH	+	+	+	-	+	+	+	-	-	-	B
N34	XY/L/BC	+	+	-	-	+	-	-	+	±	-	E
N35	?/?	±	+	+	±	+	+	±	+	-	+	F
N36	?/?	-	±	±	-	+	+	±	+	-	+	G
N37	XY/L/SH	+	+	-	+	±	+	+	-	-	+	H
N39	?/?	-	-	-	-	-	-	-	-	-	-	I
N40	XY/L/BC	+	+	-	+	-	+	+	±	-	+	J
N42	XY/L/BC	+	+	+	-	+	+	+	-	-	±	K
N43	XY/?	+	+	+	-	+	+	+	-	-	-	B
N44	XY/L/SH	+	+	+	+	-	+	+	-	+	+	L
N46	XY/Ayr	-	+	±	+	+	+	±	-	+	-	A
N48	XY/?	+	+	+	+	-	+	+	-	+	+	L
N49	XY/L/BC	+	-	+	+	+	-	+	+	+	+	M
N50	XX/?	-	+	-	-	+	+	-	-	-	+	N
N52	XY/L/BC	-	+	+	-	+	+	±	+	-	+	G
N53	XY/L/BC	+	±	±	+	-	+	+	-	-	+	K
N58	?/?	+	+	+	±	+	+	+	±	±	+	P
N134	XY/L/SH	-	+	-	+	+	+	-	-	+	+	C
N135	XX/L/SH	±	-	-	-	+	-	-	-	-	-	Q

contd..

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G.C. strain	Pt.Sex/ G.C. Source	C.T. strain 1B	2	3A	4	5	6	8	9	11	13	Inhibition pattern
N136	XX/Psy.	+	+	+	-	+	-	-	+	-	-	R
N138	XY/L/SH	±	+	+	+	+	+	±	+	+	+	P
N139	XY/L/BC	±	-	-	-	+	-	-	-	-	-	Q
N141	?/?	+	-	±	+	±	+	+	-	-	+	S
N142	XY/L/WI	+	-	-	-	+	-	-	-	-	-	Q
N144	XX/L/BC	+	±	+	+	-	+	+	-	-	+	K
N146	XY/Flk.	+	-	+	+	-	+	-	+	-	-	T
N147	XY/L/BC	+	+	-	+	+	-	-	+	-	+	U
N148	?/?	+	+	+	-	+	+	+	-	-	+	K
N149	XX/L/SH	+	+	+	+	+	+	+	+	+	+	P
N150	XX/L/SH	+	-	±	+	-	+	-	+	-	-	T
N151	XX/L/BC	+	+	-	+	+	-	-	+	-	+	U
N153	XY/L/SH	+	+	-	+	+	-	-	+	-	+	U
N155	XY/L/BC	+	+	-	-	-	+	+	+	-	-	V
N156	XY/Ncl.	-	+	-	±	+	+	+	-	-	-	W
N157	?/?	+	+	-	+	+	-	-	+	-	+	U
N159	XY/L/BC	+	+	-	-	±	-	-	+	+	-	E
N160	XY/?	+	+	-	-	+	-	-	+	+	-	E
N164	XY/L/BC	+	-	+	+	+	+	+	-	-	+	S
N166	XX/Edin.	+	+	-	+	+	-	-	±	-	±	U
N169	?/?	-	+	-	-	+	+	-	-	-	+	N
N170	XY/?	-	+	-	+	+	+	-	+	+	+	X
N173	XY/?	-	-	-	-	+	+	-	+	+	+	Y
N174	?/?	+	+	-	±	-	+	+	+	-	±	J
N175	?/?	+	+	-	-	+	-	-	+	+	-	E
N176	XY/L/BC	+	+	-	-	-	+	±	+	-	-	V

contd..

contd.

G.C. strain	Pt.Sex/ G.C. Source	C.T. strain 1B	2	3A	4	5	6	8	9	11	13	Inhibition pattern
N179a	XX/U.K.	-	±	-	-	+	+	-	-	-	+	N
N179b	XX/U.K.	-	±	-	-	+	+	-	-	-	+	N
N181	XY/L/SH	-	+	-	-	+	+	-	-	-	±	N
N190	?/?	+	±	-	-	-	+	+	+	+	+	Z
N191	XY/L/BC	+	+	+	+	+	+	+	+	±	+	P
N194	XX/Edin.	+	+	-	+	+	-	-	±	-	+	U
N195	XY/L/BC	+	-	+	±	+	-	+	+	±	+	M
N196	XY/L/BC	+	-	+	±	+	-	+	+	±	±	M
N198	XY/Hns.	-	+	-	+	+	-	NT	+	-	-	Z1
N199	XY/L/BC	+	+	+	+	+	±	+	+	+	+	P
N197	XY/L/BC	+	+	+	+	+	+	+	±	+	-	Z2
N201	?/?	+	-	-	-	-	-	±	±	-	±	Z3
N202	?/?	-	-	-	-	-	-	-	-	-	-	I
N203	?/?	+	+	-	+	+	±	+	-	-	+	H
N205	XY/?	+	+	-	+	+	-	-	+	-	+	U
N200	XY/L/BC	+	NT	+	+	±	-	+	-	+	±	Z4
N207	XY/L/SH	+	±	+	NT	+	+	NT	+	-	+	F
N206	XY/L/SH	+	+	+	NT	+	+	+	+	-	+	F
N208	?/?	+	+	+	+	+	+	+	+	+	-	Z2
N209	XX/Psy.	+	±	±	+	+	+	+	±	±	±	P
N210	XY/L/BC	+	+	+	+	+	+	+	±	±	-	Z2
N211	XY/L/BC	+	+	+	+	±	+	+	+	+	+	P
N212	XY/L/BC	+	+	+	+	+	+	+	+	+	+	P
N213	XY/L/BC	+	-	+	+	+	±	+	-	-	+	S

contd..

contd.

G.C. strain	Pt.Sex/ G.C. Source	C.T. strain 1B	2	3A	4	5	6	8	9	11	13	Inhibition pattern
N214	XY/L/BC	+	+	+	+	±	-	+	-	+	+	Z4
N215	XX/L/BC	+	+	+	±	+	+	+	+	+	+	P
N216	XY/L/BC	+	-	±	-	+	+	+	+	+	+	Z5
N217	XY/?	+	+	+	+	±	+	±	+	-	±	F
N218	?/?	+	+	+	±	+	+	+	-	+	+	Z6
N219	XY/L/BC	±	+	+	+	+	+	+	-	+	+	Z6
N220	XY/L/BC	+	+	+	+	+	+	NT	+	+	+	P
N221	XY/L/BC	-	-	-	-	-	-	-	-	-	-	I
N222	XY/L/BC	-	-	-	-	-	-	-	-	-	-	I
N223	XX/L/BC	+	+	NT	+	+	+	+	+	+	+	P
N224	XX/L/BC	+	+	+	+	-	+	+	-	-	+	K
N236	XY/Lon.	-	+	+	+	+	-	+	±	+	+	Z7
N241	XX/?	±	+	+	+	±	+	+	+	+	-	Z2
N242	?/?	+	+	±	+	+	+	+	+	+	-	Z2
N243	XY/?	+	+	+	+	+	+	+	+	-	+	F
N244	XY/L/SH	+	+	+	+	+	+	+	+	-	+	F
N245	?/?	+	+	+	+	+	+	+	±	+	+	P
N246	?/?	+	+	+	+	+	+	+	±	+	+	P
N228	XX/L/BC	+	+	+	+	-	+	+	-	-	+	K

+: Inhibition; ±: Partial inhibition; -: No inhibition;

N.T.: Not tested; XY: Male; XX: Female; ?: Unknown source;

Psy: Paisley (Scotland); Abn.:Aberdeen (Scotland); Flk:Falkirk (Scotland); Ncl.: Newcastle (England); Edin:Edinburgh (Scotland);

contd. .

contd.

Hns: Honduras; Lon. London; L/BC, L/SH, or L/WI: Strain isolated from a case who had been infected locally and was attending Black Street Clinic, Southern General Hospital or Western Infirmary (all in Glasgow) respectively.

For the purpose of this work, as already explained, Kellogg's five types (T1 - T5) were reduced to two, namely Type A, comprising T1 and T2, and Type B, to which T3, T4 and T5 were assigned. But at the very first subculture, which was invariably performed at the Glasgow City Laboratory (where the strains were isolated), almost all the Type A strains were converted to Type B; it was extremely rare for them to remain unchanged on subculture. Few strains, therefore, were obtained as Type A. Of these few two (N221 and N222) are in Group I, which also includes two Type B strains. Another Type A strain (N142) is in Group Q which again includes two other strains of Type B. It seems clear that the pattern of inhibition of a strain by colicins bears no relationship to its colonial type.

As the vast majority of the strains tested were isolated from patients reported to have acquired their infections locally, and very few cases had been infected elsewhere, evaluation of the usefulness of this typing scheme in contact tracing was limited.

By consideration of the clinic or hospital attended by the patients from whom the strains were isolated, it was possible to subdivide the locally acquired infections, broadly, into three groups, namely the cases attending three different clinics in Glasgow, respectively, the Black Street Clinic (BC), the Southern General Hospital (SH) and the Western Infirmary (WI). Despite the limited number of sources of infections there is an indication that the cases in one group could be in some way connected and, conversely, that they could be differentiated from cases in other groups.

Examples which give the indication of possible relatedness between

source of infections and pattern of the isolated strain are the following. Group F contains 6 strains, three of which were locally acquired infections and the strains were isolated from patients attending the Southern General Hospital. Group K includes 6 strains; five were from patients attending the Black Street Clinic. In Group P which includes 13 strains, seven were isolated from patients attending the Black Street Clinic and 3 from Southern Hospital patients. Two strains (N166 and N194) which were the only strains reported to be from Edinburgh are both grouped together in Group U. Groups V and Z4 each includes two strains isolated from local infections of patients attending the Black Street Clinic. Only one strain (N156) was reported to be from Newcastle and it is included singly in Group (W). So are strains number N198 and N236; both are singly included in their groups (Z1 and Z7) and they were single isolates from Honduras and London respectively.

No evidence suggests that there is any relation between sex of the patient and group of gonococcal strain.

Fig. 3. 10 illustrates some of the results given in Table 3.14. It shows the pattern of inhibition of C.T.6 and C.T.9 on the same three strains of gonococci used as indicators. Strain N212 is completely inhibited (+) by C.T.6 while N213 is only partially inhibited (±) and N214 shows no inhibition (-). When the same three strains are tested against C.T.9, N212 is again completely inhibited but the other two show no inhibition at all.

Fig. 3. 11 shows the black diametrical band which resulted from the growth of C.T.1B. Three strains of gonococci N209, N210 and N211 are all completely inhibited. The reflection of the flashlight in the surface of the medium reveals the lines left by the wire loop in the

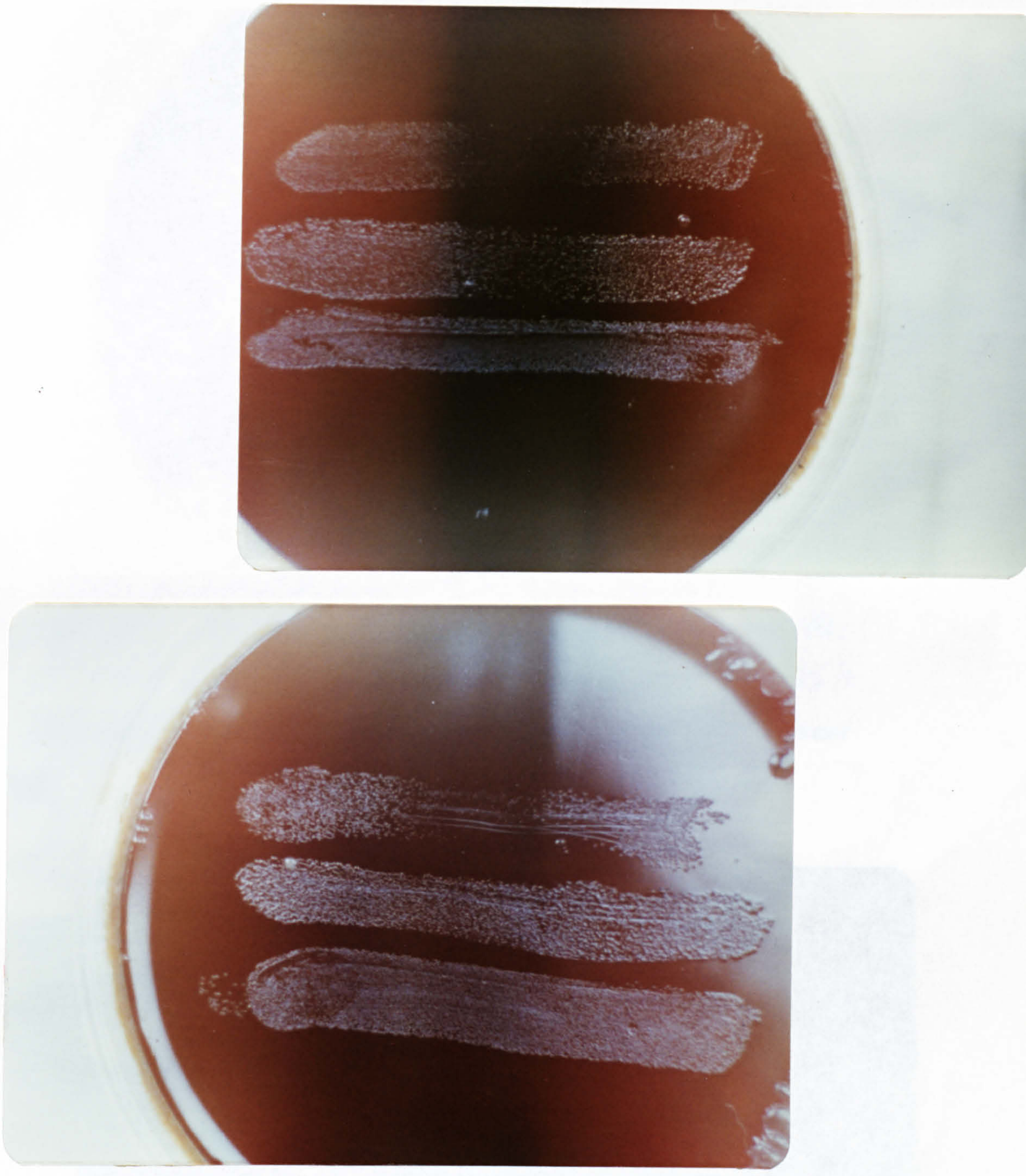


Fig. 3.10. Pattern of inhibition by C.T. strains 6 and 9 on three strains of gonococci streaked at right angles. The dark vertical area was left by C.T. strain after growth on the reverse side of the medium. The results are graded as + , ± , and - on C.T.6 respectively and + , - and - on C.T.9 (Table 3.14).

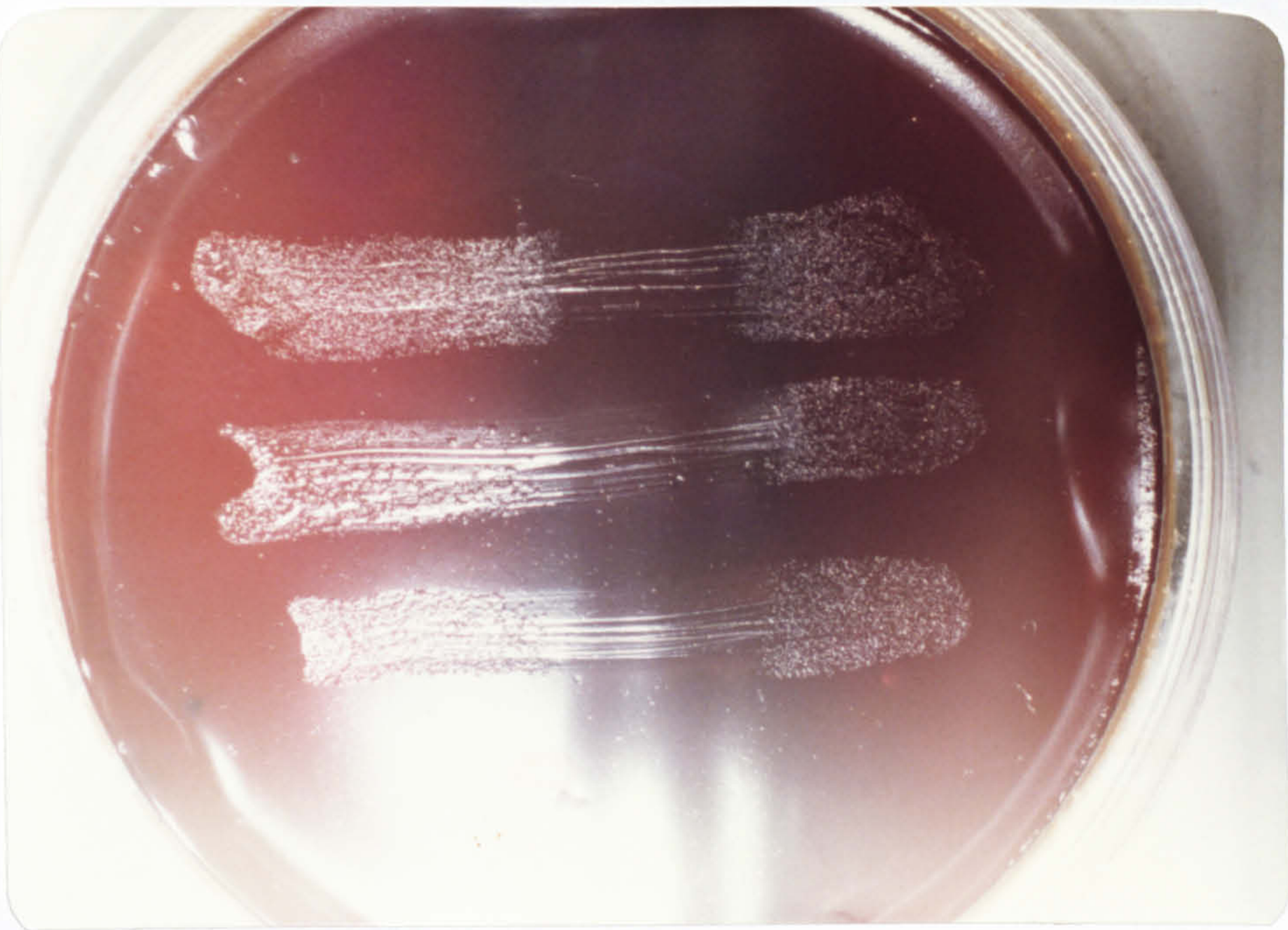


Fig. 3.11. Inhibition pattern of three gonococcal strains, N209, N210 and N211 (in downward sequence) caused by C.T. 1B which had grown on the other side of the medium (in the dark vertical diametric area).

Fig. 3.12 Inhibition (at higher magnification) caused by previous growth of a C.T. strain (streaked vertically on the other side of the medium)on a gonococcal strain (streaked horizontally)

central zones during streaking thus rendering very obvious the oomplete inhibition of growth in those areas. Fig. 3.12, photographed at higher magnification shows the black diametrical band due to the growth of a C.T. strain on the reverse surface of the medium and the growth of a gonococcal strain streaked at right angles to it. The growth of the latter has been completely inhibited in the zone overlying the previous growth (the very few colonies viable in this zone are not considered significant). It can also be seen that the gonococcal strain has been inhibited at the middle but not near the edges of the dark band.

C.T. strains growing on brain heart infusion blood agar always left these black bands around and underneath their growth. On further investigation it was shown that a similar blackening of this medium can be produced by pouring a few drops of dilute organic acids (acetic or lactic) or 20% sulphuric acid - but <u>not</u> 40% caustic potash - on its surface.

From these observations it was deduced that (a) acids produced by C.T. strains as a result of their metabolic activities caused the blackening of the medium, and (b) gonococcal strains were not inhibited by the acids in the zone of former growth of the C.T. strain - otherwise the inhibition would have extended to the full width of the band.

It was therefore inferred that the inhibition was a specific effect of the colicin.

3. 3. 2 <u>Stability of colicin production</u>

All ten C.T. strains of <u>Sh. sonnei</u> used in the typing of gonococci were checked regularly for their stability in producing colicins. This was done by observing the patterns of inhibition

by the C.T. strains, of 10 indicator strains (I.S.) of Sh. sonnei on Brain heart infusion blood agar (Oxoid). Table 3.15 shows the inhibition patterns of the 10 indicator strains which for this investigation were tested in duplicate.

Table 3.15 The pattern of inhibition by colicin type (C.T.) strains on indicator strains (I.S.) of Sh. sonnei

I.S.	C.T. 1B	2	3A	4	5	6	8	9	11	13
1	+/+	-/-	+/+	+/+	+/+	-/-	+/+	+/+	-/-	+/-
2	+/+	+/+	+/+	+/+	+/+	+/+	-/-	+/+	-/+	+/+
4	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/+
5	-/-	-/-	+/+	+/+	+/+	-/-	±/±	-/-	-/-	-/-
6	+/+	-/-	+/+	-/-	+/+	+/+	-/-	+/+	-/+	+/+
7	+/+	-/-	+/+	-/-	+/+	+/+	-/-	+ +	± ±	+ +
8	-/-	-/-	+/+	+/+	+/+	-/-	+/+	-/-	-/-	-/-
9	+/+	+/+	+/+	+/+	+/+	+/+	-/-	+/+	+/+	+/+
13	-/-	-/-	+/+	+/+	+/+	-/-	+/+	±/0	-/-	-/-
15	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	±/+	+/+

+ : Inhibition ± : Partial inhibition - : No inhibition

0 : Not tested

Duplicate results are given throughout

The indicator strain numbers correspond respectively to

Sh. sonnei 2, 56, 2M, 38, 56/56, 56/98, R1, R6, 2/15 and E.coli Row

Three of the indicator strains did not give identical duplicate results. These were I.S. 1 tested against C.T.13, I.S.2 and I.S.6 against C.T.11. However, they all showed inhibition more frequently than resistance.

3. 3. 3 Effect of extra incubation of C.T. strains on inhibition of indicator strains (I.S.) of Sh. sonnei and gonococcal strains

During tests of sensitivity of gonocin to colicins it was noticed that there was a slight difference in the pattern of inhibition of some strains when C.T. strains were incubated for about 4 days at 37°C as compared with the patterns obtained when the C.T. strains were incubated for one day only. This observation was confirmed when 17 strains of gonococcci chosen at random were tested after incubating the C.T. strains for 1 day and again after incubating them for 4 days. Table 3. 16 shows that there are differences between the patterns of inhibition obtained after 1 day's incubation and those obtained after 4 days incubation of the C.T. strains. Strains N37, N40 and N48 show these variations, each one showing greater inhibition when tested against C.T. strains which had been incubated for the longer period. It was therefore assumed that, on prolonged incubation of C.T. strains, they were either slow producers of bacteriocin, or produced other by-products which interfered with the inhibitory activity of the colicin. When, however, all the Shigella indicator strains were tested in the same way, they also showed variations in some of the inhibition patterns. In contrast, the difference was always a loss of inhibition as a result of prolonged incubation of the C.T. strains. (Table 3.17).

This showed that the increased inhibition of gonococci over four days which was demonstrated in Table 3.16 was not in fact due to further production of bacteriocin. The other possibility, that other by-products were the cause of further inhibition, seemed to be more likely and such inhibitory by-products could in fact be free fatty acids, particularly short-chain ones. Accordingly all fatty acids which were likely to be produced by C.T. strains on Brain heart infusion blood agar after 1 - 4 days were screened by gas-liquid chromatography and the results are given in Section 3.4.4.

Table 3.16 Inhibition patterns of gonococcal strains (G.C.) by C.T. strains of Sh. sonnei incubated for 1 day and for 4 days.

G.C I.D.	C.T. 1B 1/4	2 1/4	3A 1/4	4 1/4	5 1/4	6 1/4	8 1/4	9 1/4	11 1/4	13 1/4
N35	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	-/-	+/+
N37	+/+	+/+	-/+	+/+	+/+	+/+	-/+	-/+	-/+	+/+
N40	+/+	+/+	-/+	+/+	-/+	+/+	+/+	+/+	-/+	+/+
N44	+/+	+/+	+/+	+/+	-/-	+/+	+/+	-/-	+/+	+/+
N46	-/-	+/+	+/+	+/+	+/+	+/+	+/+	-/-	+/+	-/-
N48	+/+	+/+	+/+	+/+	-/+	+/+	+/+	-/+	+/+	+/+
N135	+/+	-/-	-/-	-/-	+/+	-/-	-/-	-/-	-/-	-/-
N139	+/+	-/-	-/-	-/-	+/+	-/-	-/-	-/-	-/-	-/-
N140	+/+	-/-	-/-	+/+	-/±	±/±	-/-	+/±	+/+	±/±
N141	+/+	-/-	+/+	+/+	+/+	+/+	+/+	-/-	-/-	+/+
N164	+/+	-/-	+/+	+/+	+/+	+/+	±/±	-/-	-/-	+/+
N192	+/+	+/+	-/-	-/-	+/+	-/-	+/+	+/+	+/+	+/+
N193	±/+	-/-	-/-	±/+	-/-	-/-	-/-	+/+	+/+	-/-

contd..

contd.

	C.T.									
G.C. I.D.	1B 1/4	2 1/4	3A 1/4	4 1/4	5 1/4	6 1/4	8 1/4	9 1/4	11 1/4	13 1/4
N173	-/-	-/-	-/-	-/-	+/+	+/+	-/-	+/+	+/+	+/+
N176	+/+	+/+	-/-	-/-	-/-	+/+	+/+	+/+	-/-	-/-
N179	-/-	-/-	-/-	-/-	±/±	+/+	-/-	-/-	-/-	+/+
N181	-/-	±/±	-/-	-/-	+/+	+/+	-/-	-/-	-/-	±/+

I.D. Incubation period (in days) for C.T. strains at 37°C

+ : Inhibition ±: Partial inhibition -: No inhibition

NOTE: Cross lines gather strains which were tested simultaneously

Table 3. 17 Effect of 4 - day incubation of colicin type (C.T.) strains on patterns of inhibition of Shigella indicator strains (I.S.)

I.S. \ C.T.	1B	2	3A	4	5	6	8	9	11	13
1	+	-	+	+	+	-	-*	+	-	-
2	+	+	+	+	+	+	-	+	-	+
4	-	-	-	-	-	-	-	-	-	-*
5	-	-	+	+	+	-	+	-	-	-
6	+	-	+	-	+	+	-	-*	-	+
7	+	-	+	-	+	+	-	-*	-	-*
8	-	-	+	+	+	-	+	-	-	-
9	+	+	+	+	+	+	-	+	+	+
13	-	-	+	+	+	-	-*	-*	-	-
15	+	+	+	+	+	+	+	+	+	+

+ : Inhibition - : No inhibition

* : Different results from those obtained after 1 day incubation of C.T. strains (Table 3.15).

The reasons for the loss of inhibition of the indicator strains shown in Table 3.17 were not investigated, and the only explanation which seemed feasible was that during prolonged incubation of the C.T. strains the colicin had degenerated.

To confirm that inhibition of gonococci was caused by colicin activity alone, crude colicin was tested against 4 gonococcal strains (See 3. 3. 4 below)

3. 3. 4 Examination of crude colicin of C.T.9

The sterile preparation of crude colicin of C.T.9 was tested against the indicator strains of Sh. sonnei and 4 strains of gonococci which had been previously tested by Kekessy & Piguet method. The results (shown in Table 3.18) are almost the same as those obtained by using living cultures of C.T.9 strain (See Tables 3.14 and 3.15).

Table 3.18 Examination of the activity of crude colicin of C.T.9 against indicator strains of Sh. sonnei and gonococcal strains

	I.S.									
	1	2	4	5	6	7	8	9	13	15
C.T.9	+	±	-	-	+	+	-	+	±	±

	Gonococcal strain			
	N37	N40	N48	N49
C.T.9	-	±	-	±

+ : Inhibition ± : Partial inhibition - : No inhibition

The only difference is partial inhibition (±) instead of complete inhibition (+) in a few instances. This can be seen in I.S.2 and I.S.15, as well as in N49. (See Tables 3.15 and 3.14 respectively for comparison). On the other hand N37 and N48 both show no inhibition (-), a result which had also been obtained by using a living culture of C.T.9 incubated for one day. This contrasts with the result obtained by using a C.T.9 culture incubated for four days.

The conclusions are that (a) one day living culture of C.T. strains have the same effect as their colicins on gonococci, and

(b) when the incubation of the C.T. strains is limited to one day there is no opportunity for other inhibitory substances to accumulate and interfere with colicin activity.

3. 4 Gas-liquid chromatography (GLC)

3. 4. 1 Standard solutions of volatile fatty acids: Qualitative and quantitative analysis

1 µl of a $\frac{1}{4}$ meq standard solution of volatile fatty acids (VFA) was injected on to the GLC column, adjusting the attenuater to 1×10^2 and the chart speed to 5mm/min. Fig. 3.13 shows the resulting chromatogram. All measurements of peaks are given in Table 3.19. These are the measurements required for the identification and quantitation of peaks. Quantitation was made by multiplying peak height (H) by peak width at half height (W).

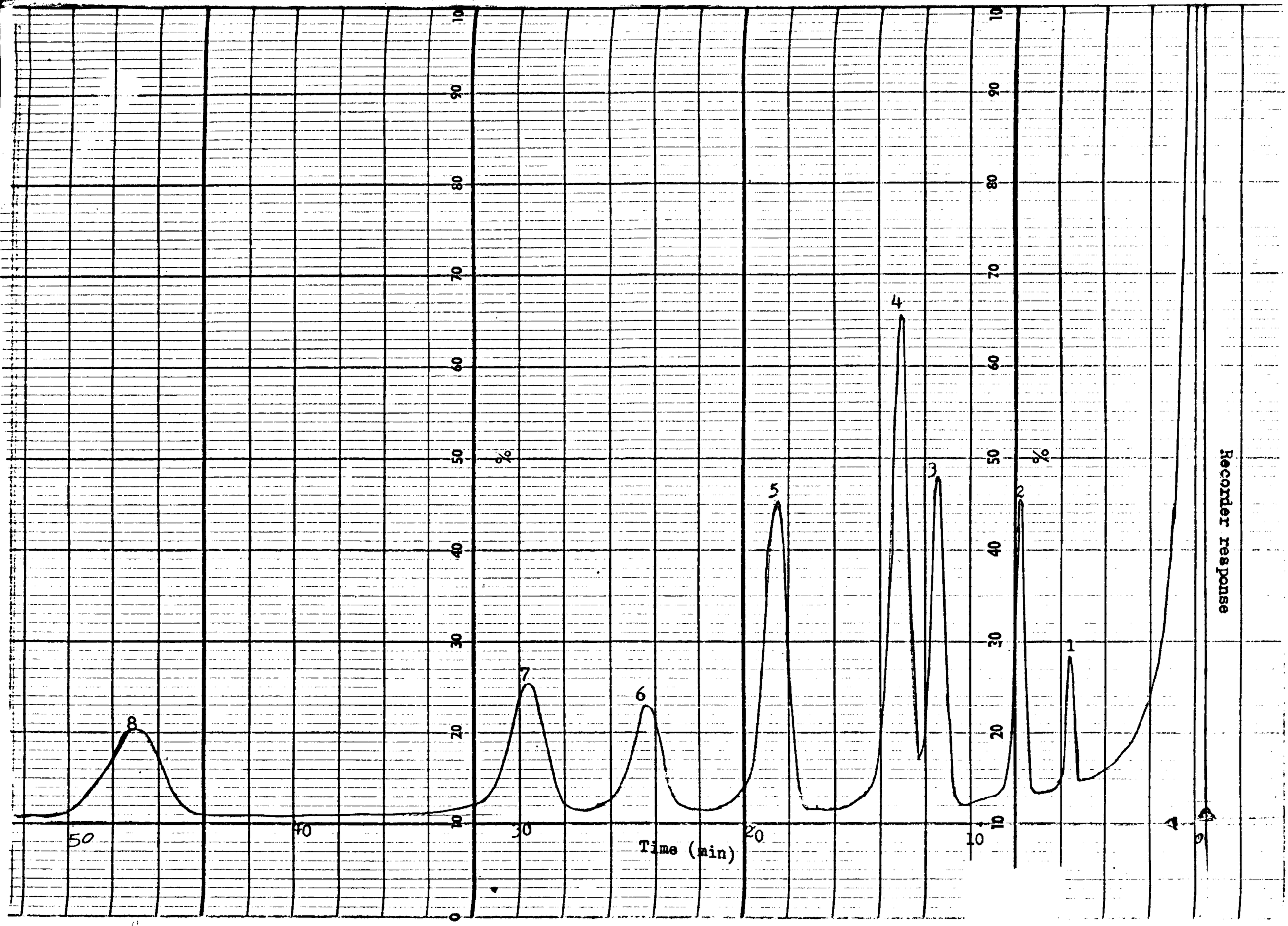
Table 3.19 Measurements and identification of peaks of Fig.3.13

(Standard solution of VFA)

Peak No.	R.T.* (min)	Peak identification	Peak Height (H) (in mm)	Peak width at half height (W) (mm)	Peak area $\left(\frac{H \times W}{mm^2}\right)$
1	5.9	Acetic acid	29.2	2.4	70.08
2	8.1	Propionic acid	66.0	2.8	184.80
3	11.2	n-Butyric acid	72.2	3.2	231.04
4	13.4	Isovaleric acid	105.2	4.0	420.8
5	18.8	n-Valeric acid	68.0	5.4	367.2
6	24.9	Isocaproic acid	24.0	7.0	168.0
7	29.9	n-Caproic acid	28.8	8.8	253.44
8	49.4	Heptanoic acid	20.0	12.2	244.0

* R.T. = Retention time

Fig. 3.13 Chromatogram of a standard solution of volatile fatty acids ($\frac{1}{4}$ meq). Attenuation: 1 x 10^2; chart speed; 5mm/min. Peak identification: 1: acetic; 2: propionic; 3: n-butyric; 4: isovaleric; 5: n-valeric; 6: isocaproic; 7: n-caproic; 8: heptanoic; acids.


Recorder response
%
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
50
40
30
20
10
Time (min)

It can be seen from the chromatogram in Fig. 3.13 and from Table 3. 19 that isobutyric acid had been omitted from the mixture. The normal position of its peak, according to the sequence of chain-length of the acids, is between the peak of propionic and that of n-butyric acid. It was omitted because its peak was totally overlapping with that of propionic acid (Fig.3.14), so that any unknown peak which had an R.T. value of about 8.1 min was identified as propionic and/or isobutyric acid. When some peaks of the analysed samples appeared at that position, some of the samples were re-analysed on a more efficient system (See below).

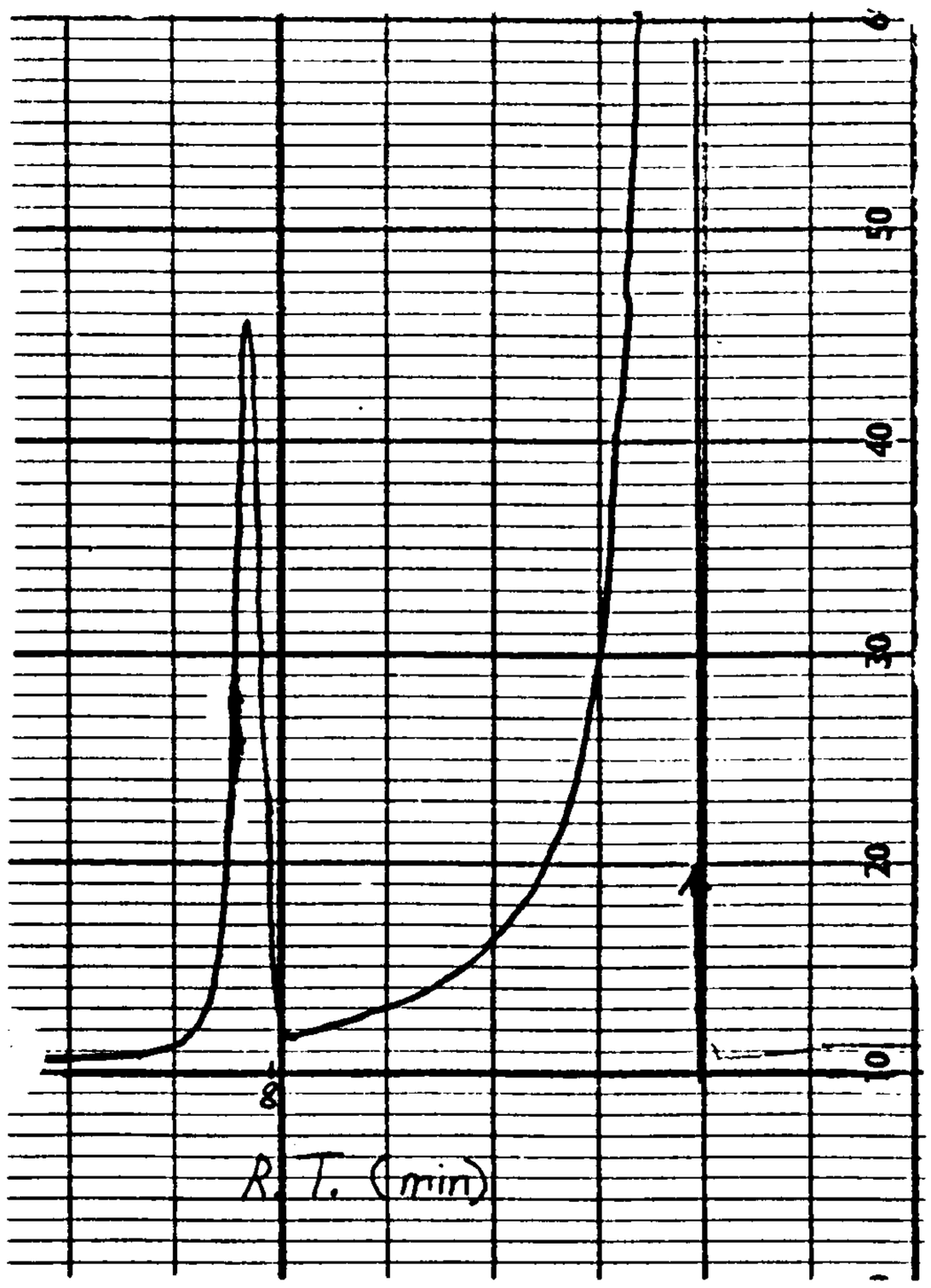


Fig. 3.14 Chromatogram of $\frac{1}{4}$ meq solution of propionic and isobutyric acids showing complete overlapping of the two peaks.

3. 4. 2 Standard solutions of non-volatile fatty acids, Qualitative and quantitative analysis

1 µl of standard solution No.1 of non-volatile fatty acids (NVFA) (1 meq) was injected on to the GLC column. The attenuator was adjusted to 2 x 10^2; the chart speed remained at 5mm/min. Fig. 3.15 shows a chromatogram of standard solution No.1. Measurements of the peaks are given in Table 3.20, where the peaks are identified and analysed quantitatively.

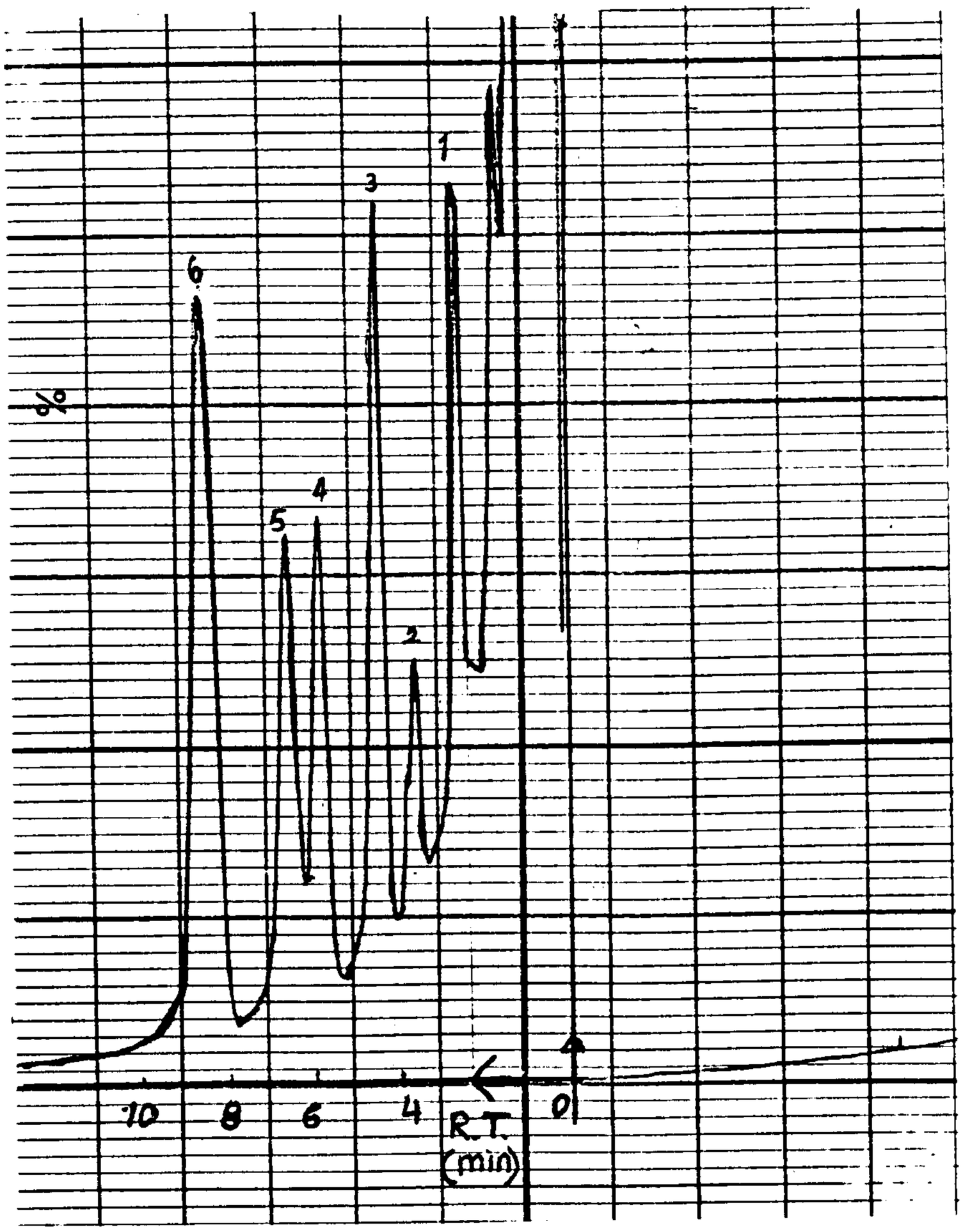


Fig. 3.15 Chromatogram of standard solution No.1 of non volatile fatty acids (1meq). Attenuation: 2x10^2; chart speed: 5mm/min. Peak identification: 1: lactic; 2:oxalic; 3:methylmalonic; 4:malonic; 5: fumaric; 6: succinic; acids.

Table 3. 20 Measurements and identification of peaks of the chromatogram on Fig.3.15 (standard solution No.1 of NVFA)

Peak No.	R.T.* (min)	Peak identification	Peak height (mm) (H)	Peak width (at H/2)(mm)	Peak area (HxW)(mm^2)
1	2.6	Lactic acid	103.0	2.0	206
2	3.6	Oxalic acid	50.0	3.0	150
3	4.5	Methyl malonic acid	102	2.2	224.4
4	5.9	Malonic acid	67.2	2.6	174.72
5	6.6	Fumaric acid	65.0	2.8	182.0
6	8.4	Succinic acid	95.4	3.0	286.2

* : R.T. = Retention time

The various fatty acids in the complete standard solution were identified by injecting a solution of each acid separately. Examples are shown in Fig. 3. 16 (A and B).

The standard solution of NVFA (No.1) was not adequate for the identification of the peaks which appeared beyond the last peak (succinic acid) in standard solution No.1. Therefore, three more acids (maleic, malic and glutaric) were prepared and injected on to the GLC column. The composite solution of these was designated NVFA - No.2. The first peak to appear in its chromatogram was that of maleic acid which was identified by injecting its solution separately into the GLC. (Fig. 3.3.16, C). In this figure the peak is enlarged because the attenuation had been altered to 1 x 10^2. As it was the only peak (in the chromatogram of solution No.2) which had an R.T. value equal to that of some unknown peaks, the measurements were taken only on it and were as follows: R.T. 14.1min; peak height:49.8mm; peak width at half height:3.5mm; peak area 174.3mm^2.

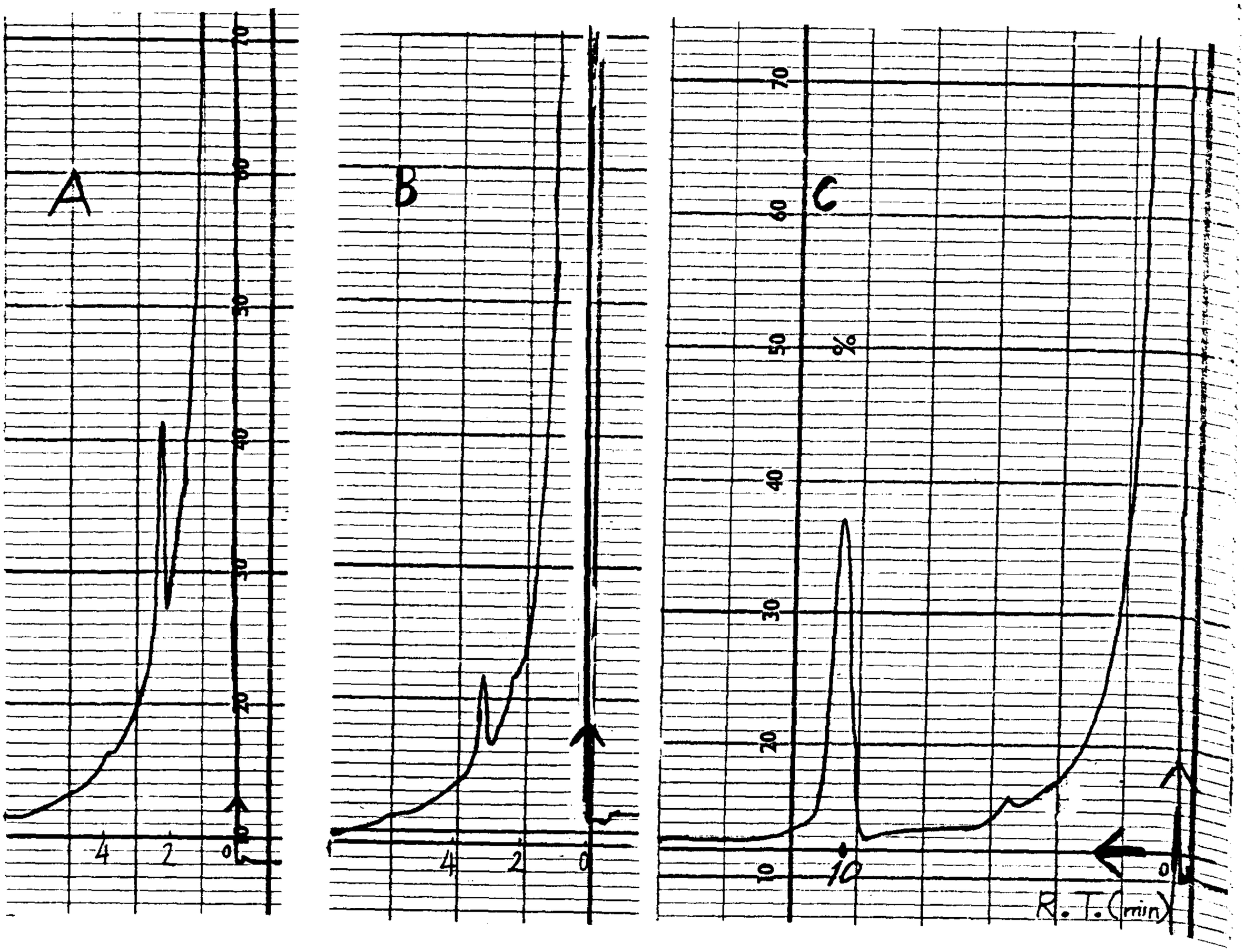


Fig. 3.16 Chromatograms of single acid solutions prepared for the identification of the appropriate acid in the complete standard solutions. (A) and (B) lactic and oxalic acids respectively (from NVFA No.1) (C) maleic acid (from NVFA No.2) Attenuation for A and B: 2 x 10^2 and for C:1x 10^2; chart speed: 5mm/min.

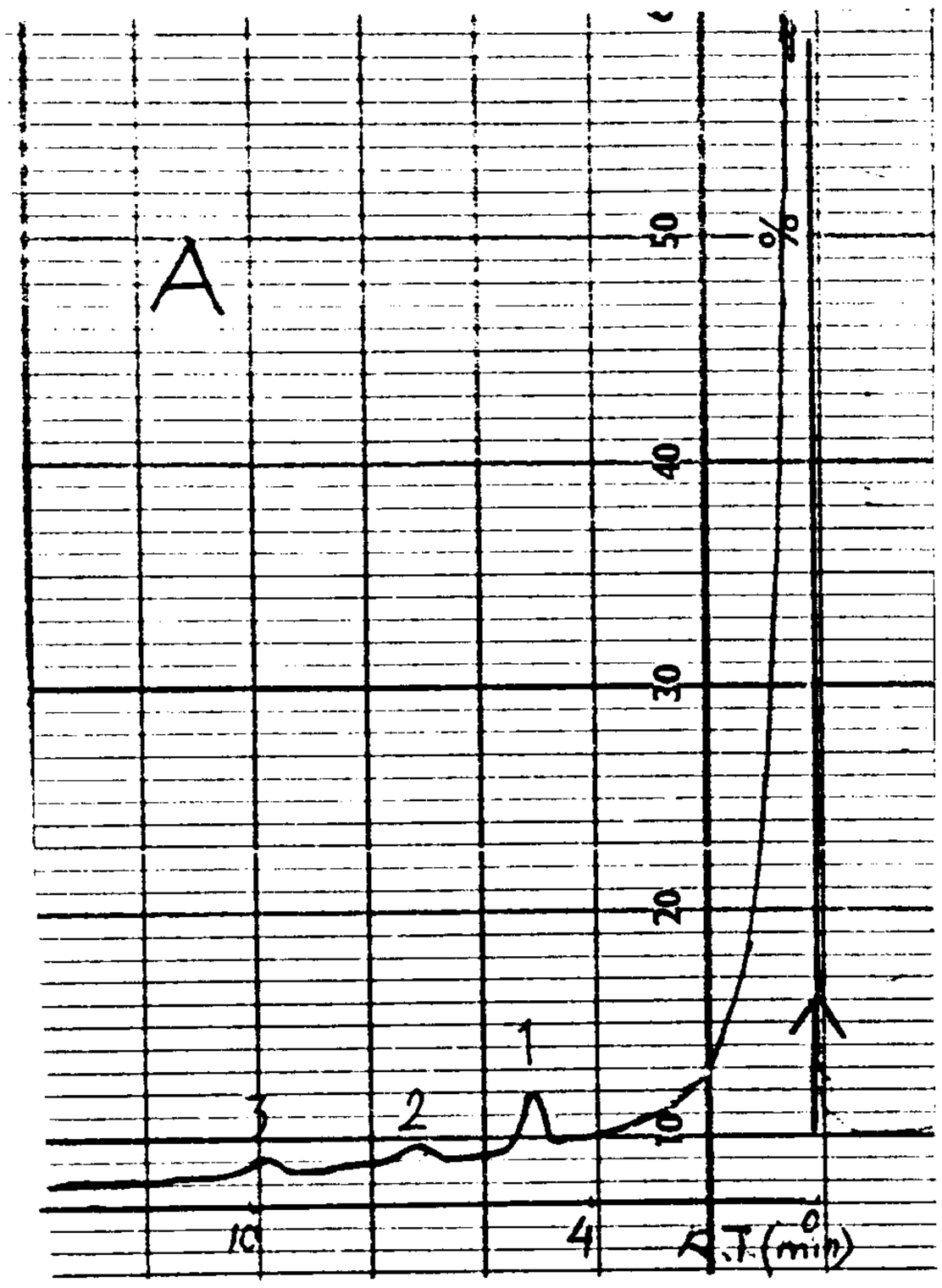
3. 4. 3 Analysis of samples of gonococcal cultures on GC medium

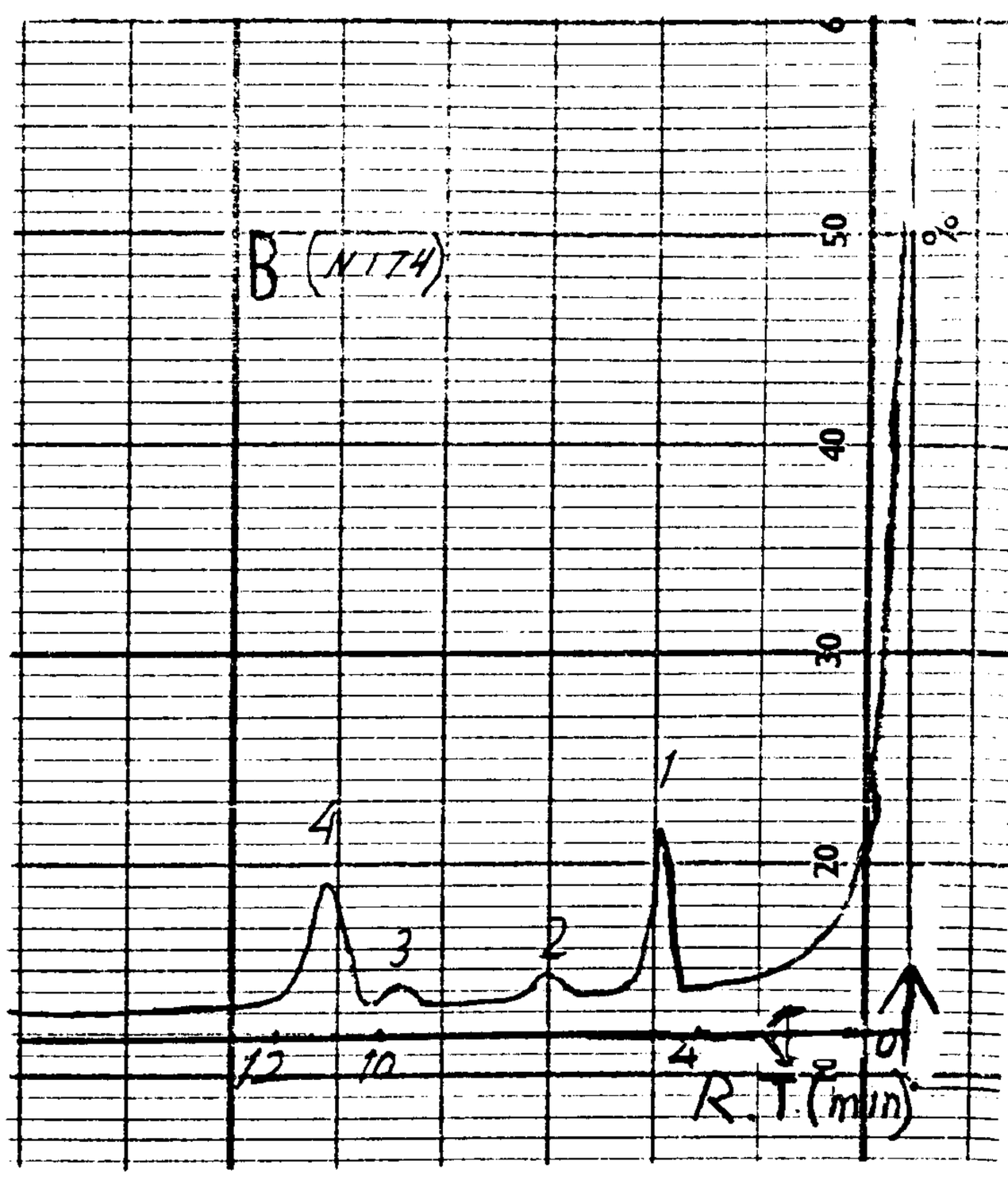
Some of the gonococcal strains which produced inhibitors on GC medium were heavily seeded on this medium and incubated for 2 days at 37°C. Samples of the medium underneath the growth were analysed by GLC. Uninoculated GC medium was also analysed. Peaks of acids which were common to chromatograms of both uninoculated GC medium and the culture samples were not of interest. Consideration was given only to those peaks which appeared in chromatograms of culture samples but were absent or much smaller in size in chromatograms of uninoculated media. These peaks represented acids produced by the gonococci. Figs. 3.17 - 3.19 represent chromatograms of VFA and NVFA for the tested gonococcal cultures on GC medium as well as for the uninoculated medium. Measurements of the most significant peaks in the samples of cultures are given in Table 3. 21.

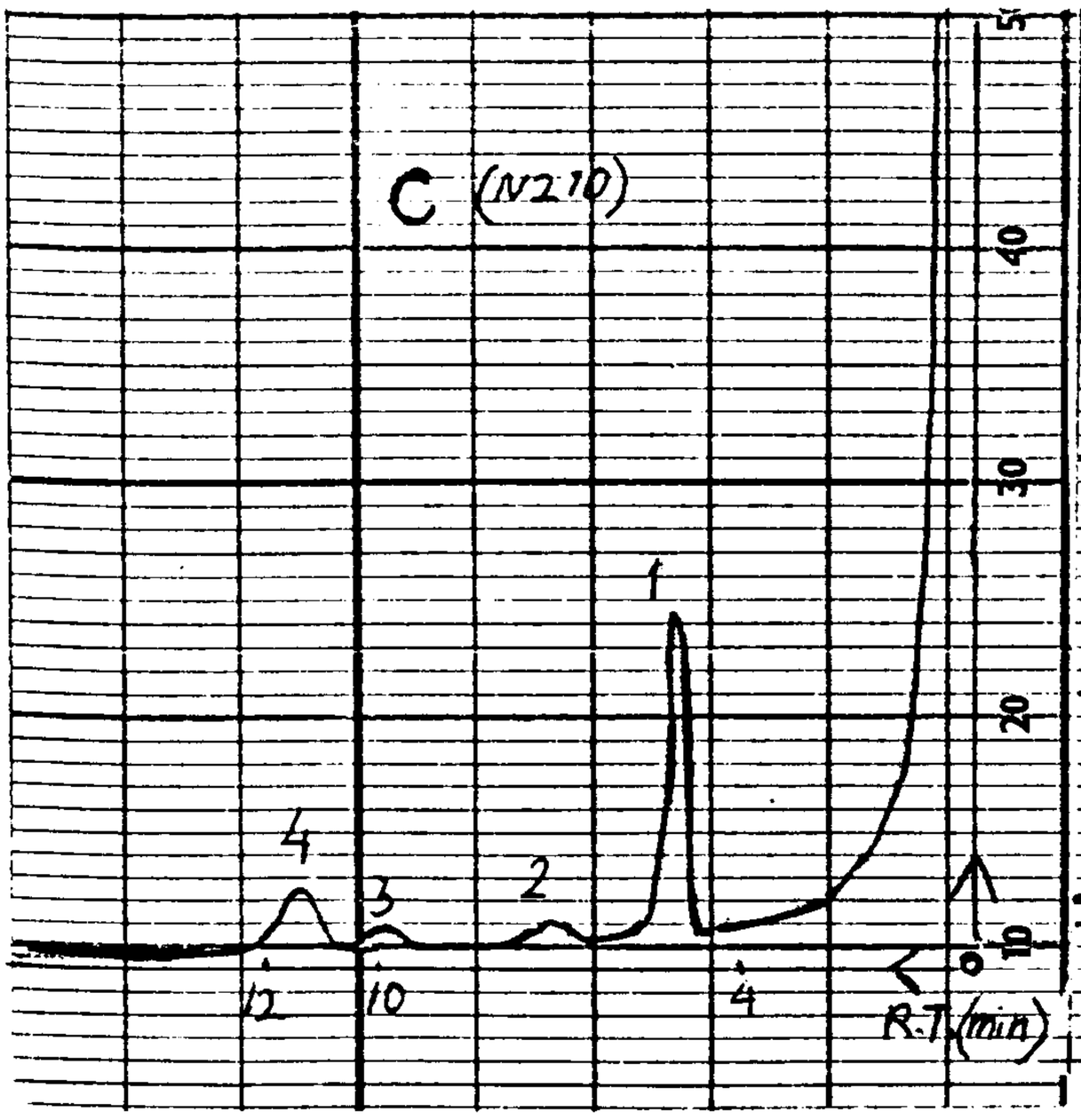
Table 3. 21 Measurements and identification of the most significant peaks in Figs. 3. 17C and 3. 17D

Fig. No.	Peak No.	R.T. (min)	Peak identification	Peak Height (mm)	Peak width at half height (mm)	Peak Area (mm^2)
3.17C	1	5.08	Acetic acid	29.5	2.2	64.9
	4	13.30	Isovaleric acid	6.2	4.2	26.04
3.17D	1	5.04	Acetic acid	18.0	2.9	52.2
	4	13.24	Isovaleric acid	24.2	4.5	108.9

It can be seen from Fig. 3. 17 and Table 3. 21 that acetic and isovaleric acids are the only two acids whose peaks are significantly large, i.e. the amounts of these two acids produced by the gonococci were appreciably greater than the amounts present in the control.







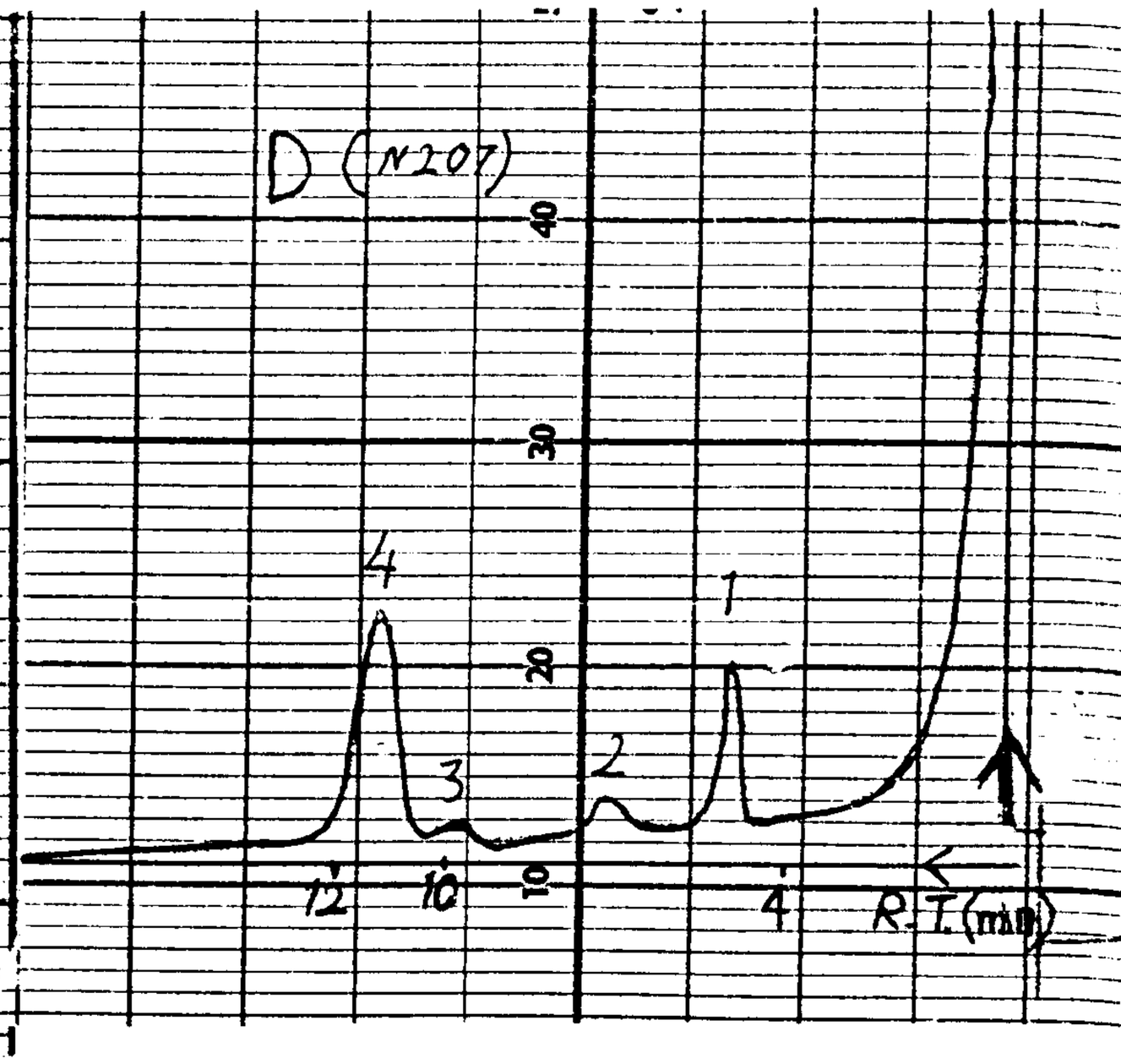


Fig. 3.17 Chromatogram of VFA of uninoculated GC medium (A), and of samples of 3 gonococcal cultures (B, C and D). Attenuation: 2 x 10^2; chart speed: 5mm/min. Identification of acids represented by peaks 1: acetic; 2: propionic; 3: butyric; and 4: isovaleric .

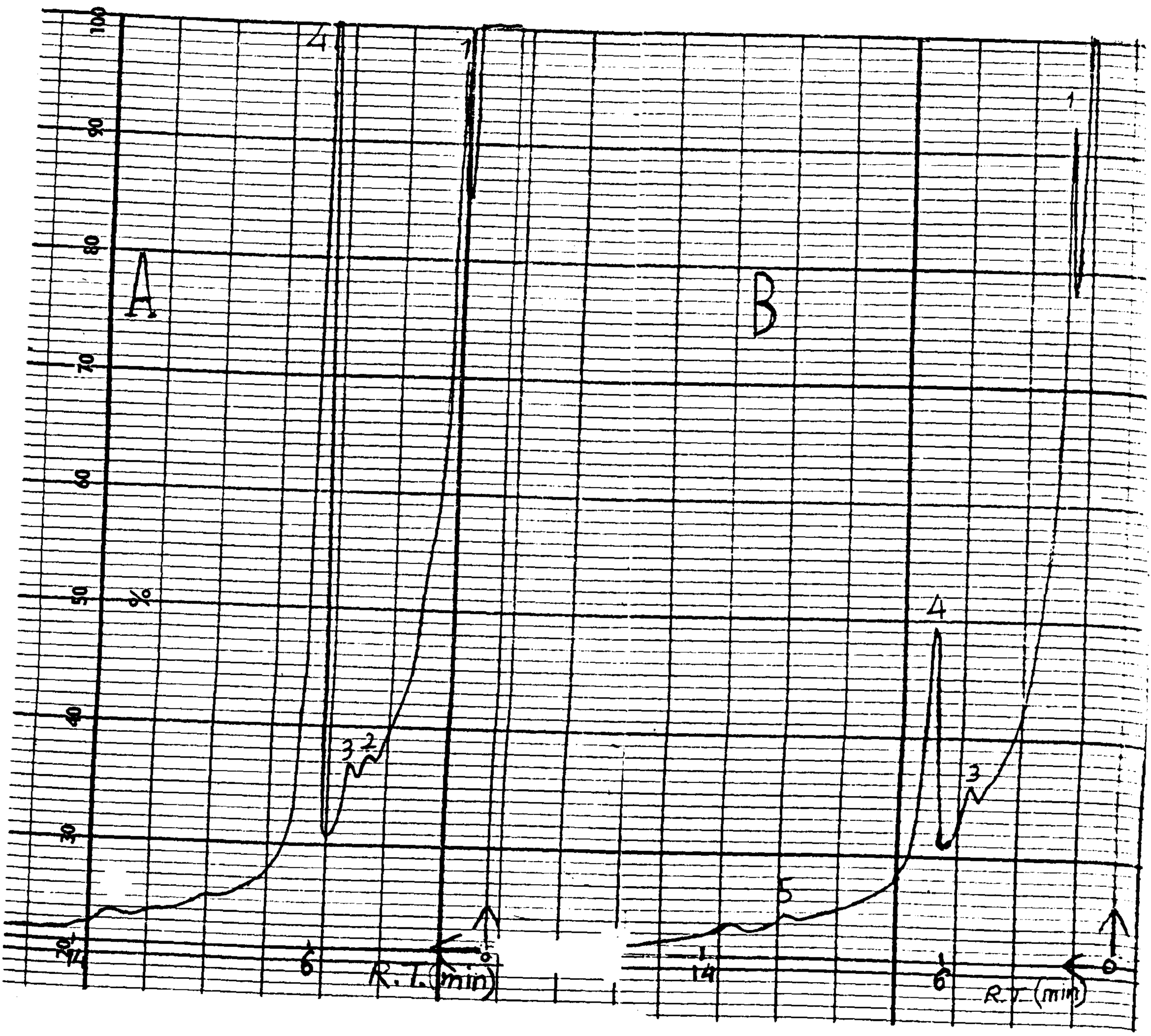


Fig. 3.18 Chromatograms of NVFA in uninoculated GC medium (A) and in a gonococcal culture (strain No. N174) (B). Attenuation : 1×10^2; chart speed: 5mm/min. Identification of peaks 1: undifferentiated peak; 2: malonic; 3: fumaric; 4:Succinic;5:maleic; acids.

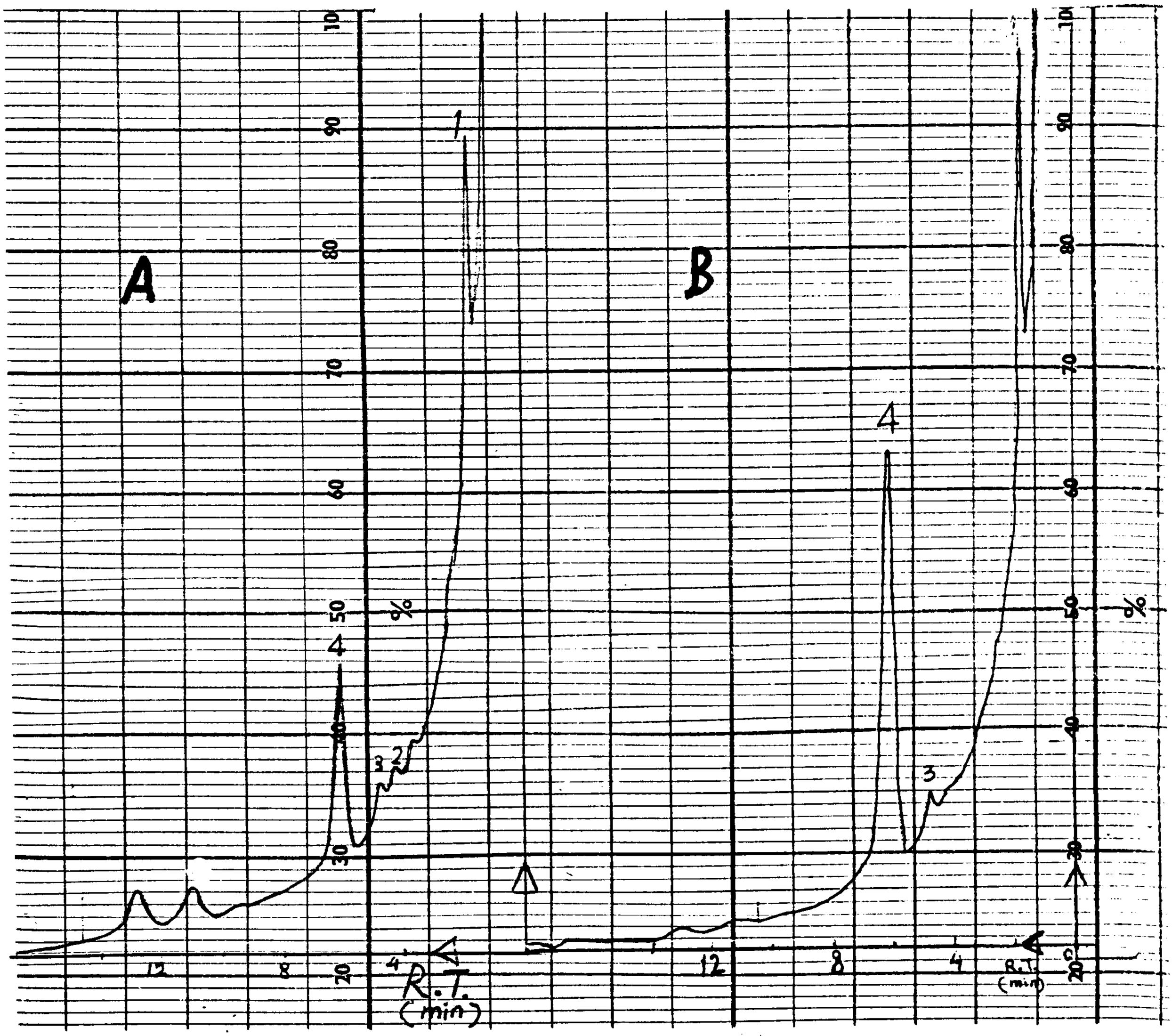


Fig. 3.19 Chromatogram of NVFA in samples of 2 gonococcal strains, N207 (A) and N210 (B). Attenuation 1 x 10^2; chart speed: 5mm/min. Identification of peaks 1: undifferentiated; 2: malonic; 3: fumaric; 4: succinic; acids.

Two GC media were prepared containing differing combinations of the two significant acids (acetic and isovaleric) as shown in Table 3.22. The concentration of each acid was determined by its amount found in the inoculated GC medium (the gonococcal culture).

Table 3. 22 The combinations of acetic and isovaleric acids which were added to GC medium.

Medium No.	Concentration of acetic (%)	Concentration of isovaleric (%)
1	0.0428	0.0128
2	0.038	0.048

It can be seen that in this way, GLC procedure allows the identification of acids produced by the peak position (R.T.) and allows determination of their amounts from the peak areas. These measurements were calibrated using an external standard solution which contains known amount of acids. The two media described in Table 3.22 were tested with other media which were prepared in the same way but with the addition of acids of significant amount produced by C.T. strains of Sh. sonnei on BHIBA medium. The results of tests of all the media are presented below (3. 4.6).

3. 4. 4 Analysis of samples of C.T. cultures of Sh. sonnei

All the C.T. strains of Sh. sonnei were analysed by GLC for the production of fatty acids after 1, 2, 3 and 4 days. Each strain was heavily seeded on to four plates of BHIBA medium (Oxoid) and

incubated at 37°C. On each day one plate was removed for analysis. Uninoculated BHIBA medium was also tested.

Figs. 3.20 - 3.35 show selected chromatograms of VFA and NVFA of some samples.

The increase in peak area of acids indicates that the production of these acids was continuous during the four days of incubation, and that the acids were accumulating more and more in the region of growth of the C.T. strains. Table 3.23 shows the measurement of the most significant peaks in chromatograms of samples of 1 day and 4 day cultures of Sh. sonnei shown in Figs. 3.21 - 3.28.

Table 3. 23 Measurements of the most significant peaks in chromatograms of Shigella C.T. cultures shown in Figs. 3.21 - 3.28

Figure No.	Chromat-ogram	Peak* No.	R.T.**	Peak Identi-fication (acid)	Peak height(+)	Peak width (+)	Peak area (++)
3.21	CT 1B/1d	1	6.4	Acetic	19.0	2.4	45.6
	"	2	8.64	Propionic(x)	25.2	2.8	70.56
	CT 1B/4d	1	6.4	Acetic	42.6	3.2	136.2
	"	2	8.64	Propionic	35.2	3.4	119.68
3.23	CT 4/1d	1	5.9	Acetic	20.0	2.3	46.0
	"	2	7.8	Propionic	20.20	3.0	60.60
	CT 4/4d	1	5.9	Acetic	25.20	2.8	70.56
	"	2	7.8	Propionic	38.0	3.2	121.6

contd.

Figure No.	Chromat-ogram	Peak* No.	R.T.**	Peak Identi-fication (acid)	Peak Height(+)	Peak width(+)	Peak area(++)
3. 24	CT 5/1d	1	6.02	Acetic	18.8	2.4	45.12
	"	2	8.44	Propionic	18.0	3.0	54.0
	CT 5/4d	1	6.2	Acetic	32.4	2.8	90.72
	"	2	8.3	Propionic	29.8	3.4	101.32
3. 25	CT 8/1d	1	6.64	Acetic	10.6	4.0	42.4
	"	2	8.66	Propionic	6.4	3.5	22.4
	CT 8/4d	1	6.62	Acetic	11.0	4.8	52.8
	"	2	8.64	Propionic	32.0	3.6	115.2
3. 26	CT 9/1d	1	6.62	Acetic	19.4	3.0	58.2
	"	2	8.2	Propionic	18.2	3.0	54.6
	CT 9/4d	1	6.62	Acetic	24.0	2.8	67.2
	"	2	8.2	Propionic	36.4	3.2	116.48
3. 27	CT 11/1d	1	6.64	Acetic	12.4	3.0	37.2
	"	2	8.4	Propionic	11.8	4.0	47.2
	CT 11/4d	1	6.6	Acetic	20.2	3.2	64.64
	"	2	8.52	Propionic	25.0	3.8	95
3. 28	CT 13/1d	1	6.4	Acetic	18.2	2.8	50.96
	"	2	8.4	Propionic	18.0	3.2	57.6
	CT 13/4d	1	6.4	Acetic	26.0	3.6	93.6
	"	2	8.4	Propionic	32.0	3.6	115.20

*: Only the most significant peaks were considered

**: Retention Time (min). (+): Measurements in mm. (++): Peak height multiplied by peak width at half height (mm^2). (x): All peaks having the R.T. value of about 8.0min were considered as propionic acid (See 3.4.5 for details). d: Time (in days) for the tested CT cultures.

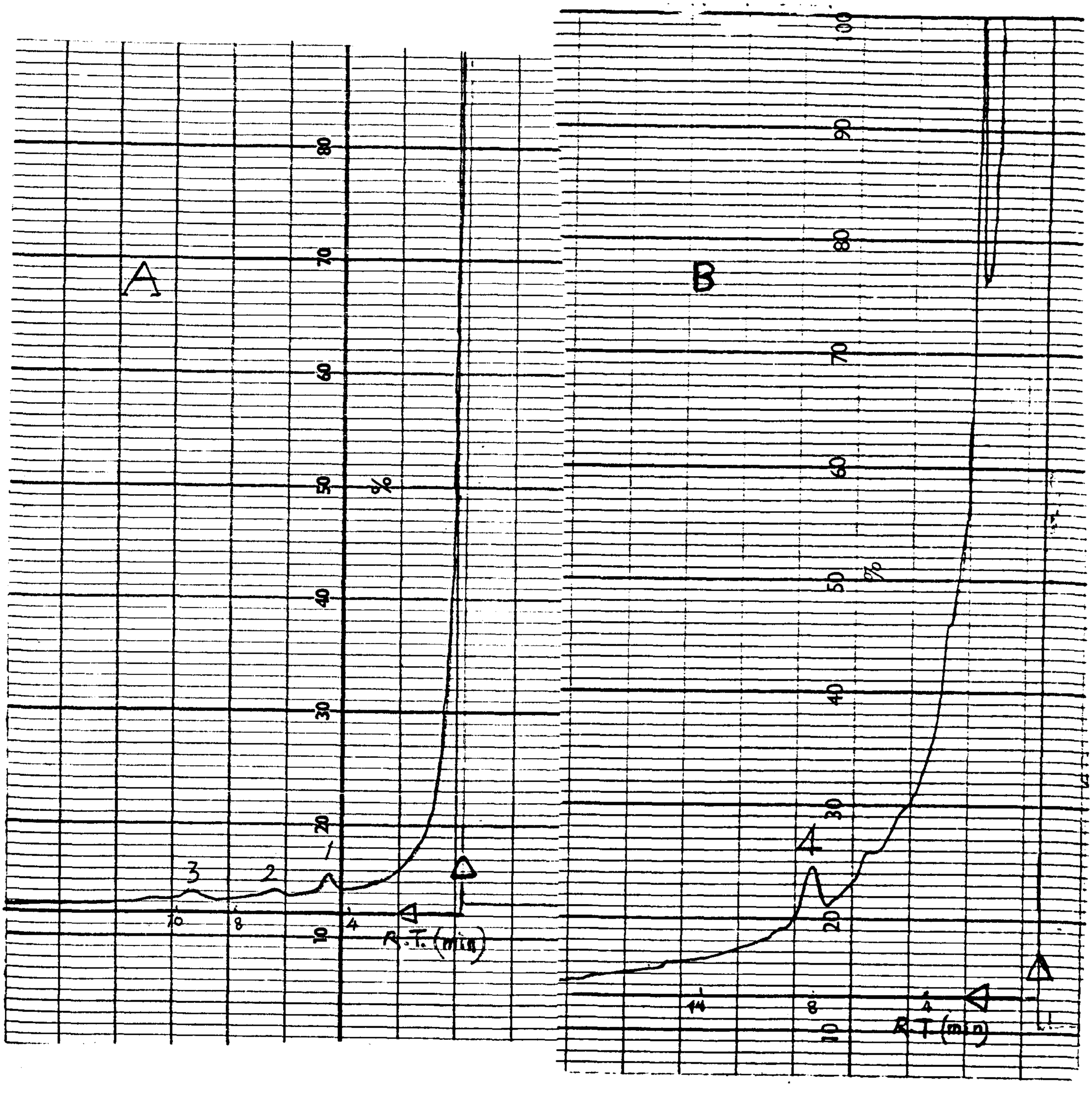


Fig. 3.20 Chromatograms of VFA (A) and NVFA of uninoculated BHIBA medium. Attenuation 2 x 10^2 (for A) and 1 x 10^2 (for B); chart speed: 5 mm/min. Identification of peaks: 1. acetic; 2. propionic; 3. butyric; 4. succinic; acids .

Fig. 3.21. Chromatograms of VFA of C.T.1B cultures incubated for 1 day (A), 2 days (B), 3 days (C), and 4 days (D). Attenuation: 2 x 10^2; chart speed 5mm/mim Identification of peaks: 1.acetic; 2.propionic; 3. isovaleric; acids.

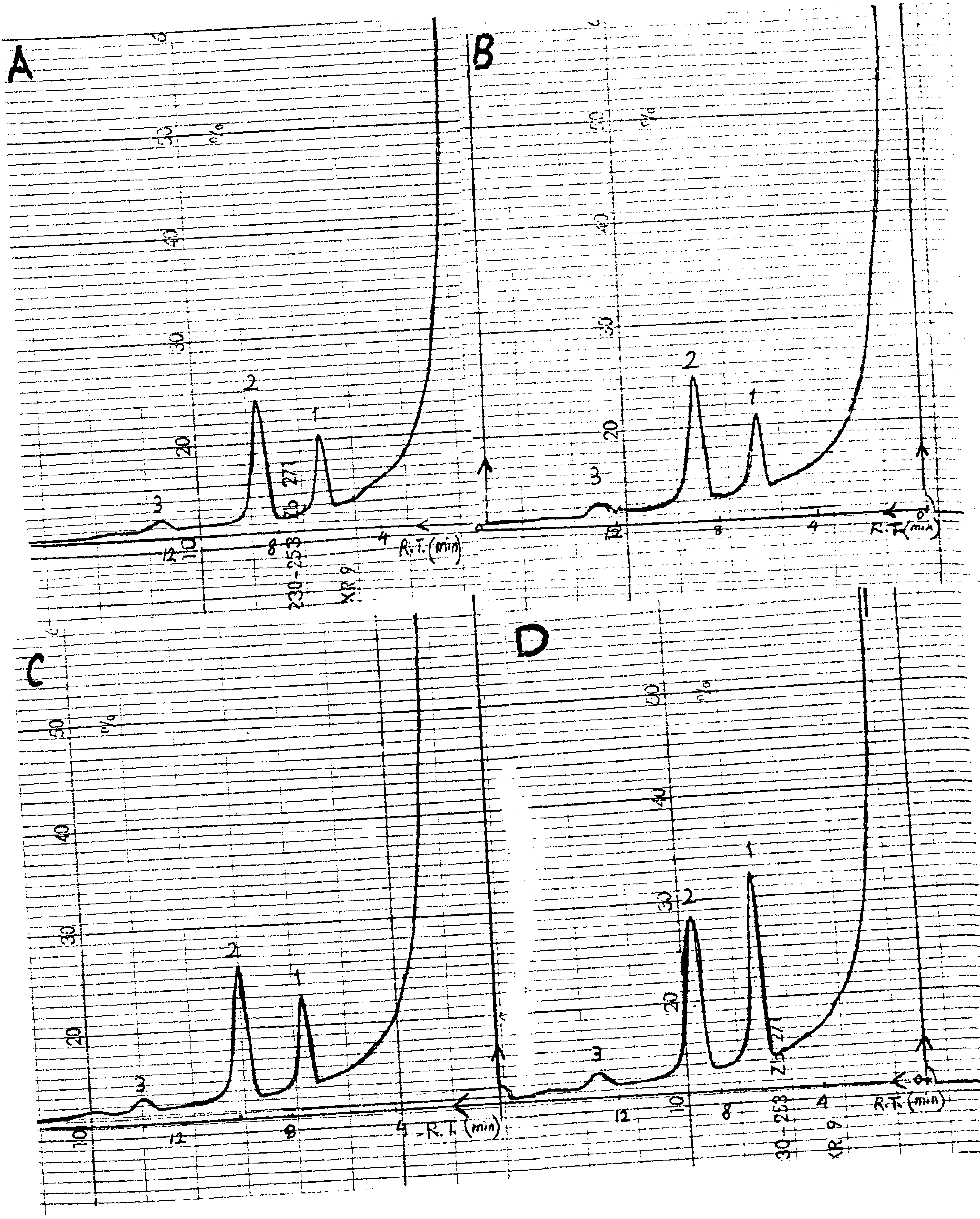
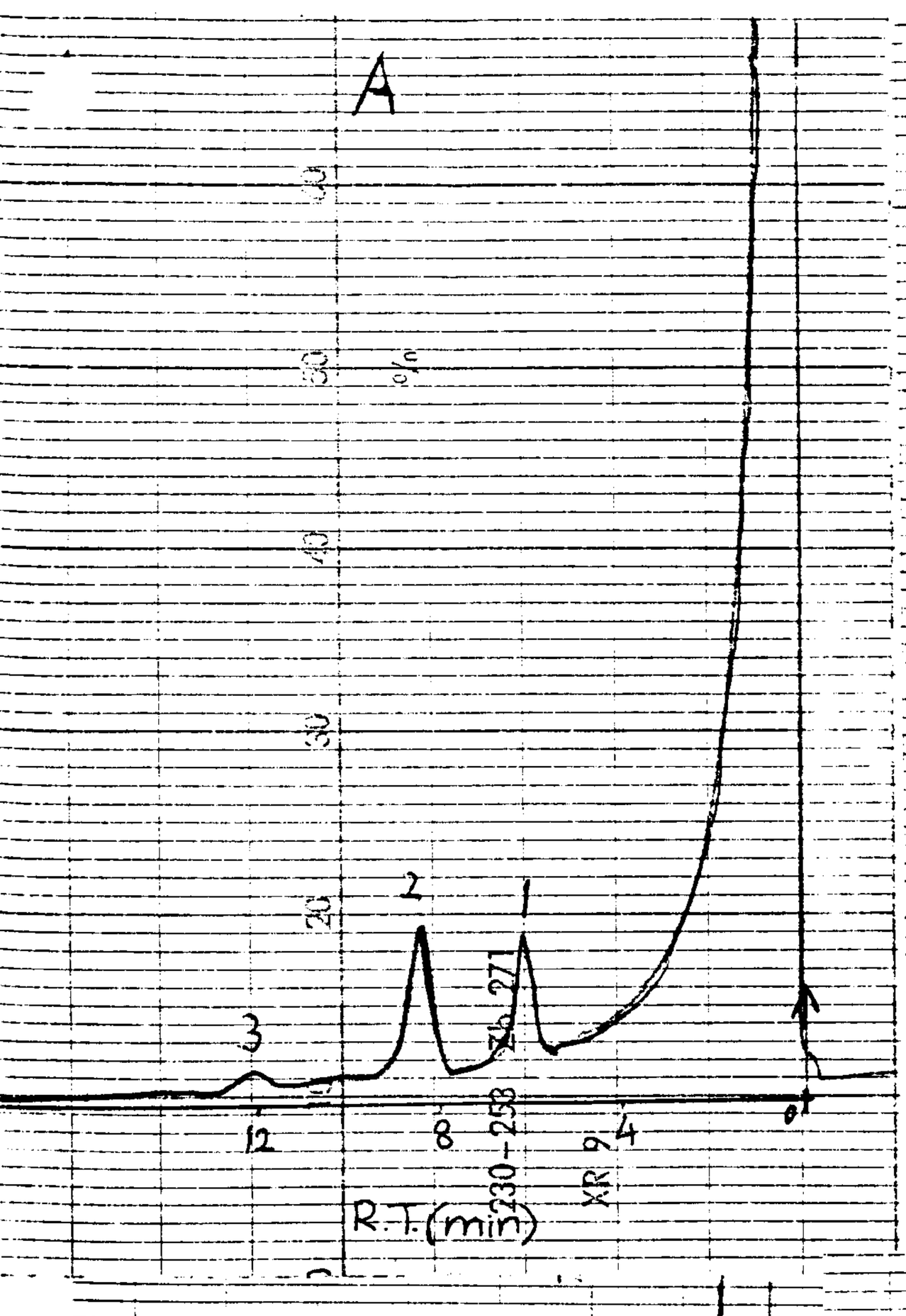
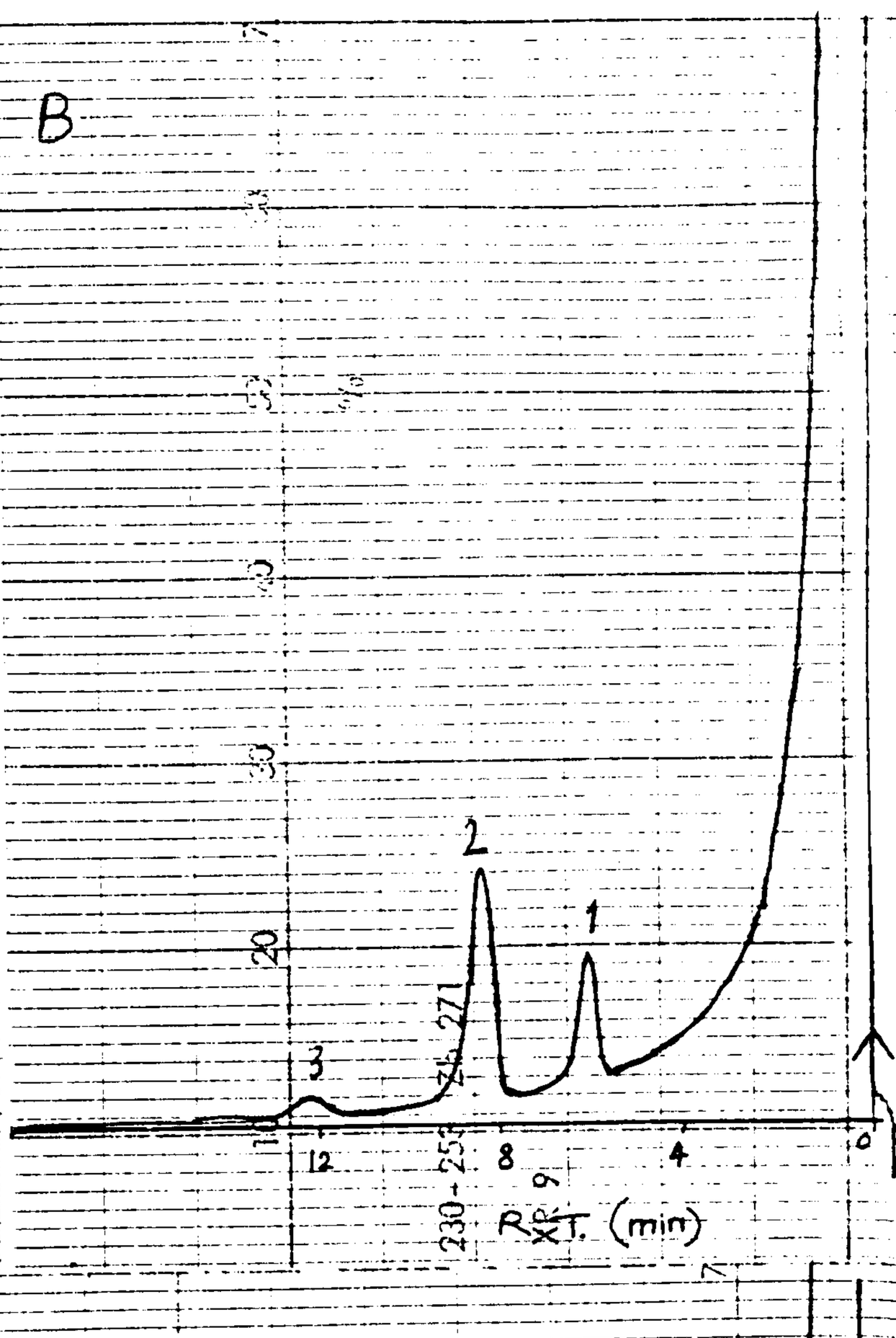

A
B
C
D
%
1
2
3
R.T. (min)
30
40
20
10
12
8
4
0
230-253
271
XR 9

Fig. 3.22 Chromatograms of VFA of C.T.3A culture incubated for 1 day (A), 2 days (B), 3 days (C) and 4 days (D). Attenuation: 2 x 10^2; chart speed: 5mm/min Identification of peaks: 1: acetic, 2: propionic, and 3: isovaleric acids

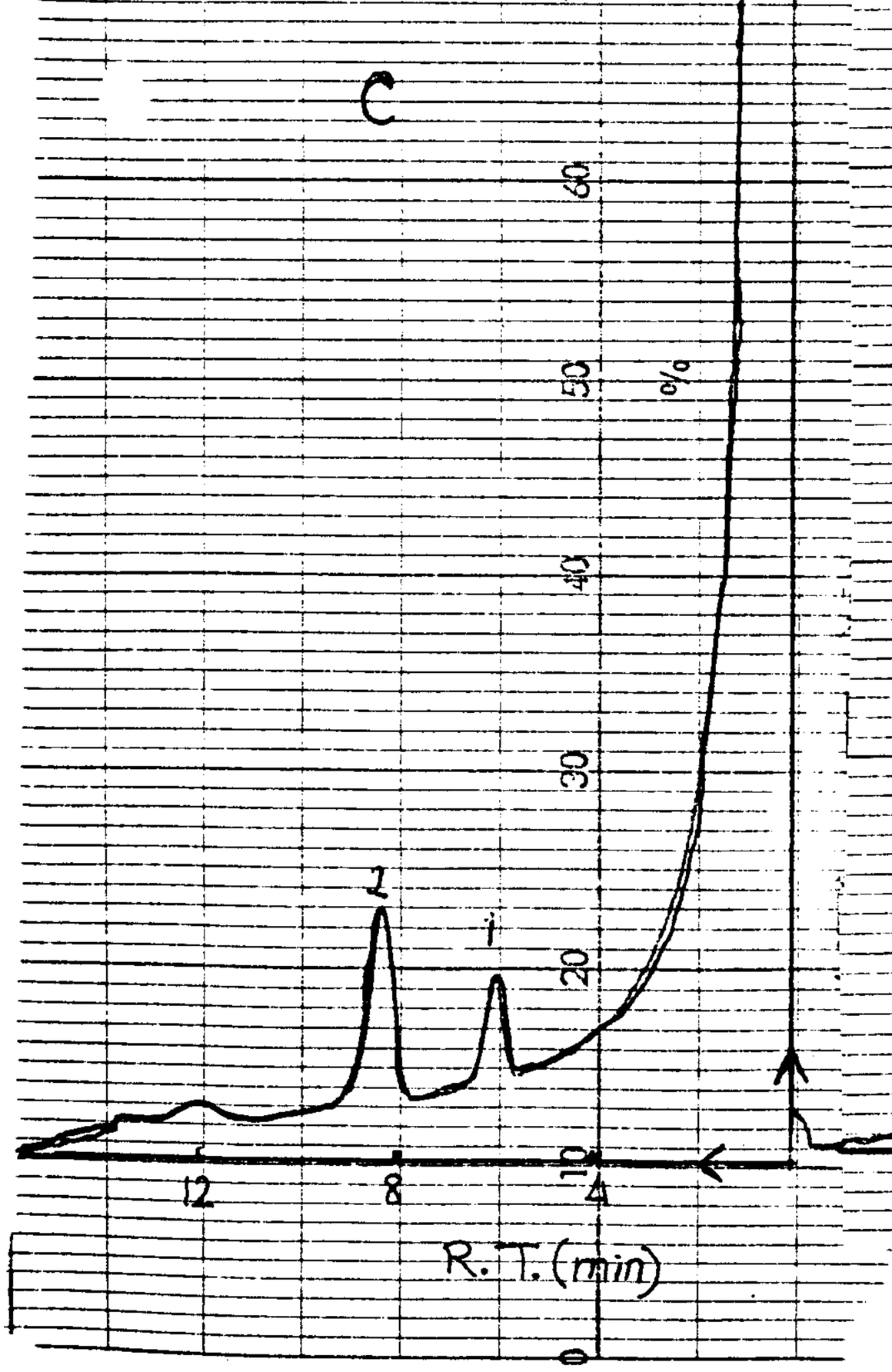
A


%
30
20
2
1
3
230-258
254 271
12
8
4
0
R.T. (min)

B


%
30
20
2
1
3
254 271
230-258
12
8
4
0
R.T. (min)

C


%
60
50
40
30
20
10
1
1
12
8
4
0
R.T. (min)

D

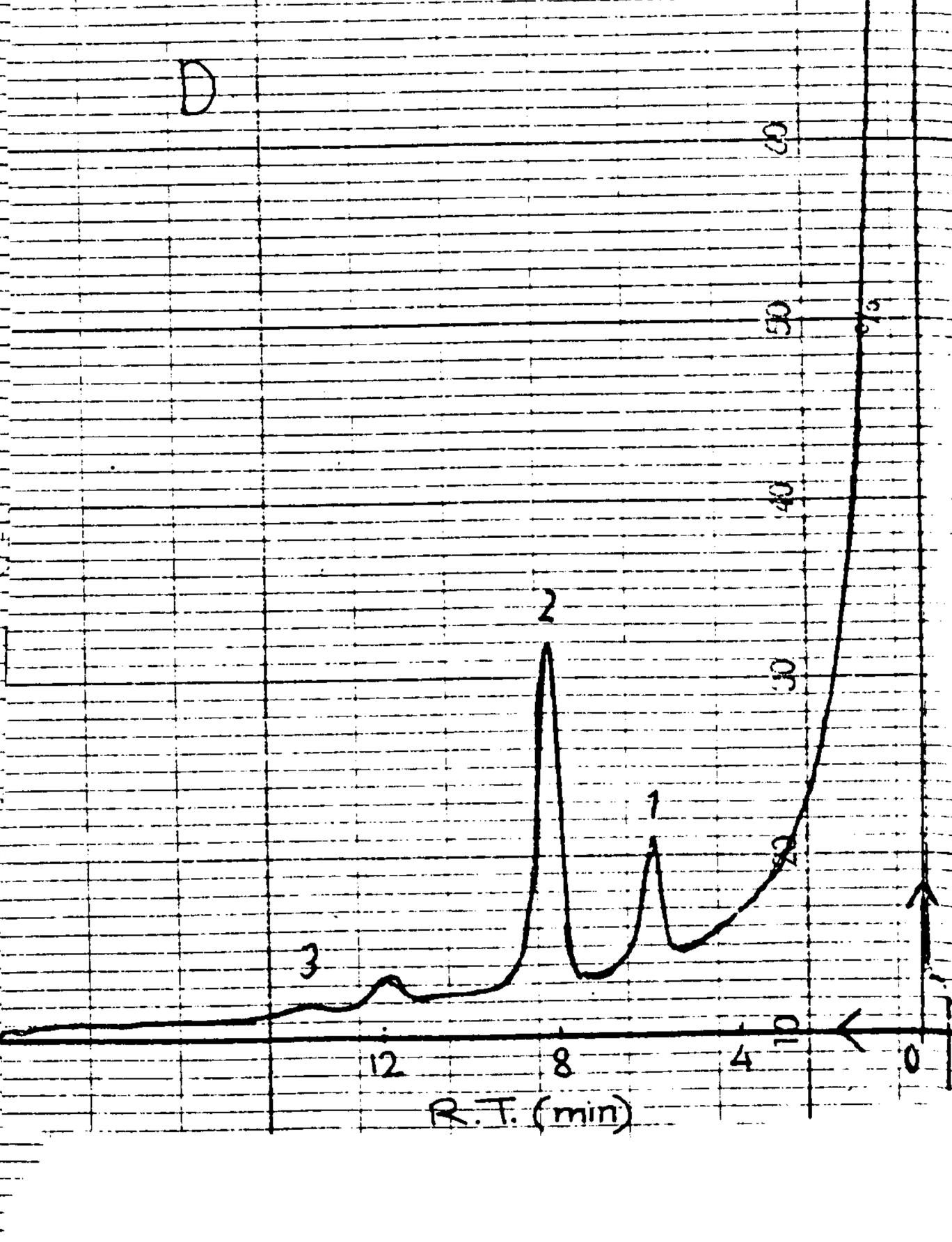

%
60
50
40
30
20
10
2
1
3
254 271
12
8
4
0
R.T. (min)

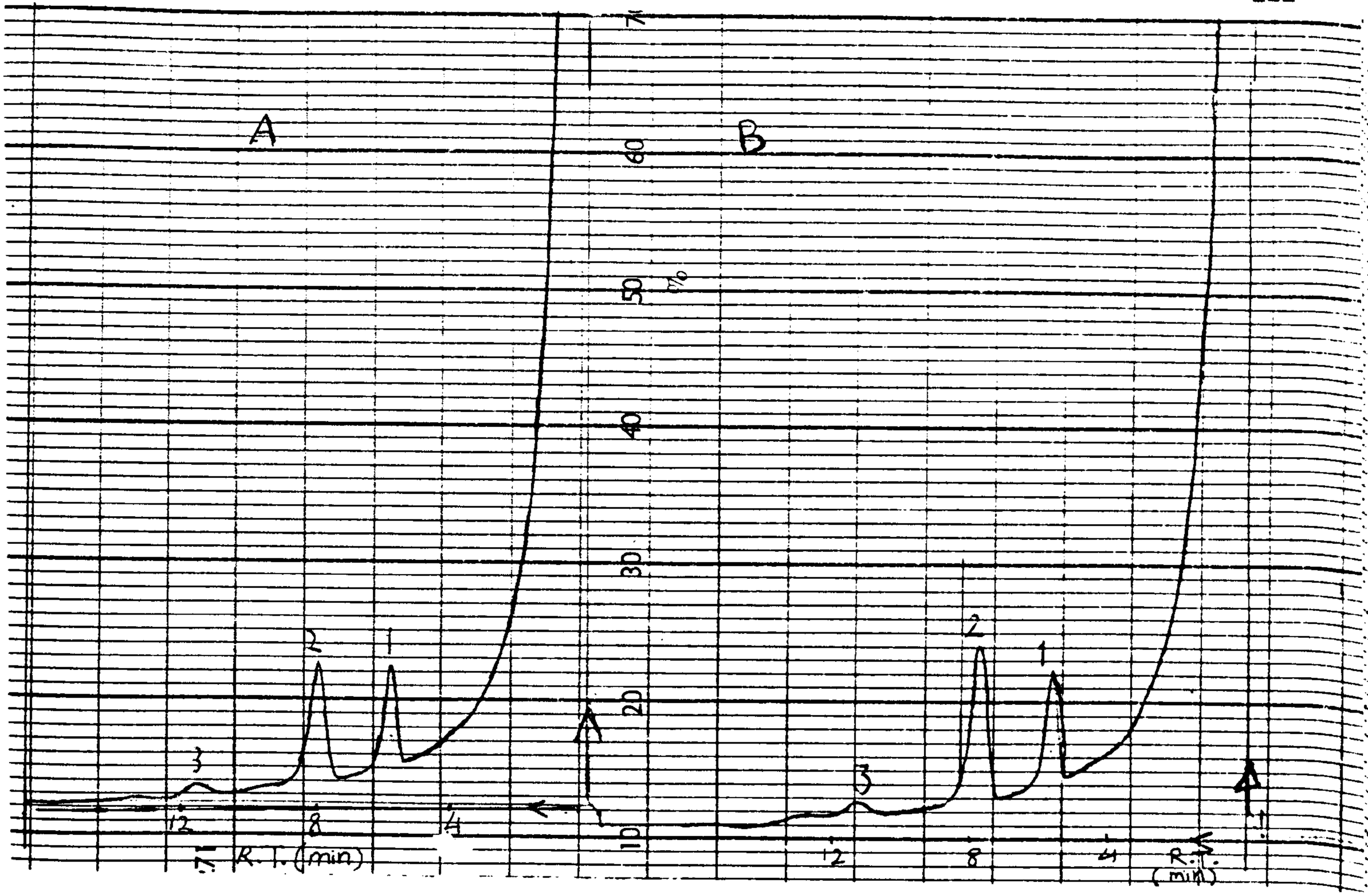
Fig. 3.23 Chromatograms of VFA of C.T. 4 cultures incubated for 1 day (A), 2 days (B), 3 days (C), and 4 days (D). Attenuation: 2 x 10^2, chart speed: 5mm/min, Identification of peaks: 1. acetic, 2. propionic, and 3. isovaleric;acids .

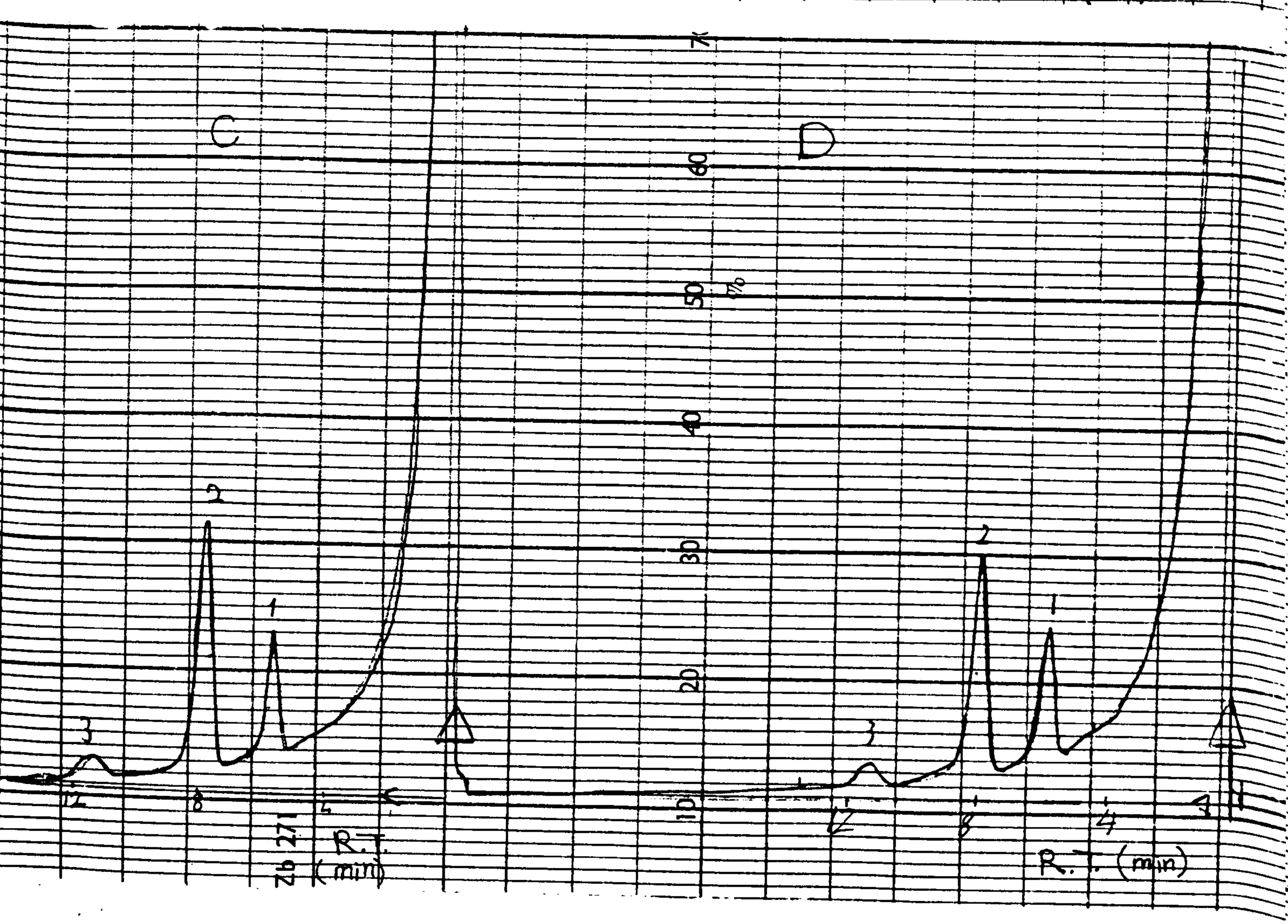
A

B

C

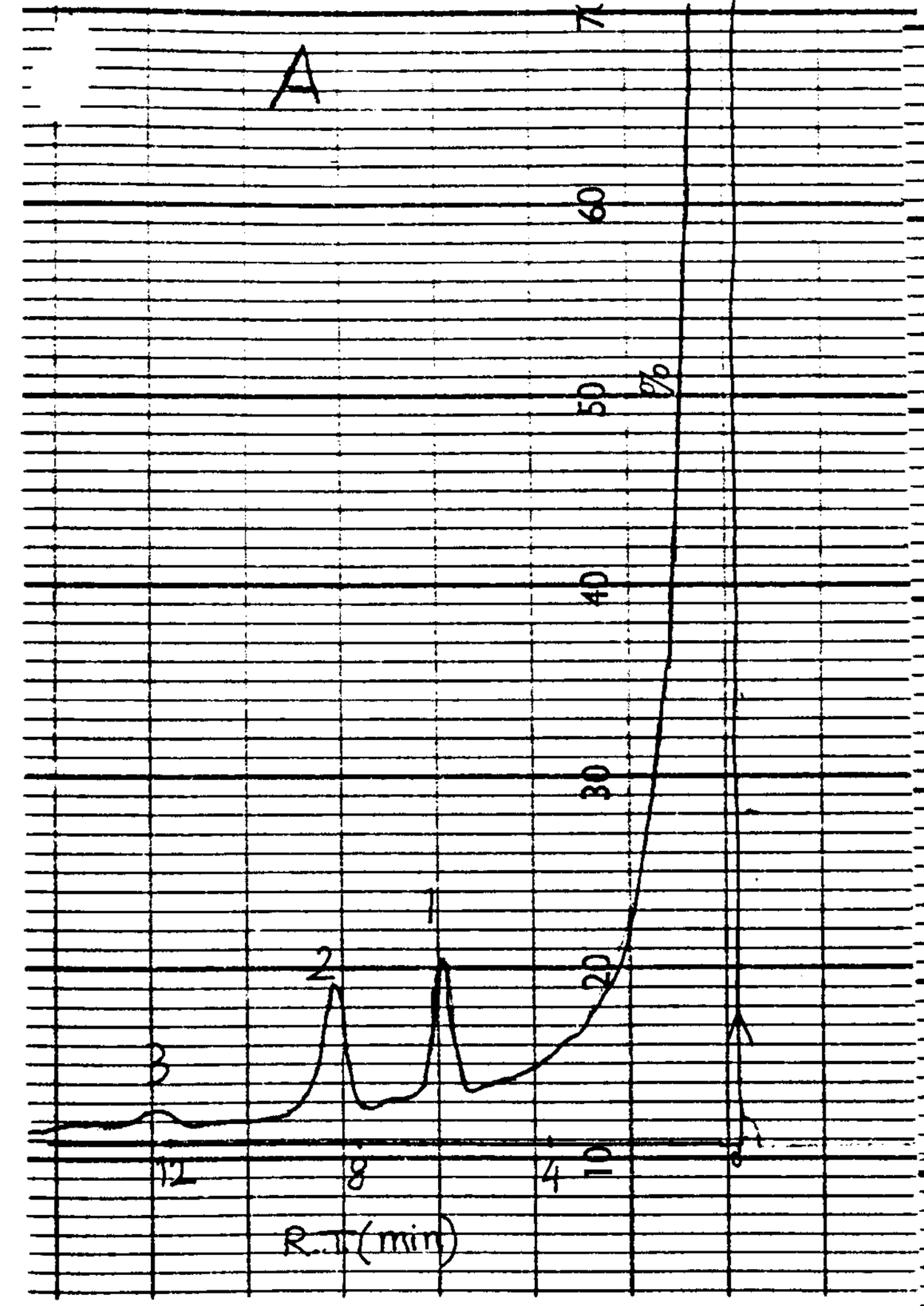
D

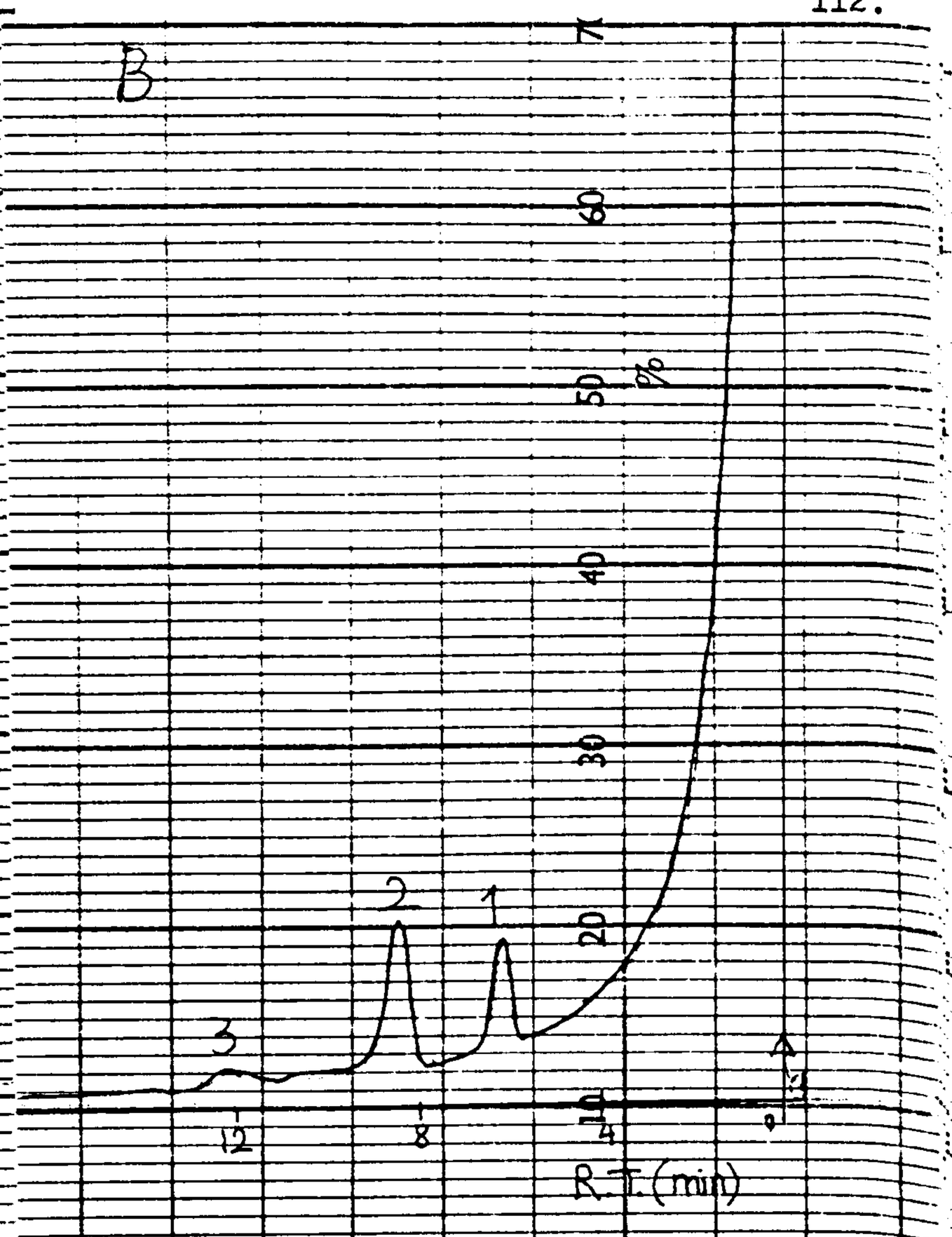


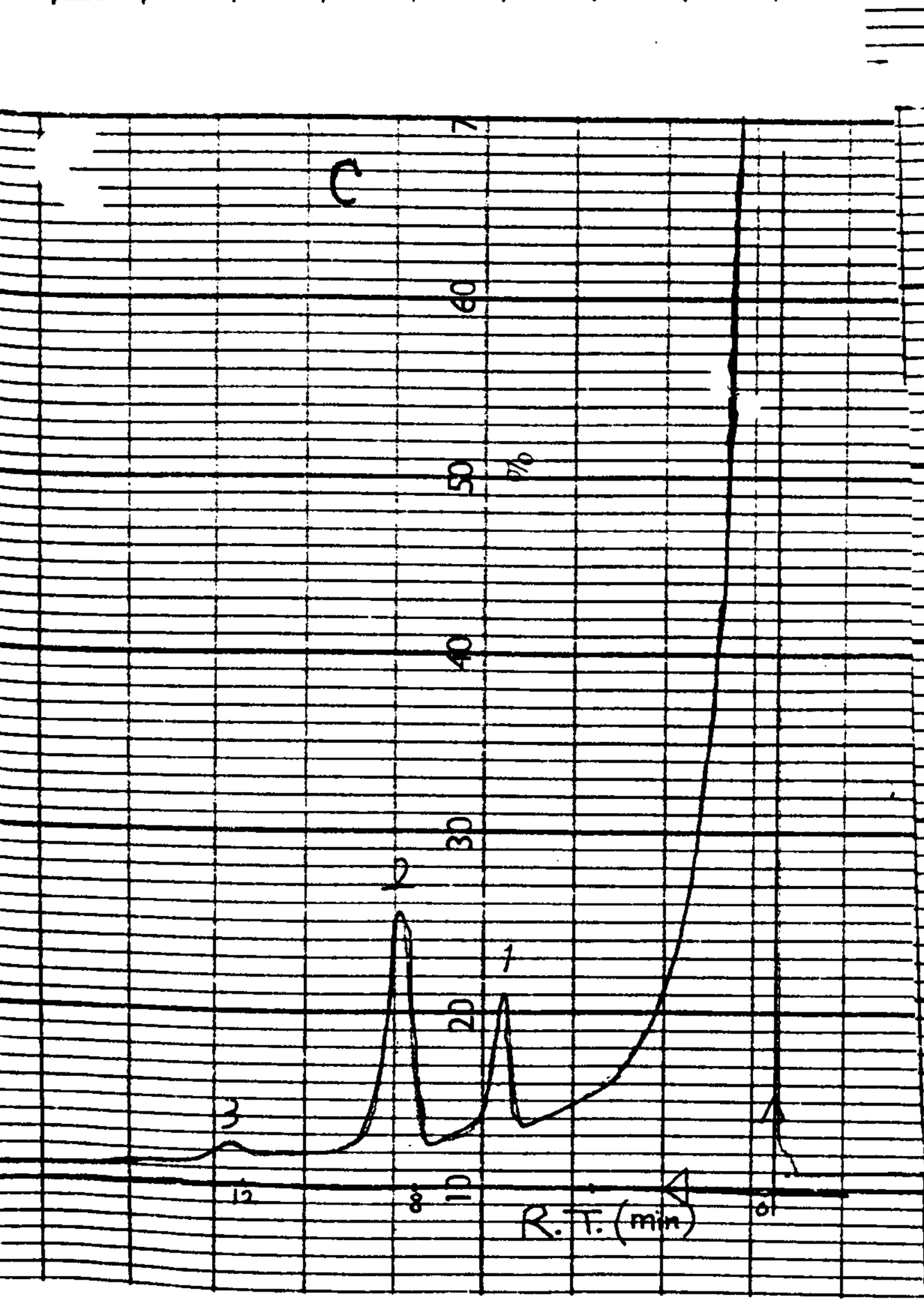


Zb 271

Fig. 3.24 Chromatograms of VFA of C.T. 5 cultures incubated for 1 day (A), 2 days (B), 3 days (C) and 4 days (D). Attenuation: 2×10^2; chart speed: 5mm/min Identification of peaks: 1. acetic, 2. propionic, and 3. isovaleric; acids.


A
%
60
50
40
30
20
10
0
1
2
3
12
8
4
0
R.T.(min)


B
%
60
50
40
30
20
10
0
1
2
3
12
8
4
0
R.T.(min)


C
%
60
50
40
30
20
10
0
1
2
3
12
8
4
0
R.T.(min)

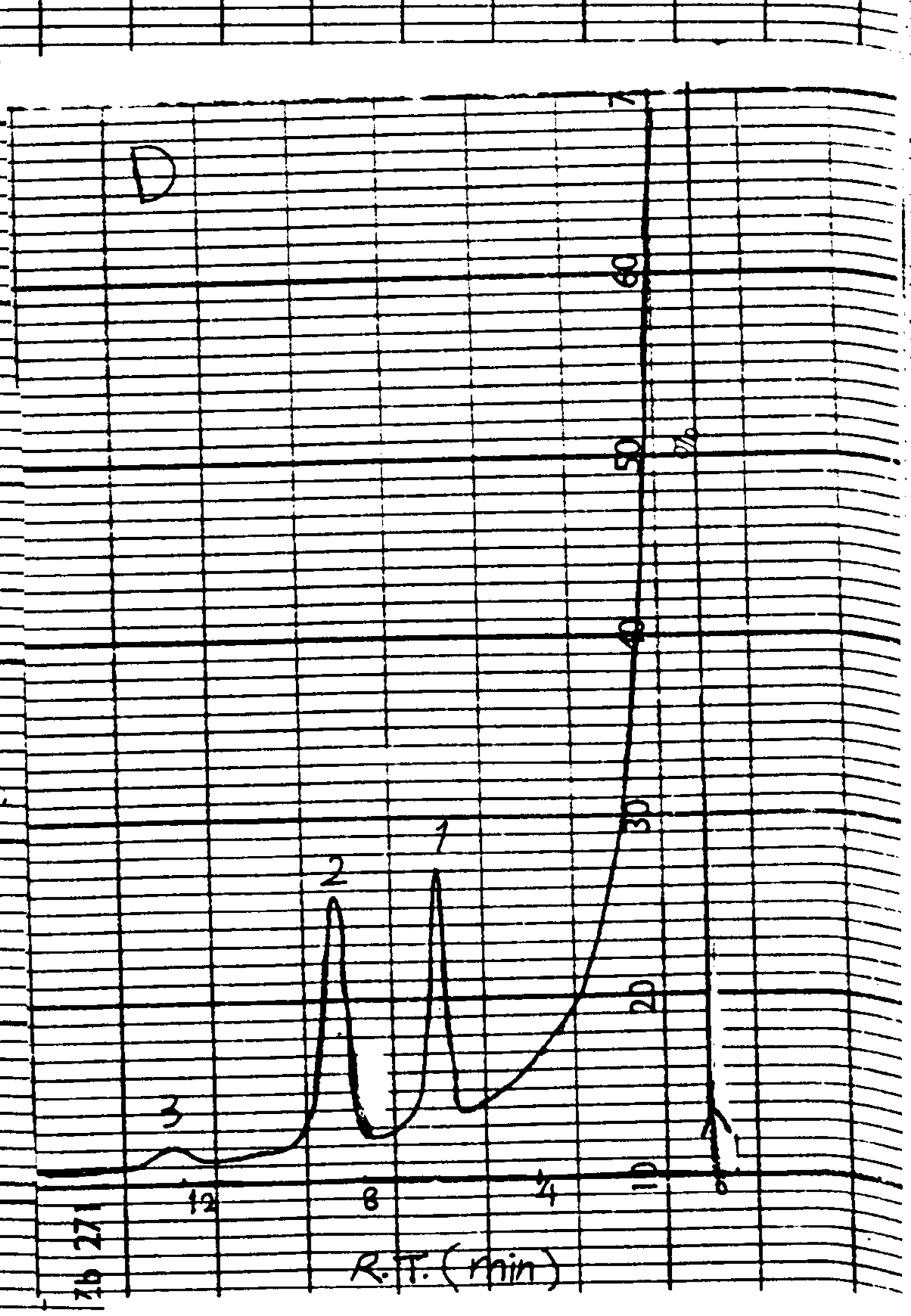
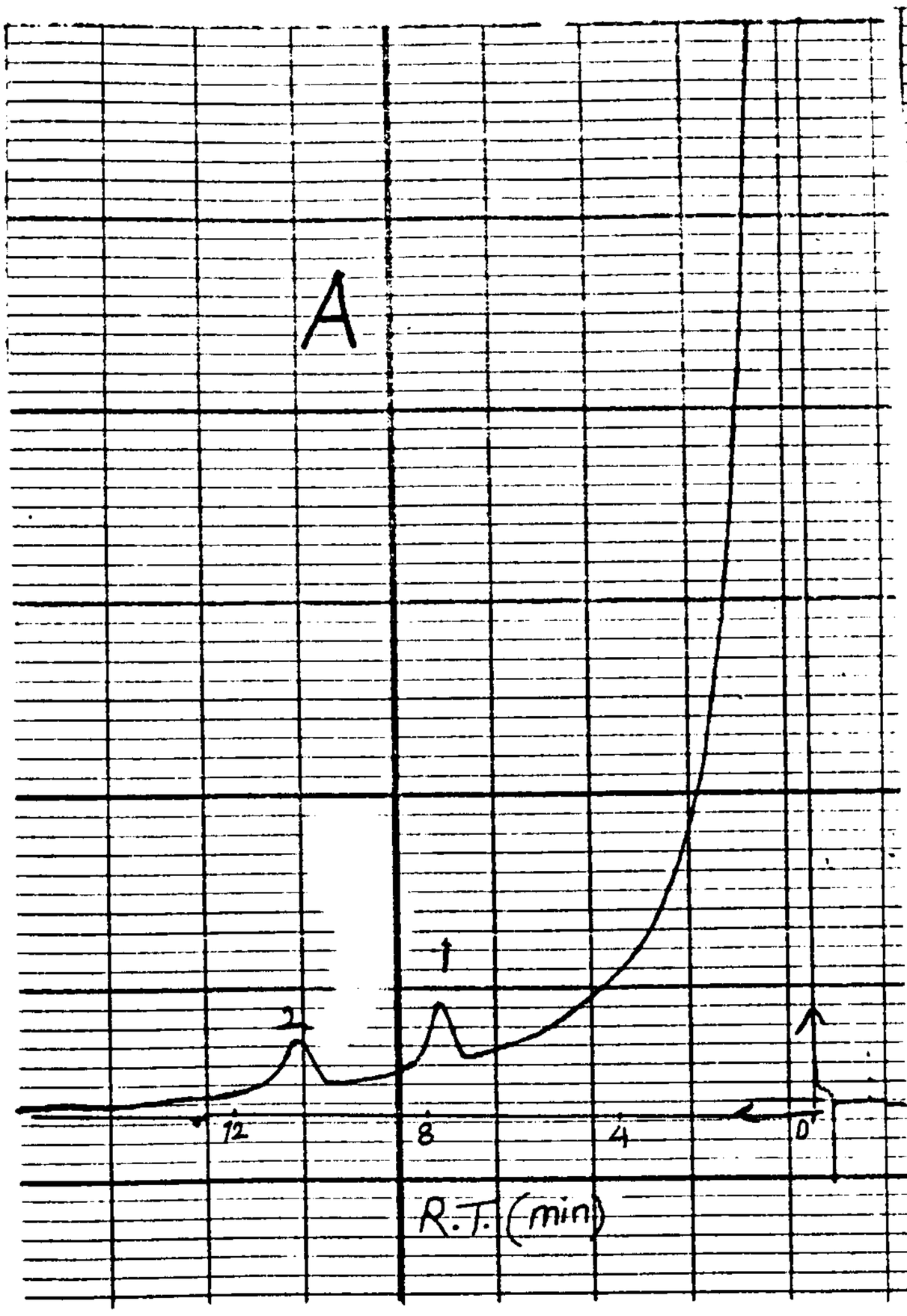
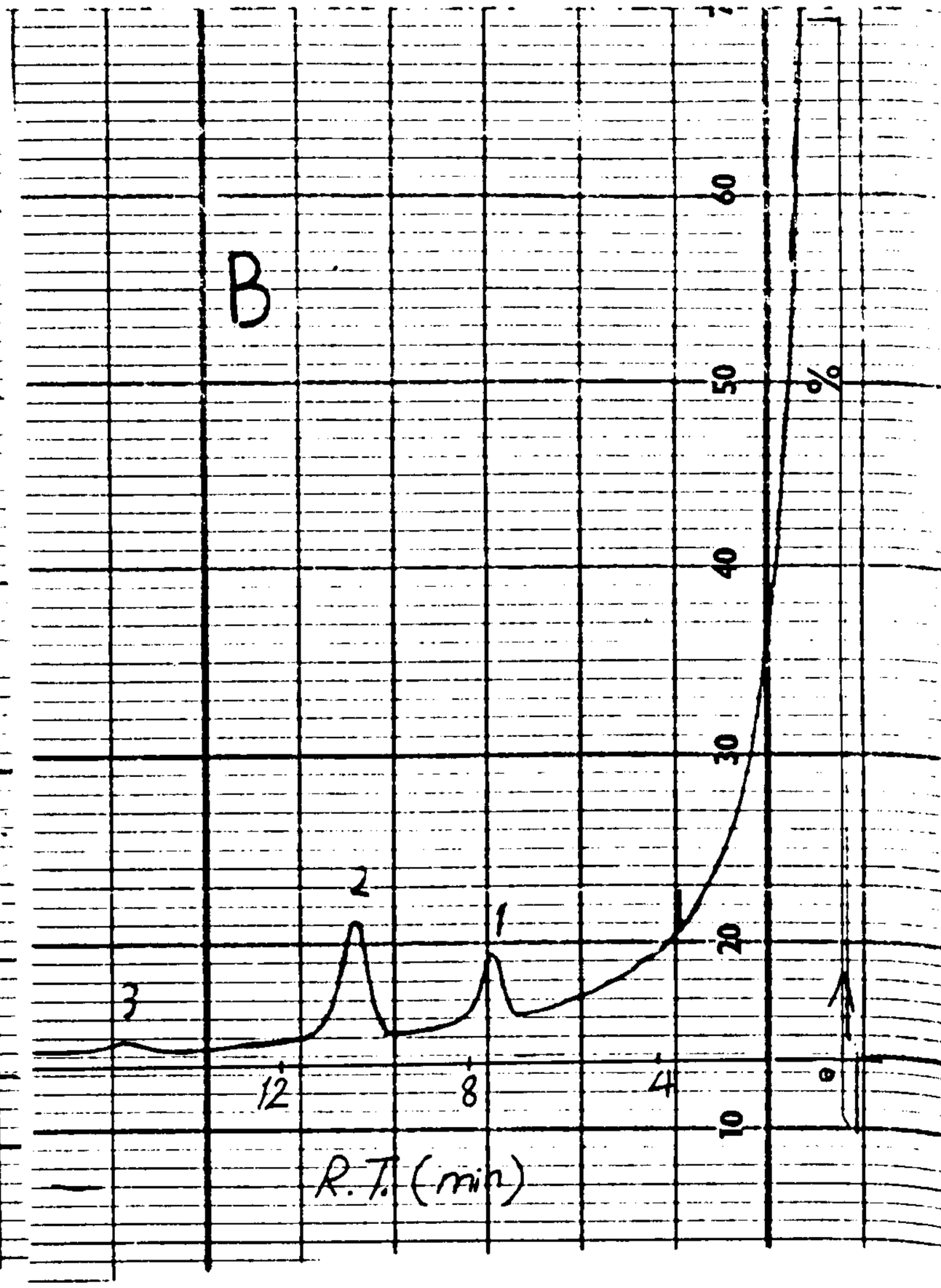

D
%
60
50
40
30
20
10
0
1
2
3
12
8
4
0
R.T.(min)

Fig. 3.25 Chromatograms of VFA of C.T.8 cultures incubated for 1 day (A), 3 days (B), and 4 days (C).

Attenuation: 2 x 10^2; chart speed: 5mm/min

Identification of peaks: 1. acetic, 2. propionic and 3. isovaleric; acids.

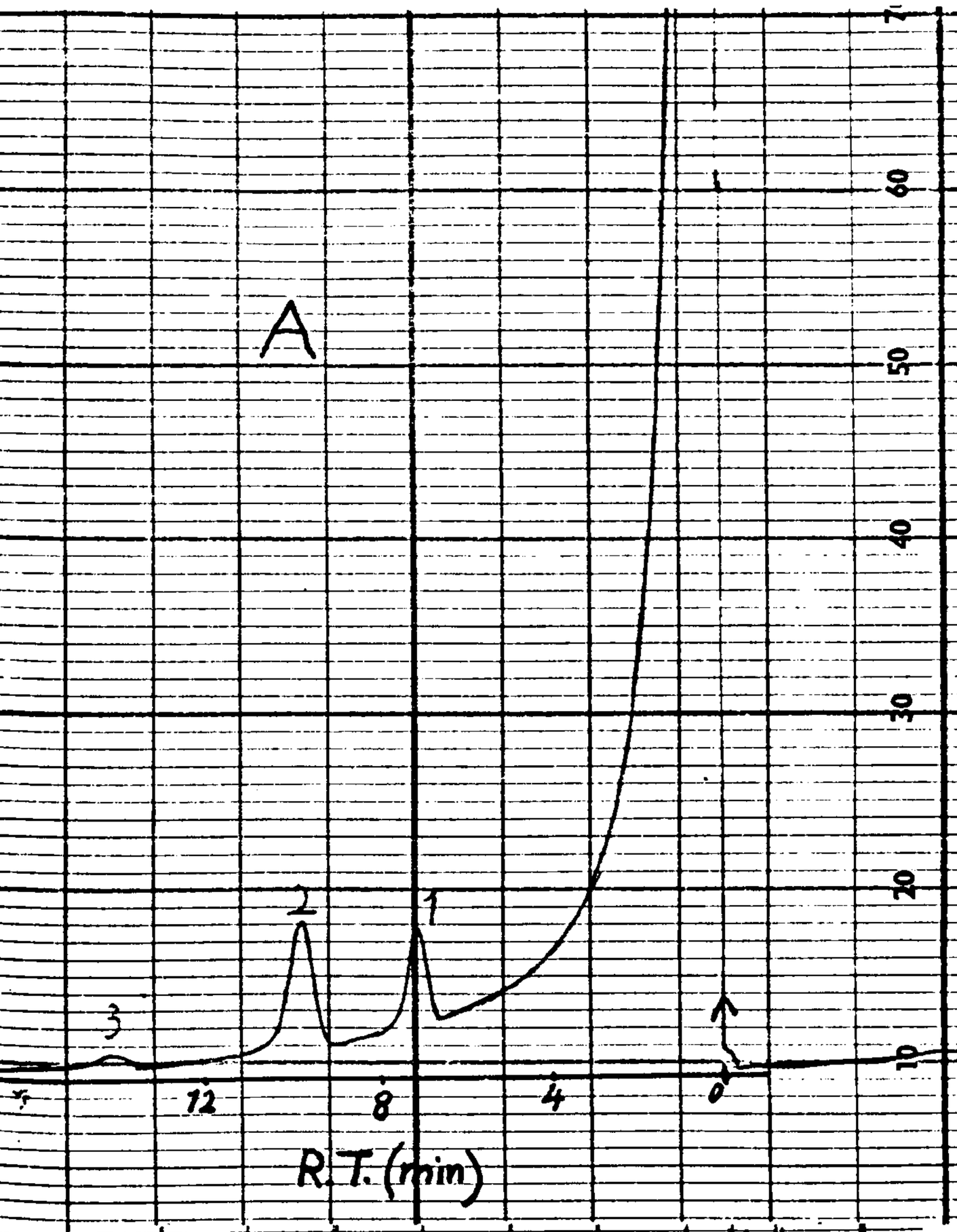

A
1
2
12
8
4
0
R.T. (min)


B
60
50
%
40
30
20
10
2
1
3
12
8
4
0
R.T. (min)

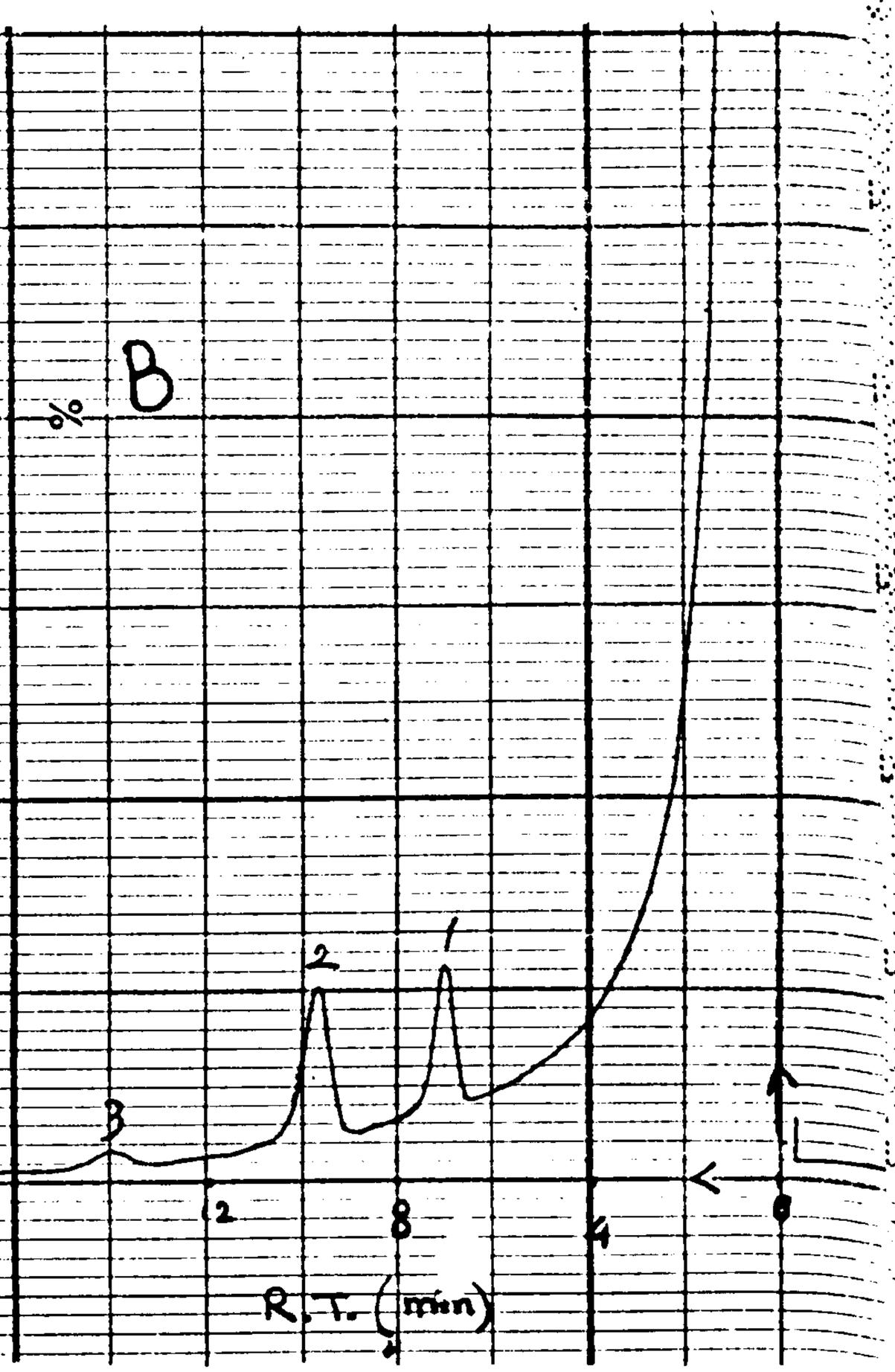
C
60
50
%
40
30
20
10
2
3
1
12
8
4
10
0
R.T. (min)

Fig. 3.26 Chromatograms of VFA of C.T.9 cultures incubated for 1 day (A), 2 days (B), 3 days (C) and 4 days (D). Attenuation: 2×10^2; chart speed: 5mm/min Identification of peaks: 1. acetic, 2. propionic, and 3. isovaleric acid.

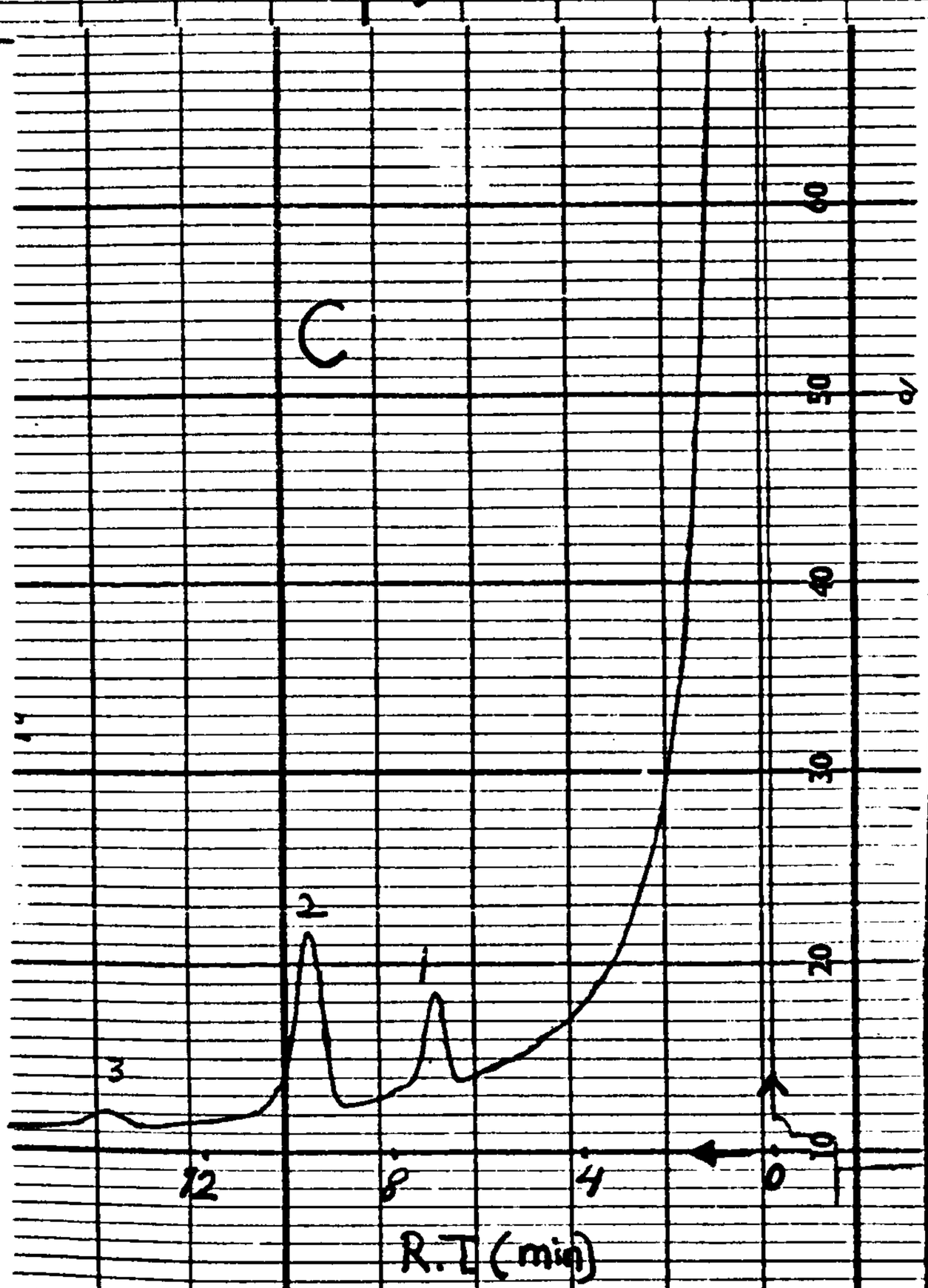
A


60
50
%
40
30
20
10
0
3
2
1
12
8
4
0
R.T. (min)

B


60
50
%
40
30
20
10
0
2
1
3
12
8
4
0
R.T. (min)

C


60
50
%
40
30
20
10
0
3
2
1
12
8
4
0
R.T. (min)

D

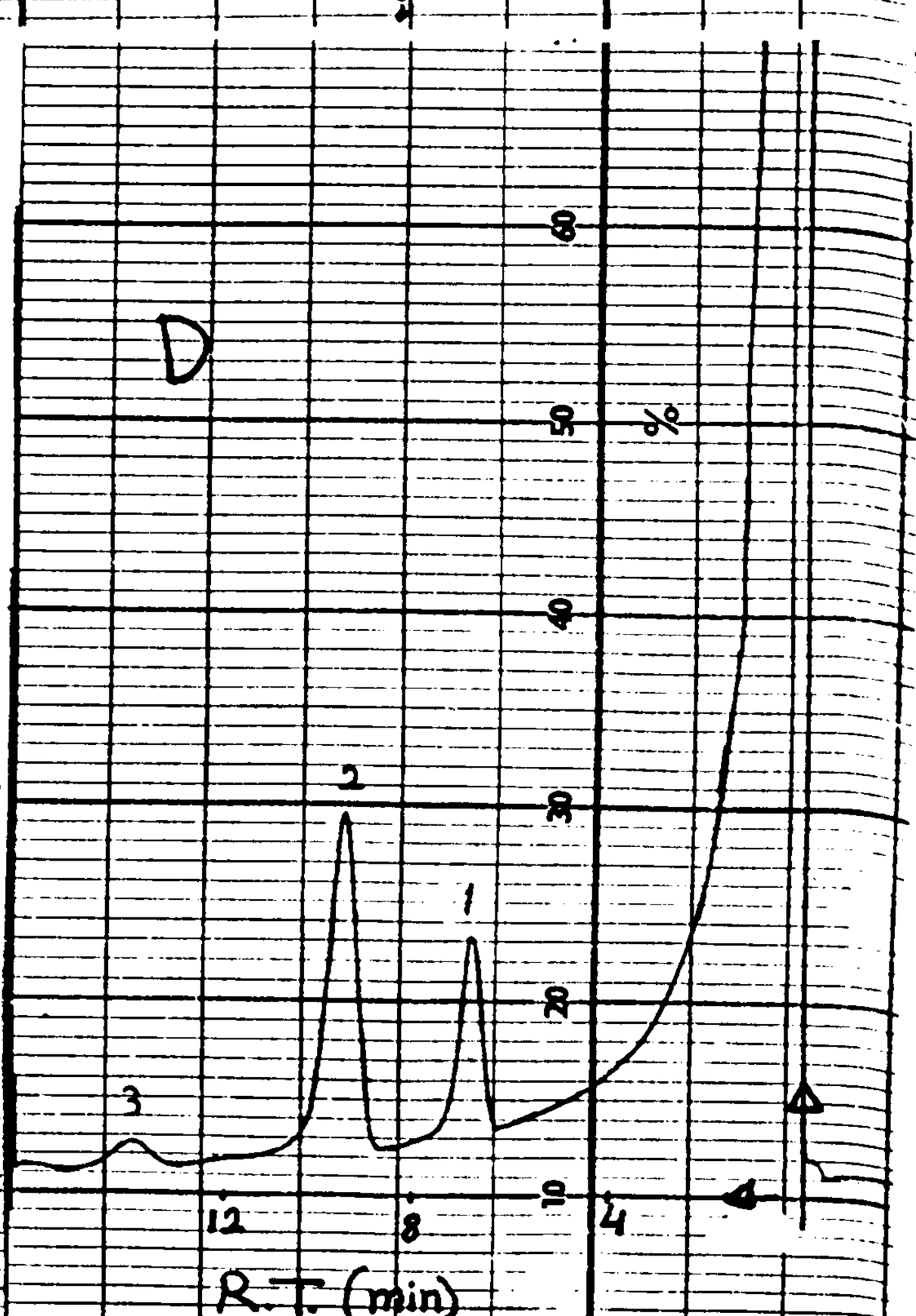
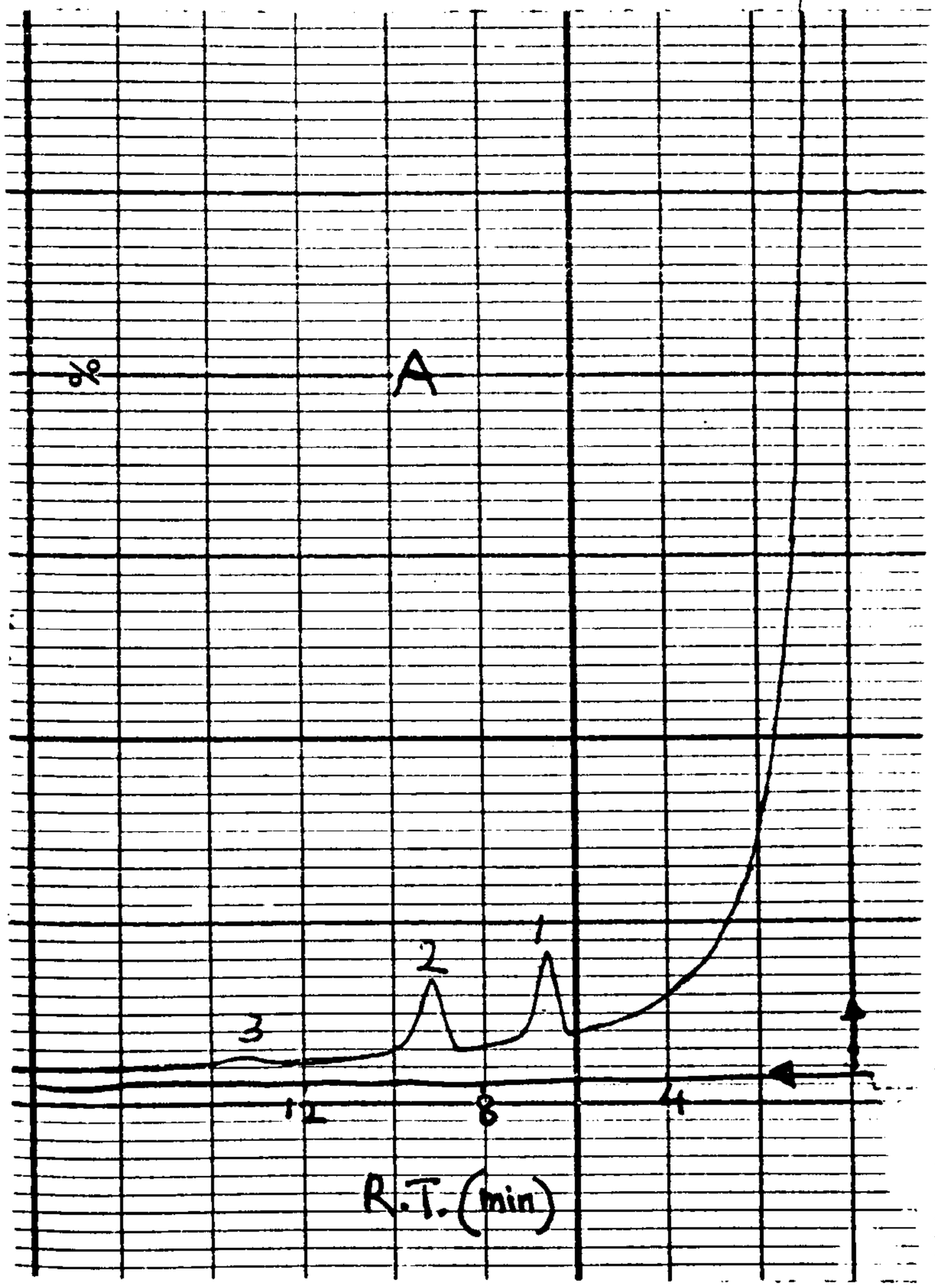
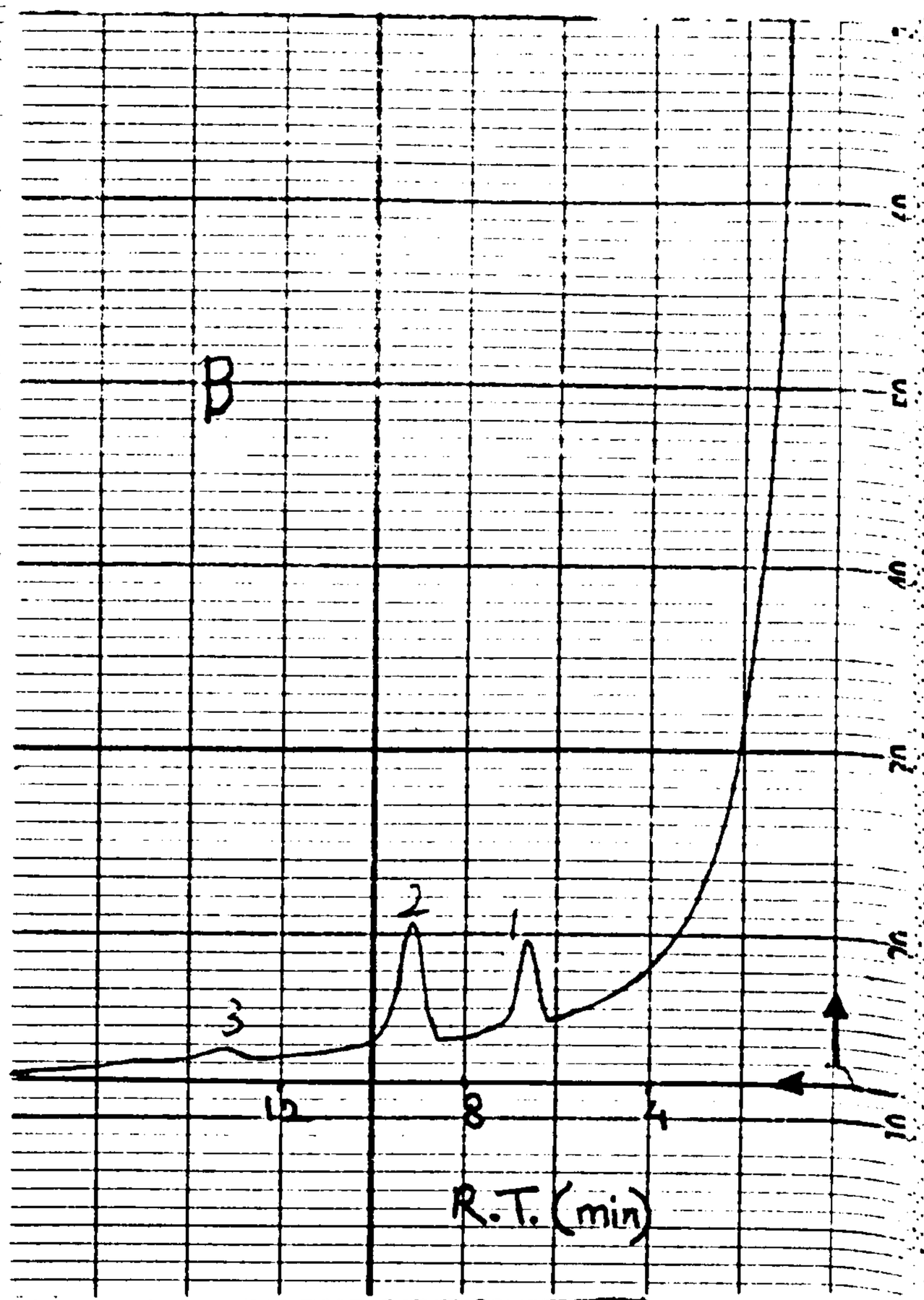

80
60
%
40
30
20
10
0
2
1
3
12
8
4
0
R.T. (min)

Fig. 3.27 Chromatograms of VFA of C.T.11 cultures incubated for 1 day (A), 2 days (B), and 4 days (C)
Attenuation: 2 x 10^2; chart speed: 5mm/min
Identification of peaks: 1:acetic, 2: propionic, and 3: isovaleric acids.

A


%
100
80
60
40
20
0
1
2
3
12
8
4
R.T. (min)

B


%
100
80
60
40
20
0
1
2
3
12
8
4
R.T. (min)

C

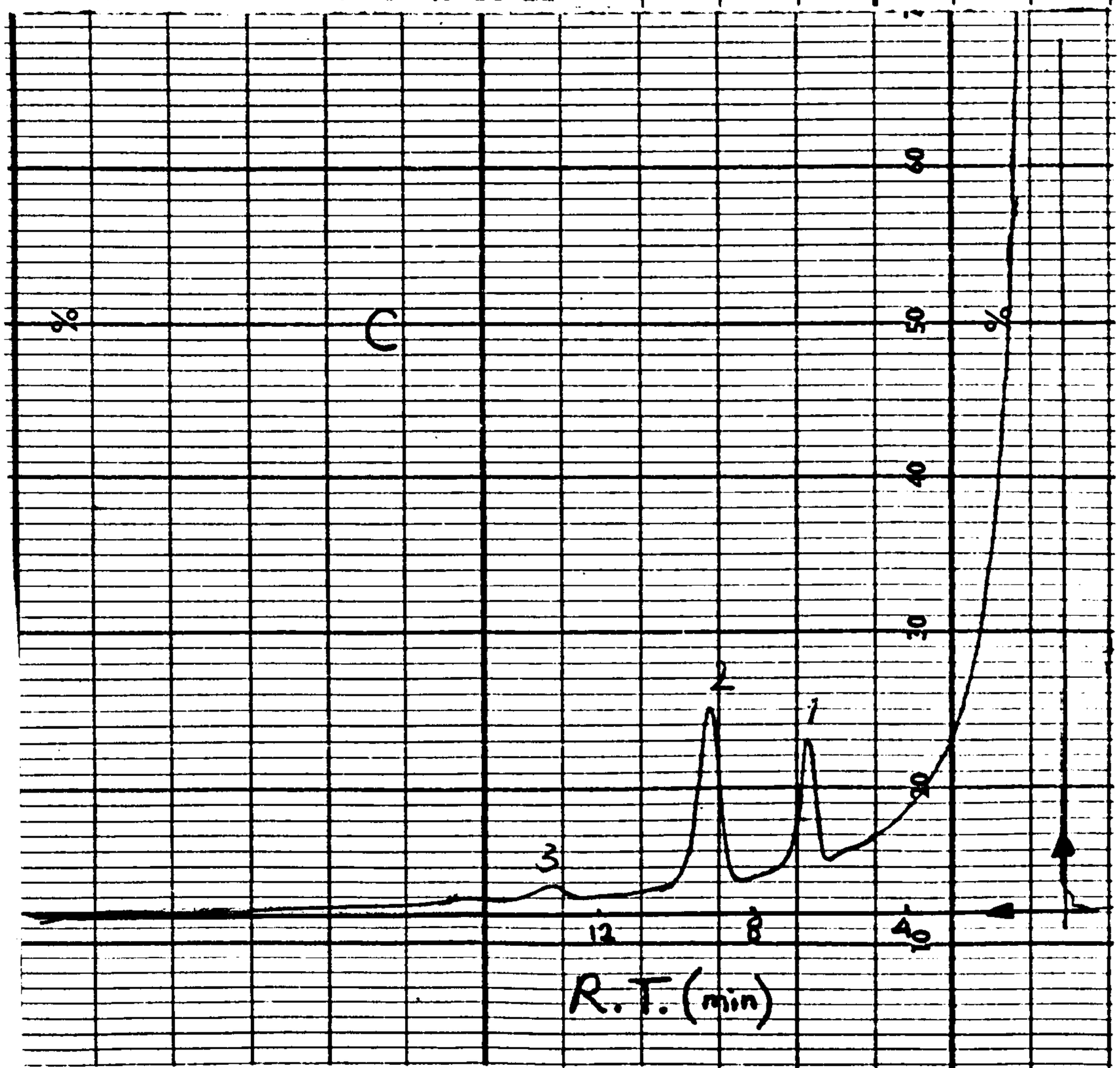
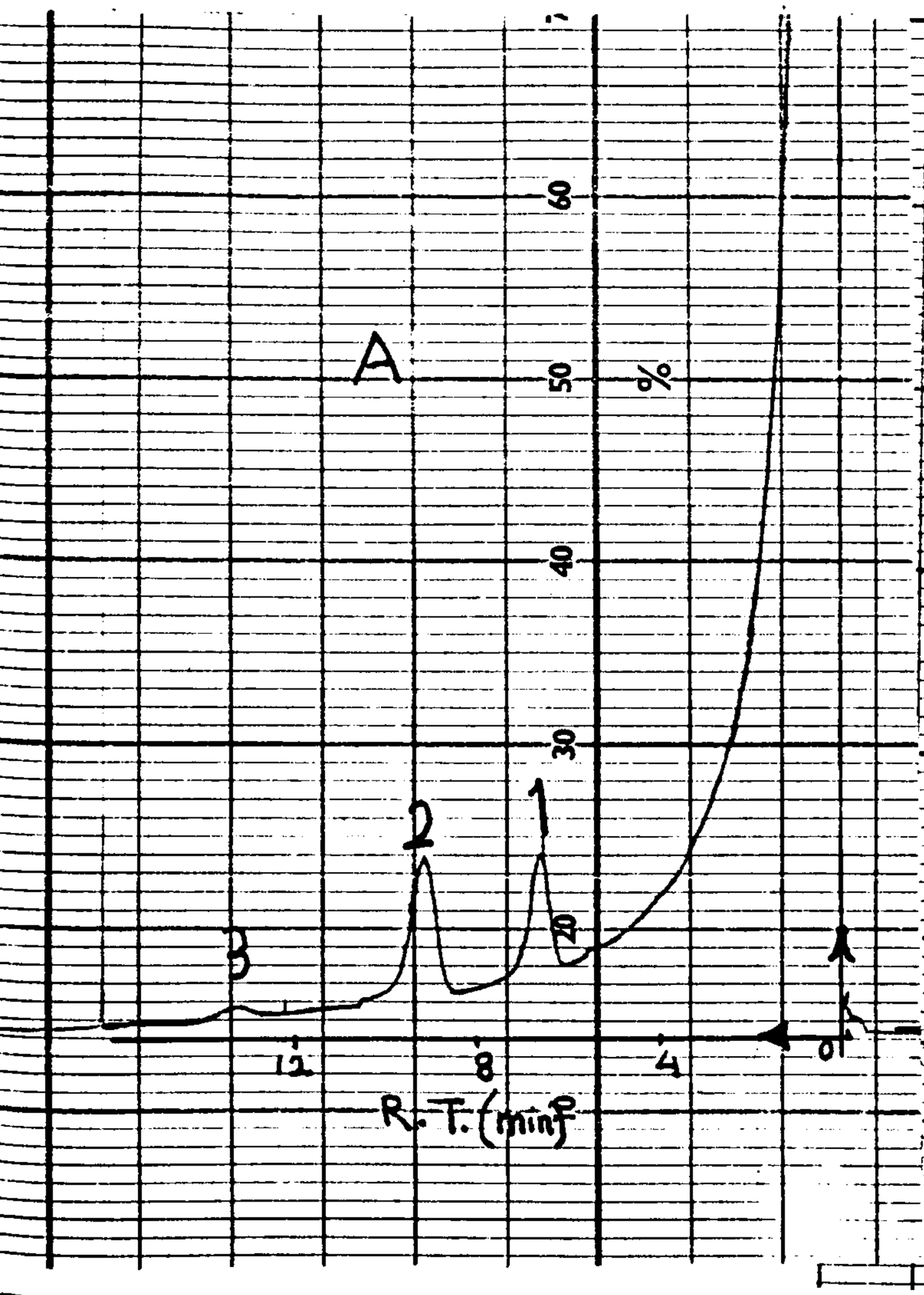
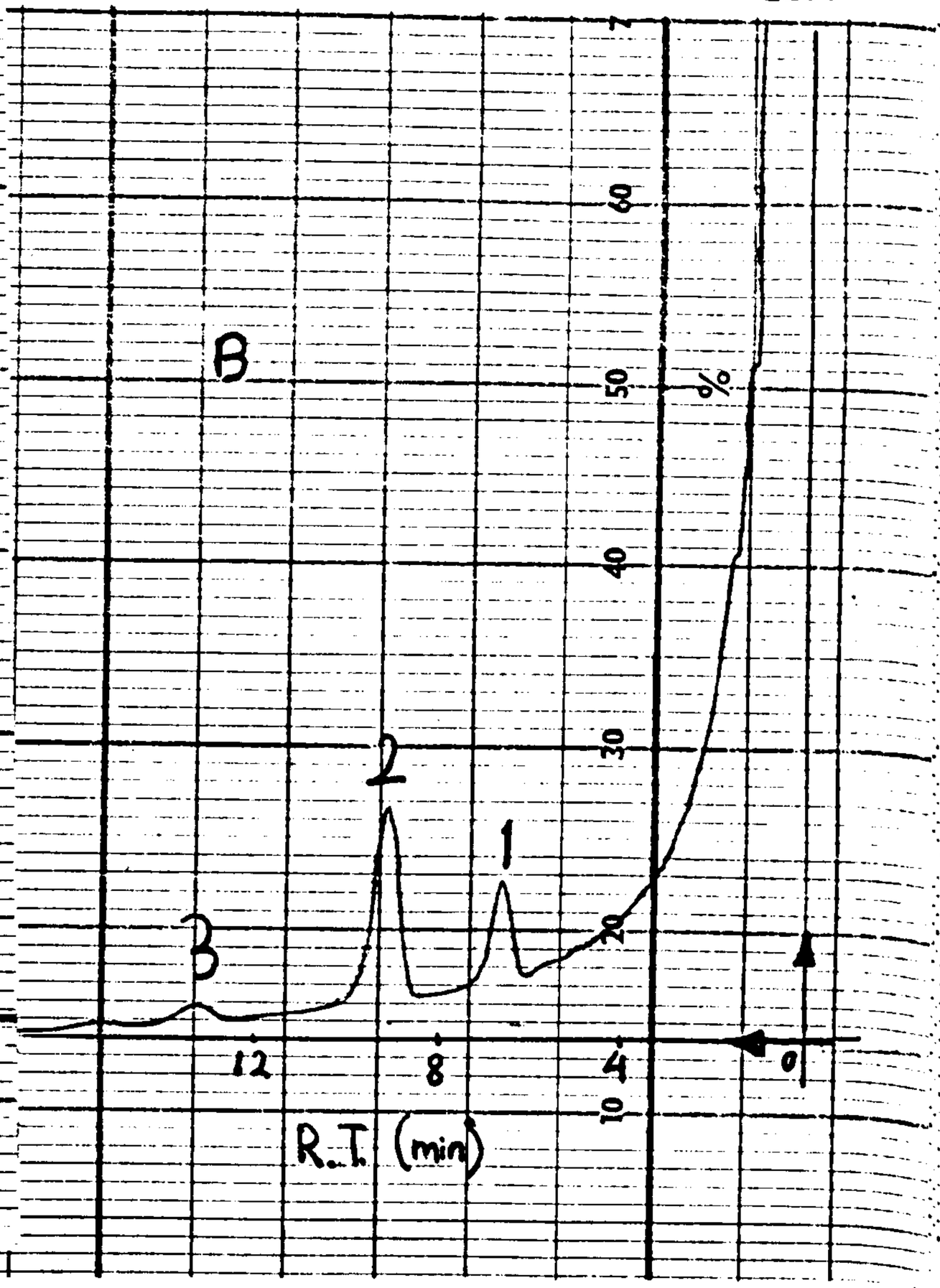
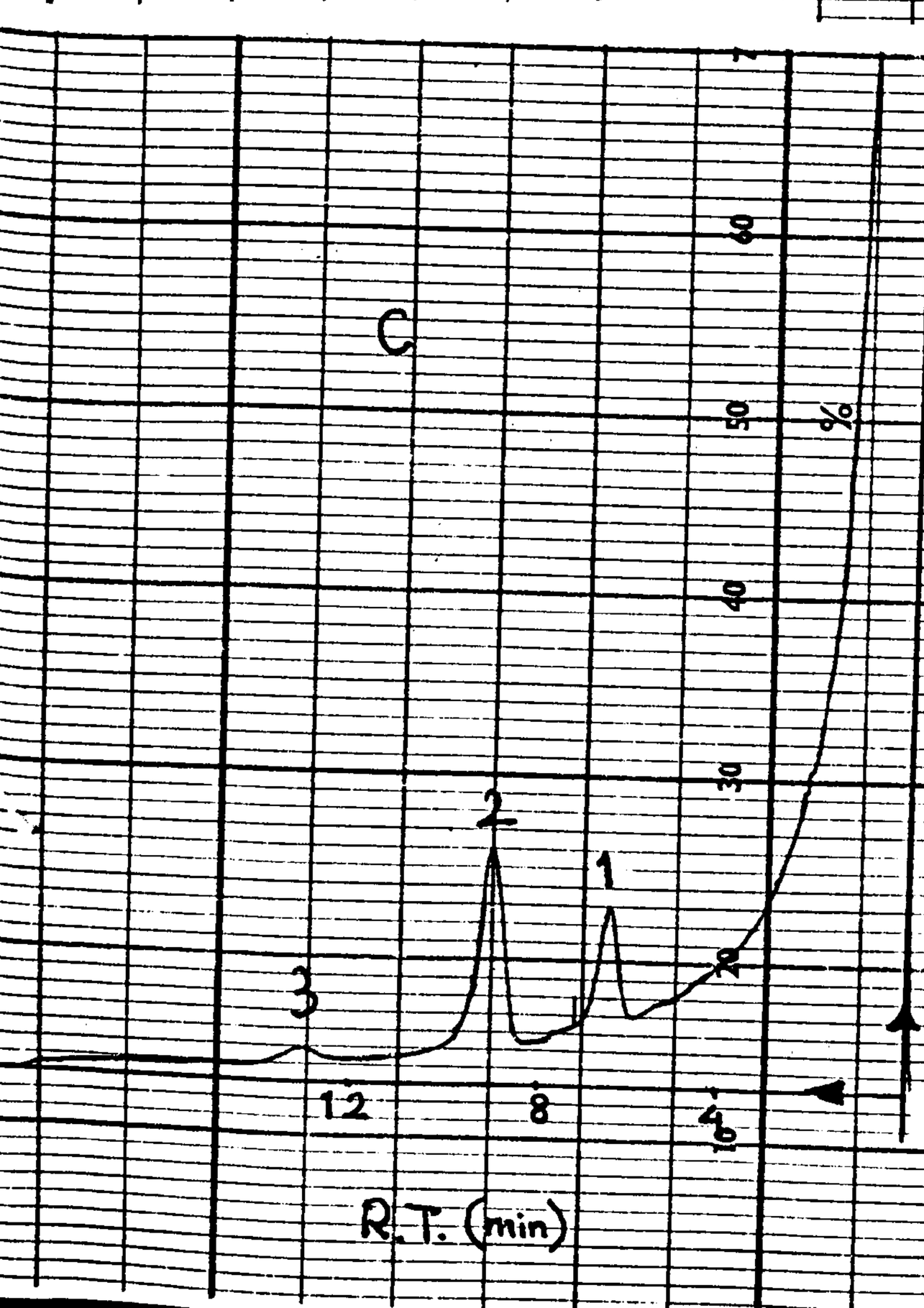

%
100
80
60
40
20
0
1
2
3
12
8
4
R.T. (min)

Fig. 3.28 Chromatograms of VFA of C.T.13 cultures incubated for 1 day (A), 2 days (B), 3 days (C), and 4 days (D). Attenuation: 2 x 10^2; chart speed: 5mm/min. Identification of peaks: 1: acetic; 2: propionic; and 3: isovaleric; acids.


A
60
50
%
40
30
20
10
0
1
2
3
12
8
4
R.T. (min)


B
60
50
%
40
30
20
10
0
1
2
3
12
8
4
R.T. (min)


C
60
50
%
40
30
20
10
0
1
2
3
12
8
4
R.T. (min)

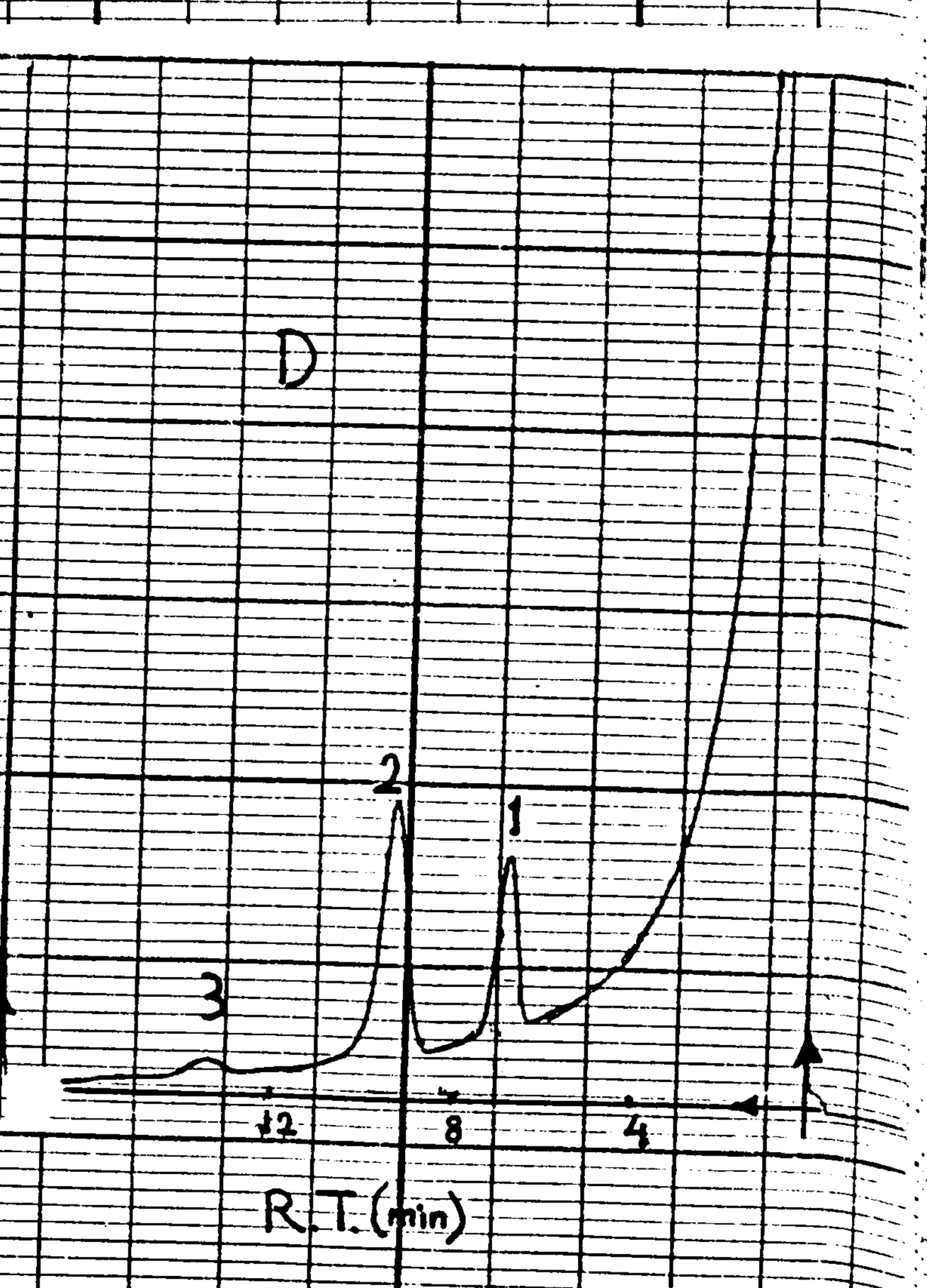
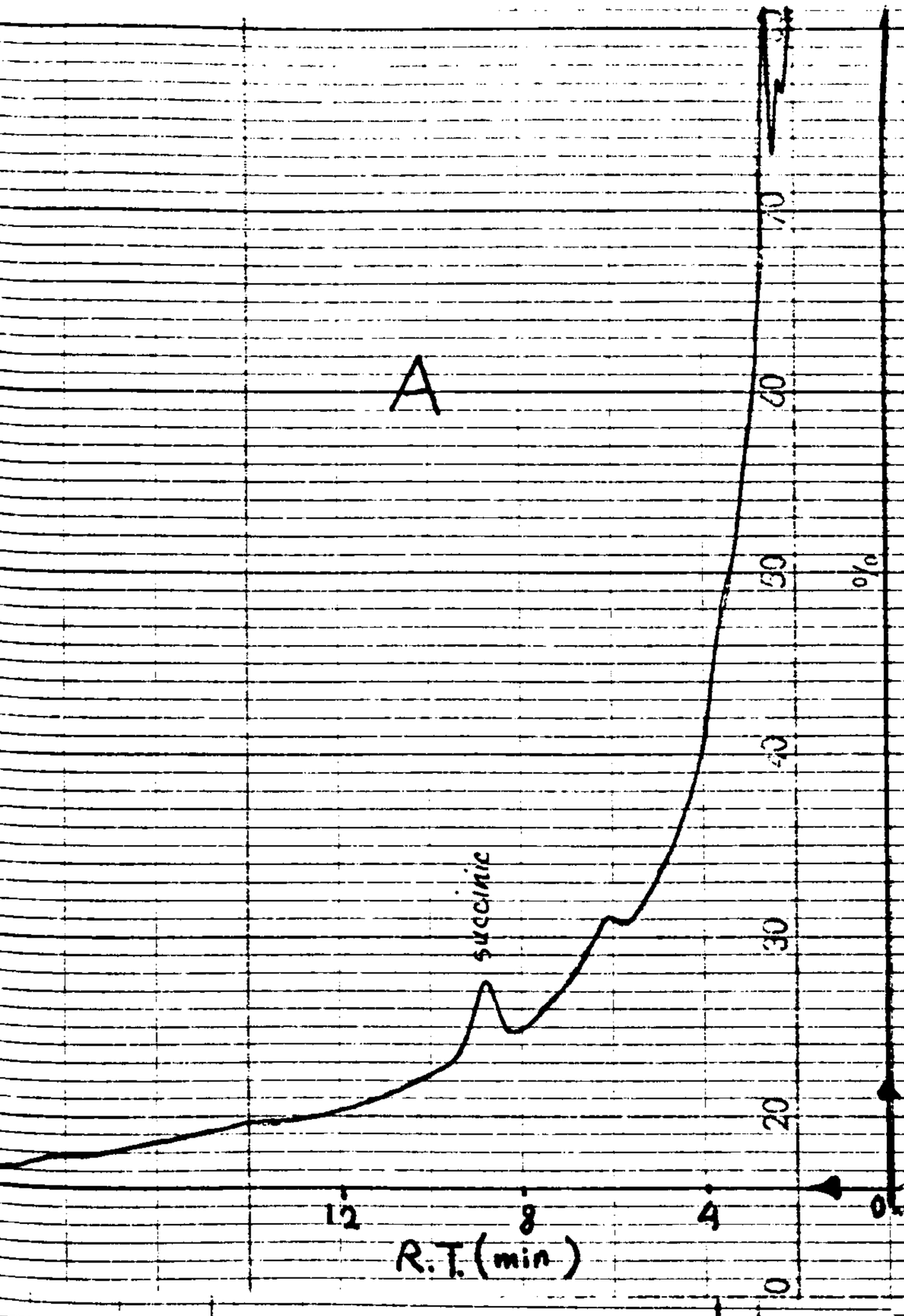
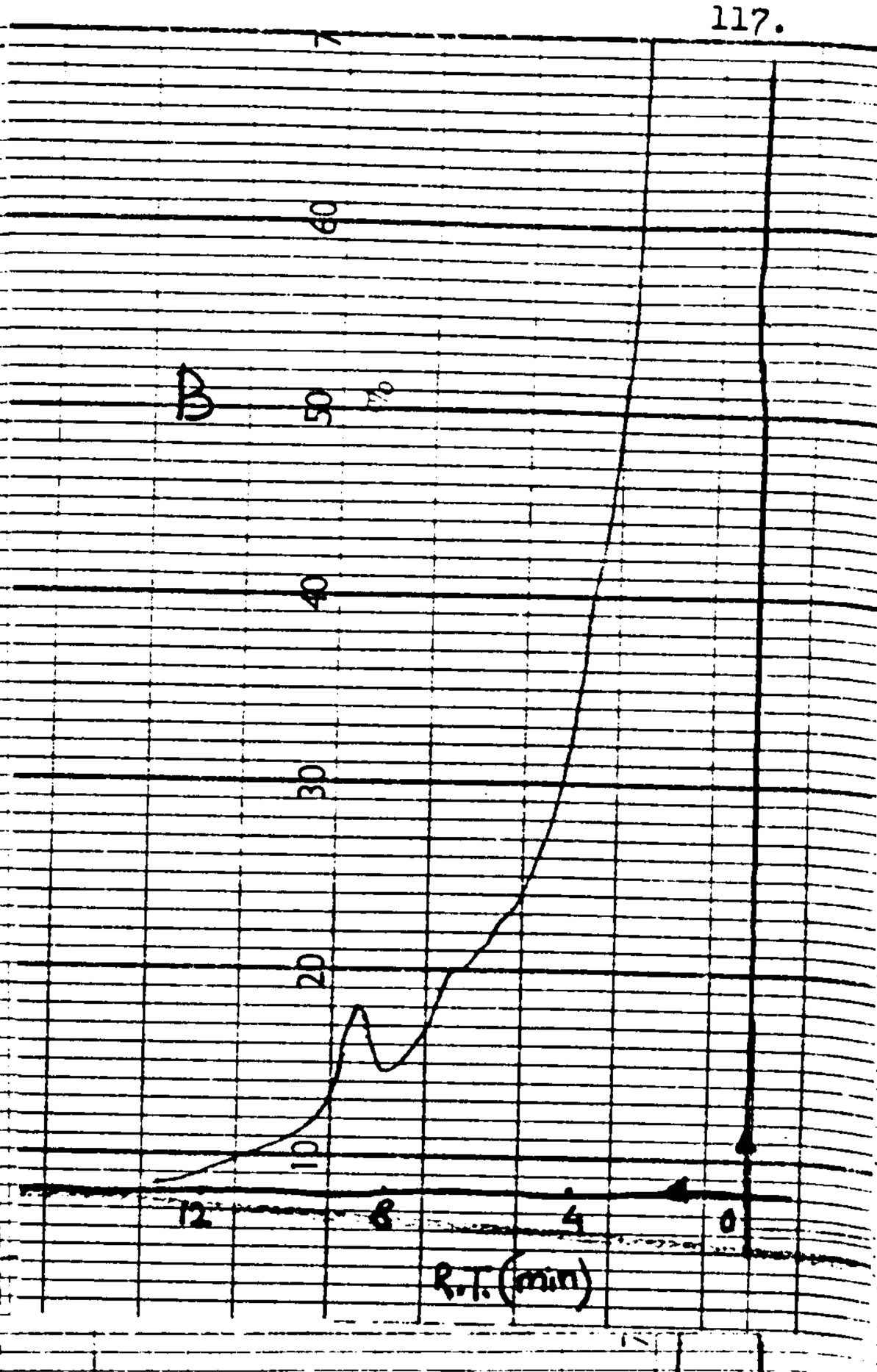

D
60
50
%
40
30
20
10
0
1
2
3
12
8
4
R.T. (min)

Fig. 3.29 Chromatograms of NVFA of C.T.2 cultures incubated for 1 - 4 days (A-D) respectively.
Attenuation: 1 x 10^2; chart speed 5mm/min.

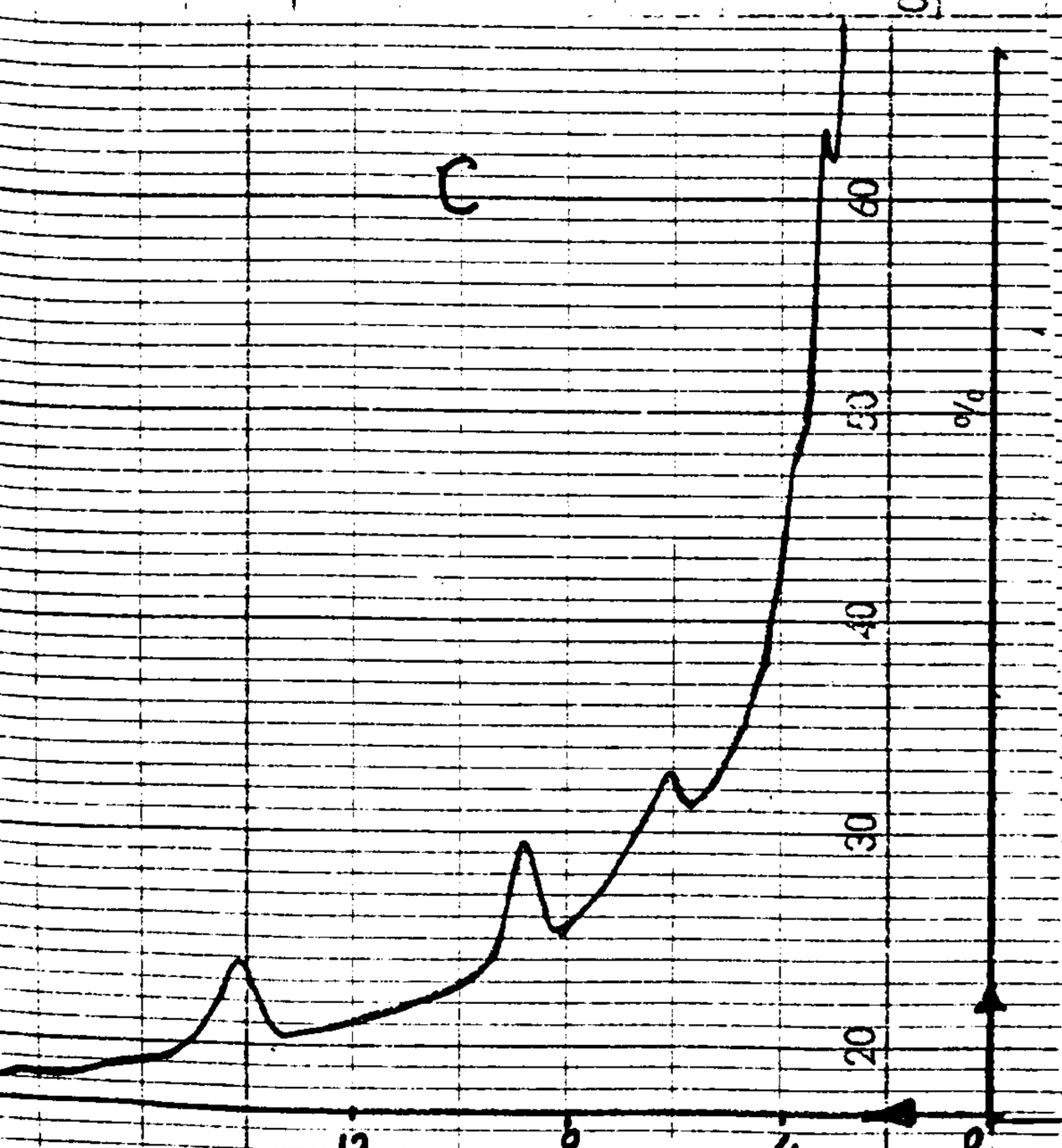
A


succinic
12
8
4
0
R.T. (min)
10
20
30
40
50
%

B


Zb 271
12
8
4
0
R.T. (min)
10
20
30
40
50
%

C


12
8
4
0
R.T. (min)
10
20
30
40
50
%

D

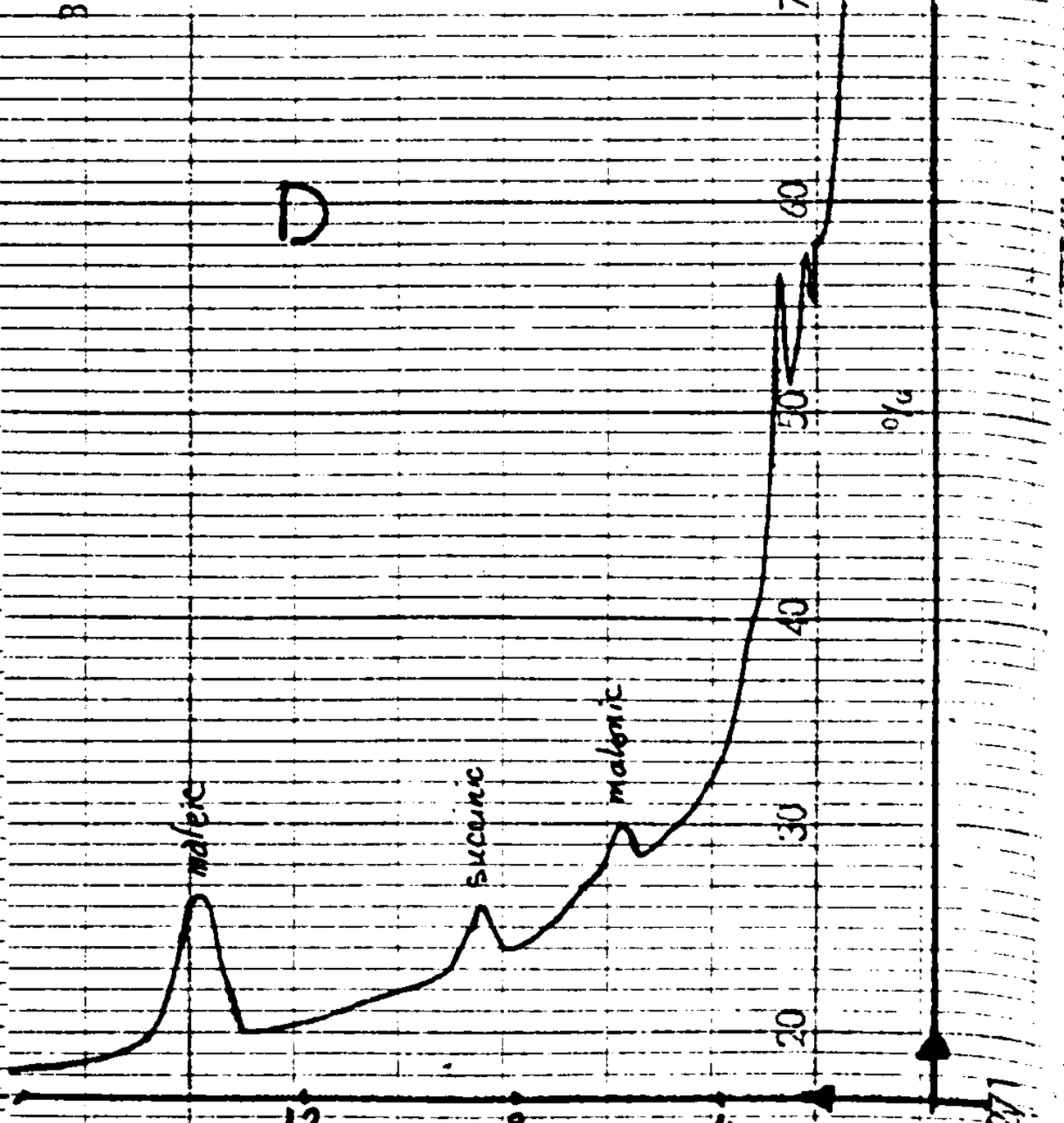
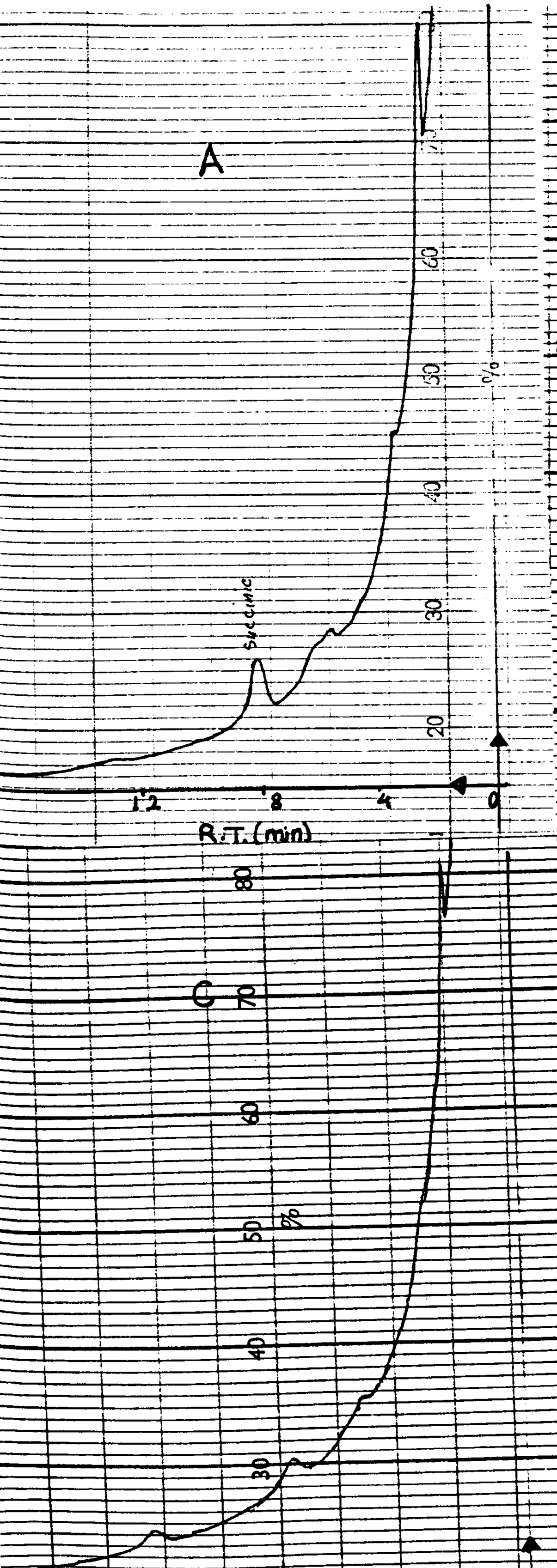
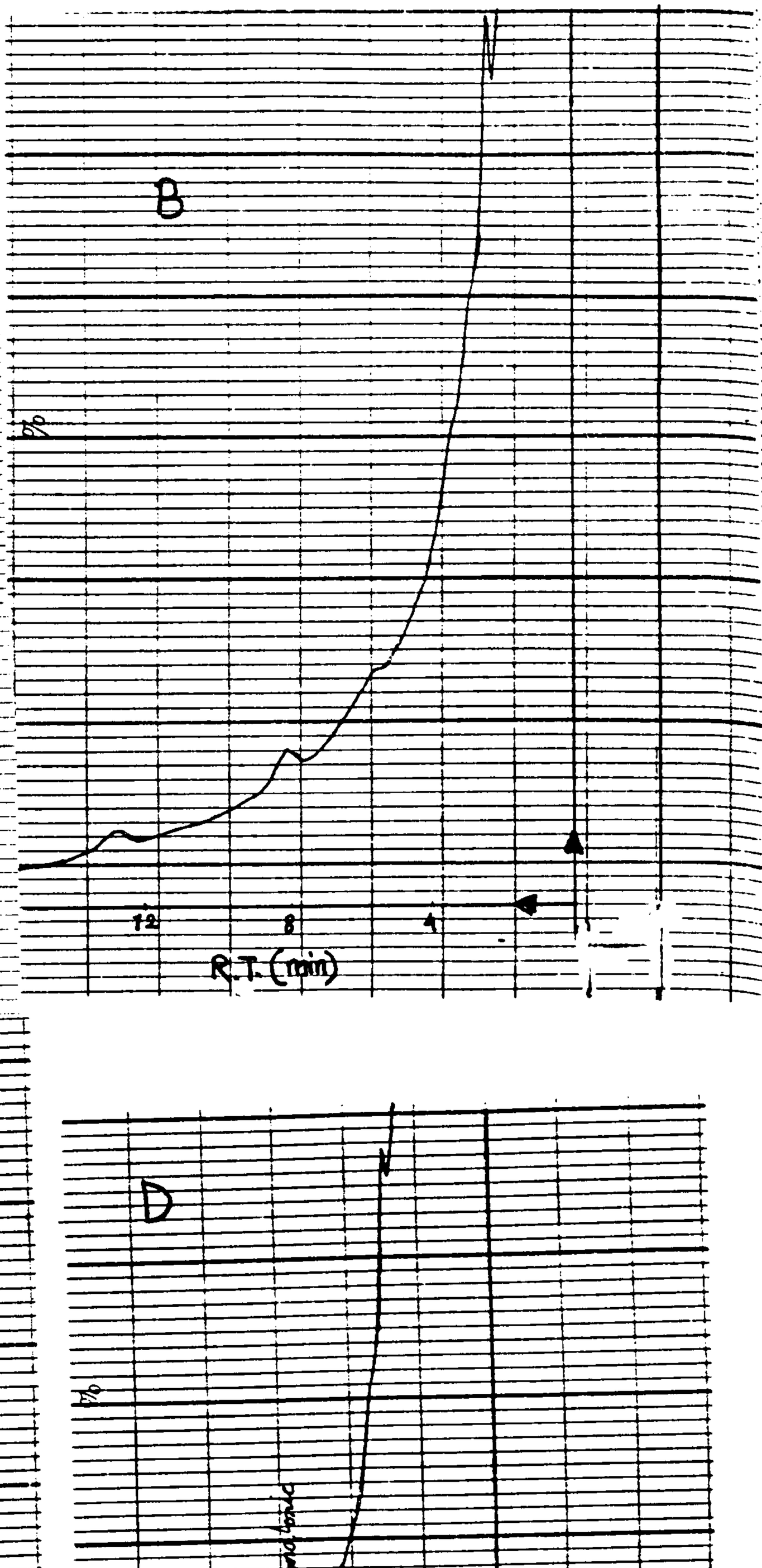

maleic
succinic
malonic
12
8
4
0
R.T. (min)
10
20
30
40
50
%
230+258 76 271

Fig. 3.30 Chromatograms of NVFA of C.T.3A cultures incubated for 1 - 4 days (A-D) respectively. Attenuation 1×10^2; chart speed: 5mm/min.

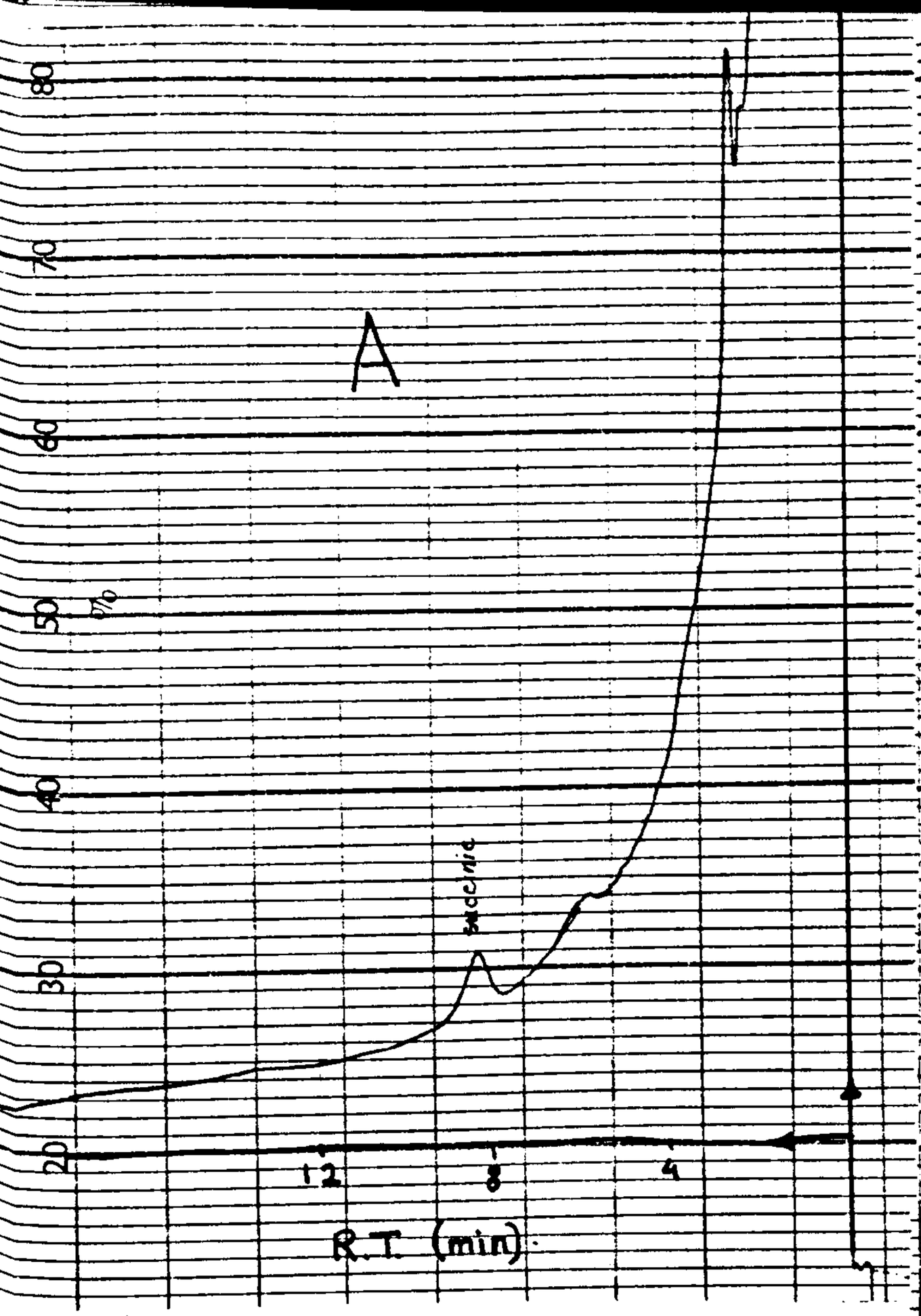

A
60
50
%
40
30
20
Succinic
12
8
4
0
R.T. (min)

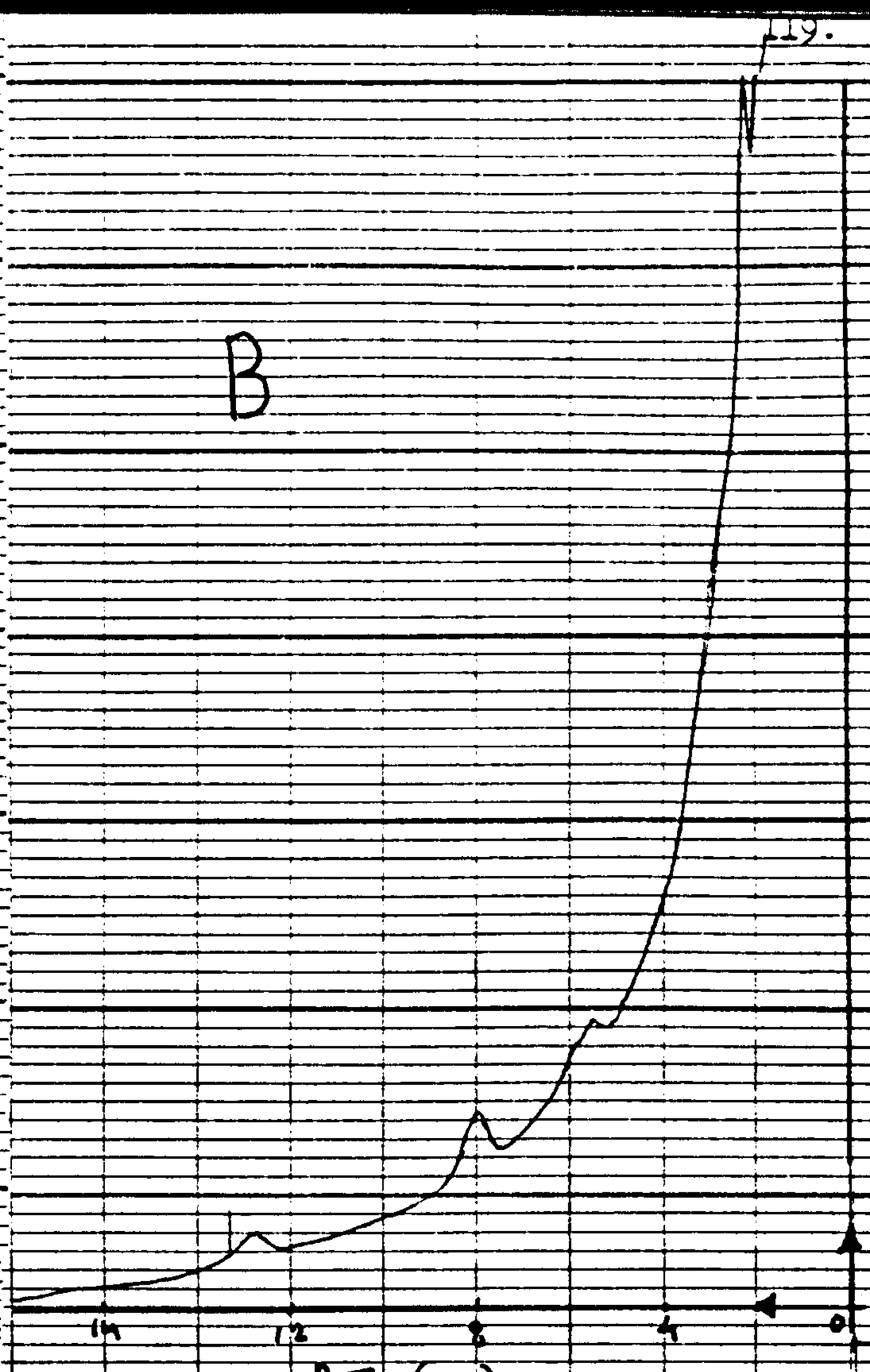

B
%
12
8
4
R.T. (min)

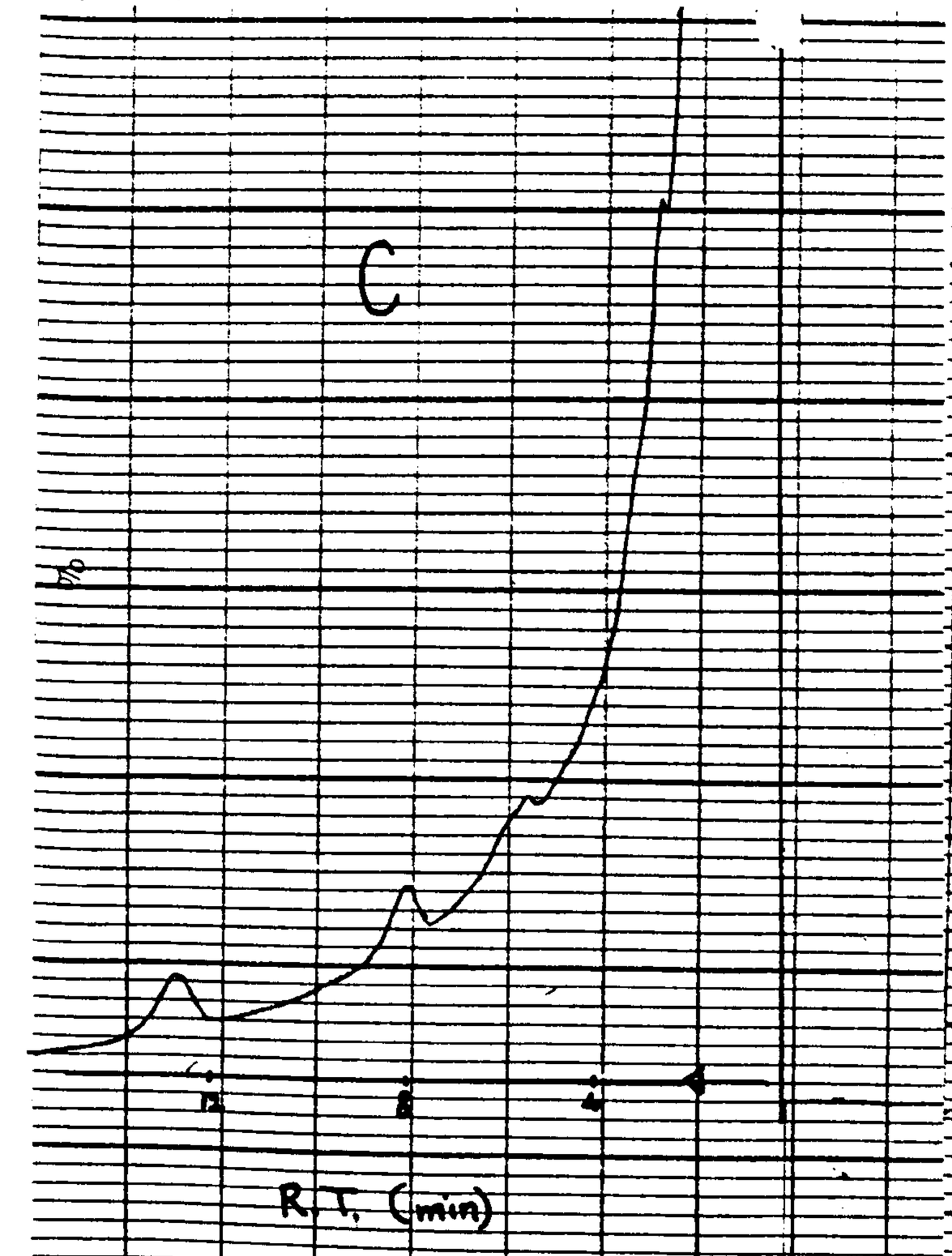
C
80
70
60
50
%
40
30
20
12
8
4
R.T. (min)

D
%
Succinic
10
8
4
R.T. (min)

Fig. 3.31 Chromatograms of NVFA of C.T.4 cultures incubated for 1 -4days (A-D) respectively. Attenuation: 1×10^2; chart speed 5mm/min.


A
%
50
40
30
20
succinic
12
8
4
R.T. (min)


B
16
12
8
4
0
R.T. (min)


C
%
succinic
malonic
12
8
4
R.T. (min)

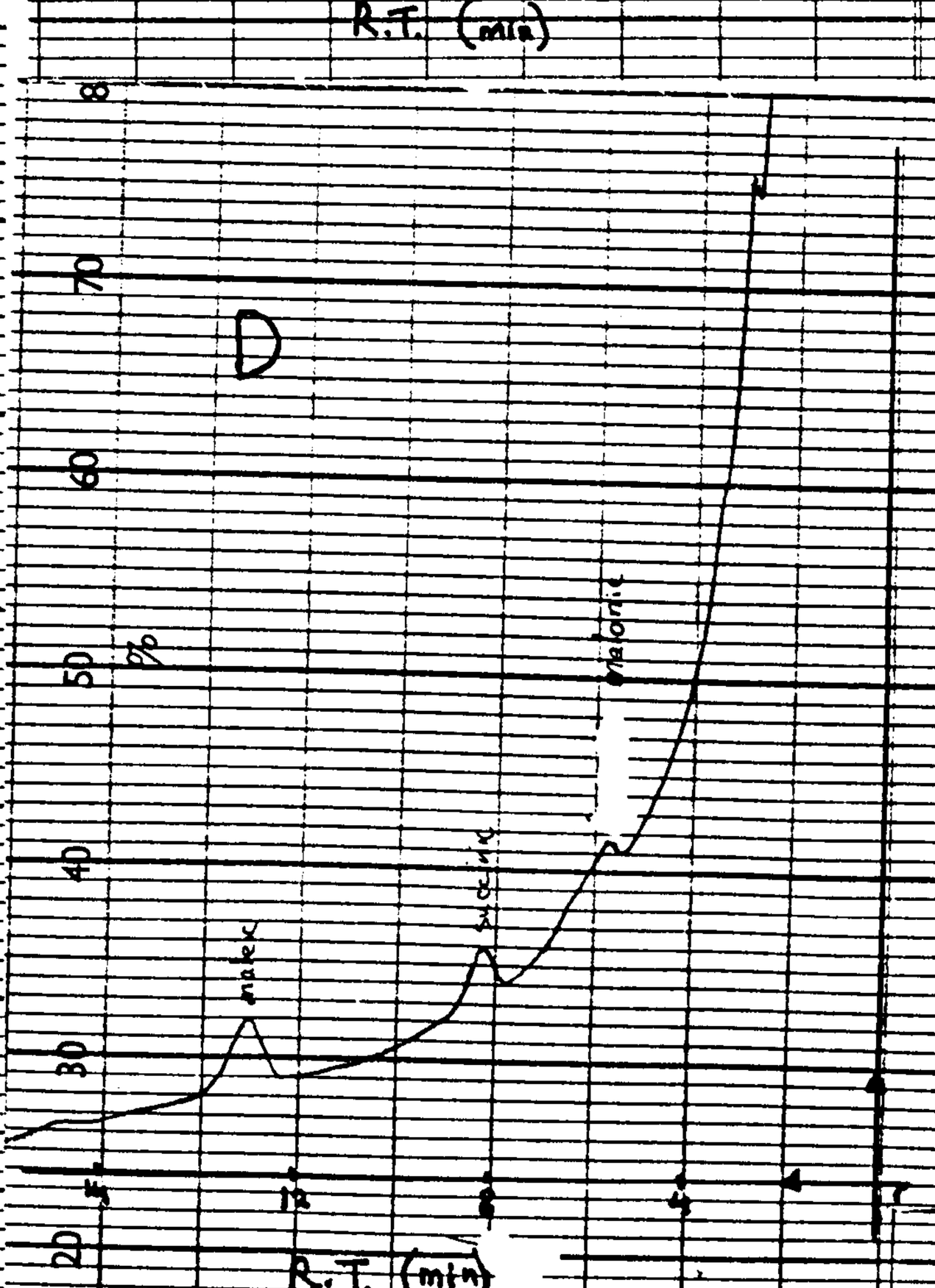

D
%
50
40
30
20
malonic
succinic
malic
12
8
4
0
R.T. (min)

Fig. 3.32 Chromatograms of NVFA of C.T.8 cultures incubated for 1 - 4 days (A-D) respectively. Attenuation: 1×10^2; chart speed: 5mm/min.

120.

A

succinic

R.T. (min)

B

R.T. (min)

C

R.T. (min)

D

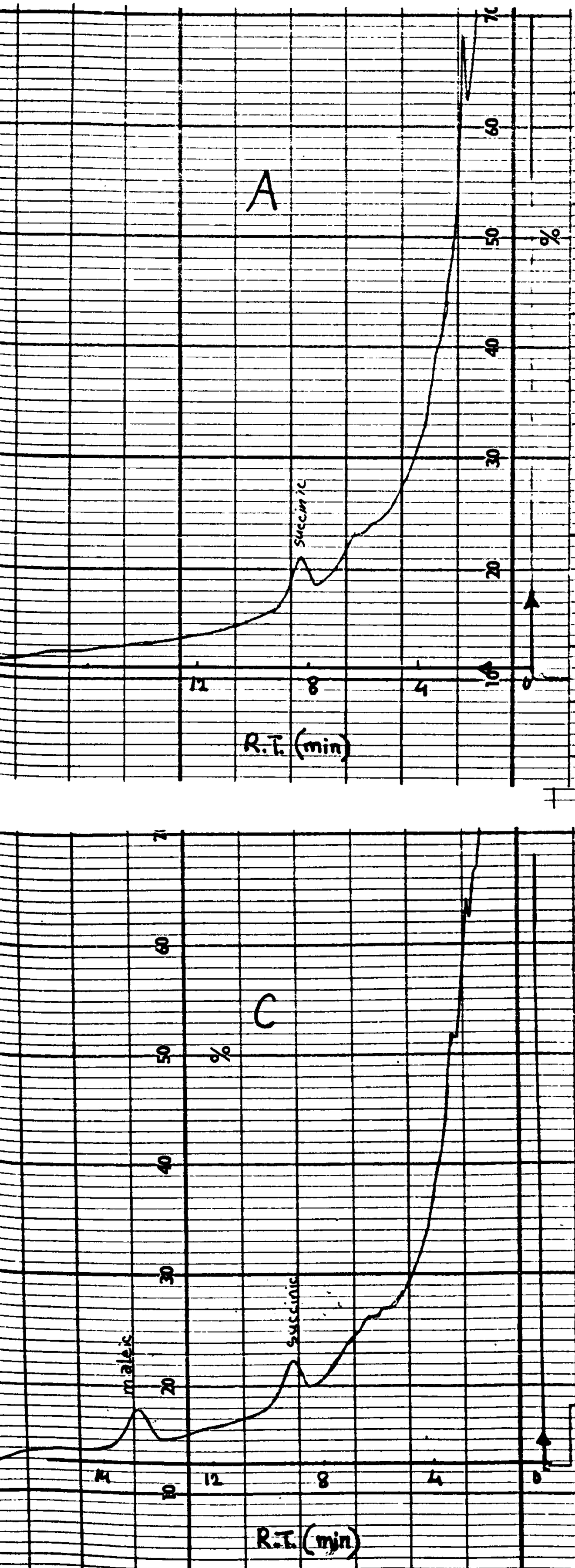
malonic

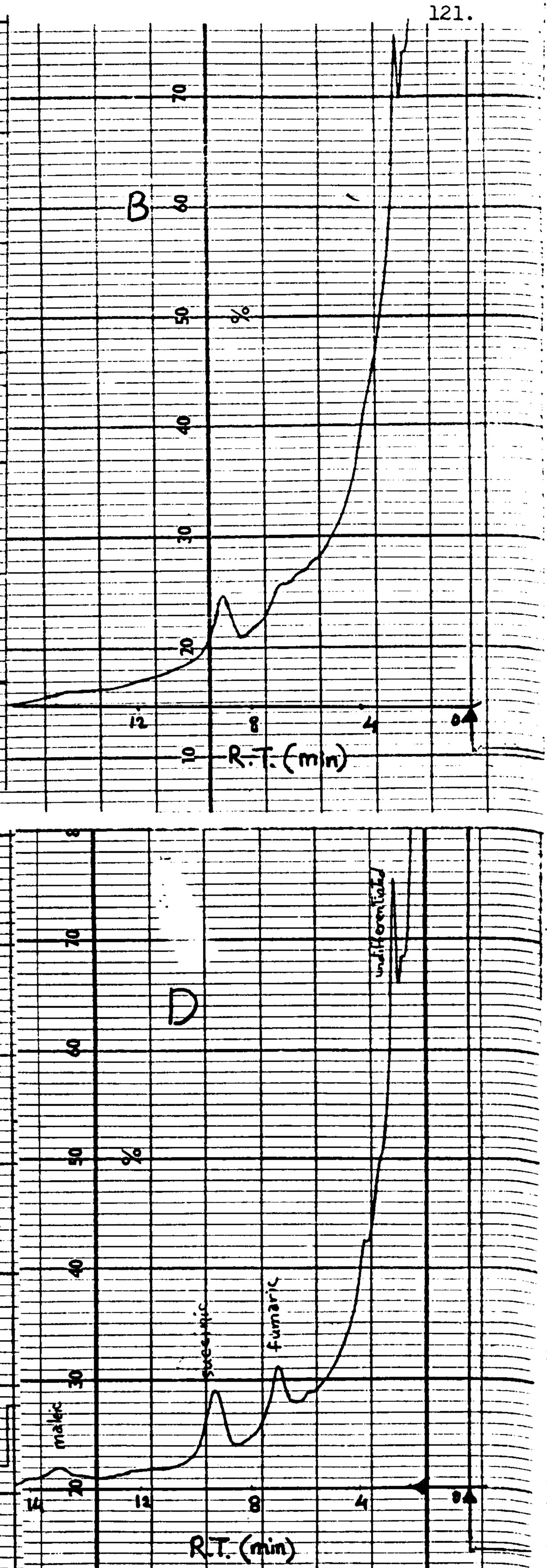
succinic

malic

R.T. (min)

Fig. 3.33 Chromatograms of NVFA of C.T.9 cultures incubated for 1 -4days (A-D) respectively. Attenuation: 1×10^2; chart speed: 5mm/min.


A
succinic
%
70
60
50
40
30
20
10
0
14
12
8
4
R.T. (min)


B
%
70
60
50
40
30
20
10
12
8
4
0
R.T. (min)

C
%
70
60
50
40
30
20
10
malic
succinic
14
12
8
4
0
R.T. (min)

D
%
70
60
50
40
30
20
undifferentiated
malic
succinic
fumaric
14
12
8
4
0
R.T. (min)

Fig. 3.34 Chromatograms of NVFA of C.T.11 cultures incubated for 1 - 4 days (A-D) respectively. Attenuation: 1×10^2; chart speed: 5mm/min.

A

B

C

D

succinic

R.T. (min)

succinic

malic

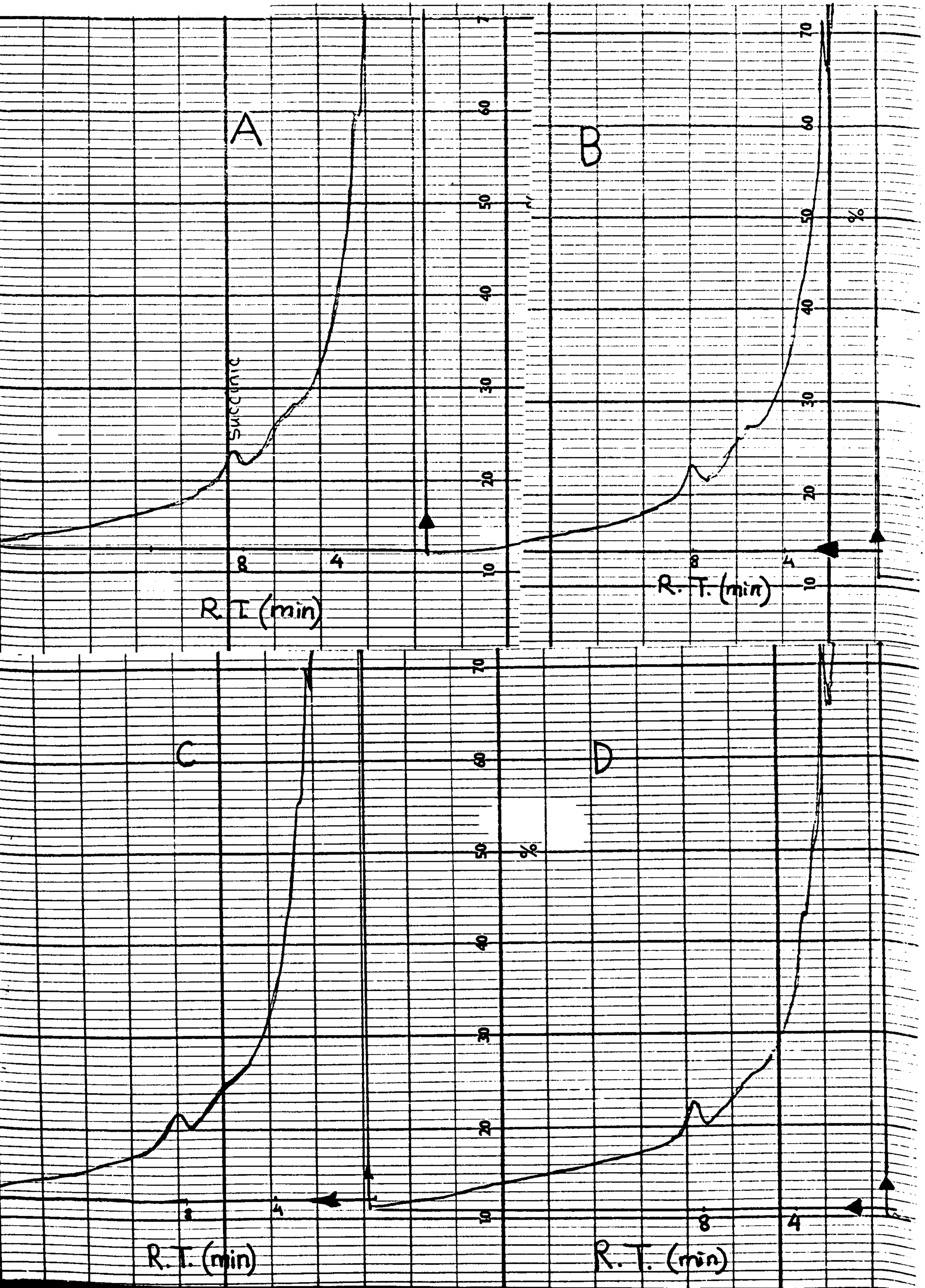
succinic

malonic

R.T. (min)

R.T. (min)

Fig. 3.35 Chromatograms of NVFA of C.T.13 cultures incubated for 1 - 4 days (A-D) respectively. Attenuation: 1×10^2; chart speed 5mm/min.


A
Succinic
70
60
50
40
30
20
10
%
8
4
R. T. (min)
B
C
D

It can be seen from the chromatograms in Figs. 3.20 - 3.28 that peaks of acetic and propionic acids are the most significant peaks in all the samples. Measurements were taken for these peaks in samples of 1 day and 4 days' old cultures (Table 3.23). The largest peak areas for acetic and propionic acids were those for C.T. 1B and C.T.4. Therefore, concentrations of these two acids in samples (of 1 day and 4 days) of the two C.T. cultures were calculated. Equivalent concentrations of both acids were added to four samples of uninoculated BHIBA media (Oxoid). These four were tested together with the two prepared in section 3. 4. 3 and the results are given in section 3. 4. 6.

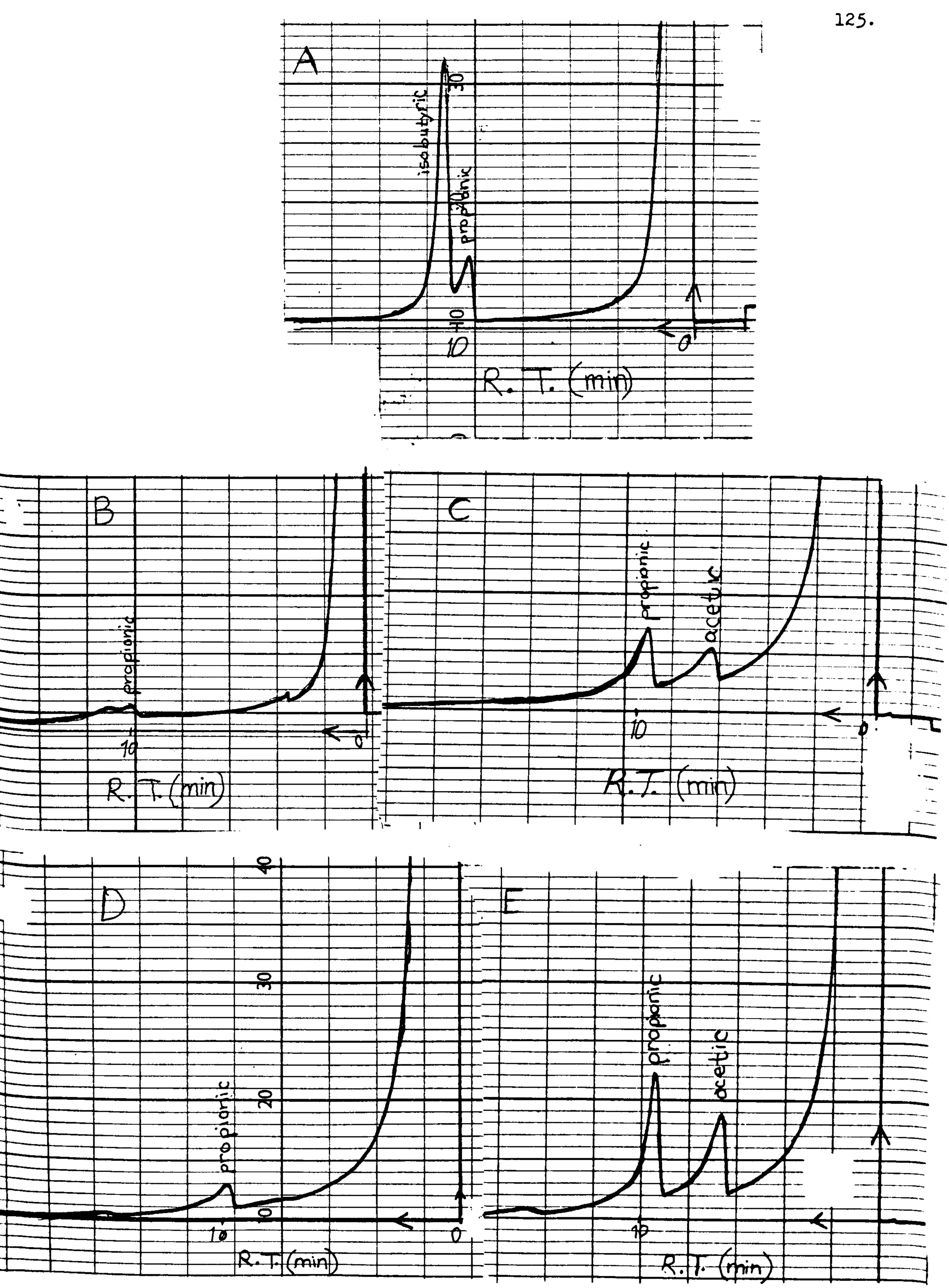
3. 4. 5 Identification of the peaks of propionic and/or isobutyric acids

The R.T. value for the peak (which had been identified previously as propionic and/or isobutyric acids) was calibrated with the R.T. values of peaks of the two acids in standard solution and was found to be propionic acid. No isobutyric acid was present. Confirmation is given in Fig. 3. 36, which shows chromatograms of a standard solution (1meq) of propionic and isobutyric acids where their peaks have been well separated by using a more efficient GLC system. When two of the C.T. strains chosen at random were analysed by the new GLC system their chromatograms showed peaks of acetic and propionic acids.

3. 4. 6 Inhibition of gonococci by fatty acids produced by gonococcal and Shigella C.T. strains as detected by GLC

Fatty acids which were produced by gonococci and Sh. sonnei C.T. strains were detected by GLC. Acids which were common and in equal

Fig. 3.36 Chromatograms of: ¼meq standard solution of propionic and isovaleric acids (A) showing the separation of peaks of the two acids; cultures of C.T.1B incubated for 1day (B) and 4 days (C); and cultures of C.T.6 incubated for 1 day (D) and for 4 days (E). Attenuation: 4 x 10 ; chart speed: 5mm/min .


A
isobutyric
propionic
30
10
0
R. T. (min)
B
propionic
10
0
R. T. (min)
C
propionic
acetic
10
0
R. T. (min)
D
40
30
20
10
0
propionic
R. T. (min)
E
propionic
acetic
10
R. T. (min)

amounts in chromatograms of both inoculated and uninoculated media were considered to have no inhibitory activity on gonococci at their calculated concentration.

On the other hand, acids which were present in appreciable quantities in samples of culture extracts, but which were absent in samples of uninoculated media, were tested for their inhibitory activity towards gonococci. These acids were produced either by gonococci or GC medium or by C.T. strains of Sh. sonnei on Brain heart infusion blood agar (BHIBA). In the former case these acids were able to mimic gonocin activity.

In the latter case it has already been established that they interfered with the colicin activity which was inhibitory to gonococci.

Table 3. 24 shows details of the acids of appreciable peak areas in the chromatogram of culture samples, and the concentration calculated for each acid. G.C. medium and BHIBA were prepared and to these were added the combination of acids in concentrations equivalent to those found in the culture samples.

The C.T. strains C.T. 1B and C.T.4 were chosen for quantitative purposes, as representatives for the ten C.T. strain cultures (1 - 4 day old) which were examined by GLC. Chromatograms of these two representatives were characterized by appreciable peak areas for acetic and propionic acids.

Media No. 1 and 2 were GC media containing acetic and isovaleric acids in the concentrations shown, and media Nos. 3 - 6 were BHIBA to which acetic and propionic acids were added.

Each of the six media, as well as blank GC medium and BHIBA, were inoculated with 9 gonococcal strains to see whether or not the acids were in concentrations sufficient to cause inhibition to gonococci.

Table 3. 24 Fatty acids produced by gonococci and Shigella sonnei C.T. strains, and media which contained the acids in the concentrations shown.

Sample tested	Fatty acid	Acid conc. (µl/ml sample)	Medium No.
Gonococci on GC medium			
N210	Acetic	0.4289	1
	Isovaleric	0.1250	
N207	Acetic	0.3795	2
	Isovaleric	0.4762	
Shigella C.T. strain on BHIBA			
C.T. 1B/1d*	Acetic	0.093	3
	Propionic	0.070	
C.T. 1B/4d	Acetic	0.273	4
	Propionic	0.012	
C.T. 4/1d	Acetic	0.121	5
	Propionic	0.059	
C.T. 4/4d	Acetic	0.134	6
	Propionic	0.141	

* d: Time (in days) of incubation for the tested C.T. culture

Table 3. 25 shows the results of the tested strains after incubation for one day at 37°C.

Table 3. 25 Inhibitory effect on gonococci of certain fatty acids at concentrations producible by cultures of gonococci and *Shigella sonnei* C.T. strains.

Medium:	GC Medium	No.1	No.2	BHI	No.3	No.4	No.5	No.6
pH (approx):	7.2	6.2	6.1	7.3	6.4	6.2	6.3	6.2
Gonococcal Strains								
N210	++	++	+	++	++	++	++	++
N214	+	+	-	+	+	+	+	+
N216	++	++	+	++	++	++	++	++
N230	++	±	-	++	++	+	++	-
N241	++	+	±	++	++	++	++	++
N242	++	++	++	++	++	++	++	++
N243	++	+	-	++	++	++	++	++
N244	++	++	++	++	++	++	++	+
N245	++	+	±	++	++	++	++	++

++: Heavy growth +: Growth ±: Scanty growth

- : No growth.

Concentrations of acids in each medium are shown in Table 3. 24

It can be seen from Table 3.25 that some gonococcal strains had failed to grow, or had grown less intensively on media No.1 and 2 as compared with their growth on a blank GC medium. Both media No.1 and No.2 contained acetic and isovaleric acids in concentrations producible by gonococci on GC medium. This gives the indication that these two acids, amongst other probable inhibitory by-products were responsible for the inhibition produced by 64% of gonococcal strains to other gonococci on GC medium. (See section 3.2). Three strains (N214, N230 and N243) failed to grow on medium No.2 whereas none of the 9 tested strains failed to grow on medium No.1 The reasons are likely to be the higher concentration of isovaleric and the lower pH value in medium No.2 as compared with medium No.1. It can also be seen from the table that none of the tested strains have shown a reduction in growth intensity on media No.3 and No.5. These two media contain acetic and propionic acids in amounts equivalent to the maximum amount which C.T. strains can produce after 24hr. incubation. This means that fatty acids in no way interfere with the colicin inhibitory activity when the C.T. strains are incubated for just one day at 37°C. On the other hand, only one strain (N230) failed to grow on medium No.6 although it grew on a blank BHIBA as well as on media No.3 and No.5. The same strain also grew less intensively on medium No.4. Both media No.4 and No.6 contained acids in concentrations producible by C.T. strains but after 4 days' incubation at 37°C. This might be the explanation as to why some gonococcal strains tested by Kekessy & Piguet method showed increased inhibition when the C.T. strains were incubated for 4 days in comparison with the inhibition they had exhibited when the C.T. strains were incubated for one day only (See section 3. 3.3).

From all these observations it is clear that (a) acetic and isovaleric acids produced by gonococci during their metabolic activities were responsible for the inhibition of gonococcal strains which was demonstrated during testing for bacteriocin production by these strains and (b) acids which were produced by Sh. sonnei C.T. strains within 24hr incubation are inadequate to interfere with the colicin activity towards gonococci.

DISCUSSION

4. DISCUSSION

4. 1 Growth and survival of gonococci in culture media

The increasing interest in laboratory studies of N. gonorrhoeae is due, in the first place, to the increase in the incidence of gonorrhoea in most countries of the world, and secondly to the realisation that a great deal remains to be ascertained about the organism itself, particularly its growth in vitro.

Since the discovery of the gonococcus about one hundred years ago, attempts have been made to maintain it in laboratory cultures. Many successful media have been formulated or modified to improve the growth yield of gonococci (Mueller & Hinton, 1941; Gerhardt & Heden, 1960; Thayer & Martin, 1966; Brookes & Heden, 1967), but they did not appreciably prolong the survival of the organisms which therefore had to be repeatedly sub-cultured. The Enriched Medium for Neisseria (EMN), devised for this purpose, is a modification of Tryptone soya broth (TSB) (Oxoid). It has been found to yield a slower growth rate but a much longer survival period than TSB and all the other media with which it was compared (Fig. 3.6, 3.7 and 3.8). The percentage survival of gonococci in EMN cultures (Table 3.1) was higher than that reported by Hafiz & McEntegart (1976) in their medium, in which survival times were said to be the longest ever recorded for gonococci in liquid media. In short, EMN supports a reasonable growth and a remarkably prolonged survival of gonococci. The long survival of cells in this medium may be due to particular ingredients. The effect of each ingredient and its probable role in prolonging the survival of cells were as follows:

i) Peptone

Peptone in a concentration of 1 - 3% is reported to favour the growth of Neisseria species (Wilson & Miles, 1966). Its concentration in EMN is 4%, the same as in Membrane enriched Teepol broth, the medium used for the detection of coliforms in the bacteriological examination of water supplies. But it has not previously been used in as high a concentration as this in media for the cultivation of gonococci. Yet there is no evidence that in a concentration of 4% it has any harmful effect on these organisms. On the contrary their growth is enhanced by some of its constituents at even greater concentrations than those at which they are present in 4% peptone. For example, the reported optimum concentration of cystine for gonococcal growth is within the range 0.025-0.075% (Boor, 1942) and a concentration of 0.05% was found to be stimulatory (Gould et al., 1944), whereas its concentration in EMN is only 0.015%. This, however, is double its concentration in TSB, which contains 2% peptone. In fact all the amino-acid constituents of peptone are present in high concentrations in EMN, and it has been shown that gonococci grow best in media in which amino-acids supply the nitrogen (Morse et al., 1977). Another important consideration is that additional calcium ions are reported to enhance the viability of N. gonorrhoeae (Bergey, 1974), and the concentration of calcium in peptones is higher than that of any other metallic constituent.

ii) Glucose

The concentration of glucose in EMN is 0.58%. It has been reported that, although glucose has little or no beneficial effect on the growth of members of the genus Neisseria, it does increase

the colony size of gonococci when used in a concentratiom of 0.2% (Wilson & Miles, 1966). But the role of glucose in the growth and survival of gonococci is more important than its effect on colony size and the comparatively high concentration of glucose in EMN could be one of the reasons for the prolonged survival of the organisms in the medium. Many studies of glucose metabolism in gonococci have been published. When the concentration of glucose in a medium is increased it stimulates the synthesis of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Holten, 1947a, 1947b). "The metabolism of glucose by N.gonorrhoeae occurred in two stages. First the glucose was dissimilated to acetate and CO_2 during active growth of the culture. Second, the acetate was oxidized via the tricarboxylic acid cycle following the depletion of glucose in the medium. During the oxidation of acetate there was little, if any, increase in the growth yield" (Morse et al, 1974). "Growth on glucose markedly reduced the levels of all tricarboxylic acid cycle enzymes", and that was why acetate catabolism was retarded until the glucose was depleted (Hebeler & Morse, 1976). From these observations it was concluded that, in EMN there was a continuous supply of carbon dioxide, first, during the utilisation of glucose whose degradation liberated CO_2 and acetate, and second, from the oxidation of acetate which started after all the glucose had been depleted. The accumulation of acetate, during the first stage of glucose metabolism, was not harmful because EMN is well buffered (Table 3.2) and there was evidence to suggest that acetic acid became less inhibitory to gonococci when the pH of the culture was between 7.5 and 7.0 (Miller et al., 1977). Starch, as will be discussed later, may also

have a protective role against fatty acids.

iii) Sodium chloride

The concentration of sodium chloride in EMN is about 1%. This is higher than the optimum for the viability of gonococci, which was stated to be 0.3% (Amies, 1967). For growth yield the optimum was found in this study to be 0.5% (Fig. 3.2). The maximum concentration tolerated by the organisms is 3% (Table 3.4), which is, however, much higher than what EMN contains.

iv) Potassium phosphate

The concentration of K_2HPO_4 (0.5%) is high enough to provide an efficient buffer for the culture without having any undesirable effect such as that found at a concentration of 0.7%, which reduced the growth rate of the cells (Fig. 3.2 and 3.3). In a glucose containing medium, both phosphate and sodium are necessary for the conversion of triose to pyruvate (Holten, 1975).

v) Sodium bicarbonate

The concentration of sodium bicarbonate in EMN is 0.01% which is less than in Thayer-Martin medium (1966) and Hafiz & McEntegart medium (1976), in which its concentration is 0.015%. Gonococci were found to be capable of utilising the bicarbonate as a substitute for carbon dioxide or even in preference to the latter (Talley & Baugh, 1975; Earl et al, 1976; Jones & Talley, 1977). It seems that its concentration in EMN is sufficient for the start of gonococcal growth until, as already described, the carbon dioxide is released in the medium during the metabolism of glucose.

vi) Starch

The concentration of starch in EMN is 0.1%, which is the concentration used in other media for cultivating gonococci.

The role of starch in the media was previously suggested to be to neutralise the inhibitory action of some unsaturated fatty acids present in the agar. The acids are adsorbed by the linear components of starch (Ley & Mueller, 1946). This might also be the case with acids produced in the medium as by-products. The starch thus helps in protecting the organisms against the harmful effect of the acids.

vii) Serum

Horse or rabbit serum was added as a supplement to the autoclaved EMN in a concentration of about 10%. It supports the growth of gonococci in that concentration (Amies & Garabedian, 1967). Its main role is nutritional. It can be substituted by hydrocele or ascitic fluids, but it has the advantage over these fluids of being commercially available. It may, however, contain antibiotics (Cruickshank et al., 1975b), and this may be the reason why, in a few instances during this study, the growth of some strains was inhibited.

4. 2 Growth and survival of gonococci at various temperatures

In general any liquid bacterial culture can be kept at 4°C without any significant reduction in the number of cells, but this does not apply to N. gonorrhoeae because it rapidly dies at such a low temperature. During this study it was thought that loss of cells could be averted by preserving cultures at a temperature below the range of growth which is from 38.5 to 30°C. But it was found that only a marginal reduction in temperature was practicable because many strains were lost at 28 - 27°C. Nevertheless the rapid loss of gonococcal cells was, to some extent, overcome by preserving the cultures at their minimal growth temperature (30°C). (Figs. 3.6, 3.7, 3.8 and 3.9).

Furthermore, two strains (N221 and N222) of colony type A (which represents Kellogg types T1 or T2) showed remarkable stability in cultures kept at 30°C. This may have been due to a genotypic characteristic in those particular strains which had prevented their conversion to type B (T3, T4 or T5) after first subculture. On the other hand it may have been caused by alteration of the incubation temperature to 30°C, an observation which supports a previous statement (Jephcott, 1972) that incubation at 30°C allows maximum stability of cells of T2 colony type. One day's incubation at 37°C prior to incubation at 30°C may be even more beneficial in preventing the loss of some strains during their lag phase at the latter temperature. At 29°C it was found that little decrease in growth intensity occurred after a second week of incubation (Table 3.12).

The composition of the medium is no less important than the temperature of incubation in prolonging the survival of gonococci (Figs. 3.6, 3.7 and 3.8). Increasing the energy sources and essential elements in the medium (as in EMN) is probably one of the main factors in prolonging cell survival (Fig. 3.9).

4.3 Production of inhibitors by gonococci: Probability of gonocin production

As Table 3.12 shows, inhibitory substances were produced by as many as 64% of gonococcal strains tested on GC medium. Other workers (Flynn & McEntegart, 1972; Walstad et al., 1974; Knapp et al., 1975; Luger, 1975) reported that the percentage of strains which produce these substances is even higher (75 - 100%). The inhibitors were claimed to be true bacteriocins (Flynn & McEntegart, 1972; Luger, 1975), but these workers did not check the

immunity of the producers to their own inhibitors. Accordingly they suggested a scheme for typing gonococci on the basis of their gonocinogeny which enabled Flynn & McEntegart to type 75% of their strains and Luger 90% of hers.

On the other hand, authors who investigated in detail the nature of the inhibitors found that the inhibition was due to non-specific by-products (Walstad et al., 1974; Knapp et al., 1975). In this study similar observations were made when gas-liquid chromatographic analysis showed that acetic and isovaleric acids were produced by some strains in appreciable amounts on the medium used for gonocin detection (See Section 3.4.3). When the same concentration of those two acids was added to a GC medium, 11 out of 18 (61%) of the strains tested were inhibited completely or partially (Table 3.25). This percentage is very close to that found for producers of inhibitory substances (Table 3.12). Walstad et al. (1974) found inhibitory free fatty acids during a gel chromatographic analysis of producer culture extracts. They also found that omission of starch from the GC medium resulted in a larger zone of inhibition. As starch neutralised the inhibitory effect of fatty acids (Ley & Mueller, 1946), this again confirms that the inhibitors were free fatty acids.

Glucose was reported to reduce the diffusion of E.coli colicin in solid media (McGeachie & McCormick, 1963), and to inhibit the production of colicin by strains of Sh. sonnei and E.coli (Lavoie et al., 1974). Accordingly, a medium lacking in glucose should increase the production of true bacteriocins. But the reverse was found to be the case; inhibitors were produced only in the presence of glucose (Tables, 3.12 and 3.13). It has already been shown that

acetic acid is formed during the degradation of glucose by gonococci (Morse et al., 1974). The acid, which is one of the main inhibitory by-products, was only produced on GC medium, which contains glucose, but was not produced on CBA medium, which lacks glucose. The producer strains, on the other hand, showed inhibitory activity on GC medium but not on CBA (Tables 3.12 and 3.13). Therefore, the production of inhibitors was associated with the presence of glucose, which in turn does not enhance the production of bacteriocins. Once again this shows that the inhibitors were not true bacteriocins.

However, there are gonocinogenic strains of N. gonorrhoeae, but they are rare, less than 1% of the strains tested in one study (Lawton et al., 1976). In this study 2 out of 50 (4%) of the strains tested were found to produce an inhibitor to which they were not themselves susceptible. If the inhibitor was a true gonocin it should have been produced more plentifully on CBA medium. Clearly, gonocin typing can only be of limited usefulness because only a few strains will be typable by gonocinogeny of the tested strains.

It will be remembered that, as described earlier, the work of Lawton et al. (1976) on 2123 strains of gonococci showed that sensitivity to gonocins was not a satisfactory method for typing them.

4. 4 Typing of gonococci by their sensitivity to the colicins of Sh. sonnei

As already mentioned, many bacteriocins or similar substances have been reported to have an inhibitory effect on

gonococci. A killer factor from C. albicans (Hipp et al., 1974, 1975) as well as the pyocins of Ps. aeruginosa (Sidberry & Sadoff, 1976; Morse et al., 1976; Blackwell et al., 1979) have both been suggested, separately, as feasible means of typing gonococci on the basis of their sensitivity to the inhibitors. No report has been published, so far, on the effect of Sh. sonnei colicins on gonococci. Another genus from the Enterobacteriaceae (E.coli) was reported to inhibit gonococci (Geizer, 1968). The inhibitory effect of Sh. sonnei colicins on gonococci (Table 3.14), suggested the possibility of using the colicins of 10 C.T. strains for typing gonococci. Certain limitations, such as gaps in the information available about sources of infection, made it difficult to evaluate adequately the usefulness of this typing scheme in epidemiological studies, but the results were at least encouraging.

There was some degree of correlation between the sources of the patients' infection and the patterns of inhibition of the gonococcal strains isolated from their discharges (See Section 3.3.1).

Ready availability of the colicin type strains in laboratories which conduct epidemiological studies of Sh. sonnei infections, the remarkable stability of the colicinogeny of C.T. strains, and the ease with which they can be maintained viable in cultures are all considered advantageous in their application to gonococcal typing.

4. 4.1 Effect of prolonged incubation of Sh. sonnei C.T. strains on patterns of inhibition of indicator strains

It has been found, in practice, that prolonging the incubation of colicin type strains of Sh. sonnei may result in changes in the inhibition patterns of some indicator strains tested (Table 3.16 and 3.17).

With gonococcal strains the inhibition was increased, but with Sh. sonnei indicator strains it was slightly reduced. Thus, it was logical to assume that the increased inhibition (of gonococci) was not due to slow production of bacteriocins by some C.T. strains because Sh. sonnei indicator strains exhibited less inhibition after prolonged incubation of the C.T. strains.

As it was previously stated, two days' incubation of C.T.2 strain resulted in weak colicin production and it was accordingly suggested that incubation of all C.T. strains should be prolonged for four days (Abbott & Graham, 1961). But later authors (Reller, 1971; Morris & Wells, 1974) followed the suggestion of Gillies (1964), who modified the procedure of Abbott & Shannon (1958) and showed that one day's incubation of all the C.T. strains is adequate for the colicin production.

It was however possible to detect some inhibitory fatty acids, namely acetic and propionic acids, which were present in some four days old C.T. cultures(See Section 3.4.4). But the amounts of these acids produced in the one day old C.T. cultures were not sufficient to inhibit gonococci. This was clearly shown when these two acids were added to an uninoculated Brain heart infusion blood agar in concentrations equivalent to their maximum production in a one day old C.T. culture, and none of the nine gonococcal strains inoculated on that medium was inhibited (Table 3.25).

Accordingly, it has been suggested that (a) incubation of Sh. sonnei C.T. cultures must not exceed one day when they are to be used for typing gonococci, and (b) the growth of the C.T. strain should be exposed briefly to chloroform vapour without removing the killed growth before inverting the medium for inoculating the gonococcal

strains, thus ensuring that no more by-products are being produced.

4. 4.2 Examination of the activity of crude colicins

As already explained it was assumed that short-chain fatty acids might be the inhibitory by-products produced by C.T. cultures after four days of incubation. This assumption was made before the analysis of culture samples by GLC. No attempt was made to detect long-chain fatty acids because it is the short-chain ones that comprise the major degradation products of carbohydrates. Long-chain acids were found mainly in gel chromatographic analysis of whole cell extracts (Walstad et al., 1974). It has been reported that the biochemical activities of Gram-negative bacteria are inhibited by short-chain (C2 - C6) fatty acids and not by the long-chain (C10 - C18) ones (Shay & Freese, 1973). If however, any inhibitory lipid other than the fatty acids had interfered with the colicin activity, it should have been washed off during the preparation of crude colicins. When crude colicin of C.T.9 was tested against strains of Sh. sonnei indicators and gonococci the results were identical to what was found when a living one day old culture of C.T.9 was tested against those same indicator strains. This excludes the possibility that any kind of by-product interfered with the colicin activity providing that the C.T. strains were incubated for only one day.

4. 5 Gas-liquid chromatographic analysis

By the aid of gas-liquid chromatography it was possible to investigate two matters:-

i. the nature of the inhibitors which were produced by many gonococcal strains on GC medium and which are capable of inhibiting the producers as well as other strains.

ii. The probability of interference by some non-specific inhibitory by-product with colicin activity towards gonococci.

The results of these investigations (Section 3.4.3 and 3.4.4) showed that first, during the metabolic activities of gonococci on GC medium, acetic and isovaleric acids were produced. The former resulted from the degradation of glucose present in the medium. Secondly, during the metabolic activities of Sh.sonnei C.T. strains both acetic and propionic acids were produced in appreciable quantities. But their amounts (produced after one day's incubation of the C.T. strains) were too little to cause inhibition to gonococcal strains.

It should not be forgotten that acetic and isovaleric or acetic and propionic acids were not the only acids which resulted from the metabolic activities of gonococci and colicin-type strains respectively. Neither were they the only acids detected by the GLC. But they were the only acids which appeared in appreciable concentrations in comparison with their concentration in samples of uninoculated media.

It should also be pointed out that the amount of acetic acid which was produced by gonococci on GC medium (Oxoid) after two day's incubation was more than that produced by Sh. sonnei C.T. strains on Brain heart infusion blood agar (Oxoid) after four day's incubation (Table 3.24). This does not necessarily mean that gonococci are more active than Sh. sonnei in glucose utilisation. Despite the higher concentration of glucose in Brain heart infusion medium as compared with GC medium, glucose in the former was autoclaved together with all other ingredients (except the blood) whereas in GC medium it was added as a sterile supplement to a GC agar base.

There is evidence to suggest that the colony size of gonococci was larger on Brain heart infusion blood agar when it was sterilised by filtration than when it was autoclaved (Seth & Wilkinson, 1976). This might also be true for Sh. sonnei. If so, then it is possible that the better glucose utilisation (indicated by acid production) by gonococci in comparison with its utilisation by Sh. sonnei, was due in fact to the state of the glucose in the medium rather than to more vigorous biochemical action by gonococci.

It can be concluded from this work, using gas-liquid chromatography, that colicins produced by Shigella sonnei may be applied in typing gonococci without the attendant fear of non-specific substances interfering with the reading of results.

APPENDIX

The Glasgow City Laboratory numbers of the gonococcal strains used in this study.

Serial No. of strain	City Lab. No.	Serial No. of strain	City Lab. No.	Serial No. of strain	City Lab. No.
N1	402701	N31	403821	N61	-
N2	-	N32	403957	N62	410763
N3	-	N33	-	N63	410777
N4	402435	N34	408119	N64	-
N5	401433	N35	-	N65	410797
N6	401640	N36	-	N66	412032.3
N7	402565	N37	408370	N67	412072.3
N8	402639	N38	408395	N68	412074.5
N9	401704	N39	-	N69	412096
N10	-	N40	408463	N70	412096
N11	402830	N41	408467	N71	412225
N12	401589.90	N42	408477	N72	412226
N13	402836	N43	408479	N73	412278.9
N14	403265	N44	408527	N74	412286.7
N15	403434	N45	408534	N75	412297
N16	403168.9	N46	408535	N76	412303
N17	403395	N47	-	N77	412304
N18	403366	N48	408742	N78	412708
N19	403293	N49	408856	N79	412776
N20	403364	N50	408867	N80	412847
N21	402839	N51	408927	N81	412848.9
N22	403282	N52	-	N82	412857
N23	403364	N53	408969	N83	412861
N24	403138	N54	-	N84	413035
N25	402639	N55	409085	N85	413038
N26	402830	N56	409088	N86	413042
N27	-	N57	409089	N87	413190
N28	403908	N58	-	N88	413195
N29	403907	N59	410673	N89	413196
N30	403979	N60	410733	N90	413284.5

contd.

contd.

Serial No. of strain	City Lab. No.	Serial No. of strain	City Lab. No.	Serial No. of strain	City Lab. No.
N91	413374	N125	417246	N159	429327
N92	413387.8	N126	417273	N160	429350
N93	413395.6	N127	417281	N161	429361.2
N94	413430	N128	417298	N162	429502
N95	413964.5	N129	417301.2	N163	429504
N96	413980	N130	417220	N164	429523.4
N97	415688	N131	-	N165	-
N98	415735	N132	417968	N166	429632.3
N99	415862	N133	428401	N167	429740
N100	415960.1	N134	428475	N168	-
N101	415982	N135	428468.9	N169	-
N102	416001.2	N136	428487.8	N170	400243
N103	416014	N137	-	N171	400265
N104	416044	N138	428578	N172	400336
N105	416064	N139	428663	N173	-
N106	416123	N140	-	N174	401199
N107	416132	N141	-	N175	-
N108	416135	N142	428707	N176	401246.7
N109	416158.9	N143	-	N177	401246.7
N110	416353	N144	428756	N178	-
N111	416439	N145	428763	N179	401258
N112	-	N146	428764	N180	401281
N113	-	N147	428769	N181	401283
N114	416479.80	N148	428792.3	N182	401284
N115	416485	N149	428802.3	N183	401535
N116	416497.8	N150	428812.3	N184	401567.8
N117	416500.1	N151	429105.6	N185	401569
N118	416558	N152	429181	N186	641
N119	416559	N153	429185	N187	401645.6
N120	416560	N154	429202.3	N188	401774
N121	416563	N155	429230	N189	401825
N122	417001.2	N156	429233	N190	401981
N123	417117	N157	429256	N191	428400
N124	417242	N158	429268	N192	428756.7

contd.

Serial No. of strain	City Lab. No.	Serial No. of strain	City Lab. No.
N193	429231	N226	415238
N194	410960	N227	415132.3
N195	410985	N228	414958.9
N196	410847	N229	415146
N197	411342	N230	415368
N198	411489	N231	415305
N199	411500	N232	415044
N200	411569	N233	415148
N201	R3	N234	415236
N202	R5	N235	415306
N203	R7	N236	415153
N204	411668	N237	415388
N205	411966	N238	415400
N206	411984	N239	415474
N207	411783	N240	415484
N208	290	N241	415840
N209	414069	N242	415250
N210	414105	N243	415866
N211	414113.4	N244	415368
N212	414129	N245	415173
N213	414233	N246	415136
N214	414231		
N215	414117.8		
N216	413415		
N217	413451		
N218	198		
N219	413652		
N220	415002		
N221	415004		
N222	415008		
N223	414235.6		
N224	414809.10		
N225	415240		

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